

Nucleic Acid Delivery: from Ultrasound-triggered Release to Autonomous Systems

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Date

Signature

"Life is not easy for any of us. But what of that? We must have perseverance and, above all, confidence in ourselves. We must believe that we are gifted for something, and that this thing, at whatever cost, must be attained."

- Marie Curie

"The only limit to our realization of tomorrow will be our doubts of today."

- Franklin D. Roosevelt

"We are what we repeatedly do. Excellence, then, is not an act, but a habit."

- Aristotle

"The only person you should try to be better than is the person you were yesterday."

- Unknown

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وَأَخِيرُ دَعْوَاهُمْ أَنِ الْحَمْدُ لِلَّهِ رَبِّ الْعَالَمِينَ

SUMMARY

Drug delivery plays a crucial role in effectively managing diverse medical conditions. However, conventional systemic drug delivery methods face challenges related to maintaining precise drug concentrations and minimizing side effects. These hurdles can be addressed through the design and refinement of smart responsive drug delivery systems that can be controlled by different types of endogenous and exogenous stimuli.

In this work, we present two innovative strategies for achieving controlled on-demand drug delivery, employing ultrasound and enzymes as physical and chemical triggers, respectively. Ultrasound emerges as a standout trigger due to its deep tissue penetration and non-invasive applicability in biological environments, surpassing other physical triggers such as light, magnetic fields and temperature. The unique ability of ultrasound to convert mechanical energy into productive work at the molecular level inspired us to adapt principles developed in the field of polymer mechanochemistry and translate them into biomedical applications. Overcoming the challenge associated with the typically high energy levels of ultrasound in polymer mechanochemistry, we develop a carrier that inherently responds to low-intensity (*i.e.*, spatial-peak temporal-average intensity (I_P) below $720 \text{ mW}\cdot\text{cm}^{-2}$) and high frequency (4 -12 MHz) medical imaging US.

Our mechanoresponsive drug delivery system, constructed from DNA nanomaterial, serves as a versatile scaffold for delivering various therapeutic nucleic acids, both DNA- and RNA-based. We characterize our system extensively, investigate its mechanical properties and evaluate the underlying mechanism of ultrasound responsiveness. We demonstrate successful delivery of CpG oligodeoxynucleotides leading to controlled immunostimulation in different cell culture models. Moreover, we validate our drug delivery system in healthy animal immunostimulation model *in vivo*, marking an unprecedented application of polymer mechanochemistry in living organisms. Additionally, we demonstrate the delivery of small interfering RNA oligonucleotides as a second cargo, resulting in controlled gene knockdown in cell culture experiments. We believe that this pioneering work positions our mechanochemically responsive drug delivery system as a universal platform, poised for loading other types of cargo, such as small molecules and proteins, further expanding its potential applications in fundamental and therapeutic research,

In the second strategy, our focus is to design DNA circuits that can be activated by the enzymatic activity of exonucleases, facilitating a precise control over the delivery of CpG oligodeoxynucleotides *in vitro*. These DNA-based nanosystems are pre-programmed to achieve distinct pharmacokinetic profile, characterized with an acute and robust immunostimulatory response, in contrast to a system lacking controlled drug release, which exhibited a slow and delayed response. Furthermore, we transformed the DNA circuit into a dissipative system, characterized by multiple cycles of activation/deactivation, operating out-of-equilibrium. Such systems are envisioned to achieve autonomous control, allowing it to seamlessly interact with and respond to the biological milieu, requiring minimal external intervention. We believe this approach paves the path for tailored drug delivery systems, in which a harmonious interplay between activation and inhibition responses becomes paramount.

ZUSAMMENFASSUNG

Wirkstoffabgabe spielt eine entscheidende Rolle bei der effektiven Behandlung verschiedener Krankheiten. Allerdings stehen konventionelle systemische Wirkstoffabgabesysteme im Hinblick auf die Aufrechterhaltung präziser Arzneimittelkonzentrationen und der Minimierung von Nebenwirkungen vor Herausforderungen. Diese Herausforderungen können durch die Gestaltung und Verfeinerung intelligenter reaktiver Wirkstoffabgabesysteme überwunden werden, die von verschiedenen endogenen und exogenen Stimuli gesteuert werden können.

In dieser Arbeit widmen wir uns zwei innovativen Strategien für kontrollierte, bedarfsgesteuerte Wirkstoffabgabe unter Verwendung von Ultraschall und Enzymen als physikalische und chemische Trigger. Ultraschall hebt sich aufgrund seiner tiefen Gewebedurchdringung und nicht-invasiven Anwendbarkeit in biologischen Umgebungen als herausragender Trigger hervor, wodurch er andere physikalische Trigger wie Licht, Magnetfelder und Temperatur übertrifft. Die einzigartige Fähigkeit von Ultraschall, mechanische Energie auf molekularer Ebene gezielt in produktive chemische Reaktionen umzuwandeln, inspirierte uns, Prinzipien der Polymermechanochemie zu adaptieren und in biomedizinische Anwendungen zu übersetzen. Um die Herausforderung der in der Polymermechanochemie typischerweise hohen Energielevel von Ultraschall zu überwinden, entwickeln wir einen Wirkstoffträger, der von Natur aus auf geringe Intensität (d. h. räumlicher und zeitlicher Mittelwert der Intensität (IP) unter $720 \text{ mW}\cdot\text{cm}^{-2}$) und hohe Frequenzen (4-12 MHz) von medizinischem Bildgebungs-Ultraschall reagiert. Unser mechanoreaktives Wirkstoffabgabesystem, bestehend aus DNA-Nanostrukturen, dient als modulares Gerüst zur Abgabe verschiedener DNA- und RNA-basierter therapeutischer Nukleinsäuren. Wir charakterisieren unser System umfassend, untersuchen seine mechanischen Eigenschaften und erforschen den zugrunde liegenden Mechanismus der Ultraschallreaktivität. Wir zeigen die erfolgreiche Abgabe von CpG-Oligodesoxynukleotiden, die zu einer kontrollierten Immunstimulation in verschiedenen Zellkulturmodellen führt. Darüber hinaus validieren wir unser Arzneimittelabgabesystem in einem gesunden Tiermodell für Immunstimulation in vivo und kreieren damit eine beispiellose Anwendung der Polymermechanochemie in lebenden Organismen. Zusätzlich zeigen wir die Abgabe von kleinen eingreifenden RNA-Oligonukleotiden als zweites Frachtgut, was zu kontrolliertem Gen-Knockdown in Zellkulturversuchen führt. Wir glauben, dass diese wegweisende Arbeit unser mechanomechanisch reaktives Wirkstoffabgabesystem als universelle Plattform positioniert, die für die Beladung mit anderen Arten von Frachtgut, wie kleinen Molekülen und Proteinen, geeignet ist, und somit ihr Potenzial in grundlegender und therapeutischer Forschung weiter ausbaut.

In der zweiten Strategie liegt unser Fokus darauf, DNA-Schaltkreise zu entwerfen, die durch die enzymatische Aktivität von Exonukleasen aktiviert werden können, um eine präzise Kontrolle über die Abgabe von CpG-Oligodesoxynukleotiden in vitro zu ermöglichen. Diese auf DNA basierenden Nanosysteme sind vorprogrammiert, um ein bestimmtes pharmakokinetisches Profil zu erreichen, das durch eine akute und robuste immunstimulierende Reaktion charakterisiert ist, im Gegensatz zu einem System ohne kontrollierte Wirkstoffabgabe, das eine langsame und verzögerte Reaktion zeigt. Darüber hinaus haben wir den DNA-Schaltkreis in ein dissipatives System umgewandelt, das durch mehrere Zyklen von Aktivierung/Deaktivierung charakterisiert ist und außerhalb des thermodynamischen Gleichgewichts arbeitet. Solche Systeme sollen eine autonome Kontrolle erreichen und ermöglichen eine nahtlose Interaktion und Reaktion mit der biologischen Umgebung bei minimaler externer Intervention. Wir gehen davon aus, dass dieser Ansatz den Weg für maßgeschneiderte Wirkstoffabgabesysteme ebnet, bei denen eine harmonische Interaktion zwischen Aktivierungs- und Hemmungsreaktionen von entscheidender Bedeutung ist.

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Aim and Motivation

In the realm of modern healthcare, the development of innovative drug delivery systems (DDSs) stands as a pivotal frontier, aiming to revolutionize the efficacy and precision of therapeutic interventions. Conventional administration of drugs suffers from several drawbacks that result in either low efficacy, high incidence of side effects or both.

This research project delves into the field of smart controlled DDSs, where advancements in material science and mechanochemistry converge with pharmaceutical science to create responsive platforms for drug administration. While conventional drug delivery approaches lack the real-time responsiveness, smart DDSs enable spatiotemporal control of drug delivery with high precision, with the ultimate goal of enhancing therapeutic outcomes while minimizing side effects.

Therapeutic nucleic acids (TNAs) in particular are a class of drugs that suffer from severe limitations related to their delivery. This results in poor translation rates of TNAs from the lab to the clinic. These challenges are related to their high instability in biological environments, poor pharmacokinetic (PK) profiles and immunogenic side effects. Therefore, it is highly desirable to develop DDS that can protect their TNA cargo and release it on-demand using a specific stimulus.

Several physical stimuli have been investigated to control smart DDSs and biological functions. These include temperature, magnetic field and light. The latter is one of the most widely employed physical stimuli, which led to the development of the fields of photopharmacology and optogenetics. However, light application is hindered by its low tissue penetration depth and its cytotoxicity to healthy tissue, especially strong ultraviolet (UV) light.

On the other hand, ultrasound (US) is a physical stimulus that has been used in the clinical setting for decades with excellent safety record. Furthermore, it can penetrate tissues deeply and can be applied conveniently in a non-invasive manner. US has been investigated as a tool to control DDSs such as micelles, microbubbles or liposomes. However, these systems usually exploited US *via* local heating, sonoporation or through coupling with sono-adjuvants such as microbubbles or mechanosensitive (MS) ion channels. Hence, the currently available systems lack the ability of addressing highly desirable functional and structural changes at the molecular level.

Conversely, the field of polymer mechanochemistry is an evolving field that studies the manipulation of polymers *via* mechanical forces, mostly employing ultrasonication. It harnesses US to induce chemical transformations selectively. The fields of sonopharmacology and sonogenetics aim to adopt concepts used in polymer mechanochemistry and exploit US for the mechanochemical control of biological function, by controlling drug delivery and gene expression, respectively. In spite of this, the frequencies of US usually employed in polymer mechanochemistry can be rarely employed *in situ* in biomedical settings, due to cytotoxicity resulting in cell and tissue destruction. Alternatively, high- and low-intensity focused US (HIFU and LIFU) could be used. Nevertheless, the use of diagnostic or imaging US in sonopharmacology and sonogenetics without the use of sono-adjuvants and its application *in vivo* remained out of reach till now.

In this thesis, I aim to bridge this gap by designing two different DDSs. The first is an US-controlled smart DDS in which we demonstrate for the first time the coupling of polymer mechanochemistry principles and *in vivo* biomedical applications, using a low-intensity diagnostic imaging US device that is licensed for human use. The second approach is based on DNA-circuits and DNA-dissipative systems that aim towards autonomous drug delivery with

the overall vision of designing a pre-programmed system that can interact with the surrounding environment and respond to it accordingly, without the need of external interventions.

The drug moieties investigated in this work include CpG oligodeoxynucleotides (CpG ODNs), which exert immunostimulatory effect that can be used for vaccination and cancer immunotherapy, as well as small interfering RNA (siRNA) that can be used to regulate gene expression at the translation level and knockdown the target protein.

In chapter 1, the state-of-the art and background information are summarized. Different topics that are related to this work are covered to set the stage for the subsequent work results and discussion. These include fundamentals of drug delivery and targeted therapy including nanotechnology used in DDSs; US and its use to control biological systems, including polymer mechanochemistry principles and applications; TNAs and their current therapeutic landscape; cancer immunotherapy including the role of CpG ODNs and their delivery challenges; and finally, gene regulation and knockdown, with a focus on siRNA as therapeutic agents.

In chapter 2, we present a mechanoresponsive DDS for controlled immunostimulation by US-mediated CpG ODN delivery. We showcase a DDS that is inherently responsive to low-intensity (*i.e.*, spatial-peak temporal-average intensity (I_p) below $720 \text{ mW}\cdot\text{cm}^{-2}$) and high frequency (4 -12 MHz) medical imaging US. This DDS consisted of high molar mass DNA polymers generated through rolling circle amplification (RCA). The DDS was fully characterized and the effects of US on the physical characteristics of the system and the underlying mechanism were studied. US-induced immunostimulation was investigated both *in vitro* using different cell lines, *ex vivo* using primary cells isolated from human blood and *in vivo* using mice as animal models. This work presents an unprecedented application of polymer mechanochemistry *in vivo* to successfully control a desired biological function.

In chapter 3, we discuss the utilization of the same DDS, which was engineered to deliver siRNA with the aim of gene knockdown. Therefore, the DDS needs to be extended compared to the system presented in Chapter 2. The DDS was characterized and its biological performance was studied in cell culture *in vitro* for the down regulation of *EGPF* expression as a model. The knockdown efficiencies and the cytotoxicity are compared to a current gold standard, *i.e.*, a cationic polymer-based transfection reagent.

In chapter 4, we present a different DNA nanotechnology strategy as we investigated DNA circuits and DNA dissipative systems for their use in controlled delivery of CpG ODNs. A dissipative system refers to a thermodynamically open system that operates beyond, and frequently significantly deviates from, thermodynamic equilibrium. This system interacts with its environment, exchanging both energy and matter. We designed these DNA-nanosystems to hybridize to CpG ODNs and deactivate it, and programmed the system to be activated by an enzyme, using either exonuclease III (ExoIII) or T7 exonuclease. Design parameters were optimized and both systems were characterized extensively. Furthermore, their biological performance was studied in cell culture *in vitro*, demonstrating a controlled PK profile with distinctive biological response.

Finally, a conclusion and outlook sections are outlined to summarize this work and give a perspective on future research and investigation.

1. Introduction

1.1 FUNDAMENTALS OF DRUG DELIVERY AND TARGETED THERAPY

1.1.1 EVOLUTION OF DRUG DELIVERY TECHNOLOGIES

1.1.1.1 Classes of therapeutics and their delivery challenges

Overtime, the landscape of therapeutics has undergone substantial evolution. In the preceding decades, the realm of therapeutic intervention was primarily governed by small-molecule drugs (<900 Daltons). However, the targets of these agents encompass only 2-5% of the entire human genome^{1,2}. Consequently, the demand arose for alternative classes of therapeutic agents capable of bestowing novel therapeutic functionalities. New generations of proteins, peptides, monoclonal antibodies (mABs), nucleic acids and live cells have emerged. The development of these new classes has introduced a corresponding array of additional challenges (Fig. 1.1). As such, drug delivery strategies and technologies, which were previously centred around enhancing the *in vivo* performance of small molecules, must now adapt rapidly to address the evolving requisites and intricacies of drug delivery of other classes. Regardless of their classification, whether small molecules or any of the aforementioned newer classes, the ultimate objective of drug delivery remains the maximization of therapeutic efficacy while simultaneously curtailing off-target side effects of therapeutic drugs.

Small-molecule drugs have been identified and utilized in pharmaceuticals since the late 1800s³. Their delivery is greatly influenced by their physicochemical properties. Their small size facilitates their rapid diffusion through biological fluids, enabling interaction with various tissues throughout the body, provided that they are freely soluble in such biological fluids^{4,5}. Therefore, solubility-related challenges have constrained the full therapeutic potential of small-molecule drugs⁶, as inadequate solubility hinders their absorption, and therefore their overall bioavailability. Moreover, permeability is another important parameter, as even soluble drug must effectively cross biological

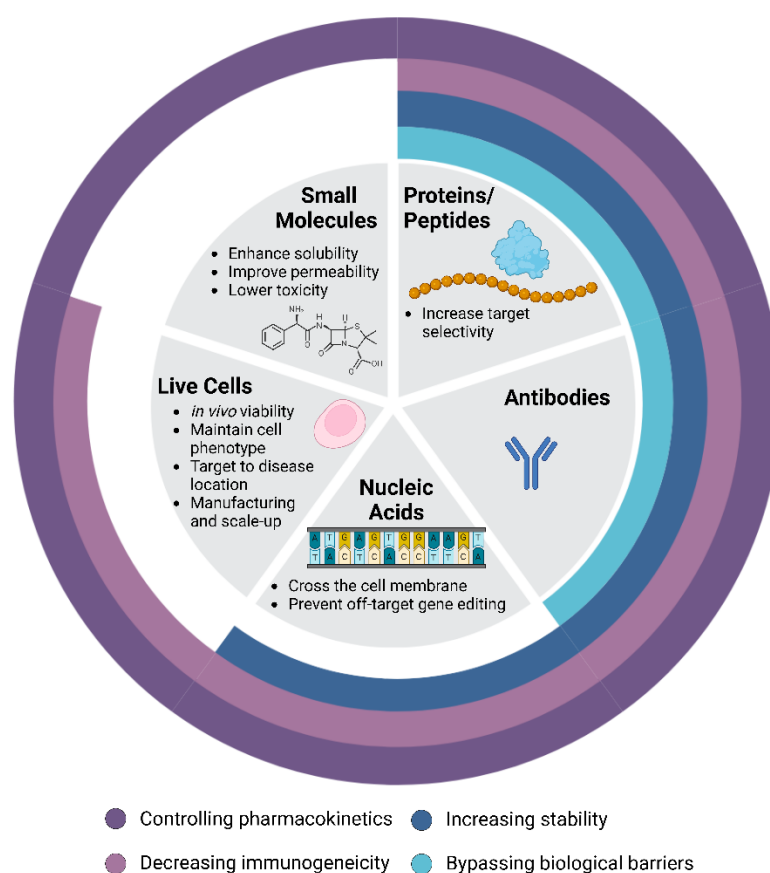


Fig. 1.1. Five classes of therapeutics (small molecules, proteins and peptides, antibodies, nucleic acids and live cells) and their associated delivery challenges. Some challenges are shared among different classes, and others are unique to a given therapeutic class.

membranes. The rapid metabolism and elimination of drugs from the body result in a short half-life, leading to a short duration of therapeutic effect. Endeavours to improve drug transport within the body led to the establishment of pharmacokinetics (PK) and pharmacodynamics (PD)^{7,8}. Heightened knowledge in these domains led to the understanding of the factors governing the efficacy, duration of action and toxicity of small-molecule drugs. Achieving a controlled PK/PD profile characterized by predictable parameters became the fundamental pursuit in advancing the delivery of small-molecule drugs, which laid the foundation for the development of controlled drug delivery systems (DDSs).

The second therapeutic class comprises peptides⁹ (consisting of 2-50 amino acids) and proteins¹⁰ (composed of 50 or more amino acids). These entities exhibit larger and more complex molecular structures compared to small-molecule drugs. While they offer advantages, such as high specificity towards particular targets, improved potency and reduced side effects¹¹, they also present several formidable challenges. Main challenge resides in the inadequate stability exhibited by peptides and proteins¹², which arises from their susceptibility to degradation or denaturation, triggered by factors such as enzymatic hydrolysis, pH changes and temperature fluctuations. In combination with their rapid clearance from the body, this consequently results in their short half-lives. Additionally, these biomolecules can trigger immune responses within the body¹³, potentially compromising the efficacy of the therapeutic intervention. Efforts to surmount these challenges¹⁴⁻¹⁶ involve enhancing the physicochemical stability of peptides and proteins¹⁰, manipulating their PK parameters, circumventing biological barriers and mitigating their potential to provoke immunogenic reactions. Through these innovative delivery strategies, the therapeutic potential of peptides and proteins can be harnessed more effectively¹⁷.

Antibodies (ABs) represent the third category of therapeutics, distinguished by their unique properties that confer highly specific interactions with target molecules¹⁸. Nonetheless, this complexity and interplay with the biological milieu provoke substantial challenges in their effective delivery¹⁹. Their large molecular size can often restrict their penetration and distribution²⁰, necessitating predominantly intravenous (IV) administration, which is often accompanied by patient inconvenience and mandates hospital or clinic visits. Furthermore, their half-life can be variable across diverse individuals, thereby influencing dosing schedules and therapeutic outcomes²⁰. Engagement with the immune system constitutes another facet of antibody action, often entailing immunogenicity²¹⁻²³. This tendency to incite immune responses underscores the potential for eliciting anti-antibodies, which in turn may cause potentially serious side effects and diminish therapeutic efficacy. Prolonged exposure to such treatments could even exert influence over the normal immune responses in patients. Progress in the domains of AB manufacturing and engineering²⁴, coupled with diverse drug delivery strategies^{25,26}, has led to prolonged half-life, enhanced penetration into targeted tissues, and mitigation of immunogenic tendencies²⁷.

The next class of therapeutics comprises nucleic acids, including both deoxyribonucleic acids (DNA) and ribonucleic acids (RNA²⁸). Therapeutic nucleic acids (TNAs), particularly with the advent of advanced genome editing tools like Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)²⁹, offer the potential for modulating cellular functions at various stages of gene expression. The first TNA to get the approval of the United States Food and Drug Administration (US FDA) was fomivirsen, an antisense oligonucleotide (ASO) utilized in the treatment of cytomegalovirus retinitis in immunocompromised patients³⁰. Although many TNAs followed and were approved by the US FDA, when comparing TNAs researched in laboratories with those approved for human use, a notable disparity in success rates becomes evident³¹. This can be attributed to the inherent challenges associated with TNAs delivery^{32,33}. TNAs have a short half-life due to their susceptibility to degradation and hydrolysis³⁴. Additionally, their immunogenicity is a concern³⁵, as the human immune system

can effectively differentiate foreign DNA and RNA sequences³⁶. Furthermore, TNAs must be delivered to their site of action within the cell, either in the cytoplasm or inside the nucleus³⁴. This requirement adds complexity, involving the need for endosomal escape and crossing multiple membranes to reach their target³⁷. To address these challenges, chemical modifications of nucleic acids^{38,39} as well as advancements in drug delivery technologies⁴⁰⁻⁴² have significantly improved TNAs' *in vivo* performance, by increasing their stability against degradation, reducing their immunogenicity and enhancing their cellular uptake^{38,43}. A recent milestone highlighting this therapeutic class and the accompanying advancements in its delivery is the approval of messenger RNA (mRNA)-based vaccines for combating coronavirus disease 2019 (COVID-19)^{44,45}.

Finally, the last and the newest class of therapeutic is live-cell therapy, in which certain types of live cells are harnessed to interact with the host cells and modulate their functions. Examples of these cells include pluripotent stem cells⁴⁶, immune cells^{47,48} and microbes⁴⁹. This innovative approach allows for the genetic engineering of cells prior to their introduction to the host system. A notable example is the development of engineered chimeric antigen receptor (CAR) T cells, which received clinical approval in 2017 for targeted cancer immunotherapy⁵⁰. Beyond cancer treatment, engineered cells can also be designed to function as pulsatile or stimuli-responsive drug factories, attempting to mimic the natural physiological profiles⁵¹. However, the successful delivery of live-cell therapies faces numerous challenges. These cells are significantly larger than other therapeutic agents, making them prone to entrapment and excretion before reaching their intended target tissues⁵². Additionally, the viability, persistence of desired phenotypes and immunogenicity of these cells are heavily reliant on host-related factors^{53,54}. As a result, autologous cell therapy, which utilizes patients own cells, presents a safer profile but is cumbersome, less scalable and consequently more expensive^{55,56}. Last but not least, the long-term effects of live cell therapies are not fully known yet. Recently, the US FDA and the European Medicines Agency (EMA) have both started a review of data and reports on secondary malignancies related to T-cells for several approved CAR T-cell medicines⁵⁷. All these challenges currently limit the widespread adoption of live-cell therapy in clinical practice⁵⁸.

1.1.1.2 Three paradigms of drug delivery

Over the span of several decades, three fundamental strategies have been employed to improve drug delivery: drug modifications, microenvironment modifications and DDSs (Fig. 1.2). These approaches have remained pivotal across various therapeutics classes.

The first strategy, drug modifications, involves altering the chemical structure of the drug itself or conjugating it with other moieties. This manipulation aims to regulate the interaction between the drug and different components of the body, including molecules, cells, tissues and drug targets, leading to controlled PKs, PDs or both. Chemical modification of small molecule drugs has laid the foundation for the field of medicinal chemistry⁵⁹, which eventually led to development of high throughput screening⁶⁰ methods allowing the rational design and optimization of drug structures based on identified structure-function relationships (SARs)^{61,62} and their associated guidelines^{63,64}. Additionally, chemical modifications have led to the development of prodrugs⁶⁵, which are initially inactive but become activated upon exposure to certain host factors, often enzymes or pH changes, reversing the chemical modification and leading to enhanced drug stability and selectivity, and reduced side effects⁶⁶.

In the realm of peptides and proteins, display technologies have been utilized to identify therapeutic molecules with desired properties. Conjugation strategies have been also employed to extend their circulation time and reduce immunogenicity. Notably, long-acting insulin delivery has been improved through rational modifications aiming to mimic natural insulin secretion^{67,68}. Various rational modifications have been employed to improve ABs binding

affinity, extend their half-life and enhance their overall efficacy⁶⁹. These encompass changes in glycosylation patterns⁷⁰, modifications in amino acids within the fragment of crystallizable (Fc) domain⁷¹, cross-isotype or cross-subclass exchanges⁷², the development of antibody-drug conjugates (ADCs)²⁶ and bispecific antibodies (bsAbs)⁷³.

For nucleic acids, chemical modifications of building blocks or conjugation with other moieties have been instrumental in controlling drug activity and improving PK profiles⁷⁴. Examples include chemically modified ASOs³⁹ employing modifications like 2'-ribose and O-methoxyethyl (MOE) modifications⁷⁵, locked nucleic acids⁷⁶ (LNAs) and phosphorothioate (PS) linkage to replace the non-bridging oxygen group with a sulfur atom in the TNA backbone⁷⁷. Other examples include incorporating modified nucleobases like N1-methylpseudouridine (m1Ψ) in synthetic mRNA vaccines^{78,79} and conjugating N-acetyl galactosamine (GalNAc) ligand to small interfering RNA⁸⁰ (siRNA) in the commercially available drug Givosiran⁸¹. The knowledge of drug modification strategies developed for various therapeutic generations is being adapted in the context of live-cell therapy. For instance, cells' surfaces can be chemically modified to impart new properties and functions without altering viability or phenotype⁸².

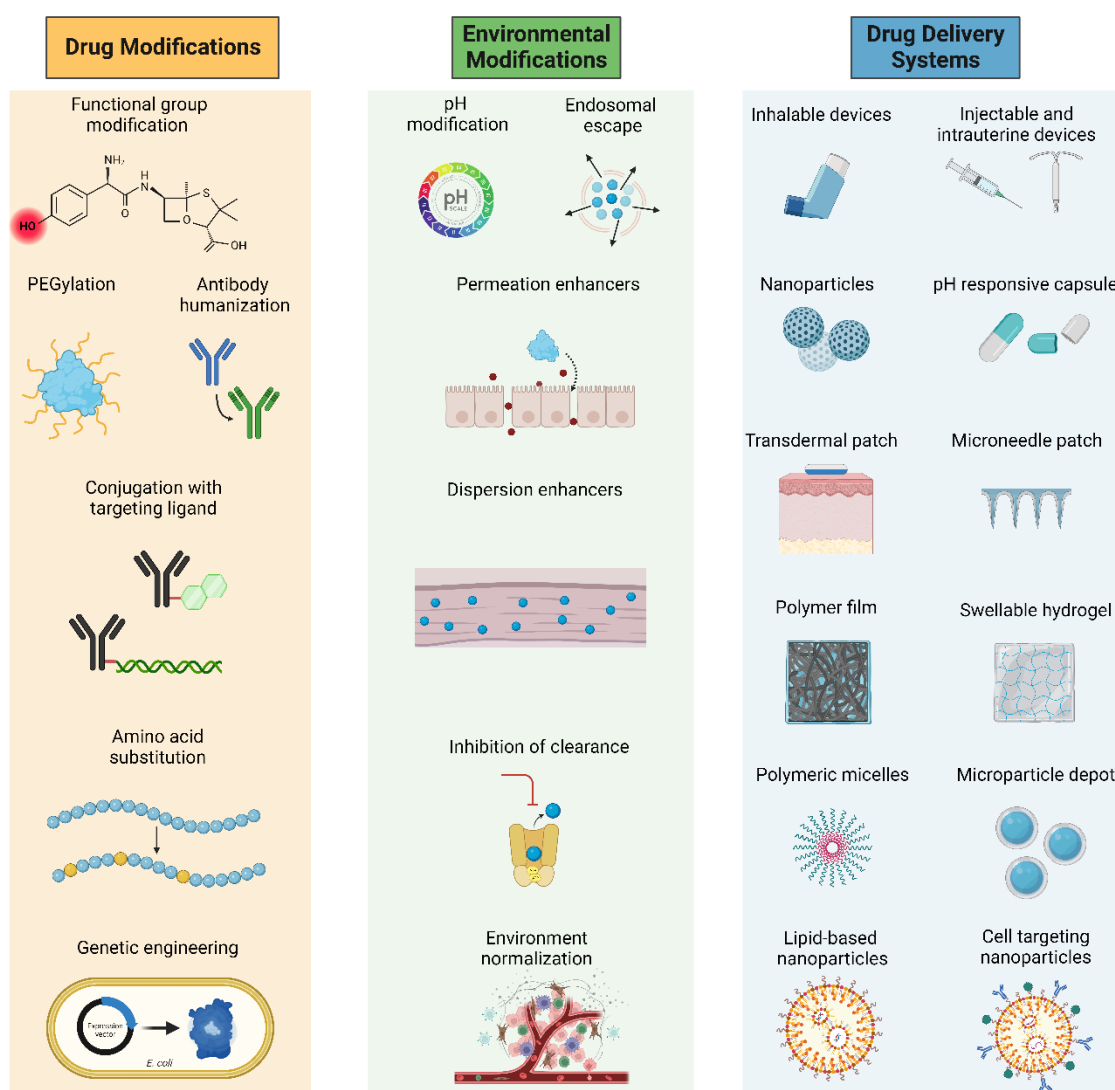


Fig. 1.2. Regardless of the class of therapeutic, three delivery paradigms were developed to improve therapeutic function: the modification of the drug or of its environment, and the design of drug delivery systems. Drug delivery systems have adopted one or more strategies for drug modification or environmental modification. Created with BioRender.com

The second paradigm in drug delivery involves modifying the microenvironment using a wide array of tools to facilitate *in vivo* navigation of therapeutic drugs. For small molecule drugs, formulation scientists have utilized excipients (*i.e.*, non-active ingredients) to enhance drugs' performance⁸³. Solubilizing agents altering biological pH have been used to improve dissolution behaviour and thereby the oral absorption of drugs with pH-dependent solubility^{84,85}. Other excipients can inhibit drug's clearance pathways, thereby prolonging circulation time.

In the case of peptides, proteins and ABs, microenvironment modifications have involved protease inhibitors⁸⁶, pH-modifiers, permeation enhancers⁸⁷, subcutaneous (SC) dispersion enhancers⁸⁸ and immunomodulators⁸⁹. For nucleic acids, efforts to improve cellular uptake⁹⁰ have included modifying endosomal pH⁹¹, incorporating cell-penetrating peptides⁹² and cationic lipids⁹³. Similar strategies have been adopted in live-cell therapy, where signalling molecules, immunomodulators⁹⁴ and tumor microenvironment modifiers⁹⁵ have been employed to achieve better therapeutic outcomes. Notably, live-cell therapeutics possess the unique ability to interact with and modify the microenvironment themselves⁹⁶⁻⁹⁹, offering promising avenues for further therapeutic advancements.

The use of DDSs constitutes the third paradigm in drug delivery, serving as a crucial interface between drugs and their microenvironment¹⁰⁰ with a notable advantage of physically shielding drugs from hostile environments. In their foundational stages, DDSs were designed to regulate the release rate of drugs from their carriers¹⁰¹, thereby controlling essential PK parameters such as its biodistribution, half-life, maximum concentration and total drug release over time¹⁰². Controlled release was achieved through four fundamental mechanisms: dissolution, diffusion, osmosis and ion exchange, and employed hydrogels, polymeric materials and nanoparticles^{103,104}.

Although initially developed for small molecules, controlled DDSs have found application across the entire therapeutics' spectrum¹⁰⁴. Peptides, in particular, have greatly benefited from sustained-release DDSs, a subset of controlled-release systems with prolonged maintenance of drug concentrations over time¹⁰⁵. In the case of ABs, controlled-release DDSs allowed replacing IV injections with SC ones¹⁰⁶, leading to enhanced patient acceptance and convenience. In the realm of nucleic acids, lipid-based DDSs have played a pivotal role in their clinical advancement¹⁰⁷. Detailed discussion on DDSs for TNAs will follow in subsequent chapters. Moreover, DDSs have found innovative application in live-cell therapies. For instance, chemically modified polymer-based implants have been utilized to safeguard and prolong the viability of insulin-secreting β -cells, offering a promising treatment for type-1 diabetes¹⁰⁸. Additionally, ongoing clinical trials are exploring live-cell DDSs¹⁰⁹, showcasing the evolving landscape of this cutting-edge technology.

1.1.1.3 Nanotechnology in drug delivery systems

In modern medicine, the use of nanotechnology for DDSs has sparked a transformative revolution. Biocompatible nanomaterials have exceptional properties such as high invasion rate, slow-, controlled- and targeted-release of therapeutic drugs¹¹⁰. Nanotechnology, the science of manipulating matter at the nanoscale, is a very broad multidisciplinary area and the term was first coined in 1974¹¹¹. Nanomaterials made of polymers, lipids, metals or organic materials, offer a unique platform for encapsulating drugs, genes or diagnostic agents. Their extremely small size and high surface area-to-volume ratio empower them to navigate biological barriers, penetrate cells and interact with specific molecular targets, all with remarkable precision¹¹². The use of nanomaterials can also protect therapeutic drugs from degradation or metabolism, improve their solubility, extend their half-lives and deliver them to the target site of action¹¹³. Consequently, therapeutic outcomes can be maximized and systemic adverse effects can be minimized. Additionally, multifunctional DDSs can be designed using nanomaterials. Examples include theranostics in which simultaneous imaging diagnosis and

treatment takes place¹¹⁴. Moreover, stimuli-responsive nanomaterials can be designed to enable on-demand drug release in response to a certain chemical or physical stimulus^{115,116}. In this dynamic landscape, nanocarriers of various types are being investigated for application in the context of different diseases including cancer¹¹⁷, infectious¹¹⁸, cardiac¹¹⁹, respiratory¹²⁰, neurodegenerative¹²¹ and autoimmune diseases¹²².

Particle size and size distribution of nanomaterials are critical parameters that determine their physicochemical properties and therefore shape their performance and efficacy¹²³. Small size scale offers high surface area-to-volume ratio, enabling improved drug loading and ability to penetrate biological barriers. Uniform size distribution usually leads to better stability of the system, more controlled drug release kinetics and predictable therapeutic outcomes¹²⁴. Additionally, particle size and size distribution have direct influence on the enhanced permeability and retention (EPR) effect, a phenomenon exploited in passive targeting in cancer therapy¹²⁵. When nanoparticles are administered within an optimal size range, they can accumulate in tumor tissues due to their compromised and leaky vasculature, as well as impaired lymphatic drainage¹²⁶. On the other hand, active targeting can be achieved by decorating the surface of nanoparticles to identify and interact with specific components in the site of action. Both passive and active targeting strategies will be discussed in more details in the following chapters.

Another parameter of paramount importance is nanoparticles' surface charge, as it determines their drug loading ability, cellular uptake and various interactions with the biological environment. Zeta potential range of -30 to 30 mV was demonstrated to exhibit colloidal stability without particles aggregation¹²⁷. Positive surface charge is often associated with enhanced cellular internalization, due to the negative charge of the cell membrane¹²⁸⁻¹³⁰. On the other hand, negative surface charge helps nanoparticles evade rapid clearance and prolong their circulation time¹³¹.

Opsonization, the process of tagging foreign materials by serum proteins called opsonins¹³², plays an important role in the fate of nanoparticles *in vivo*. Once opsonins recognize and bind to nanoparticles¹⁰², they render them recognizable by phagocytes leading to their elimination. Surface modifications to prevent opsonization and evade immune system recognition¹³³ include coating nanoparticles with hydrophilic polymers such as polyethylene glycol (PEG)^{134,135} and other polymer types¹³⁶.

The distinctive attributes that render nanomaterials invaluable for drug delivery also result in unique toxicological profiles distinct from those exhibited by their bulk counterparts. They can trigger the formation of reactive oxygen species (ROS)¹³⁷, leading to oxidative stress, inflammation and potential damage to biological components. Various nanoparticles, such as silver¹³⁸, quantum dots¹³⁹, zinc oxide^{140,141}, gold¹⁴² and titanium oxide¹⁴³ have been reported to exhibit toxic effects, raising concerns about nanomaterials' long-term safety in humans. Consequently, extensive research is imperative to guarantee the use of safe materials and mitigate risks associated with both short- and long-term exposure to nanomaterials.

1.1.1.3.1 DNA Nanotechnology as a platform for controlled drug delivery

DNA nanotechnology offers a rapidly growing toolbox to fabricate materials and devices that mimic living systems in their complexity and sophistication. Nucleic acids exhibit several features that distinguish them from other abiotic materials and biomaterials¹⁴⁴⁻¹⁴⁶. High predictability and programmability of its structures and interactions¹⁴⁷, system control permitted by the availability of a wide range of sequence-specific enzymes, accessibility by well-established synthesis methods and biocompatibility qualify DNA an attractive material for *in vitro* and *in vivo* applications¹⁴⁸.

One of the distinctive features of DNA is its ability to undergo complementary base pairing, which leads to the formation of different shapes and architectures in a precise manner^{149,150}. This feature is utilized in DNA self-assembly reactions to generate different types of nanomaterials. Dynamic DNA nanostructures can be designed to respond to different exogenous stimuli, including pH¹⁵¹, light¹⁵², temperature¹⁵³ and metal ions¹⁵⁴. The resulting response, usually in the form of controllable conformational changes, can be used for biosensing, bioimaging and drug delivery¹⁵⁵.

Different types of DNA self-assembled nanomaterials were the subject of extensive investigations (Fig. 1.3). These include DNA origami, nanocages and hydrogels. DNA origami objects are assembled short DNA strands into 2D and 3D structures¹⁵⁶⁻¹⁵⁸. The sequences can be optimized using computational methods with feasible site-specific modifications. The size of DNA origami structure can be up to 100 nm approximately. Although they have been investigated for drug delivery, their mass production remains a challenge for clinical application.

DNA nanocages encompass several structures such as tetrahedra, cubes, truncated octahedra, triangular pyramids, dodecahedra, icosahedra and others¹⁵⁹. DNA tetrahedra are among the most widely used DNA nanocages, due to their simple structures and minimal requirements of raw materials (Fig. 1.3b). They can be formed by assembling either one long strand or four short oligonucleotides. This assembly results in more stable structure that can resist nuclease degradation compared to single stranded DNA (ssDNA) strands. The 5'-end at the apex of the tetrahedron is free, allowing for modifications that help in drug delivery. For instance, attaching tumor penetrating peptides resulted in higher uptake of the tetrahedron loaded with doxorubicin by glioblastoma cells¹⁶⁰.

Another type of DNA-based material used for drug delivery is DNA hydrogels. These are hydrophilic 3D networks that can exhibit desired properties by tuning parameters such as swelling volume, crosslinking density and mechanical strengths (Fig. 1.3c). Two types of DNA hydrogels have been developed: pure DNA hydrogels formed by DNA crosslinking itself, and hybrid DNA hydrogels formed by crosslinking of natural or synthetic polymer chains combined with DNA¹⁶¹. DNA hydrogels can be produced *via* several methods such as using X-, T- or Y-shaped DNA units and nuclease amplification reactions.

Several techniques were developed over the years to produce DNA nanomaterials. Toehold-mediated strand displacement (TMSD) is the process by which a ssDNA invader strand binds to an overhang of a substrate strand in a double stranded DNA (dsDNA) and displaces an incumbent strand *via* a branch migration process¹⁶². Catalytic hairpin assembly (CHA) reaction is another method (Fig. 1.3d) in which two partially complementary DNA hairpins are designed with stem-loop structure¹⁶³. The loop of one hairpin is complementary to a target ssDNA sequence, and the loop of the other hairpin is designed to act as a catalyst for the assembly process. Once the target ssDNA strand binds to the first hairpin loop, it opens up the hairpin structure, which in turn binds to the catalyst loop of the second hairpin. This binding opens up the catalyst hairpin structure to reveal a sequence that can displace the target ssDNA that initiated the process. The free ssDNA now can hybridize with another hairpin loop, thereby initiating a chain reaction. At the end, amplified DNA products are accumulated, which can be used for a certain desired application.

Moreover, hybridization chain reaction (HCR) is another DNA polymerization process that can be initiated by short ssDNA strands¹⁶⁴. HCR enables the assembly of long DNA polymers from short DNA hairpin monomers (Fig. 1.3e). Two hairpins are needed, where each has a complementary sequence to the other in their stem regions, and a toehold sequence that acts as a binding site for the initiator ssDNA strand. This binding between the initiator strand and

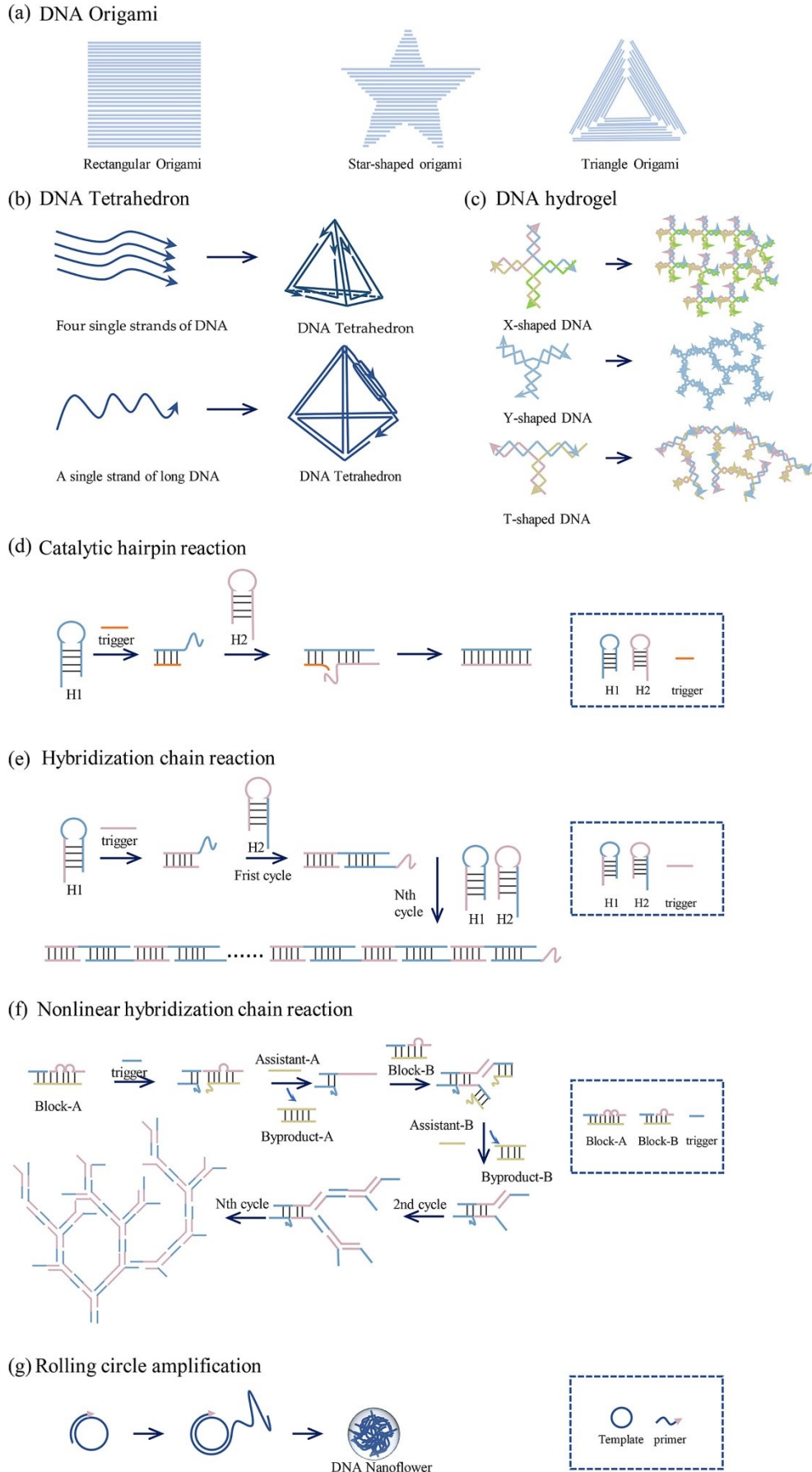


Fig. 1.3. Schematic presentation of different DNA self-assembly methods. Each method is explained in the corresponding text in section 1.1.1.3.1 (Reprinted from¹⁶⁵, Copyright (2023), with permission from Elsevier).

toehold sequence opens up the structure of the first hairpin exposing its complementary sequence that then hybridizes with the second hairpin leading to its opening as well. The chain reaction proceeds with multiple hairpins opening and hybridizing in sequential order leading eventually to a long DNA polymer. This method can be used for controlled drug delivery by using an aptamer as an initiator that could target the cells, and adriamycin could be loaded on the resulting DNA polymer¹⁶⁶. Nonlinear HCR was introduced to design branched or dendritic DNA structures that exhibit higher stability and more binding sites for drug loading (Fig. 1.3f).

Rolling circle amplification (RCA) is another isothermal method (Fig. 1.3g) that utilizes enzymatic activity to generate extremely long ssDNA chains¹⁶⁷. This method will be discussed in detail in the following section of this chapter.

1.1.1.3.2 Rolling circle amplification

The amplification of nucleic acids is considered to be one of the most enabling technologies during the last few decades. Modern molecular biology and medicine depends on the amplification of DNA and RNA. Polymerase chain reaction¹⁶⁸ (PCR) is utilized for the majority of DNA amplification. However, cycling through specific temperature steps is required. Therefore, PCR has several constraints that led to growing interest in simpler isothermal amplification methods. The use of biologically-inert temperature allows the performance of such amplifications in the presence of biological molecules, without compromising their stability *via* heating needed in PCR. Several isothermal DNA/RNA amplification methods have been developed including RCA, rolling circle transcription (RCT), nucleic acid sequence-based amplification (NASBA)^{169,170}, strand displacement amplification (SDA)¹⁷¹ and loop-mediated isothermal amplification (LAMP)¹⁷². RCA and RCT are considered among the earliest and most widely utilized isothermal amplification methods, and are the only ones that involve the use of a circular template¹⁷³. Therefore, the outstanding feature of RCA/RCT lies in their ability to create numerous copies of DNA/RNA sequences, presenting distinctive opportunities and applications.

The circular template needed for RCA can be obtained *via* padlock ligation, in which linear ssDNA template hybridizes to both ends of a padlock probe¹⁷⁰, which then can be joined by DNA ligase, resulting in circular ssDNA template. If designed appropriately, a single mismatch between the probe and the template can prevent circularization and therefore the RCA process. Afterwards, a DNA polymerase enzyme initiates the polymerization reaction utilizing monomers to build a long ssDNA chain complementary to the sequence of the template (Fig. 1.4). The DNA polymerase used should have high processivity (defined as the average length of DNA the enzyme synthesizes before dissociating), which is a critical criterium for maximizing RCA output. An outstanding example of such an enzyme is the bacteriophage enzyme Phi29 DNA polymerase with a processivity of tens of thousands of nucleotides¹⁷⁴. Therefore, it's among the most widely used enzymes in RCA. Furthermore, Phi29 polymerase was found to be able to accommodate several types of modified nucleotides¹⁷⁵, enabling the production of functionalised ssDNA or fluorescently labelled one. RCA polymers can be designed to contain sequences with defined functions, such as aptamers¹⁷⁶, drug loading sites, ASOs¹⁷⁷ and DNazymes^{178,179}. The resulting extremely long ssDNA polymers undergo sequence-independent liquid crystallization to form DNA–inorganic hybrid nanoparticle system composed of high molar mass DNA and magnesium pyrophosphate (Mg₂PPi) that is generated as a by-product through the reaction¹⁷⁶. Due to their characteristic architectures, these particles are termed DNA nanoflowers (NFs). Due to their long and non-nicked DNA backbone, together with dense DNA packaging, the DNA NFs show high resistance towards nucleases, heat, urea and dilution¹⁷⁸. Such stability is highly desirable in biomedical applications.

RCA has found extensive applications in genomics, diagnostics and oligonucleotide production. For example, the resulting RCA polymer can be used as a repeating probe that concentrates the signal in a localized area. The probe can also be anchored on solid surfaces¹⁸⁰ such as glass, beads, microwell plates, paper and microfluidic devices, by

modifying the 5'-end of the primer with binding groups such as biotin. This strategy has been employed for the detection of antigens¹⁸¹ and cell-surface markers¹⁸². Another approach is to convert the RCA into exponential process by adding one or more additional primers during the reaction. This led to the hyperbranched RCA (HRCa)¹⁸³, also called ramification amplification (RAM)¹⁸⁴. Artificial telomere elongation could also be achieved using RCA process directly on the human chromosome^{185,186}. Furthermore, encoding a restriction enzyme site into the circular template allows to cut the resulting long ssDNA by the restriction enzyme into predefined unit lengths^{187,188}. Members of the microRNA (miRNA) family that differ with single nucleotide can be discriminated using padlock RCA probes.

As a branch of DNA nanotechnology, we utilize the RCA strategy in this research as it provides a platform for nanoscale regulation and rational design of DNA. RCA-based nanomaterials can be assembled with other components such as cationic polymers, peptides and lipids. RCA sequences are programmable and perfectly defined, leading to enhanced and predictable effects. To further boost the application of RCA-based nanomaterials, several challenges should be addressed. These include the immunogenicity and metabolism of RCA polymers in living organisms, scale production and batch variations.

1.1.1.3.3 DNA circuits and dissipative systems

As another example of DNA-based nanomaterials, DNA circuits are artificial nanodevices constructed from short strands of DNA and designed to perform computational or signal processing tasks to achieve a desired output^{189,190}. They found different applications in nanotechnology and biotechnology due to their high programmability, specificity and operation at the nanoscale level¹⁹¹. These DNA nanodevices can be designed to respond to particular inputs, such as changes in temperature¹⁹², pH^{193,194}, light¹⁹⁵ or the presence of specific small molecules, enzymes or other DNA or RNA strands¹⁹⁶⁻¹⁹⁸. DNA circuits can be designed using various strategies¹⁹⁰ such as the involvement of DNA strand-displacement reactions¹⁹⁸⁻²⁰¹ or DNazymes²⁰²⁻²⁰⁴. Additionally, some circuits employ protein-based enzymes in their components such as polymerases or exonucleases to perform the programmed operation^{197,205,206}. DNA circuits can be time responsive, as their output changes with the change of the input signal over time. This ultimately leads to kinetic control over the desired output of the system, as a pre-programmable feature of the DNA-based nanosystems that distinguishes them from other DNA-based nanosystems.

DNA circuits can perform signal processing functions such as logic gates that perform Boolean operations²⁰⁷⁻²⁰⁹, signal amplification^{196,210,211}, neural network computation²¹²⁻²¹⁴, oscillations generation²¹⁵ and molecular motors and walkers that convert chemical energy into mechanical motion²¹⁶. In the realm of biological applications, DNA circuits were integrated with living

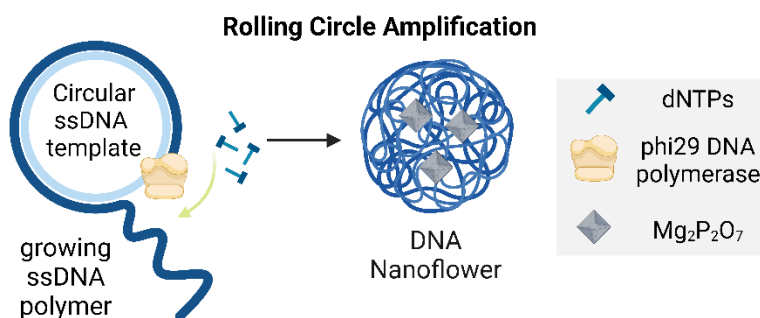


Fig. 1.4. Rolling circle amplification process in which the enzyme phi29 DNA polymerase uses a circular template to synthesize a ssDNA polymer. The polymer co-crystallize with magnesium pyrophosphate to form hybrid nanoflowers. Created with BioRender.com

cells for biosensing of disease biomarkers²¹⁷⁻²²⁰, control over gene transcription²²¹⁻²²³ and drug delivery^{205,220,224}.

In the latter, approaches used included incorporating aptamers that bind to specific targets on the cell surface, followed by the release of loaded small molecule drug cargo²²⁵⁻²²⁸. Another strategy is to trigger drug release in response to a specific endogenous or exogenous stimulus¹⁸⁹. Since DNA circuits enable kinetic control over drug release, they exhibit the potential to improve the efficacy and safety of therapeutics. Nevertheless, the application of DNA circuits to control drug release kinetics is still in its infancy. More research is needed to design DNA nanodevices with optimized structure and function to deliver different drugs to living tissues. Furthermore, the application of DNA circuits in drug delivery is focused on using the DNA sequences as information processing units while delivering small molecule drugs as cargo^{220,229}. Little effort has been dedicated to deliver nucleic acid-based sequences, that are part of the DNA circuit, as drugs themselves in living cells for therapeutic purposes.

Some DNA circuits are use-once circuits while others are renewable circuits that can revert to their original state¹⁹¹ either with external intervention or autonomously without such interference. When DNA circuits are programmed to exhibit renewable cycles that operate far- and out-of-equilibrium, they are termed DNA dissipative systems. Several examples of dissipative systems in nature where living activities are maintained by out-of-equilibrium processes with the consumption of certain types of energy can be found. For instance, the GTP-fueled assembly of tubulin into microtubules^{231,232} that play important roles in intracellular transport²³³, form mitotic spindles crucial for mitosis and meiosis²³⁴ and maintain cellular shape and structure as part of the cytoskeleton²³⁵ (Fig. 1.5). Another example is the ATP-driven polymerization of actin filaments^{236,237} that participates in muscle contraction²³⁸, cell division and cytokinesis²³⁵, cell motility²³⁹, movement of organelles and vesicles²⁴⁰, cell signaling²⁴¹ and cell shape maintenance²³⁵.

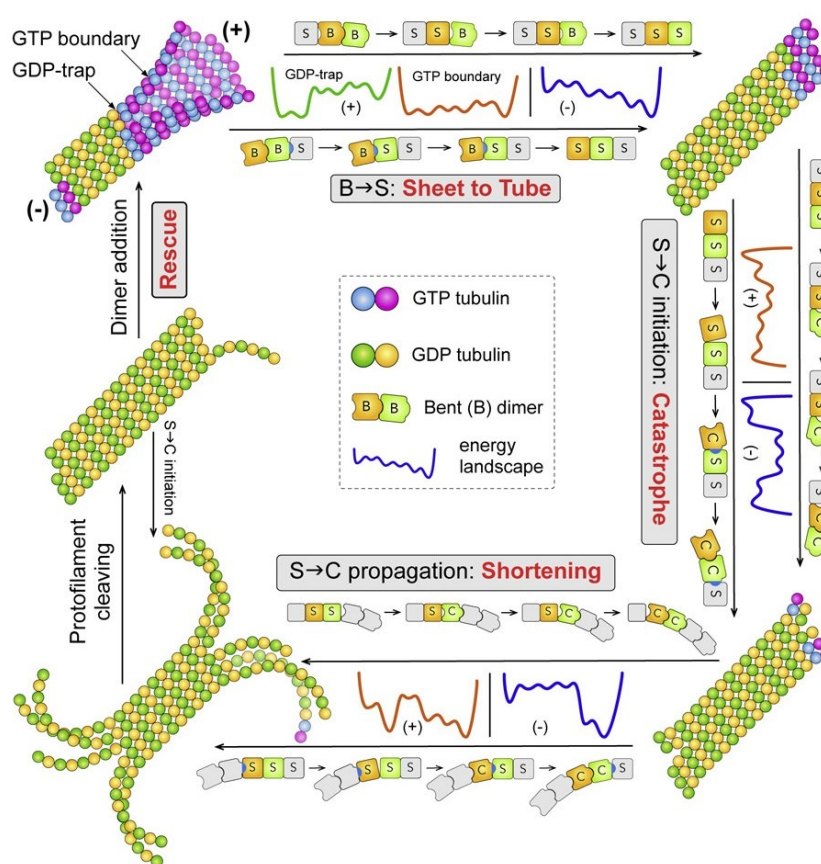


Fig. 1.5. An example of dissipative systems that exist in nature. Microtubules are the backbone of the cytoskeleton and vital to numerous cellular processes. All functions of microtubules are driven by dynamic instability, which is driven by non-equilibrium transitions between the bent (B), straight (S), and curved (C) forms of tubulin monomers and longitudinal interfaces in the two-dimensional lattice of microtubule. The resulting kinetic pathways for these transitions and their corresponding energy landscapes control the different phenomena of microtubules (Reprinted from²³⁰, Copyright (2020), with permission from Elsevier).

The emerging field of dissipative DNA nanotechnology is inspired by such living processes and aims at the development of life-like artificial systems utilizing energy dissipative reactions to drive the assembly/disassembly of programmable DNA-based structures²⁴²⁻²⁴⁴. Kinetic rather than thermodynamic control facilitates the maintenance of the non-equilibrium state²⁴². Researchers have designed DNA-based dissipative circuits^{221,245-247}, walkers^{206,248-250}, switches²⁵¹, nanostructures²⁵²⁻²⁵⁵, polymer materials²⁵⁶⁻²⁵⁹ and molecular devices that can load and release molecular cargoes driven by DNA fuel²⁶⁰⁻²⁶². However, examples demonstrating that DNA-based dissipative systems can interact with living cells to trigger a biological response are elusive. The use of dissipative systems in the context of drug delivery offers autonomous and temporal control over the activity of the therapeutic moiety and allows manipulation of their PK profiles to achieve control over the onset, duration and intensity of drug's action.

1.1.2 TARGETED DRUG DELIVERY SYSTEMS

1.1.2.1 Key challenges addressed by targeted drug delivery approaches

Drug delivery represents a continuously evolving domain within pharmaceutical sciences, encompassing different generations of DDSs. Among these, targeted DDSs are designed with the objective of delivering drugs to a specific site within the body, thereby circumventing systemic dissemination. The overall goal is to maximize therapeutic efficacy while minimizing undesirable side effects. The origins of targeted DDSs can be tracked back a century ago to Paul Ehrlich's visionary concept of a 'magic bullet'²⁶³ – a drug that directly and selectively targets a pathogen without harming host tissues. Achieving this goal necessitates the identification of disease-specific targets, the development of potent therapeutic agents and the creation of stable delivery system to transport the drug to its intended site of action with high precision. Despite significant progress, the realization of a true 'magic bullet drug' remains a formidable challenge²⁶⁴.

The demand for targeted DDSs arose from the limitations of conventional drug delivery methods, which often exhibited unsatisfactory PK, PD, pharmaceutical and pharmacotherapeutic properties²⁶⁵. An ideal drug-targeting complex should possess several attributes, including non-toxicity, non-immunogenicity, biocompatibility, biodegradability, physicochemical stability, reproducibility, cost-effectiveness, ease of elimination from the body, minimal drug leakage during transit and a predictable and controllable drug release profile²⁶⁶. Promising applications of targeted DDSs have been demonstrated in diverse fields, including cancer therapy^{267,268}, vaccination²⁶⁹, ocular^{270,271}, brain²⁷²⁻²⁷⁴ and transdermal delivery²⁷⁵, as well as the delivery of oligonucleotides^{276,277} and DNA. These applications have shown significant advantages, such as reduced toxicity, enhanced uptake, prolonged circulation time and improved bioavailability. Despite these advancements, continuous research efforts are required to refine these systems and address the intricate challenges associated with achieving the ultimate goal of precise and effective targeted drug delivery.

1.1.2.2 Active and passive targeting strategies

There are various ways to categorize targeted DDSs. One approach is to classify them to either active or passive targeting DDSs (Fig. 1.6). In passive targeting, the body's natural responses to certain physicochemical properties of the drug or its carrier complex are harnessed to lead to drug accumulation at the intended site of action²⁷⁸. This process is significantly aided by the enhanced permeability and retention (EPR) effect, in tumor tissue in particular¹²⁵. In this context, passive drug targeting takes advantage of the leaky blood vessels and poor lymphatic drainage in tumors, allowing nanoparticles to enter the tumor microenvironment more

effectively and thus improving the localization and concentration of anticancer agents within the cancerous tissue²⁷⁹. Utilizing the EPR effect has become a fundamental strategy in targeted drug delivery for cancer treatment²⁸⁰, enabling higher concentrations of cytotoxic drugs at the tumor site while reducing exposure to healthy tissues. Nevertheless, some studies show that although EPR effect has been confirmed in human tumors, its presence exhibits significant heterogeneity among individuals²⁸¹. Hence, despite their use to enhance therapeutic efficacy, EPR-dependent strategies still have unsatisfactory clinical outcomes²⁸¹. The limited impact on the clinical therapeutic outcome may promote a re-evaluation of the validity of the EPR effect in treating tumors in medical practice.

On the other hand, active targeting strategies employ a specific ligand-receptor interaction at the interface between the target cells and the drug-carrier complex²⁸². Ligands found on the drug-carrier complex vary between small molecules, nucleic acids, proteins, peptides and polysaccharides²⁸³. Such strategies are predicted to result in better uptake of targeted drug-carrier complex than passive targeting mechanisms. Within the active targeting, further different orders of targeting can be broadly defined, namely first, second and third²⁸⁴ targeting orders. These are assigned to actively targeting organs/tissues, cells and organelles, respectively. A fourth level of active targeting is assigned to the molecular level.

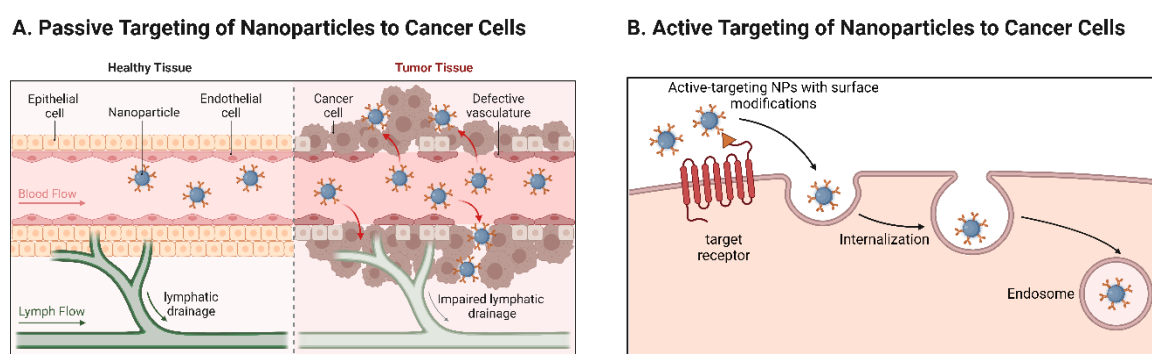


Fig. 1.6. Comparison between passive and active targeting strategies. **A.** Passive targeting of cancer cells depends on the enhanced permeability and retention effect due to compromised vascular structure and lymphatic drainage. **B.** Active targeting of cancer cells utilizing nanoparticles that have surface modifications with targeting ligands that can interact with specific target receptors on the surface of the cancer cell.

An innovative approach towards targeted DDSs involves the utilization of ‘smart’ responsive DDSs²⁸⁵. These systems are designed to encapsulate drugs leading to their inactivation, and then in response to a specific stimulus, on-demand drug release with high spatiotemporal precision can be achieved. Such stimuli can be either physical or chemical and are categorized as internal or external depending on their origin²⁸⁶. Internal stimuli include changes in pH²⁸⁷ or temperature²⁸⁸, specific enzymatic activities²⁸⁹ and changes in redox conditions²⁹⁰. External stimuli involve light²⁹¹, temperature, magnetic field²⁹² and ultrasound^{293,294}. The upcoming section will delve deeper into the discussion of external triggers employed for responsive controlled DDSs.

1.1.2.3 External triggers for controlled drug release.

The strategic use of external triggers to control DDSs offers precise and on-demand release of therapeutic drugs. Researchers have developed sophisticated DDSs that respond to various external triggers, such as temperature, light, magnetic field and ultrasound (Fig. 1.7). These smart DDSs represent promising approaches in the realm of controlled drug delivery due to

their ability to provide spatiotemporal control over drug release therefore optimizing therapeutic effects and minimizing unwanted side effects.

Thermo-responsive DDSs have been extensively investigated over the last decades, capitalizing on temperature-induced changes to achieve controlled drug release. The development of thermosensitive polymers has notably revolutionized the design of these DDSs²⁹⁵. These polymers, when integrated into carriers, enable them to maintain drug payloads at body temperature (37 °C) and release the cargo upon localized heating (approximately at 39-43 °C). Localized hyperthermia can be induced by several external triggers, such as radiofrequency thermal ablation, microwave hyperthermia or high-intensity focused ultrasound (HIFU). This temperature-triggered phase transition involves a reversible shift from a molecularly dissolved hydrated state to a dehydrated hydrophobic state²⁹⁶. Such sharp transition from a globular to coil-like structure prompts the release of therapeutics from the polymer-based carrier. Depending on their critical solution temperature (CST), thermoresponsive carriers can be categorized into two groups²⁹⁷: polymers with lower CST (LCST) that shift from solvated to collapsed state upon heating to release their cargo, and polymers with upper CST (UCST) that undergo coil-to-globule transition upon cooling. Such DDSs have been successfully used to deliver diverse drugs such as anticancer drugs, antibiotics and insulin²⁹⁷. In the context of DNA nanotechnology, gold nanoparticles were combined with DNA hydrogels to transfer the heat generated by photons to the adjacent DNA material, disrupting the complementary binding of the strands and leading to controlled drug release²⁹⁸. DNA origamis that can be designed to respond to temperature change were investigated. Assembly and disassembly of the origami structure could be beneficial for drug delivery applications²⁹⁹

The use of light as an external trigger to control DDSs have gained wide interest due to the high spatiotemporal resolution of light and the ability to fine-tune the system through adjusting various parameters such as wavelength, polarity, irradiation intensity and exposure duration³⁰⁰.

Photoresponsive nanocarriers undergo specific chemical or physical changes in response to either ultraviolet (UV) light (200-400 nm), visible light (400-700 nm) or NIR light (700-1000 nm)³⁰¹. UV light can

trigger different light-responsive mechanisms in nanomaterials, including photocleavage, photoisomerization and photocross-linking/-decross-linking³⁰². In most examples, Rhodamine B and Nile red (NR) dyes were frequently used as hydrophilic and hydrophobic drug models, respectively, to showcase the potential of these photoresponsive systems in the realm of drug delivery²⁹¹. Nevertheless, researchers could demonstrate *in vitro* UV-triggered drug delivery of some drugs such as nintedanib³⁰³, doxorubicin^{249,304}, paclitaxel³⁰⁵, 5-fluorouracil³⁰⁶, quercetin³⁰⁷ and various photosensitizers for photodynamic therapy (PDT)^{308,309}.

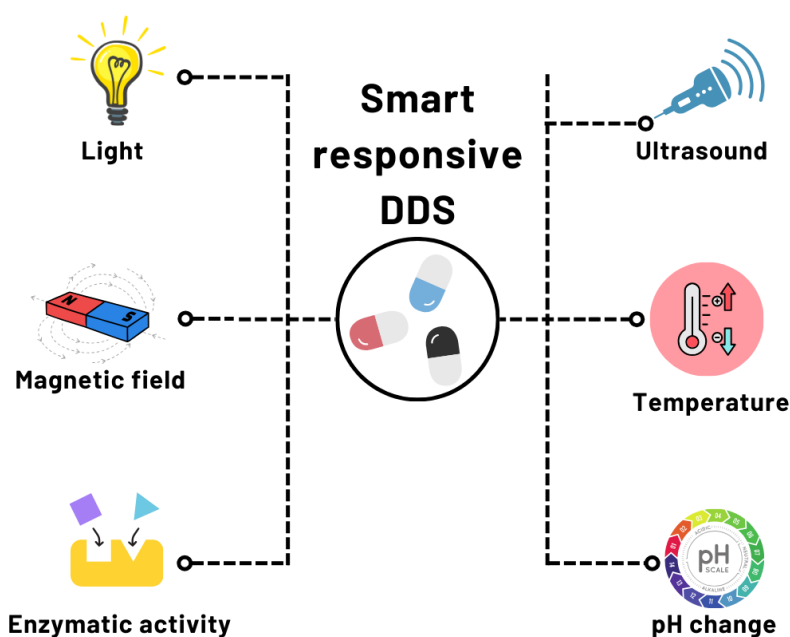


Fig. 1.7. Smart drug delivery systems can be designed to respond to different internal and external triggers.

For DNA-based nanosystems, different wavelengths can be used to modulate oligonucleotide hybridization. UV light is commonly used for photodegradation and photocleavage as it delivers high energy and can induce DNA damage. For instance, a protein-coated DNA origami was designed to respond to UV light to cleave a modified AB fragment and releasing it. On the other hand, photoisomerization utilizing molecular photoswitches is another approach in which azo-benzene, spiropyran, stilbene and diaryl ethylene can convert between different forms leading to a desired outcome. Azobenzene-functionalized DNA strands were assembled on up-conversion nanoparticles (UCNP). Photoisomerization of the azide triggers cyclic DNA hybridization and leads to controlled doxorubicin release³¹⁰.

Although UV light is the most commonly used source of irradiation in light-responsive DDSs design, it has several limitations such as poor penetration depth and high phototoxicity that result in poor clinical translation. On the other hand, visible and NIR light have better safety profiles and deeper penetration depth, but are more difficult to use due to their lower energy that is often insufficient to trigger a photo-reaction³¹¹. To circumvent this limitation, researchers have employed either two-photon absorption (TPA)³¹² and NIR-to-UV upconversion processes,³¹³ the latter converting lower-energy NIR photons to higher-energy UV/Vis photons locally. These innovative nanomaterials show potential as safer options for light-controlled DDSs for application in deeply sited diseases. The utility of such systems has been shown in cancer treatment by the delivery of doxorubicin³¹⁴⁻³¹⁹, camptothecin³²⁰⁻³²² and cis-platin³²³, mostly *in vitro* with few *in vivo*³²³⁻³²⁶ studies. Despite the progress that has been made in this rapidly evolving field, many challenges need to be properly addressed, such as non-specific photoreactions in normal cells as well as the safety and biodegradability of most nanocomposites and photoresponsive compounds.

Magnetic fields exhibit excellent penetration capabilities within intact organisms and are commonly employed in magnetic resonance imaging (MRI) for diagnostic purposes. Beyond imaging applications, magnetic-responsive DDSs have been developed to achieve controlled drug release from carriers through incorporating magnetic nanoparticles²⁹². This technique finds significant utility in cancer therapy³²⁷, where magnetic targeting enhances drug accumulation in tumors³²⁸, thereby improving therapeutic efficacy while minimizing systemic exposure and associated side effects. Particularly noteworthy are superparamagnetic iron oxide (Fe₃O₄) nanoparticles (SPIONs), which were extensively studied for drug delivery, diagnostic applications and their role as contrast agents in MRI³²⁹. Upon the application of an external magnetic field, these magnetic nanoparticles accumulate precisely at the target site. Coating of such nanoparticles with polymers can extend their circulation time and allow loading of drugs as well³³⁰. Magnetic-responsive DDSs have demonstrated successful delivery of various drugs, including doxorubicin³³¹, rifampicin and adriamycin³³². Notably, some systems are being investigated in clinical trials³³³⁻³³⁷, underscoring their potential for real-world therapeutic applications.

Ultrasound (US) serves as a valuable external trigger for controlled DDSs³³⁸. Transducers are utilized to convert electrical signal to mechanical vibrations that propagate through a medium as US waves. The frequency range of ultrasound spans from 20 kilohertz (kHz) to several gigahertz (GHz). In biomedical applications, frequencies typically range from 1 to 10 megahertz (MHz), as these frequencies have exhibited a safe profile in humans and promote rapid energy exchange at the molecular level³³⁹. US offers distinctive advantages over other external stimuli. It is non-invasive, allows deep tissue penetration and provides precise spatiotemporal control over drug delivery³⁴⁰. The upcoming chapter will delve into the detailed mechanism and application of US in the context of DDSs, representing our trigger of choice in the presented research of this thesis.

1.2 ULTRASOUND-MEDIATED CONTROL OF BIOLOGICAL SYSTEMS

1.2.1 ULTRASOUND IN MEDICINE

US is a widely employed imaging and diagnostic tool that has revolutionized the field of medicine over the past few decades and continues to shape modern healthcare practices. US utilizes high-frequency sound waves beyond the range of human hearing and offers a non-invasive and real-time imaging of internal structures of intact organisms. It provides insights into anatomical details, blood flow and tissue characteristics.

All acoustic energy with a frequency above human hearing (20 kHz) is termed US. However, typical diagnostic sonographic scanners used in medical practice operate in the frequency of 2 to 18 MHz, hundreds of times higher than the limit of human hearing. These higher frequencies are associated with shorter wavelengths; therefore, they allow imaging of smaller details.

Diagnostic sonography, also known as ultrasonography, is an US-based diagnostic imaging technique used to visualize different body structures such as tendons, joints, muscles, vessels and internal organs. Typically, a hand-held probe, called a transducer, is placed directly on and moved over the patient, in combination with a water-based gel to couple US between the transducer and the patient. In addition to the transducer, US devices are consisted essentially of a transmitter pulse generator, compensating amplifiers, a control unit for focusing, digital processors and systems for image display.

The main technology used in US diagnostics is the reflection technology (echo) that registers the reflected pulse from the boundary of two structures differing in their acoustic resistance, which is essentially based on principle of a functioning sonar. The sound wave is produced by a piezoelectric transducer encased in a probe, that receives strong, short electrical pulses from the US pulse generator and ring at the desired frequency accordingly. US waves can be focused either by the shape of the transducer, a lens in front of it or a complex set of control pulses from the US pulse generator. The arc-shaped waves travel into the body and come into focus at the designated depth.

There are four different US modes used in medical imaging. These are A-mode, B-mode, M-mode and Doppler mode. A-mode is the simplest among them, in which a single transducer scans a line through the object with the echoes plotted on the screen as a function of depth. Therapeutic FUS that aims to destroy specific tumor or calculus operates in A-mode, which allows for pinpoint accurate focus of the destructive wave energy. In B-mode US, a linear array of transducers scans a plane simultaneously, and the result can be viewed as a 2D image on a screen. The M in M-mode stands for motion, in which a rapid sequence of B-mode scans take place and their images are sequentially displayed on a screen. This allows physicians to visualize and measure range of motion relative to the probe. Finally, the Doppler mode makes use of the Doppler effect in visualizing blood flow. It can assess if blood is moving towards or away from the probe and its relative velocity, which is of particular benefit in cardiovascular medicine. The measured Doppler shift falls in the audible range and can be presented audibly using stereo speakers as a synthetic pulsing sound.

US has found various uses across different medical disciplines. One of the earliest and most frequently utilized application is in obstetrics and gynecology for monitoring fetal development and detecting abnormalities during pregnancy. In cardiology, echocardiography is used to assess heart function and diagnose different vascular disorders. Abdominal US is commonly used to image internal organs such as the liver, kidneys and pancreas. Musculoskeletal US is utilized for diagnosis and guiding treatment of soft tissue injuries and joint-related medical

issues. Furthermore, US plays an important role in guiding minimally invasive medical procedures such as biopsies and injections. In emergency medicine, US is critical for assessing trauma and guiding interventions safely and quickly.

Besides diagnostics, US is used in therapeutic applications. For instance, FUS-guided surgery is a therapeutic approach that utilizes the precision of FUS for non-invasive surgical procedures. The convergence of multiple US beams at a focal point generates high heat sufficient to induce thermal ablation at the targeted tissues. This approach offers several advantages when compared to traditional surgical procedures such as reduced infection risk, faster recovery and less damage to healthy tissue. It has been medically used for different applications including cancer cell killing^{341,342}, uterine fibroid removal³⁴³, neurosurgery^{344,345} and kidney stone fragmentation³⁴⁶.

Finally, the US-FDA defines maximum allowable US parameters³⁴⁷ based on the mechanical index (MI) and two intensity metrics: the spatial-peak pulse-average intensity (I_{SPPA}) and the spatial-peak temporal-average intensity (I_{SPTA}). MI is calculated as $p \cdot f^{-1/2}$, where p is the peak negative pressure in megapascal (MPa) and f is the US frequency in MHz. I_{SPPA} is defined as the time-average intensity over the duration of a given pulse, while I_{SPTA} is the time-average over a longer time frame, which can be applied to continuous excitation as well. The maximum permissible intensities are defined as $I_{SPTA} = 720 \text{ mW/cm}^2$ and $I_{SPPA} = 190 \text{ W/cm}^2$ or $MI = 1.9$. Higher values result in a temperature increase *via* absorption and thus can lead to cell death (above 43°C).

1.2.2 ULTRASOUND-INDUCED MECHANICAL EFFECTS

Generally, a transducer that converts electrical signals into vibrations is used to generate an acoustic wave. These vibrations are transferred into the medium and propagate as mechanical waves of compression and expansion. Such mechanical waves can interact with boundaries and objects present in the medium. Overall, through these interactions, acoustic waves can transport energy or information to a certain receiver (*e.g.*, a responsive material), which in turn can be converted into other forms of productive work. The mechanical motion of acoustic waves creates regions of compression (high density) and refraction (low density) that travel through the medium. Several factors play different roles in determining how effectively energy can be transferred by acoustic waves in a given medium. These include acoustic and geometrical properties of the medium. Commonly, acoustic impedance is used to describe the material's acoustic properties. Furthermore, the material's attenuation coefficient is an important parameter that describes the irreversible loss of acoustic energy into heat, which can occur *via* different mechanisms such as viscosity or molecular relaxation³⁴⁸.

One of the important goals when using US for smart systems is to utilize the acoustic waves for non-acoustic work; for instance, moving objects, starting chemical reactions and triggering biological processes. Towards that goal, several mechanisms are required to convert acoustic energy into more useful form of energy to get eventually the desired final outcome. Common mechanisms involve piezoelectricity, acoustic streaming, acoustic radiation forces (ARF) and cavitation.

Piezoelectricity is the ability of certain materials to generate an electric polarization in response to applied mechanical stress. Consequently, this can be used to produce motion and therefore acoustic waves. This phenomenon can be observed in crystals such as quartz, ceramics such as lead zirconate titanate and polymers such as polyvinylidene fluoride (PVDF).

Non-linear acoustic mechanisms, such as acoustic streaming and ARF, are used to directly convert US waves into steady forces on objects. The momentum transfer of energy from the acoustic waves to the fluid results in fluid flow driven by the acoustic waves, known as acoustic

streaming. Boundary layer streaming (also known as Schlichting streaming) is induced by the acoustic energy dissipation driven by the steep velocity gradient at the fluid boundary layer along vibrating structures³⁴⁹. A flow in the bulk fluid, known as Rayleigh streaming, occurs due to the shear of this boundary layer streaming³⁵⁰. Rayleigh streaming is strongest when an object is excited on resonance and causing maximal vibrations. The resonance frequencies of elastic materials depend on their size, geometry and mechanical properties³⁵¹. Rayleigh streaming can also occur when surface acoustic waves (SAW) propagate along a solid boundary, which has a higher frequency than resonant microstructures³⁵². In the presence of acoustic streaming, particles or structures in the flow will experience a drag force resulting from the viscosity of the fluid. At small length scales, viscous effects typically dominate the fluid flow. The shear stress from a fluid is proportional to its viscosity and the gradient of the velocity, which is also known as the strain rate. Microparticles in the flow will be carried along unless they are held in place by other forces. Soft structures and macromolecules can be deformed by the dragging forces and if the shear rate is high enough, they can be broken³⁵³.

The second non-linear mechanism to create steady forces from acoustic energy is the ARF, which can be exerted on surfaces, structures or microparticles upon exposure to US. For microparticles, the ARF depends on several factors related to properties of both the particles (*e.g.*, size and acoustic properties) and the acoustic field (*e.g.*, intensity and frequency)³⁵⁴. ARF on compressible particles, such as bubbles, are also called Bjerkens forces. However, these are generated *via* a different mechanism.

The final mechanism is cavitation, during which acoustic waves cause bubble formation, followed by their oscillation and potential collapse. Since bubbles respond strongly to US, a significant amount of energy can be transferred from US waves into bubbles' motion during cavitation, leading to both thermal and mechanical effects. During a bubble collapse, transient temperature can reach several thousands of kelvin and pressure is on the order of tens of megapascals (for less than 1 μ s). This leads to plasma formation and the production of radicals which emit electromagnetic radiation in a phenomenon known as sonoluminescence³⁵⁵.

Bubbles form when the rarefaction negative pressure of the acoustic wave is low enough to pull dissolved gases out of solution. This occurs more likely at lower frequencies, since the duration of strong negative pressure is longer in each cycle when compared to higher frequencies. In the case of readily-available nucleation sites in medium, such as micro- or nano-particles, or rough surfaces, then cavitation is easier to take place. MI is a parameter that indicates the strength of cavitation in aqueous systems³⁵⁶ and is commonly reported without units. When MI is below 0.1, microbubbles only scatter the US signal in a linear manner. Intermediate MI values between 0.1 to 0.4 indicate that bubbles' response becomes non-linear, and this regime is referred to as stable cavitation³⁵⁷. Slow bubble destruction by diffusion of gas can be induced in this case. For high MI values exceeding 0.4, microbubbles violently oscillate to the point of their collapse. This is known as inertial or unstable cavitation³⁵⁸, which can be accompanied by a shock wave^{355,359}. Microstreaming and bubble collapse exert shear forces on nearby structures. At high MI, collapsing bubbles near a surface can generate liquid microjets that can damage solid structures.

In addition to mechanical effects, cavitation can convert acoustic energy into a thermal one³⁵⁹. Depending on the bubbles size, US parameters and medium viscosity, three mechanisms may be involved in heat generation. First, is heating the surroundings due to non-linear acoustic radiation, which typically dominates in biomedical applications. Second is heating through viscous dissipation during bubble motion in the liquid. Third is heat conduction through the gas core during bubble compression.

Due to their mechanical properties and geometry, gas bubbles are unique acoustic objects that are heavily used in US technologies. Their response to acoustic fields can be tuned and

measured. Gas bubbles are highly compressible and behave like a spring upon exposure to US by storing energy in compression and expansion cycles, ultimately releasing it as kinetic energy to the surrounding fluid. Their response can be amplified by exciting them at their resonance frequency, which leads to large amplitude motion of the bubble and a stronger scattering response. Impressively, it was shown that a resonant bubble can reflect more than 100 times more energy than expected considering its size alone³³⁸. As a result, synthetic microbubbles have been employed as US contrast agents in medical diagnosis for decades³⁶⁰.

Nevertheless, microbubbles face several challenges related to their size and stability. This in turn limited their adoption in molecular imaging where the observation of dynamic cellular process is of interest³⁶¹. Therefore, a novel class of air-filled protein nanostructures was proposed in 2014, namely gas vesicles (GVs), to be used as contrast agents for biomolecular imaging³⁶² and have been employed in US imaging in oncology³⁶³ and neuroscience³⁶⁴. GV's have cylindrical or spindle-like shape, with lengths of 100 nm to 2 μ m and a 2-nm-thick amphiphilic protein shell. They are selectively permeable to gas and impermeable to water. GV's have a remarkable stability and pressure resistance, as they can withstand up to 1.3 MPa's before being disrupted³⁶⁵. GV's can be produced in living cells by the expression of acoustic reporter genes (ARGs), which have been developed as acoustic analogues to optical reporter genes. The contrast detected signal of GV's can be correlated to ARGs' expression³⁶⁶. The genetic engineering of various GV's variants enabled the modulation and tuning of their US response, thereby allowing multiplex imaging. Furthermore, ARGs were expressed in mammalian cells for the first time in 2019, which allowed high-resolution imaging of gene expression in living organisms³⁶⁷. More recently, a genomic mining approach was utilized to develop a second-generation ARGs for both eukaryotic and prokaryotic cells³⁶⁸. These have enhanced properties compared to their first-generation counterparts, such as stronger US contrast, the ability to generate distinguishable non-linear signals even in the presence of background tissue, as well as the maintenance of long-term stable expression. These novel ARGs enabled non-invasive *in situ* imaging of tumor colonization and tracking of gene expression and tumor growth in breast cancer mouse model *in vivo*³⁶⁸.

Beyond its established clinical use, US and its associated mechanical effects have been exploited in different scientific fields. One particular field of interest is polymer mechanochemistry, where mechanical forces obtained mostly employing US are utilized to induce molecular-level changes in polymers. The subsequent section delves into this emerging field and its applications.

1.2.3 POLYMER MECHANOCHEMISTRY: PRINCIPLES AND APPLICATIONS

Polymer mechanochemistry is an evolving field at the intersection of materials science and chemistry. It focuses on the study and manipulation of polymers *via* mechanical forces, relying mostly on ultrasonication techniques³⁶⁹. The unique aspect of polymer mechanochemistry lies in the ability to induce chemical transformations based on mechanical force exclusively. This opens avenues for investigating environmentally friendly and energy-efficient methods with more emphasis on sustainable materials and green chemistry.

When polymers were widely introduced as commodity materials, it was necessary to investigate their behaviour under mechanical forces. In 1934, Staudinger and Heuer demonstrated that the degradation of polystyrene and natural rubber decreased their viscosity and molar mass. They attributed this observation to mechanochemical depolymerization of the polymer backbone³⁷⁰. This hypothesis was confirmed by Kauzmann and Eyring in 1940, where they offered a kinetic description of the mechanochemical bond scission in polymers³⁷¹. Macroradicals were also

produced during this process and could be further utilized for secondary polymerization reactions^{372,373}.

Recently, a rapid growth of polymer mechanochemistry can be witnessed, where designer polymers have been fabricated with force-sensitive functional molecular structures named mechanophores^{374,375}. These mechanophores can be manipulated by mechanical forces to induce specific molecular transformations leading to a certain desired function. In early work, random peroxide bonds were inserted along a polymer backbone, leading to more rapid degradation after ultrasonication³⁷⁶ compared to the original polymer. True mechanochemical selectivity was observed by inserting a single azo bond in the center of a PEG polymer³⁷⁷. Ultrasonication led to faster degradation rate and precise cleavage almost perfectly in the middle of the polymer, indicating that bond scission occurred at the azo linkage. This set the stage for modern polymer mechanochemistry, in which studies aim to harness the mechanical energy of US for productive bond scissions using mechanophores embedded in polymer chains (Fig. 1.8a).

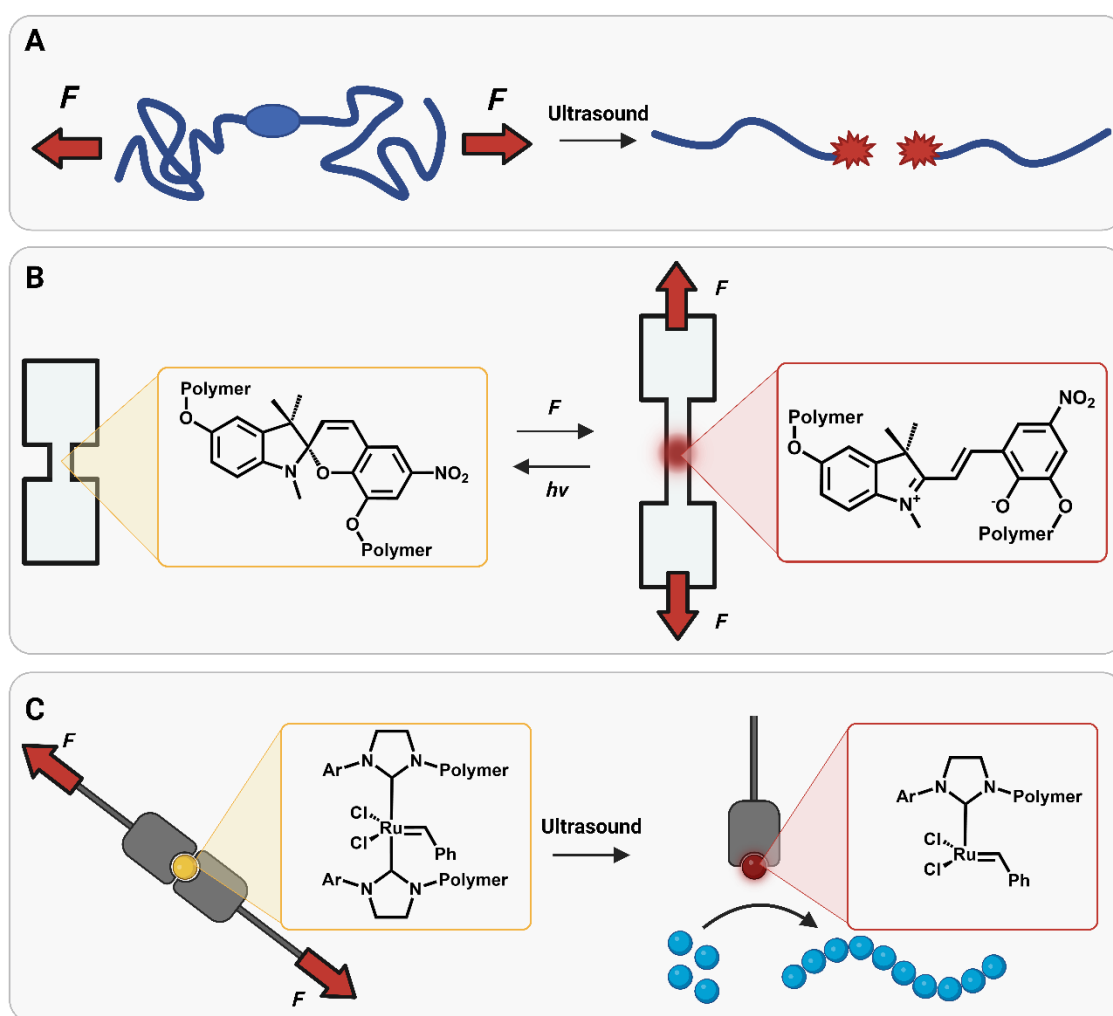


Fig. 1.8. Principles and examples from polymer mechanochemistry. **A.** The application of force to a polymer backbone results in its stretching and its ultimate scission, preferentially in the middle. **B.** Schematic diagram of 'dog bone' synthetic polymer specimens with spiropyran as a mechanophore. Upon application of tensile force, the colourless spiropyran converts to coloured merocyanine, while the conversion is reversed upon the exposure to visible light, figure recreated from reference³⁷⁸. **C.** Ultrasonication-induced mechanochemical scission of a metal complex with polymeric ligands, leading to the activation of a catalyst in which the metal center catalyzes polymerization figure recreated from reference³⁷⁹. Created with BioRender.com

Force-induced chemical responses go beyond bond scission to other novel mechanochemical reactions. For instance, a benzo cyclobutene mechanophore was inserted in PEG polymer and subjected to US, leading to electrocyclic ring opening and the formation of orthoquinodimethide, a formally disallowed ring opening according to the well-established Woodward-Hoffman rules³⁸⁰. This opened the door for the exploration of unprecedented reactivity in polymer materials with exciting potential applications.

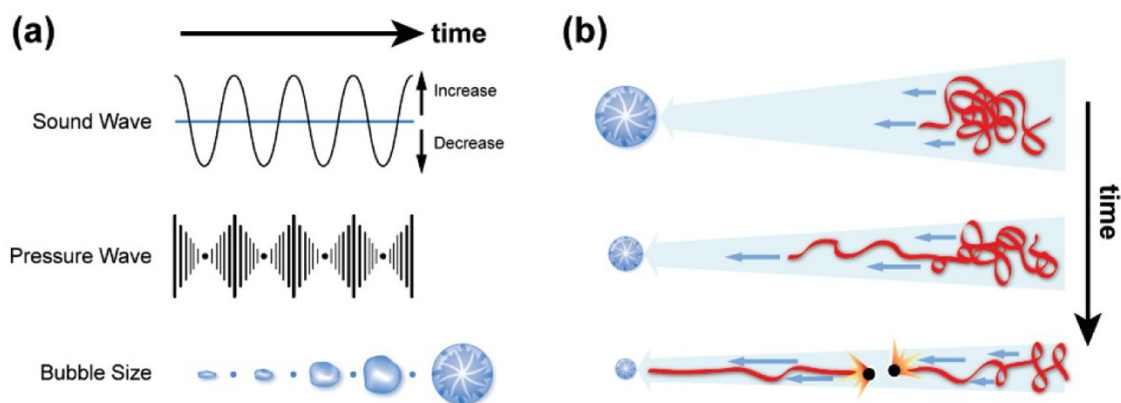


Fig. 1.9. Schematic presentation of the mechanism underlying US-induced polymer chain scission. **A.** Pressure variations induced by the acoustic field lead to the gradual formation of microbubbles. **B.** The rapid collapse of microbubbles generates solvodynamic shear forces that unfold and stretch the polymer (Reprinted (adapted) with permission from³⁸¹, Copyright (2009) American Chemical Society).

US has been used as a tool to study polymer degradation as early as 1933³⁸². The application of US to solutions *via* ultrasonic probes that are submerged directly into the sample became the method of choice for screening mechanophores' reactivity. A cooling mechanism is needed due to heat generation during ultrasonication. Cavitation is considered to be one of the main mechanisms of converting US waves to energy that exerts force on a polymer chain (Fig. 1.9). Cavitation involves the nucleation, growth and collapse of bubbles in a liquid³⁸³⁻³⁸⁵. Implosion of bubbles create voids that pull proximal chain ends *via* solvodynamic shear forces, leading to bond cleavage within 15% of the center of the polymer chain³⁸⁵. Further cleavage and reduction of molecular weight is possible, until a lower molecular weight threshold (M_{lim}) is reached, below which no further scission is observed^{386,387}. The scission rate and M_{lim} depend on effective strain rate that is determined by the dynamics of microbubble collapse. Other parameters that affect the rate of ultrasonic induced chain scission include US power intensity, polymer's concentration and molecular weight, solvent's vapor pressure and gas solubility, temperature and viscosity. Increase in either US power intensity or polymer's molecular weight lead to an increased scission rate, while the other parameters have negative influence³⁸⁸ on the scission rate.

Applications of polymer mechanochemistry

Polymer mechanochemistry has found different applications. For instance, optical force probes (OFPs) can be obtained by utilizing chromogenic mechanophores that undergo a molecular transformation accompanied by alteration of its absorption and/or emission spectra³⁸⁰. This can be applied to study real-time *in situ* mechanical failure mechanisms in different polymer architectures, with the aim of designing materials with better properties (Fig. 1.8b). The OFP type and its positioning within a material determine the accessible mechanical information in terms of spectral properties, force range and spatiotemporal resolution in different states (*i.e.*, in solution, bulk and at interfaces). Therefore, OFPs must be tailored to the specific demand of

the material they are incorporated within, to obtain data that is meaningful and representative. Qualitative visualization of force-induced reactions in polymers was first obtained by early examples of OFPs such as spiropyran^{380,389}. More recently, quantitative measurements of mechanical changes in soft matter is more widely investigated³⁹⁰⁻³⁹², in combination with high-resolution microscopy methods such as confocal laser scanning microscopy^{393,394}.

Another application is the incorporation of transition metals in the central region of linear polymers, which resulted in catalysts that can be activated by mechanical forces (Fig. 1.8c). Moreover, polymer mechanochemistry facilitates reactions that are otherwise unattainable under standard and traditional reaction pathways³⁹⁵. In the area of sustainable polymer chemistry, chemists usually perform research in three directions: to prolong the material's life cycle, to achieve sustainable synthesis of polymer and to degrade and/or recycle polymers efficiently. Mechanical forces can be potentially used in this context, especially in structural materials. Self-healing is one approach to prolong the polymers' lifecycle³⁹⁶. In 2013, it was demonstrated that bond scission of gem-dibromo cyclopropane mechanophores could be utilized to cross-link the activated chains and subsequently increasing the material's strength³⁹⁷. This demonstrated a unique property of polymer mechanochemistry in which the stimulus that causes material failure is utilized to repair or reinforce the same material on the molecular level.

Furthermore, ball milling processes, which are widely employed in industry, are investigated for sustainable polymer synthesis³⁷³, post-synthetic modifications³⁹⁸ and molar mass distribution tuning³⁹⁹. The same process of ball milling is also investigated for controlled mechanochemical degradation of polymers⁴⁰⁰, while US was used to mechanochemically unzip a polymer to its monomers⁴⁰¹. Additionally, polymer degradability can be gated by two stimuli to confer further control over the material's lifecycle. For instance, a hydrolytically stable polymer with a cyclic ether and a cyclobutene mechanophore motif was designed. Mechanochemical stimulation would induce the ring-opening of the cyclobutene, leading to poly (enol ether) derivatives. These in turn can be degraded by acids as a second stimulus⁴⁰². Using serial stimuli to logically gate the programmed degradability is a promising avenue, but it requires careful design and consideration of stimuli that are orthogonal to the environmental conditions.

1.2.4 BIOMEDICAL APPLICATIONS OF ULTRASOUND-RESPONSIVE SYSTEMS

1.2.4.1 Opening biological barriers by US

US has been used to open different types of biological membranes and barriers. Namely, the cell membrane, the endothelial barrier, the blood brain barrier (BBB) and tumor-specific barriers, which will be discussed briefly in this subsection.

Sonoporation, the opening of the cell membrane by US (Fig. 1.10), can be attributed to cavitation and streaming in the vicinity of the membrane⁴⁰³. Large shear stresses deform the cell's membrane and form pores^{404,405} that lead to passive diffusion of molecules across the membrane. Sonoporation could be temporary or permanent, and the pore size depends on US pressure. Acoustic pressures less than 100 kPa were connected to stable cavitation and a subsequent increase in cellular endocytosis, but without opening pores in the cell membrane⁴⁰⁶⁻⁴¹¹. Nevertheless, if microbubbles are in direct contact with the cell, pore formation was demonstrated due to continuous pushing and pulling of the membrane which creates cyclic tangential stress^{403,412,413}.

Acoustic pressures in the range of 190 to 480 kPa could create pores in the size of 1 nm to 4.3 μm . Most studies demonstrated that inertial cavitation is mainly responsible for such pore formation. Self-healing of the cellular membrane was observed in cells when pressure

amplitude was below 250 kPa⁴¹⁴. Presence of microbubbles in the systems also enhanced the membrane permeability⁴¹⁵. Furthermore, the cellular uptake of different drugs that belong to various classes was shown to be enhanced by sonoporation *in vitro*⁴¹⁶⁻⁴¹⁹.

Additionally, the pore size determines if cells are able to reseal their membranes after sonoporation, which is critical to cell's viability as cells would die if the pores cannot be sealed and sonoporation is permeant. It was observed that pore resealing within 2 min allowed for cell recovery, while longer resealing time resulted in cell death^{413,420-422}. The opened pores promote intracellular calcium influx which determines the biological outcome⁴⁰³. Slight increase in calcium concentration stimulates cell membrane endocytosis to rapidly seal the pore^{403,408,410,423-425}, while intermediate increase in intracellular Ca^{2+} levels leads to exocytosis rather than endocytosis, in an attempt to reseal the pores *via* patch formation^{403,413,423,426}. When pores are too large, intracellular Ca^{2+} concentration could exceed the cell death threshold^{403,427}, and the degree to which this threshold is exceeded determines if cell death would be necrotic or apoptotic^{428,429}. Generally, the induction of cell death is undesirable in most of drug delivery applications. Exceptions in which cell death is of benefit include cancer therapy. Therefore, it is crucial to maintain high cell viability and recovery after sonoporation. Finally, sonoporation-induced Ca^{2+} influx was found to have an impact on adjacent cells^{407,430-432}, by transfer of calcium waves *via* gap junctions^{431,433}.

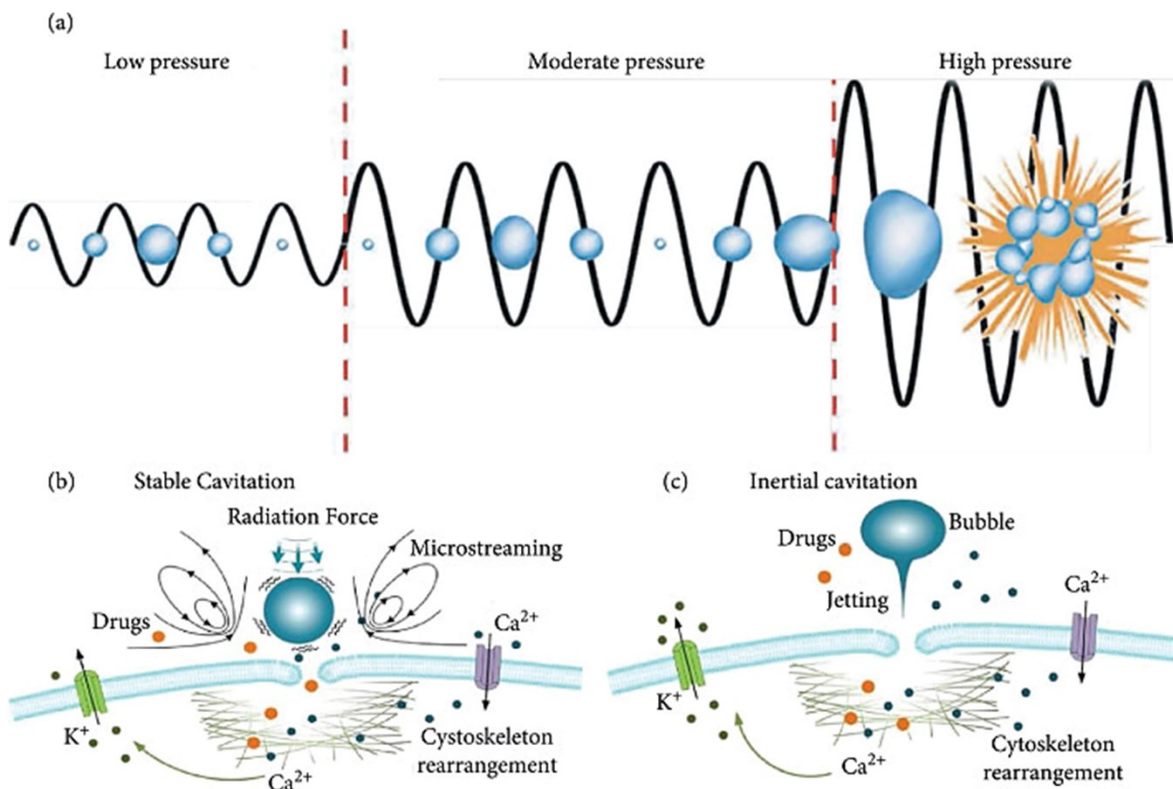


Fig. 1.10. Schematic overview of the main biophysical effects occurring during sonoporation generated by microbubbles. A. Low acoustic pressure triggers stable cavitation of microbubbles that lead to symmetrical linear oscillations. B. Microbubbles undergoing stable cavitation generate volumetric oscillations to exert radiation force and microstreaming-induced shear forces. C. Microbubbles undergoing inertial cavitation produce microjets. These different physical forces can result in the permeabilization of the cell membrane, rearrangement of the cytoskeleton and transmembrane ion transport such as K^+ efflux and Ca^{2+} influx (adapted from Ref. ⁴³⁴, CC BY 4.0).

Moreover, the cellular cytoskeleton can be affected by sonoporation, since pore formation can actively disrupt actin and tubulin networks⁴³⁵⁻⁴⁴⁰, which was shown to be reversible in most of the cells⁴⁴¹. It is noteworthy that most studies investigating sonoporation evaluate cell viability is over 12 to 24 h after sonoporation, in terms of viability or proliferation assays⁴³². However, general safety of sonoporation cannot be estimated by such short-term assays alone. Potential effects of sonoporation on the genome, cytoskeleton long-term function, cell turnover time and upregulation of cellular stress-associated molecules are not commonly investigated^{442,443}.

Sonopermeation is defined as the breaking of tight junctions between endothelial cells⁴⁴⁴⁻⁴⁴⁸. This increases the endothelial layer permeability and allows drugs to cross this barrier. Such opening can extend up to 10 min after US exposure^{444,449,450}. This phenomenon is related to sonoporation and the resulting intracellular Ca^{2+} influx and cytoskeleton disruption as described earlier⁴³⁷. However, we have limited knowledge about the effects of chemical components on cell membrane and tight junction poration till now. Moreover, it was demonstrated that the blood vessel diameter affects the behaviour of microbubbles significantly. Microbubbles can impact the endothelial cell layer more violently in smaller vessels with diameter ranging from 10 to 20 μm , as they are more confined and closer to the vessel wall⁴⁵¹⁻⁴⁵⁷. Furthermore, the lack of smooth muscles in small capillaries render them less resistant to mechanical forces^{458,459}. Caution should be taken when US and microbubbles are utilized to increase vascular permeability. More dangerous biological effects could occur, such as vascular ruptures, haemorrhages, vascular occlusion and thrombosis^{407,460,461}.

The brain is considered to be one of the most difficult regions to be accessed in the human body. It is protected from toxic substances by a special endothelium barrier, named the BBB, that prevents non-selective crossing of soluble molecules from the general blood circulation to the central nervous system (CNS)^{421,462-464}. To overcome this barrier, drugs have been designed to bind to specific transporter carriers to allow their transcytosis towards the brain^{462,464-467}. This improved drug delivery to the brain to some extent, but its efficiency remains quite low⁴⁶⁸. The use of US and microbubbles is proposed as an alternative approach to increase the BBB permeability temporarily and allow passive diffusion of different therapeutics. This can be achieved using unfocused or focused US in combination with microbubbles.

US parameters must be critically selected to enable the penetration of the intact skull and the exposure of the underlying tissue with accurate precision⁴⁶⁹. Skull shape and structure strongly differ between patients. Therefore, patient-specific US beam profiling has been developed, based on specific 3D scanning of the skull with high-resolution scans such as MRI or computed tomography (CT) scans to be used to deduct the skull's acoustic properties and precise tumor margins⁴⁷⁰⁻⁴⁷⁴. Subsequently, the focal areas can be precisely defined to minimize collateral damage in the brain's vital areas. Increased permeability of the BBB by cavitation bubbles is a result of disruption of tight junctions in its endothelium⁴⁷⁵. Subsequently, resealing of the BBB takes place after a certain time. Reversible opening of the BBB was observed when bubbles in the size range of 1-2 μm were used at 0.45 MPa acoustic pressure with recovery time of 24 h. On the other hand, 5 days were needed for BBB recovery after using 6-8 μm bubbles at 0.6 MPa acoustic pressure⁴⁷⁶. Two additional effects were reported, including increased transports of vacuoles through the endothelial cell lumen^{477,478}, and reduction in the P-glycoprotein efflux pumps^{407,479,480}. The latter was responsible to significantly enhance small molecular weight drugs delivery across the BBB.

In general, the disruption of the BBB was demonstrated to improve the bioavailability of several types of drugs in the brain. These include small molecules such as chemotherapeutics and MRI contrast agents, macromolecular drugs such as dextrans, antibodies and pDNA, as well as stem cells⁴⁸¹⁻⁴⁸⁷. Clinical trials in humans showed that MRI-guided FUS could safely deliver chemotherapeutics across the BBB in patients with primary brain tumors^{488,489}.

To allow safe use of US-mediated BBB opening, the potential of vascular damage and neuroinflammation must be investigated extensively. Minor red blood cells (RBC) extravasations have been reported, but not considered to compromise the safety of this approach since no apoptosis or ischemia were observed⁴⁷⁵. On the other hand, a sterile inflammatory response was reported after BBB disruption, similar to cases of brain ischemia or mild traumatic brain injuries⁴⁹⁰. Reports have demonstrated that single treatment may have minimal long term (7 to 13 weeks) effects. However, repeated procedures were associated with severe damage that corresponds to pathological trauma injuries, such as vascular damage, inflammation and neurodegeneration⁴⁹¹. In preclinical studies, dexamethasone administration after US treatment reduced the risk of tissue damage due to secondary inflammation caused by BBB opening⁴⁹².

In tumours' treatment, drugs must cross several barriers before reaching their target sites. After crossing the endothelium layer, drugs need to cross through the interstitium, also known as the tumor microenvironment (TME). TME is a dense biological matrix in which several physiological processes take place^{493,494} that aid in the development, progression and metastasis of tumor cells. With huge variation between tumors, TME usually has a rigid extracellular matrix (ECM) composed of proteins, glycoproteins, polysaccharide and proteoglycans as well as different cell types including stromal cells, pericytes, cancer associated fibroblasts (CAFs) and immune cells⁴⁹⁵. The ECM is a hurdle that prevents nanoparticles transport and penetration towards cancer cells⁴⁹⁶.

US and microbubbles can be used to aid in TME penetration and improve drug delivery to tumor sites. Although the exact mechanical effects of cavitation on the TME remain largely unexplored⁴⁹⁷, it was demonstrated that it resulted in increased intratumoral drug concentrations of both small molecules and nanomedicines⁴⁰⁷ as well as immunomodulating proteins⁴⁹⁸ in numerous studies. It remains unclear if the observed effects are due to enhanced sonopermeation, TME penetration or both. Other biological responses that are still unknown might play a role too. Moreover, bubble cavitation could increase TME oxygenation as the oxygen core is dissolved to the tumor vasculature and subsequently to the TME⁴⁹⁹⁻⁵⁰¹, thereby improving drug efficacy and immune cell infiltration⁵⁰¹.

1.2.4.2 Payload transport and delivery

US can be used for the remote activation of drugs in the field of smart responsive DDSs. Four main types of carriers are used for this purpose; microbubbles, phase-change nanodroplets, nanocarriers and emerging carriers. Microbubbles were initially used as US contrast agents, and later developed as targeted drug carriers³⁵⁹ (Fig. 1.11). The extent of microbubble response to US depends on the surrounding medium as well as the microbubbles structure, namely the type of gas in their core and the composition of their shell. For DDS, the microbubbles' shell is typically composed of lipids encompassing perfluorocarbon gas. The payload can be loaded within the shell, or directly linked on the surface⁵⁰². Additionally, shells can be made of proteins, polymers or surfactants, and cores can be filled with other gases such as oxygen. Inertial cavitation of drug-loaded microbubbles leads to shell rupture and drug release. To improve targeting, microbubbles can be modified with ligands to bind specifically to a target site⁵⁰³. Moreover, a combination of magnetic and acoustic fields can be used to aggregate bubbles functionalized with SPIONs, and then excite them with US⁵⁰⁴.

Phase-change nanodroplets emerged as an alternative to microbubbles, characterized with improved stability and longevity⁵⁰⁵. Nanodroplets are basically vesicles with a core of phase-changeable material with a size range of 400 to 800 nm in diameter. This size is comparable to that of the gap between endothelial cells (*i.e.*, 380-780 nm), which may facilitate drug uptake into tumor tissues. Perfluorocarbons (PFCs) are common materials used in the core, due to their nontoxicity and low solubility in water that results in longer lifetime of the droplets⁵⁰⁶. When these liquid droplets are exposed to physiological temperatures, they become

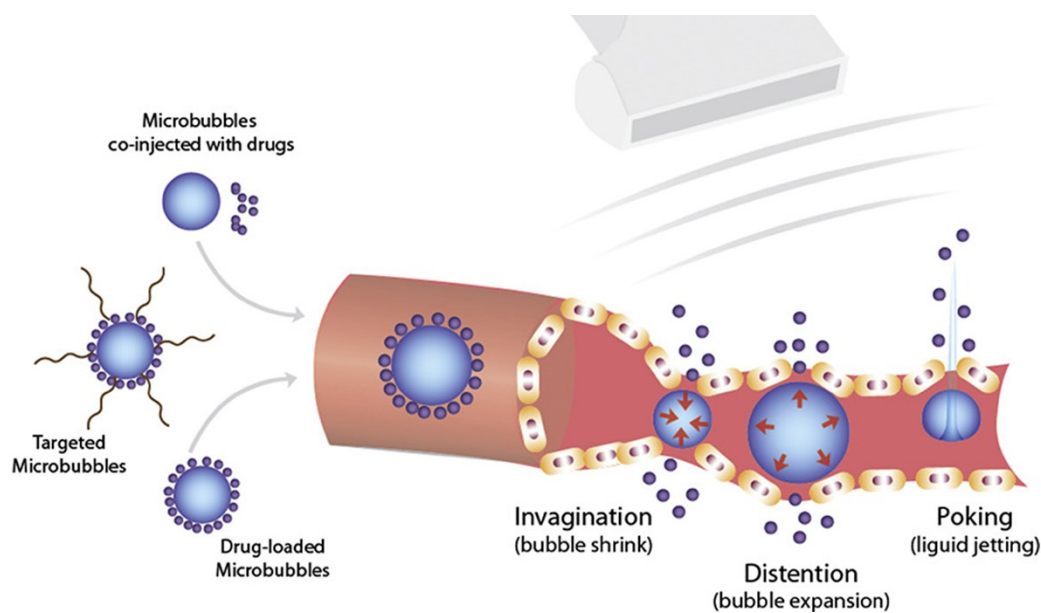


Fig. 1.11. US- and microbubble-mediated drug delivery. Microbubbles are either co-injected or loaded with the drug. Targeted microbubbles can be achieved by surface modifications. The cavitation of the microbubbles upon US exposure leads to drug release. Furthermore, liquid jetting enhances membranes permeability and therefore increasing drug crossing the membrane (Reprinted from⁵⁰⁷, Copyright (2020), with permission from Elsevier).

metastable and rapidly transition into the gas phase upon excitation with US⁵⁰⁸, in a process named acoustic droplet vaporization^{508,509}. Subsequently, dramatic size changes take place and liquid droplets with 200 to 300 nm diameter size can expand into gas bubbles with 1 to 5 μm in diameter. PFC nanodroplets were investigated for US imaging, drug delivery, sonothrombolysis and BBB opening, mostly utilizing high intensity US⁵¹⁰. Efforts to further understand the effects of nanodroplets vaporization at the target site are ongoing to predict and control their behaviour *in vivo* for potential clinical applications in the future⁵¹¹.

Nanocarriers, both organic and inorganic, can be utilized as DDSs to be triggered by US. Inorganic nanocarriers mostly transport their payload either adsorbed or conjugated to their surface⁵¹². They include mesoporous silica particles (MSNPs), gold nanoparticles, SPIONs and carbon nanotubes. MSNPs and carbon nanotubes can be used to enhance cavitation in response to low-frequency US (20 to 90 kHz), as they are able of retaining air stably inside their hollow hydrophobic structures⁵¹³. Self-assembled organic nanocarriers such as nanoemulsions, liposomes⁵¹⁴⁻⁵¹⁶, polymeric micelles⁵¹⁷ and polymersomes⁵¹⁸ can be used to encapsulate payloads in their core. Their response to US might be triggered by thermal or mechanical effects, depending on their specific characteristics. A common approach is to combine liposomes with microbubbles. Another less popular combination is MSNPs with US-responsive block copolymers⁵¹⁹. In general, surface chemistry is needed to couple the payload to its carrier. Nevertheless, supramolecular host-guest complexes can be utilized as an alternative that does not require chemical modifications.

Finally, emerging carries are materials that provide promising new directions and potential for payload delivery. One example is antibubbles, which consist of a liquid core surrounded by a thin gas layer to separate it from the surrounding fluid. These antibubbles can oscillate asymmetrically which leads to higher harmonics with non-linear scattering strengths similar to those of microbubbles contrast agents^{520,521}. Antibubbles can be responsive to magnetic fields by dispersing magnetite particles in their liquid core⁵²². Hydrogels that respond to US represent

another emerging material which allow for stepwise release of a payload, rather than one-time release that is usually demonstrated for microbubble and nanocarrier-based systems⁵²³⁻⁵³⁰.

1.2.4.3 Sonopharmacology and sonogenetics

The use of US for the mechanochemical control of biological function has been investigated in the fields of sonopharmacology and sonogenetics, by controlling drug delivery and gene expression, respectively.

Sonopharmacology

Sonopharmacology is an emerging field in which principles of polymer mechanochemistry are utilized for drug activation and delivery. Since US is used as a tool to deliver shear forces to polymer chains in solution³⁶⁹, polymers have been developed to be activated using polymer mechanochemistry principles to be utilized in biomedical applications.

Towards this end, strong covalent or supramolecular mechanophores were incorporated in polymers as drug releasing and drug activating moieties. For example, US-induced selective scission of disulfide bonds centered in a polymer leads to the generation of thiol-terminated polymers. These undergo a Michael-type addition to Diels-Alder adducts of furylated drugs and acetylenedicarboxylate derivatives, resulting in downstream release of small molecule drugs *via* a retro Diels-Alder reaction⁵³¹. A similar controlled mechanochemical disulfide cleavage strategy was employed to release camptothecin, an alkaloid anticancer drug, from its beta carbonate linker *via* intramolecular 5-*exo-trig* cyclization⁵³². Furthermore, bifunctional polymers with disulfide bonds were designed not only to release anticancer drugs after US exposure, but also release a fluorophore alongside the drug molecule. The simultaneous release of fluorescent probes such as *N*-butyl-4-hydroxy-1,8-naphthalimide and umbelliferon enable non-invasive imaging of drug delivery and uptake⁵³³.

Another approach utilized high molecular weight RNA polyaptamers to bind to neomycin B, an aminoglycoside antibiotic, and deactivate it (Fig. 1.12a). Upon US exposure, phosphodiester bond scission in the RNA polymer backbone as well as destruction of non-covalent bonds between the aptamers and neomycin B lead to the release of the latter⁵³². Vancomycin, a peptide antibiotic, was deactivated by binding to its peptide target sequence within the frame of a gold nanoparticle-polymer or gold nanoparticle-nanoparticle assemblies that held the drug in its inactive form. Force-induced scission of hydrogen bonds between vancomycin and its complementary peptide target activated the drug⁵³². Mechanophore-decorated polymer brushes were designed to be loaded with higher content of vancomycin, and the same approach towards inhibiting its antibiotic activity by binding to its complementary peptide target was utilized⁵³⁴.

Gold nanoparticles connected by dsDNA were utilized for the mechanochemical activation of doxorubicin. The drug is known to intercalate dsDNA *via* non-covalent interactions, leading to its deactivation and quenching of its fluorescence. After ultrasonication, these bonds were cleaved and doxorubicin was activated⁵³⁵.

Polymer chains carrying a supramolecular coordination palladium (Pd) cage were used to non-covalently encapsulate different drugs, such as ibuprofen and progesterone, and were then activated by US leading to rupture of the Pd cage and release of the drug cargo⁵³⁶.

However, in these previous examples, the applied US doses were biomedically incompatible, in terms of frequency, power intensity and sonication times. To address these challenges, one approach was the design of force-sensitive molecular moieties in a way that they require less mechanical energy to break. A second approach was to change topology of polymer structures to increase their mechanochemical reactivity, thereby reducing the needed US doses. This could reduce the sonication times using a classical 20 kHz probe sonicator by 1-2 orders of magnitude; from hours to seconds.

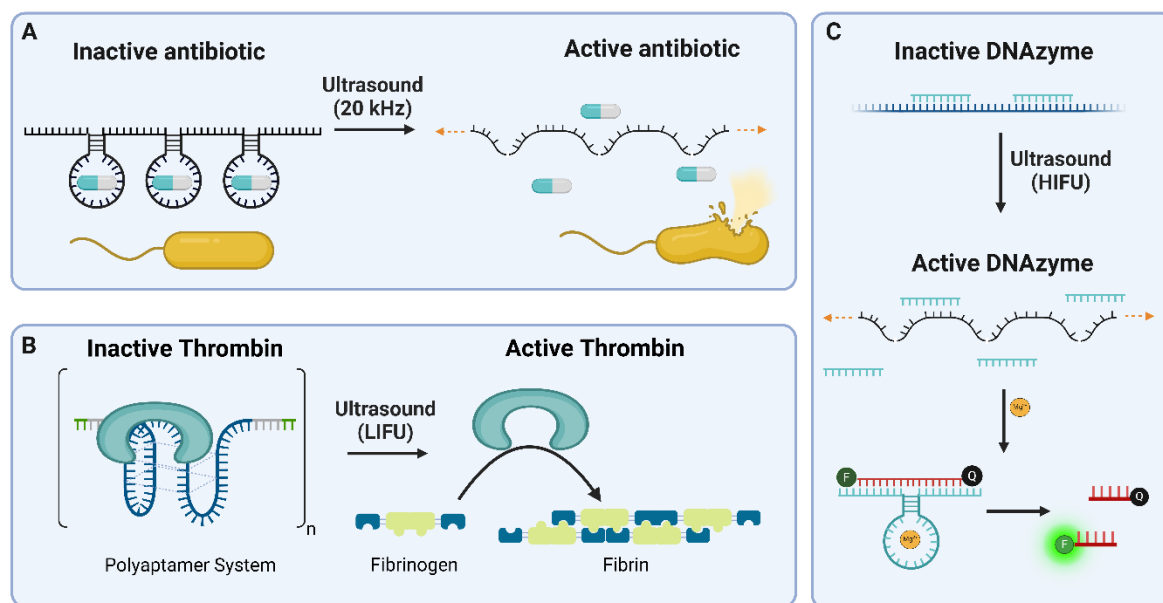


Fig. 1.12. US-mediated activation of different drugs based on polymer mechanochemistry principles. Different cargo were loaded onto polynucleic acids carriers leading to their deactivation, and subsequently they become activated by US. **A.** Neomycin B activation by 20 kHz US leads to restoring its antibacterial properties. **B.** Thrombin was activated by LIFU and could catalyze the formation of fibrin from fibrinogen. **C.** DNAzymes were activated by HIFU to exert their catalytic activity on labelled probes that become fluorescent after being specifically cleaved by DNAzymes in presence of magnesium ions. Figures recreated from references^{532, 537} and ⁵³⁸, respectively. Created with BioRender.com

In spite of this, the frequencies of US usually employed in polymer mechanochemistry can be rarely employed *in situ* in biomedical settings, due to cytotoxicity. Therefore, efforts to utilize medically-applicable US devices, such as LIFU and HIFU⁵³⁹, were investigated. A polyaptamer DNA polymer was designed to bind to and deactivate thrombin (Fig. 1.12b). Upon ultrasonication with LIFU, thrombin was released into its active form and able to catalyse the conversion of fibrinogen to fibrin⁵³⁷. Furthermore, gold nanoparticles decorated with thrombin split aptamers were designed and fabricated. Upon thrombin addition, they self-assembled into aptamer tertiary structures inducing nanoparticles aggregation and thereby deactivating thrombin. After LIFU exposure, reversible disassembly of the nanoparticles was achieved and thrombin was released into its active form⁵³⁷. Finally, DNAzymes were liberated from their polynucleic acid carrier using HIFU⁵³⁸ (Fig. 1.12c).

Nevertheless, the use of diagnostic or imaging US in polymer mechanochemistry remained out of reach till now. In this thesis, we address this shortcoming by exploiting polymer mechanochemical principles for biomedical applications using a low-intensity diagnostic imaging US device, which is licensed for human use.

Sonogenetics

Sonogenetics is a relatively new technique that was first observed by Heureaux *et al.* in 2014⁵⁴⁰, but it was not until 2015 that Ibsen *et al.* coined the term, thereby introducing this innovative technique to the scientific community⁵⁴¹, in which US is utilized to modulate biological activity. In sonogenetics, either genetically-encoded US-responsive elements or engineered sono-responsive proteins are used to connect US and molecular function. Mechanosensitive (MS) ion channels were identified as sono-adjuvants that aid in sonogenetics⁵⁴². Approaches used in sonogenetics utilize either thermal or mechanical effects of US (Fig. 1.13).

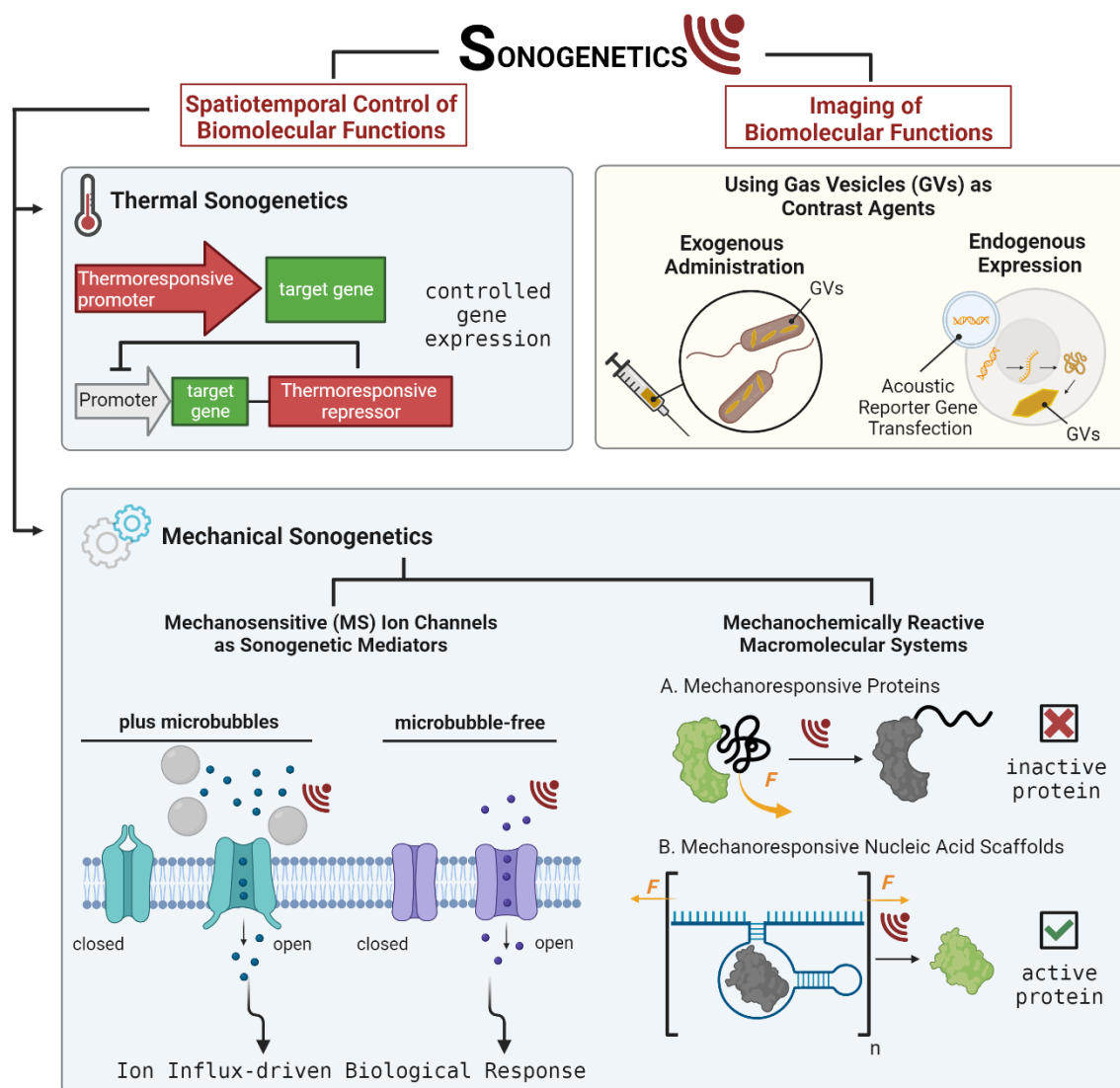


Fig. 1.13. A general schematic summary of the state-of-the-art sonogenetic tools used for controlling or imaging biomolecular functions. Thermal and mechanical sonogenetics can be used to manipulate cellular functions. In thermal sonogenetics, heat-responsive promoters and repressors are exploited, while mechanical sonogenetics utilize mechanosensitive (MS) ion channels and mechanochemically reactive macromolecular systems. Such strategies eventually result in the control of protein activity, signalling pathways or gene expression. On the other hand, gas vesicles (GVs) have been developed as acoustic contrast agents to image biomolecular functions. Towards that end, GVs are either administered exogenously or produced endogenously after genetically engineering the target cells (adapted from Ref. ⁵⁴³, CC BY 4.0).

In thermal sonogenetics, focused US (FUS) is used to deliver energy to a specific target area. It can be used to control gene expression in combination with thermoresponsive promoters and repressors. One prominent example is the mammalian heat shock protein 70 (HSP70) promoter that can be induced by mild heating protocols that don't usually damage the tissue⁵⁴⁴⁻⁵⁴⁹. Thermal logic circuits can be designed to operate under the control of temperature-dependent transcriptional repressors. This was utilized for *in vivo* control of microbial therapeutics⁵⁵⁰. Other applications of thermal sonogenetics in the field of cancer immunotherapy involve US-driven expression of cytotoxic T lymphocyte antigen 4 (CTLA-4) and programmed death-ligand 1 (PD-L1) in engineered microorganisms⁵⁵¹, and the heat-inducible chimeric antigen receptor (CAR) expression in T cells⁵⁵².

In mechanical sonogenetics, the use of MS ion channels as US mediators has been the most widely investigated strategy, either in the presence or absence of microbubbles as sono-adjuvants. The addition of microbubbles to the system lowers the threshold of US energy and sensitizes the cells towards US. This approach was widely used for various applications in different biological systems.

Various types of MS ion channels were discovered in a wide range of living organisms, spanning from bacteria to humans⁵⁵³⁻⁵⁵⁶. They possess unique structures and mechanotransduction properties that facilitate their biological functions⁵⁵⁷. The roles of MS ion channels are essential for physiological processes, such as cellular signalling, sensory perception and mechanical stress adaptation⁵⁵⁸. Upon exposure to US, both excitatory and inhibitory effects of MS ion channels have been demonstrated⁵⁵⁹. Multiple types of naturally occurring MS ion channels in mammalian cells have been identified. This include the two-pore potassium channel (K2P) family that consists of six subfamilies including TWIK (tandem of pore domains in a weak inward-rectifying K⁺ channel) and TREK (TWIK-related K⁺ channel) subfamilies. In particular, three key members: TREK-1, TREK-2 and TRAAK (TWIK-related arachidonic acid activated K⁺ channel) are primarily found in sensory neurons and respond to different mechanical stimuli⁵⁶⁰. The Piezo family is another crucial MS ion channel family that has diverse roles in mechanosensory, developmental and regulatory processes. Transient receptor potential (TRP) channels have also been postulated to be directly activated by mechanical stimuli. Furthermore, acid sensing ion channel 1a (ASIC1a) stands as the sole member within its family known to be activated by US⁵⁶¹, and there is some emerging evidence suggesting that ASIC3 may also be US-responsive⁵⁶².

The first demonstration of US-mediated opening of MS ion channels was reported by Heureaux *et al.* for the bacterial large conductance mechanosensitive ion channel (MscL) expressed in mammalian cells. A G22S mutant of MscL that can be activated at a lower US threshold, in combination with integrin-bound microbubbles⁵⁴⁰. One year later, the term sonogenetics was introduced by Ibsen *et al.*, in a study revealing that neural circuits of *Caenorhabditis elegans* (*C. elegans*) responded to low-pressure US stimulation when coupled with microbubbles⁵⁶³. The role of TRP-4 MS ion channel was revealed as its absence leads to a reduced US sensitivity. Later on, other MS ion channels were activated in *C. elegans*, such as MEC-4 and MEC-6, resulting in reversal behaviour⁵⁶⁴. Further evidence suggested the cooperative roles of TPR-4 and MEC-4 in eliciting neuronal responses to US⁵⁶⁵. The MscL family is found in most microorganisms, as they function as osmotic release valves in response to osmotic shock⁵⁶⁶. The best studied example is the MscL from *Escherichia coli* (*E. coli*) and efforts to express it in mammalian cells opened new opportunities for introducing new mechanotransduction pathways and employing sonogenetics in mammalian systems.

Integrin-bound microbubbles were used in combination with G22S mutant of MscL in retinal pigment cells⁵⁴⁰. Biotinylated microbubbles were used to target streptavidin-coated T cells that express Piezo1 ion channels endogenously. US-activation of the MS ion channel led to calcium influx that triggered calcium-dependent phosphatase calcineurin⁵⁴². This in turn phosphorylated NFAT transcription factors leading to their translocation to the nucleus to activate their promotor, which in turn triggered the expression of anti-CD19 CAR gene in T cells. Activated T cells could engage with cancer cells and ultimately resulted in their apoptosis.

In the context of neuroscience, the auditory sensing protein Prestin was engineered in target neurons to respond to US both *in vitro* and *in vivo*⁵⁵⁵. An ultrasensitive mutant of Prestin enabled transcranial US targeting of neurons in deep brain regions⁵⁵⁶. Furthermore, biotinylated microbubbles conjugated to avidin can be bound to microbubbles functionalized with biotinylated anti-Piezo1 antibodies⁵⁶⁷. This significantly reduced US intensity needed to stimulate the sensitized neurons.

Microbubble-free stimulation of MS ion channels is also feasible. However, a higher frequency of US or a highly sensitive mutant of the ion channel is needed. MscL was sensitized further to US *via* the gain-of-function mutation I92L. Engineered neurons could be excited on demand using low-pressure US pulses⁵⁵⁴. The same MS ion channel mutant was used for inducing cell apoptosis in several cancer cell lines⁵⁶⁸. A different mutant of MscL (G22S) was expressed in primary neurons and could be stimulated at lower voltage gating threshold by US. The stimulation of MscL G22S in excitatory neurons in mice cortices led to a strong muscular response in the limbs⁵⁶⁹. This work demonstrated the first MscL-based neuromodulation in the right cortex and right dorsomedial striatum of mice brain *in vivo* using US without affecting any other regions. Recently, MscL could be stimulated by music characterized by low-frequency (*i.e.*, as low as 50 Hz) in transgenic cells, for the modulation of insulin levels in type 1 diabetic mice to mimic the wild-type blood concentration⁵⁷⁰.

Furthermore, engineered Prestin was utilized for sonogenetic neuromodulation for the amelioration of Parkinson's disease in mice. Dopaminergic neurodegeneration was reduced 10-fold upon US stimulation, and symptoms were alleviated by about 4-fold⁵⁷¹.

Piezo1 ion channel was utilized in many different studies. It was shown that US alone could stimulate Piezo1 in primary neuron culture, leading to calcium influx and a subsequent increased nuclear c-Fos expression. This led to increased expression of several proteins such as phospho-CaMKII, phospho-CREB, and c-Fos in the neuronal cell line⁵⁷². A vertically deployed SAW platform was used to trigger Piezo1 channels *in vitro*, demonstrating that the ion channel's response depends on the pulse repetition frequency or the burst duration of US⁵⁷³. Moreover, Piezo1 expression in osteoblast precursor cells resulted in their selective stimulation that triggers downstream signalling pathways. This resulted in increased cell migration and proliferation. Additionally, structural remodelling was observed after US stimulation of Piezo1-transfected cells, as seen by the accumulation of F-actin filaments in the perinuclear region⁵⁷⁴. Recent investigations to understand the biological mechanisms underlying US neuromodulation revealed functional expression of Piezo1 in different regions in the brain at different levels of sensitivity⁵⁷⁵.

Although not widely applied yet, US neuromodulation of TRAAK ion channels was observed in genetically engineered neurons⁵⁵³, thereby opening the door for further studies for using K2P ion channel family as tools for sonogenetics in the future. In the TRP family, FUS was used to activate a Na⁺-containing TRPC1 current and stimulate cyclooxygenase-2 (COX2)-dependent pathways that eventually induced mesenchymal stem cell (MSC) tropism to kidneys and muscles⁵⁷⁶. Within the same family, TRPA1 is particularly expressed endogenously in astrocytes in different brain regions such as the hippocampus, cortex and trigeminal caudal nucleus. TRPA1 channels were used for US neuromodulation where they drive calcium influx that results in Best1-mediated release of gliotransmitters, such as glutamate. Glutamate can stimulate NMDA receptors in the nearby neuronal synapses and initiate their action potential. Direct mechanical processes that affect the cells such as brain concussion might involve the TRPA1-Best1-NMDAR pathway in its pathological manifestations⁵⁷⁷.

On the other hand, FUS was utilized *in vivo* for selective activation of genetically modified neurons expressing TRPV1 ion channels in the brain. This stimulation results in locomotor behaviour and FUS did not affect neuronal integrity or triggered inflammation or apoptosis⁵⁷⁸.

Finally, ASIC1a MS ion channels were found to play a crucial role in mechanotransduction in the activation of cultured neurons by low-intensity US. This role was found to be more profound than that of Piezo1 or TRP MS ion channels. *In vitro*, the proposed mechanism indicates that the activation of the sodium channel ASIC1a leads to the activation of voltage-gated calcium channels, which increases intracellular concentration of calcium. On the other hand, a different mechanism was proposed *in vivo*, in which ASIC1a activation was related to

a cytoskeletal-dependent mechanism due to mechanical effects of acoustic pressure exerted on ECM proteins⁵⁶¹.

In a novel and distinctive approach towards sonogenetics, macromolecular systems that can inherently respond to US were developed. These systems obviate the need of using MS ion channels or co-administering nano- and microbubbles as sono-adjuvants. Therefore, this strategy broadens the definition of sonogenetics and expands its scope. These approaches were inspired by the principles of polymer mechanochemistry discussed earlier and involve two strategies to control cellular activity by US at the protein level.

One strategy is the *de novo* design of mechanoresponsive proteins to involve a long flexible domain that renders them sensitive to ultrasonication since these unfolded chains can transfer shear forces from solution to the protein scaffold. The shear forces originate from collapsing cavitation bubbles induced by US. Especially, supercharged elastin-like polypeptide chains (SUPs)⁵⁷⁹ were fused to target proteins⁵⁸⁰ to enhance their sensitivity to US. For instance, a green fluorescent protein (GFP) variant modified with an internal long unfolded pentapeptide repeat unit (VPGE_G)_n was linked to the 11th beta-strand that is part of the beta-barrel⁵⁸¹. Shear forces lead to changes in the protein tertiary structure, displacing the beta strand from the barrel and switching off fluorescence. Another example is the deactivation of trypsin *via* a genetically engineered inhibitor fused with a SUP domain. The latter played the role of a mechanochemical transducer of US. Protein-protein interactions were cleaved upon ultrasonication leading to the activation of trypsin⁵⁸¹.

1.3 THERAPEUTIC NUCLEIC ACIDS: A NEW HORIZON IN TARGETED DRUG DELIVERY

Therapeutic nucleic acids (TNAs) represent a rapidly evolving frontier in medicine. They are nucleic acids themselves or closely related compounds used for disease treatment, and they can be either DNA- or RNA-based. As a therapeutic class, they fall somewhere between small molecules and biologics with different physicochemical properties and characteristic instability in the biological environment. TNAs can be classified broadly into DNA therapeutics (ASO, CpG oligodeoxynucleotides (CpG ODNs), DNA aptamers and gene therapy) and RNA therapeutics (micro RNA (miRNA), siRNA, ribozymes, RNA aptamers and decoys).

1.3.1 DNA THERAPEUTICS

1.3.1.1 ASOs and aptamers

ASOs are short single-stranded (ss) DNA sequences that range from 8 to 50 nucleotides (nt) in length. They bind to their target mRNA sequence by means of standard Watson-Crick base pairing, resulting in blocking mRNA translation either by the degradation of the mRNA by endogenous RNase H or by steric hinderance of the process⁵⁸². Aptamers, also called chemical antibodies, are ssDNA or ssRNA molecules that bind specifically to their target with high affinity⁵⁸³. Aptamers are developed through a process called systematic evolution of ligands by exponential enrichment (SELEX) from a large pool of nucleic acids⁵⁸⁴. DNA aptamers that bind to coding sequences for thrombin, vascular endothelial growth factor (VEGF), prostate specific antigen (PSA), human immunodeficiency virus trans-acting responsive element, interferon-gamma (IFN- γ), hemin, dopamine and heat shock factor are under clinical development⁵⁸⁵.

First-generation ASOs included unmodified nucleic acids that were highly susceptible to nuclease degradation. Second-generation ASOs were chemically modified to enhance their stability, specificity, binding affinity and safety⁵⁸⁶. The most commonly employed chemical

modification to ASOs was the PS modification in which one of the non-bridging oxygen atoms in the backbone is replaced by a sulfur atom. This improved ASOs resistance to nucleases and enhanced their cellular uptake. To further increase their stability, the ribose sugar moiety was modified at the 2' position, usually with either 2'-O-methyl (2'-OMe) or 2'-O-methoxyethyl (2'-MOE). Alternatively, non-sugar furanose rings can be used to modify ASOs and improve their PK profiles⁵⁸⁷. In third-generation ASOs, locked nucleic acid (LNA), peptide nucleic acid (PNA) and phosphorodiamidate morpholino oligomers (PMO) are commonly used⁵⁸⁸.

Although several ASO candidates are being explored for the treatment of neurodegenerative diseases as well as other diseases in the eye, liver and muscles, only nine ASOs have been approved by the US FDA for therapeutic use as of November 2023. Fomivirsen was approved in 1998 to treat cytomegalovirus (CMV) retinitis in acquired immunodeficiency syndrome (AIDS) patients³⁰. However, it was withdrawn from the market in 2001 as the introduction of highly active antiretroviral therapy (HAART) reduced the incidence rate of AIDS CMV retinitis significantly. Four of the approved ASOs are developed to treat Duchenne muscular dystrophy (DMD), which is a genetic disorder characterized by progressive muscle degeneration and weakness due to the alterations of a protein called dystrophin that helps in keeping muscle cells intact. These are eteplirsen (2016)⁵⁸⁹, golodirsen (2019)⁵⁹⁰, viltolarsen (2020)⁵⁹¹ and casimersen (2021)⁵⁹².

Other ASOs target liver genetic disorders, such as mipomersen (2013)⁵⁹³, inotersen (2018)⁵⁹⁴ and volanesorsen (2019)⁵⁹⁵ were developed for treating homozygous familial hypercholesterolemia (HoFH), hereditary amyloidogenic transthyretin (hATTR) and Familial chylomicronaemia syndrome (FCS), respectively. Finally, nusinersen (2016)⁵⁹⁶ is approved for treatment of spinal muscular atrophy (SMA) in the CNS.

1.3.1.2 Gene therapy

Gene therapy aims to replace the function of an abnormal or a dysfunctional gene with a functioning variant. The current treatment landscape of gene therapy covers several genetic disorders such as adenosine deaminase deficiency, α -1-antitrypsin deficiency, cystic fibrosis, familial hypercholesterolemia, Gaucher's disease and haemophilia B⁵⁹⁷. A vector, usually a retrovirus, is needed to aid the delivery of the gene in the form of a therapeutic gene expression cassette. This is composed of a promoter, the transgene of interest and a termination signal⁵⁹⁸. Two main approaches of gene therapy exist. First is the germ line therapy in which the desired gene is introduced into germ cells leading to transmission to the next generation. The second is the somatic gene therapy in which the desired gene is transferred to somatic cells that are not heritable^{599,600}.

Several gene therapies were approved for human use by different regulatory authorities. These include Gendicine to treat patients with head and neck squamous cell carcinoma linked to mutations in the *TP53* gene⁶⁰¹, Luxturna for inherited retinal disorder⁶⁰², Zynteglo for beta thalassemia⁶⁰³, Zolgensma for SMA⁶⁰⁴, Strimvelis for adenosine deaminase deficiency (ADA-SCID)⁶⁰⁵, Hemgenix for haemophilia B⁶⁰⁶, Imlygic for multiple solid tumors⁶⁰⁷, Glybera for lipoprotein lipase deficiency (LPLD)⁶⁰⁸, Skysona for cerebral adrenoleukodystrophy (CALD)⁶⁰⁹, as well as several CAR T cell therapeutics for different types of lymphoma and myeloma including Breyanzi⁶¹⁰, Tecartus⁶¹¹, Carvykti⁶¹², Kymriah⁶¹³ and Abecma⁶¹⁴. In the case of cell-based therapy, the gene transfection is done *ex vivo* rather than *in vivo*. Then, cells are transferred back to the patient to exhibit their therapeutic effects.

1.3.2 RNA THERAPEUTICS

Two approaches were used for the development of RNA drugs. The first is RNA interference (RNAi) strategy in which short RNA sequences recognize and bind to their complementary targets in endogenous RNA transcripts and alter their processing^{615,616}. The second approach is using mRNA to encode certain target peptides or proteins and induce their transient expression in the cytoplasm. While the first strategy leads to protein downregulation, the second results in upregulation of the target protein, for instance to replace a defective protein or present antigens for vaccination^{617,618}.

Several major hurdles must be overcome to develop RNA therapeutics, specifically the rapid degradation of exogenous RNA by RNases that are abundant in the biological environment and tissues, the delivery of the negatively charged RNA across the hydrophobic cytoplasmic membrane and the strong immunogenicity of exogenous RNA that result in cell toxicity and impaired mRNA translation.

These hurdles have been substantially overcome with recent advancements in different fields, including RNA biology, bioinformatics, chromatography and nanotechnology, thereby facilitating the recent rapid development of RNA therapeutics. Advantages of RNA-based drugs include their ability to act on targets that are otherwise “undruggable” for a small molecule or a protein, their rapid and cost-effective development, in comparison to that of small molecules or recombinant proteins and the rapid ability to alter the sequence of the mRNA construct for personalized treatments or to adapt to an evolving pathogen.

1.3.3 CHALLENGES IN DELIVERING NUCLEIC ACIDS

In general, TNAs suffer from several common challenges that limit their success in the therapeutic setting. First, unique stability issues arise due to their complex and fragile nature, which renders them prone to hydrolysis and digestion by nucleases. Either chemical modifications, protection through a DDS or ultra-cold storage conditions are needed to keep the TNAs intact and stable. Incompetent delivery to target organs is also another key challenge for TNA therapeutics. DDSs that can be controlled or targeted present potential platforms that aid in delivering TNAs to their target site. Finally, immunogenicity and side effects must be minimized for a successful delivery of TNAs. We will discuss the specific challenges and the various strategies used to overcome each challenge for the TNA subclasses that are of interest for the presented work. These are the CpG ODNs and siRNA, which will be discussed in section 1.4.8 and section 1.5.7, respectively.

1.4 CANCER IMMUNOTHERAPY

1.4.1 IMMUNOTHERAPY IN CANCER TREATMENT: SHIFTING THE FOCUS TO HARNESSING THE IMMUNE SYSTEM

Immunotherapy-based strategies are emerging as powerful weapons in the battle between human and cancer. The rapid advancement in the field is augmented by the success of several treatment approaches in clinical trials and more treatments are being approved by the US FDA. The research has been on the rise to increase our knowledge about how to harness components of the immune system to defend the body against the internal threat of cancer. Cancer immunotherapy has been highlighted in *Science* in 2013 as the ‘Breakthrough of the Year’⁶¹⁹. Furthermore, in the last decade, two Nobel prizes in Physiology (2011) and Medicine (2018) were awarded to scientists contributing to the rapidly growing field of immunology.

Although cancer cells are less immunogenic than pathogens, the immune system is capable of recognizing and eliminating tumour cells through delicate and complicated interactions. The ‘Cancer-Immunity Cycle’ was described by Chen and Mellman⁶²⁰ as a seven-steps process (Fig. 1.14), that starts by the release of tumor-associated antigens (TAAs) which get captured by antigen presenting cells (APCs), mainly dendritic cells (DCs). Next, DCs present these antigens on major histocompatibility complex (MHC) I and II molecules to T cells. This results in the priming and the activation of effector T cell responses against the cancer-specific antigens. Two types of T cells exist in this stage; T effector cells and T regulatory cells, and the ratio of those is critical to determine the outcome; a shift towards T effector cells is needed if anti-tumour activity is desired. Finally, T cells traffic to and infiltrate the tumour site, where they recognize and bind to cancer antigens and kill the malignant target cells. This results in the release of additional TAAs, which ultimately amplifies the immune-cancer cycle. Each step requires coordination between inhibitory and stimulatory factors, to keep the overall process in balance and prevent autoimmunity.

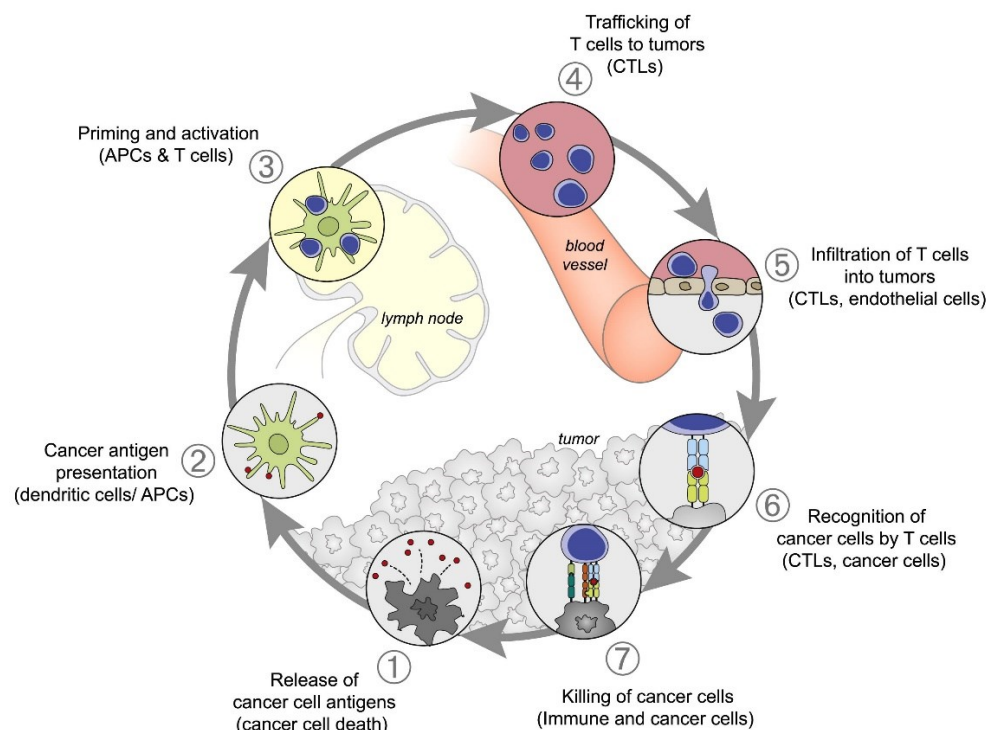


Fig. 1.14. The cancer-immunity cycle, in which the process of generation of immunity to cancer can self-propagate and lead to the accumulation of immune-stimulatory factors through seven major steps that start with cancer cells releasing antigens and end with killing of cancer cells (Reprinted from⁶²⁰, Copyright (2013), with permission from Elsevier).

However, in cancer patients, this cycle does not perform optimally. Any step could be dysfunctional; TAAs may not get detected, antigen presentation may be suboptimal, antigens may be treated as self rather than as foreign, trafficking of T cells into the tumour site may not be efficient or T cells may be inhibited by factors present in the TME⁶²¹. The goal of cancer immunotherapy is to induce or reinitiate a self-maintained cycle of cancer immunity that can be amplified and propagated, but in a controlled way to avoid unrestrained autoimmune self-destructing responses. These immunotherapies fall into several classes, including checkpoint blockers, lymphocyte activating cytokines, CAR T cells and other cellular therapies, agonists, cancer vaccines and immune adjuvants, oncolytic viruses and bispecific antibodies⁶²².

1.4.2 IMMUNE ADJUVANTS AND THE CENTRAL ROLE OF APCs

Cancer vaccines and immune adjuvants are based on the ability of certain types of immune cells, namely CD8⁺ and CD4⁺ T cells, to recognize TAAs in a similar way that pathogen-associated antigens (PAAs) are recognized. As explained earlier, one of the critical steps in the immune-cancer cycle is the presentation of TAAs on the surface of the APCs. Two specialized APCs play important roles in the anti-tumour immunity process; DCs⁶²³ and macrophages⁶²⁴. DCs are highly effective APCs that are capable of stimulating T cells to attack cancer cells. Hence, many strategies were developed to modulate DCs function to improve cancer immunotherapy. The first approach is to activate DCs *in situ*; by the administration of different DC-stimulating agents. The second is to use cell therapy to develop DC-based vaccines. Nevertheless, cell-based vaccination is expensive, cumbersome and the subsequent cell transplantation could lead to poor lymph node-homing and antigen presentation may not be as effective as expected. On the other hand, DC stimulation *in situ* is less challenging, more practical and less expensive. The most challenging aspect in this approach is to overcome the limitations associated with the direct administration of immunostimulants to the system, such as rapid clearance, non-specificity and possible side effects⁶²³. These limitations can be addressed by the design of an advanced DDS that can be controlled using a suitable stimulus.

1.4.2.1 Toll-like receptors and their role in cancer immunotherapy

The activation of APCs involves several families of receptors. One of these is the family of toll-like receptors (TLRs), which belongs to the broader class of pattern recognition receptors (PRRs). The TLR family includes ten members identified in humans to date, each with variable expression pattern amongst different cell types. Each TLR responds to a different type of pathogen-associated molecular patterns (PAMPs), such as lipids, bacterial proteins and nucleic acids from both viruses and bacteria⁶²⁵. Activation of TLRs initiates the downstream signalling pathways and functions as an alarm signal that triggers the appropriate innate and adaptive immune responses^{626,627}. We are interested in TLR9 in this thesis, which detects unmethylated CpG dinucleotides in pathogens' DNA (Fig. 1.15). Usually, these motifs tend to be highly methylated when present in vertebrate DNA, and are less prevalent than in bacterial and viral DNA⁶²⁸. TLR9 is expressed in B cells, plasmacytoid DCs (pDCs) and macrophages, and it is localized in the endoplasmic reticulum (ER) inside the cell^{629,630}.

Synthetic CpG ODNs contain CpG motifs resembling those found in bacterial DNA, and stimulate the same immune response that skews the immune milieu towards T helper 1 (Th1) -cell response and pro-inflammatory cytokine production⁶³¹. At first, CpG ODNs are internalized by immune cells, in a process that is facilitated by Class III phosphatidylinositol 3-kinase (PI3K)⁶³². Meanwhile, TLR9 redistributes from the ER to the lysosomal compartments containing the CpG ODN. The binding of CpG ODN to its ligand-binding site in TLR9 triggers a MYD88-dependant signalling pathway⁶³³.

This results in the transcription of inflammatory cytokines induced by Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), such as tumour necrosis factor (TNF), interleukin 6 (IL-6)^{634,635}, interleukin 12 (IL-12) and type I interferons (type I IFNs)⁶³⁶. The immune response of DCs to PAMPs leads to their activation and maturation, and as a result they express chemokine receptors and costimulatory molecules. Among these, C-C chemokine

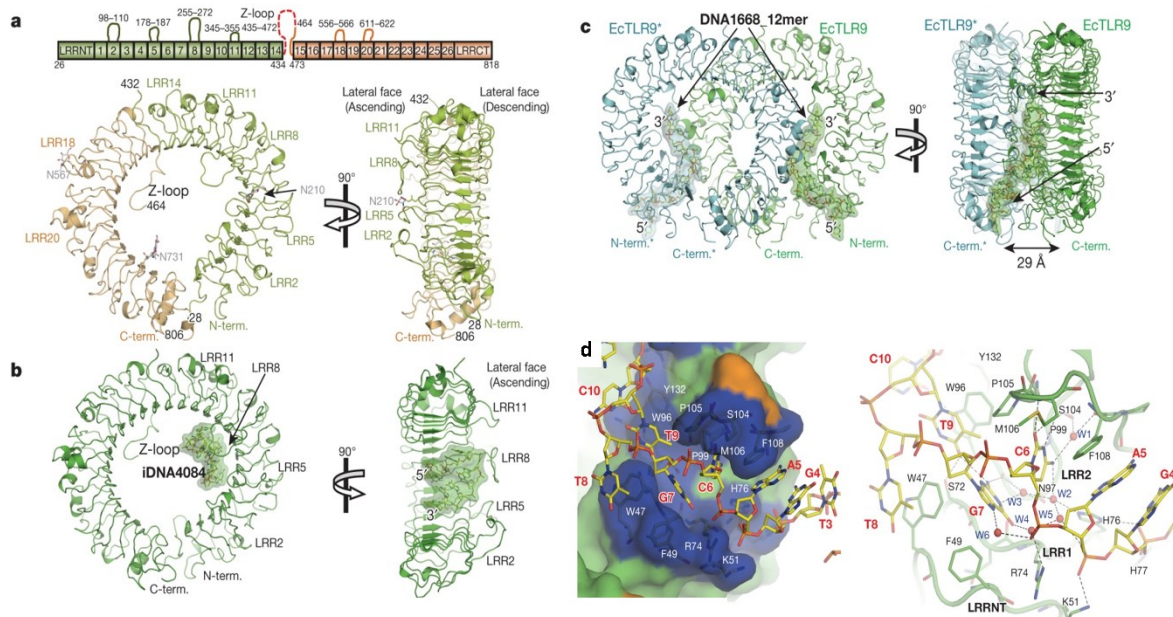


Fig. 1.15. A. The monomer structure of unliganded EcTLR9 showing the lateral face (left) and the convex face from the N-terminal side (right). The N-glycan residues are shown in stick representations with their O, N and C atoms coloured red, blue and grey, respectively. The N- and C-terminal halves of EcTLR9 are coloured light green and orange, respectively. **B.** Structure of EcTLR9 in complex with an inhibitory DNA showing the lateral face (left) and the concave face of the N-terminal half (right). Bound inhibitory DNA is depicted in stick representation and semi-transparent surface representation, respectively. **C.** The dimer structure of the complex of EcTLR9 and 12mer CpG ODN1668. EcTLR9 and its dimerization partner EcTLR9* are shown in green and cyan, respectively. The second TLR9 within the dimer and its residues are indicated with asterisks. The CpG ODN 12mer is shown in stick representation and semi-transparent surface representation. **D.** The binding groove of CpG-DNA (Left). An overview of CpG ODN recognition by TLR9 (Middle). A magnified view of the CpG ODN recognition (Right). Reproduced with permission from Springer Nature⁶³⁷.

receptor type 7 (CCR7) is critical for the migration of DCs to tumour-draining lymph nodes⁶³⁸, and the recruitment of DCs to the TME⁶³⁹. In parallel, the costimulatory molecules CD80 and CD86 are secreted by mature DCs and interact with CD28 and CTLA4, respectively, to control the activation or suppression of T cells^{640,641}. The activation and trafficking of anti-tumor T cells leads to the desired anti-tumor activity.

The interaction between CpG ODNs and TLR9 is highly specific, as the inversion of CpG motif (*i.e.*, GpC) results in the complete antagonism of the receptor⁶³² (Fig. 1.15b). Native DNA has a phosphodiester backbone, leaving it prone to hydrolysis by nucleases that are present in blood and inside cells. Therefore, synthetic CpG ODNs are made of nuclease-resistant PS bonds in their backbone, which improves the PK profile and increases the half-life of ODNs in the blood⁶⁴². CpG ODNs were broadly investigated for their therapeutic potential. Four major applications were developed including protective immunity, vaccine adjuvants, reduced allergic responses and cancer immunotherapy⁶³¹. The administration of CpG ODNs either alone or in combination with other therapies was proved to elicit the immune cascade needed for the activation of natural killer (NK) cells and cytotoxic T lymphocytes (CTLs) that contribute to cancer immunotherapy⁶³⁴. In the following section, we will discuss further in details the history of CpG ODNs development and their current therapeutic landscape.

1.4.3 A BRIEF HISTORY OF CPG ODNs AND THEIR CURRENT CLINICAL APPLICATIONS

Over thirty years ago, Krieg and Klinman showed that certain sequence motifs in the bacterial DNA are responsible for foreign DNA stimulation of the immune system⁶⁴³. These were composed of a central unmethylated CpG dinucleotide between specific flanking bases. Then, it was shown that CpG ODNs were recognized by TLR9 *via* MyD88-dependent signalling pathway. Unmethylated CG motifs are much less common in the mammalian DNA compared to bacterial DNA. This is attributed to both CpG suppression (as the mammalian stop codons contain CG dinucleotides) and CpG methylation⁶⁴⁴. Moreover, TLR9 is located in the ER and endo-lysosomal cellular compartments. Since bacterial and viral components are subject to phagocytosis, they end up in the same compartment of TLR9, unlike self-DNA⁶⁴⁵.

Synthetic CpG ODNs were developed in 1995 and different sequences were found to stimulate TLR9 with varying number and location of CpG dimers, as well as different base sequences flanking them⁶⁴⁶. CpG ODNs can be classified into four categories based on their sequence, secondary structures and effect on peripheral blood mononuclear cells (PBMCs). These are Class A (Type D), Class B (Type K), Class C and Class P. Notably, most CpG ODNs are named using arbitrary numbers that come from testing a large number of potential CpG ODN sequences. Their classification into different categories came at a later stage, when it became evident that certain characteristics of CpG ODNs lead to specific responses. Moreover, some papers use different names to refer to CpG ODNs that have been described before, which further complicates the naming convention.

Class A CpG ODNs are characterized by the presence of a poly G sequence either at the 5'-end, 3'-end or both. Their CG dinucleotides are contained within an internal palindrome sequence that can be 4 to 8 nt long. Finally, they have a partially PS-modified backbone, typically with 7 to 10 PS bonds at one or both ends. In terms of biological activity, Class A CpG ODNs stimulate the production of large amounts of type I IFNs, namely interferon- alpha (IFN- α). They induce the maturation of plasmacytoid dendritic cells and strongly activate NK cells *via* indirect cytokine signalling and they have no effect on B cells⁶⁴⁷.

Class B CpG ODNs are typically 18 to 28 nt long and usually have a fully PS-modified backbone. Their CG dimer is found in a 6-mer sequence, and the strongest CpG ODN of this class has three of these 6-mer sequences. They are characterized by their ability to trigger monocytes maturation, pDCs differentiation and production of TNF, and B cells stimulation to proliferate and produce immunoglobulin M (IgM)⁶⁴⁸. Since these CpG ODNs can strongly induce a humoral immune response, they were widely investigated as vaccine adjuvants.

Class C CpG ODNs resemble class A in containing palindromic CpG motifs, but resemble class B in being composed entirely of PS-modified backbone. They can stimulate both B cells and pDCs, to secrete IL-6 and IFN- α , respectively⁶⁴⁹. Finally, class P CpG ODNs contain double palindromes that lead to highly ordered structures. This results in inducing the strongest type I IFN production among all CpG ODN classes⁶⁵⁰. CpG ODNs have been investigated clinically as adjuvants for both infectious diseases and cancer immunotherapy. We will briefly discuss both applications and the advancements made in both fields.

1.4.3.1 CpG ODNs as vaccine adjuvants for infectious diseases

CpG ODNs have been evaluated clinically for use in combination with vaccines against several infectious diseases, such as malaria, hepatitis B virus (HBV), pneumococcus, influenza, human immunodeficiency virus and anthrax. Furthermore, few clinical studies evaluated the use of CpG ODNs as a stand-alone anti-infectious agent, without additional vaccines.

Heplisav B became the first approved drug containing CpG ODN by receiving a marketing authorization valid throughout the EU on February 2021⁶⁵¹. It contains 20 µg rHBsAg and 3000 µg CpG 1018. It is indicated for active immunisation against HBV caused by all known subtypes of HBV in adults 18 years of age and older. It is administered in two doses, one month apart. The need for a booster dose has not been established. However, it is recommended to evaluate subjects who are immunocompromised or who have chronic renal failure, as they may require a booster dose.

A third-generation anthrax vaccine is being developed using CpG 7909 as an adjuvant, called NuThrax. It is intended to be used both postexposure and as a prophylactic vaccine. This combination resulted in faster, higher magnitude and longer duration of immune response in animals, compared to the licensed anthrax vaccine (*i.e.*, BioThrax). These properties are advantageous especially for biodefense preparedness⁶⁵². Furthermore, the mice receiving NuThrax maintained anthrax resistance for more than one year⁶⁵³. In humans, phase I clinical trials demonstrated an accelerated and enhanced immune response in healthy volunteers^{654,655}.

Malaria vaccines that have CpG ODNs as adjuvants are also under clinical investigation. They are designed against the merozoite surface protein 1 (MSP 1) or apical membrane antigen 1 (AMA1) of the parasite. Co-delivery of the vaccine and the CpG ODN led to significantly stronger antibody response against these malaria antigens⁶⁵⁶⁻⁶⁵⁸. In the context of influenza, the addition of CpG ODNs to commercially available vaccines improved immunogenicity in animal models⁶⁵⁹ and in phase Ib clinical trials in humans as well^{631,660}.

As a stand-alone infection therapy, CpG ODN was shown to be effective against *Listeria*, *Francisella*, *Klebsiella* and other pathogens. Moreover, CpG 10101 was administered subcutaneously to patients with chronic hepatitis C virus (HCV) infection⁶⁶¹. However, with the introduction of curative viral protease inhibitors for HCV treatment, it is unlikely that further evaluation of CpG ODNs for this indication will be pursued.

1.4.3.2 CpG ODNs as adjuvants for cancer immunotherapy

CpG ODNs are also being evaluated for their potential role in cancer immunotherapy, either as an adjuvant or as stand-alone therapy. The use of antigenic peptides to activate T cells against specific types of cancer cells was found to be insufficient to illicit enough CTLs to attack the tumor^{662,663}. One approach to overcome this issue is to use adjuvants, such as CpG ODNs. Towards this goal, preclinical and clinical studies have demonstrated that antigen-specific CD4⁺ and CD8⁺ T cells were stimulated by CpG-adjuvanted cancer vaccines.

For example, most melanomas and several other tumors express melanoma-associated antigen recognized by T cells 1 (MART1)⁶⁶⁴. Vaccination with a MART1-derived peptide, incomplete Freund's adjuvant and CpG 7909 resulted in an increased frequency of antigen-specific CD8⁺ T cells in all patients. On the other hand, whole recombinant proteins were investigated as they provide greater variety of tumor epitopes. Studies showed higher antigen specific responses in patients with different types of cancer⁶⁶⁵⁻⁶⁶⁷.

As a monotherapy, CpG ODN administration did not lead to clinical responses in patients with chronic lymphocytic leukaemia⁶⁶⁸ and non-Hodgkin lymphoma⁶⁶⁹. Partial responses were reported for using CpG ODNs in metastatic melanoma⁶⁷⁰, cutaneous T cell lymphoma⁶⁷¹ and stage IV renal cell carcinoma⁶⁷². Improvement in activity was demonstrated when intratumoral injection was used rather than systemic administration of CpG ODNs, in both preclinical studies in mice⁶⁷³ and in clinical trials in human patients⁶⁷⁴.

The combination of CpG ODNs and chemotherapy has demonstrated inconsistent results among different clinical trials. Combining CpG 7909 with platinum-based chemotherapy in patients with stage IIIb-IV non-small cell lung carcinoma (NSCLC) improved the tumor

response and prolonged survival, along with a significant tumor infiltration of CD8⁺ T cells⁶⁷⁵. In contrast, combining the same CpG ODN with standard first-line chemotherapy of advanced NSCLC did not show improvement over monotherapy. Similarly, no improvement was demonstrated for combining CpG ODN with paclitaxel/carboplatin⁶⁷⁶. Little difference in median overall survival and no difference in median progression free survival were demonstrated when CpG ODN was combined with gemcitabine/cisplatin. Moreover, a higher proportion of grade 3 adverse effects was reported in patients receiving CpG ODN.

Radiotherapy- CpG ODNs combination were also evaluated in preclinical and clinical trials. In murine experiments, CpG ODNs were found to enhance the response of immunogenic tumors towards radiation treatment with or without chemotherapy⁶⁷⁷. In clinical trials, intratumoral injection of CpG ODN combined with radiation therapy improved the generation of tumor-specific CD8⁺ T cells in lymphoma and melanoma patients and led to systemic tumor regression⁶⁷⁰. CpG ODNs are also under investigation for augmenting antineoplastic effects of local irradiation in subjects with Hodgkins and non-Hodgkins lymphoma⁶⁷⁸.

Another potential combination is with therapeutic mABs. CpG ODNs were shown to upregulate CD20 expression in malignant B cells and increase IFN-inducible genes' expression in preclinical studies. Therefore, it was used in combination with rituximab (anti-CD20 mABs) in clinical trials that demonstrated five clinical responses and four partial responses among the treatment group^{679,680}. Another clinical trial that used a combination of CpG 7909 and erlotinib (EGFR inhibitor) did not show significant benefit compared to erlotinib alone, in patients with advanced recurrent EGFR + NSCLC⁶⁸¹.

Immune checkpoint inhibitors such as anti-PD-1 and anti-CTLA4 antibodies have shown significant improvement in cancer immunotherapy⁶⁸². Combining this class with CpG ODNs was also investigated, with preclinical studies showing improved tumor eradication⁶⁸³. mABs against CTLA4 such as ipilimumab and tremelimumab were investigated with CpG ODNs in patients with advanced solid tumors (including melanoma). Pembrolizumab (anti-PD-1 antibody) is being investigated in combination with CpG ODN in patients with metastatic melanoma. In a similar direction, CpG ODNs were also combined with immunosuppressive cytokines such as anti-IL-10 antibodies showing enhanced antitumor activity⁶⁸⁴.

In summary, CpG ODNs were demonstrated to increase the immunogenicity of vaccines when targeting various types of tumors. In general, patients developed stronger antigen-specific serum antibodies and higher CD8⁺ T cell responses. However, the magnitude of the immune effects varied between trials and inconsistent effects in other immune parameters were observed. Moreover, the improved immune responses rarely correlated with a better clinical outcome. To overcome these limitations, it is recommended that vaccination should be initiated early rather than waiting for other conventional therapeutics to fail. Better outcomes are also anticipated in patients with small and slow-growing tumors rather than with large tumor burdens. Finally, enrolling patients with similar cancer type and clinical presentation would aid in results analysis and rational design of clinical trials in the future.

1.4.3.3 Drug delivery systems for CpG ODNs

Different approaches in development of DDSs to improve CpG ODNs delivery have been investigated. These include self-assembled CpG ODNs, conjugation of CpG ODNs with proteins or peptides and complexation of CpG ODNs with nanomaterials (Fig. 1.16). We discuss these approaches and their progress in the field.

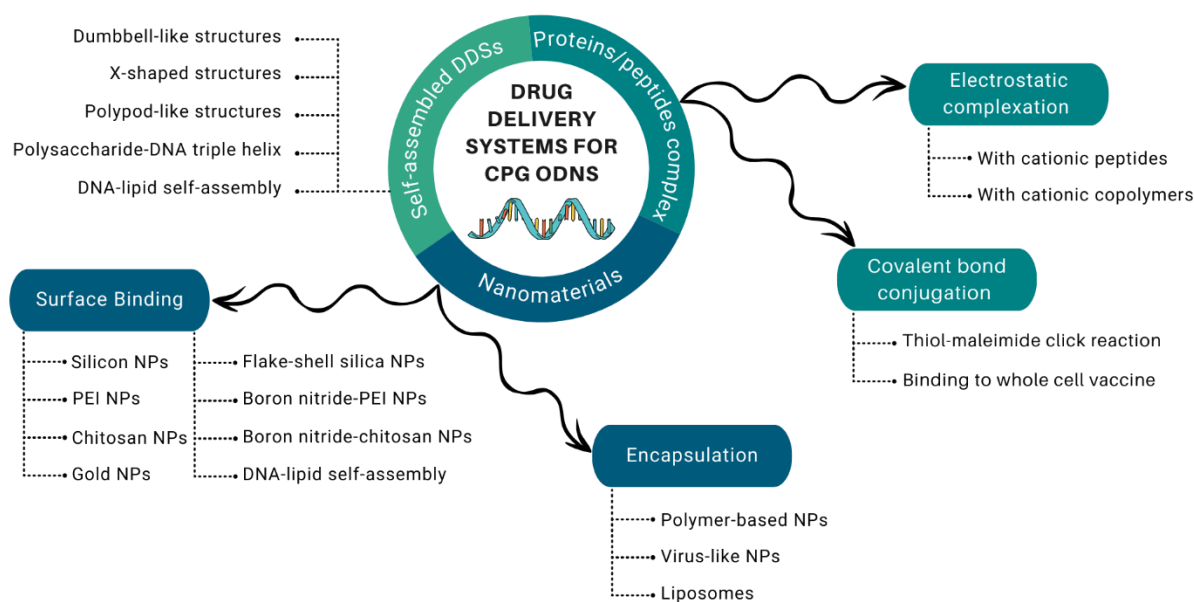


Fig. 1.16. Schematic presentation summarizing three different approaches used in the development of drug delivery systems for CpG ODNs. These include self. Assembled CpG ODN DDSs, conjugation/complexation of CpG ODN with proteins or peptides and CpG ODN complexation with nanomaterials.

Self-assembled CpG ODN DDSs

These systems include nanosized CpG ODN complexes that are assembled based on the complementary base pairing between DNA bases. This complexation resulted in higher resistance to DNases and improved cellular uptake⁶⁸⁵. Therefore, the use of the natural phosphodiester backbone was feasible as an alternative to using the PS modified backbone. This is advantageous since the PS-modified DNA was demonstrated to induced prolonged blood clotting time⁶⁸⁶⁻⁶⁸⁸, disruption of cell signalling⁶⁸⁹ and activation of the complement system^{690,691}. Furthermore, it could cause anaemia, neutropenia, thrombocytopenia and arthritis in animal models^{692,693}.

For instance, the ligation of two CpG ODN molecules to form a dumbbell-like structure with two ss-loops and one ds-stem resulted in the immunomodulator MGN1703 which is highly resistant to nucleases. MGN1703 could induce IFN- α , IL-6, IFN- γ , IL-12 p40, IL-2 and TNF production by PBMCs⁶⁹⁴. This mixture of cytokines and ILs indicated that MGN1703 could activate both cellular and humoral immunity. It was demonstrated that the immunostimulatory activity of MGN1703 was dependent on the CpG motif position, the loops' size and the stem's length. Phase I clinical trials confirmed the antitumor efficacy and safety, and determined the dose to be 60 mg twice a week⁶⁹⁵. Moreover, phase II clinical trial for the treatment of metastatic colorectal carcinoma showed significant improvement in progression-free survival⁶⁹⁶.

Another example is X-shaped DNA, composed of four CpG ODNs with an ACGT sticky end sequence to enable their self-assembly. This molecule resulted in higher expression levels of CD80 and CD86 in mouse bone marrow-derived primary dendritic cells (BMDCs) when compared to the nonself-ligated single X-DNA units. The co-administration of the self-ligated X-DNA and doxorubicin to mice with induced colitis-associated colorectal cancer resulted in a significant reduction in the number of colonic polyps larger than 3 mm was observed in comparison to the administration of doxorubicin alone⁶⁹⁷.

Self-assembled polypod-like structures composed of 3 to 8 CpG ODN molecules were investigated⁶⁹⁸. The secretion of proinflammatory cytokines, such as TNF and IL-6, was induced in macrophages cell line. The higher the number of ODNs in the polypod-like structure, the higher the level of stimulated cytokines. Furthermore, these polypod-like CpG ODNs could be used to form a hydrogel through increasing their concentration, increasing the solvent's salt concentration or associating them with other DNA strands⁶⁹⁹. The resulting hydrogels could be loaded with an antigen or allergen. For example, a CpG ODN hydrogel containing the Japanese cedar pollen allergen Cryj1 was demonstrated to be effective in treating or preventing pollen allergies⁷⁰⁰. Loading the CpG ODN hydrogel with ovalbumin (OVA) as a model resulted in OVA-specific CTL response⁶⁹⁹.

Similar strategy in using polypod-like structures was adopted to deliver lipid-modified CpG ODN to the lymph nodes. A polypod-like DNA with three pods was selected to provide the needed structure to bind to CpG ODNs modified with stearic acid. The resulting complex could bind more efficiently to plasma proteins and be taken up more efficiently by macrophages, when compared to complexes with non-modified CpG ODNs. SC injections in mice showed high IL-12 p40 production in the draining lymph nodes and low levels of IL-6 in plasma, indicating better targeting of the lymph nodes⁷⁰¹.

An additional example of a self-assembled CpG ODN system are hybrid nanoparticles formed by assembling polysaccharides and CpG ODNs, in a triple-helical complex composed of one molecule of CpG ODN and two molecules of soluble β -glucan schizophyllan (SPG). These nanoparticles were shown to induced both humoral and cellular immune responses in mice. Therefore, they were investigated as an adjuvant to influenza vaccines. The antibody production increased by more than a 100-folds compared to the standard vaccine alone. Moreover, the survival rate after a lethal viral challenge was 100% in mice that received both the vaccine and the adjuvant, and 10% only in mice that received the vaccine alone. Further studies confirmed the activity in cynomolgus monkeys.

Furthermore, DNA sequences modified with lipids can self-assemble into micelles, and CpG ODNs extended with complementary sequences can be loaded into the system. This DDS was administered *via* IV injection to mice and were taken up by DCs in the spleen. Activated DCs presented co-stimulatory molecules such as CD40/80/86 and MHC class I/II. Subsequently, pro-inflammatory cytokines such as IL-6, IL-12p40 and TNF were produced in dose-dependent manner⁷⁰². Furthermore, six routes of administration of this DDS were investigated, including oral route, IV injection, SC injection, intranasal, intramuscular injection and intraperitoneal injection. It was shown that SC and intranasal routes of administration could promote specific activation of CD8 α^+ DC in the lymph nodes, while IV injection promotes this activation in both the lymph nodes and the spleen. Using the SC route, leads to less CpG ODN in the systemic circulation, therefore fewer side effects would be expected⁷⁰³.

Similarly, the amphiphilic lipid-DNA complex was used to hybridize with both CpG ODNs and antigen peptide-bearing PNA (Fig. 1.17). The DDS exhibited efficient SC delivery of both the antigen and the immune adjuvant to CD8 α^+ DCs in the tumor draining lymph nodes *in vivo*. DC activation promoted strong antigen cross-presentation to CTL and inhibited tumor growth in melanoma and carcinoma models⁷⁰⁴.

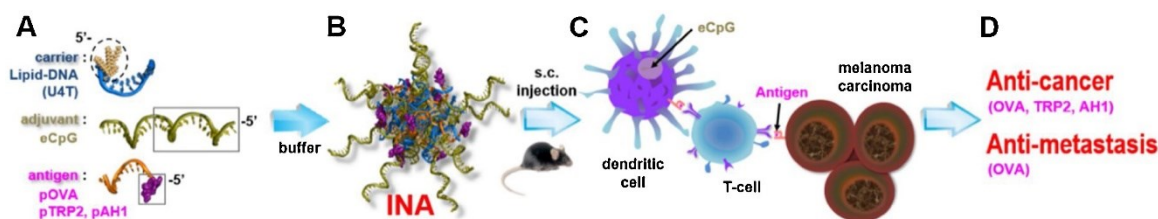


Fig. 1.17. A. Schematic representation of a drug delivery system based on self-assembly of lipid-modified DNA that consists of eight regular deoxyribonucleotides and four dodec-1-ynyluracil (U) nucleobases at the 5'-end. To allow its hybridization, CpG ODN was extended with 12 nucleotides complementary to the lipid-DNA. The antigen is ovalbumin peptide epitope (pOVA) that is covalently bound to peptide nucleic acid that is also complementary to the lipid-DNA. **B.** The components self-assemble in aqueous media to form loaded immunotherapeutic nucleic acid (INA). **C.** After subcutaneous injection into tumor mouse models, an antigen-specific immune response is triggered *via* DCs and CD8 α ⁺ cells. **D.** This results in successful therapeutic activity (Reprinted from⁷⁰⁴, Copyright (2019), with permission from Elsevier).

Conjugation/complexation of CpG ODN with proteins or peptides

Either covalent or non-covalent electrostatic interactions were used to conjugate CpG ODNs with a protein or peptide. The covalent bonding of CpG ODN with an antigen resulted in the uptake of the CpG ODN into the same APC which takes up the antigen as a vaccine. Chemically modifying the CpG ODN and the protein/peptide with a maleimide and a thiol group, respectively, enable their binding *via* click chemistry. For example, OVA–CpG-B ODN conjugates were obtained by reacting the maleimide-modified OVA with the thiolated-CpG ODN. Upregulated expression of MHC-II, CD86 and CD40, enhanced secretion of proinflammatory cytokines such as IL-12p40 and TNF and activated OVA-specific CTL responses were reported⁷⁰⁵. The latter was demonstrated to lead to reduced tumor size in mice transplanted with OVA-expressing tumor cells⁷⁰⁶. Another conjugate was prepared through using a thiolated-CpG ODN in combination with a N-succinimidyl 4-(maleimidomethyl)cyclohexanecarboxylate (SMCC)-modified Japanese cedar pollen allergen Cryj2 T-cell epitope peptide. An improvement in allergic symptoms was demonstrated when this conjugate was injected into mice that were subjected to a Cryj2 nasal challenge⁷⁰⁷.

In the context of cancer immunotherapy, the surface of apoptotic tumor cells (used as whole inactivated vaccines) was modified by CpG ODNs. When this cell-based vaccine was administered to mice, the expression of costimulatory molecules in DCs and secretion of immunostimulatory cytokines was induced, and tumor-specific CTL response was activated. This led to reduced tumor size and prevented metastatic spread⁷⁰⁸.

On the other hand, electrostatic bonds can be used to complex CpG ODNs with cationic proteins/peptides. The complexation of CpG ODNs with an HIV transactivator of transcription (Tat)-derived peptide (net charge of +8 mV) and a human cathelicidin derived-antimicrobial peptide (LL-37, net charge of +6 mV) was investigated. The resulting complexes have a zeta potential that depends on the molecular ratio of CpG ODNs to the cationic peptides. It was shown that the higher the positive charge density, the higher levels of IFN- α induction by PBMCs⁷⁰⁹.

Electrostatic complexation of CpG ODNs and positively-charged modified neoantigen peptide (Adpgk) derived from MC-38 colon carcinoma was investigated for its potential role in immunotherapy. The complex was shown to be taken up effectively by DCs and triggered their maturation *in vitro*. It could also activate CTLs *in vivo*. This DDS offered both prophylactic

effects in colorectal tumor challenge and therapeutic effect in established colorectal tumor model with significant prolongation of survival rate in mice⁷¹¹.

Additionally, cationic molecular bottlebrushes made from copolymers of 2-methacryloyloxyethyl phosphorylcholine and 2-methacryloyloxyethyl trimethylammonium chloride were fabricated to be complexed with CpG ODNs *via* electrostatic interactions (Fig. 1.18). The resulting DDS was shown to increase production of IL-6 and TNF in macrophages *in vitro*⁷¹⁰.

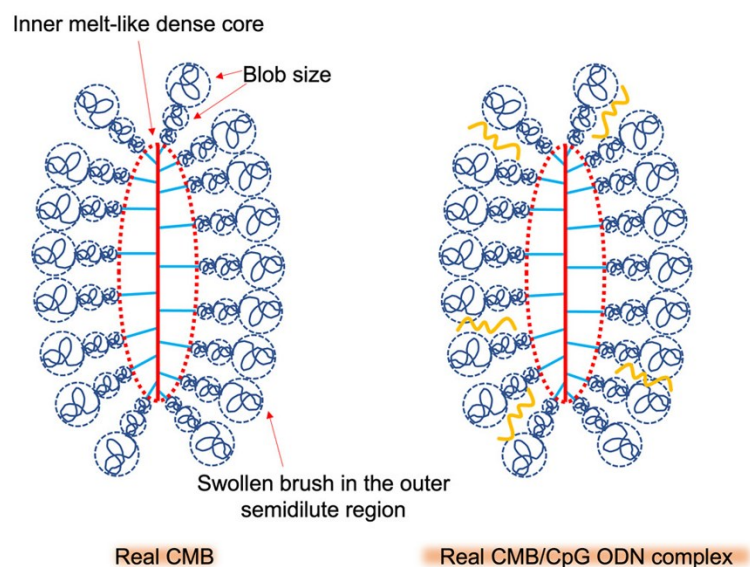


Fig. 1.18. Schematic illustrations showing CpG ODN drug delivery system based on the electrostatic complexation of CpG ODN with cationic molecular bottlebrush (CMB). **A.** Presentation of the CMB in which brush chains are spread from the backbone. **B.** Presentation of the CMB-CpG ODN complex in which the latter are trapped on the outer layer because of less crowding chains (Reprinted (adapted) with permission from⁷¹⁰, Copyright 2023 American Chemical Society).

CpG ODN complexation with nanomaterials

CpG ODNs can be either encapsulated within nanomaterials or bound to their surface. One of the candidate materials for CpG ODN formulation intended for clinical application is PLGA, since it's a polymer approved by the US FDA. CpG ODNs could be encapsulated inside PLGA particles using the double emulsion solvent evaporation method, and then coating the surface of the particles with the house dust mite allergen Der p2. Three different particles' size were obtained, namely 300 nm, 1 μm and 9.2 μm . All three types of particles were shown to inhibit airway hyperresponsiveness in mice models. However, the percentage of eosinophils in bronchoalveolar lavage fluids and the ratio of Der p2-specific IgG2a and IgG1 (IgG2a/IgG1) were higher with the smaller particles (*i.e.*, 300 nm and 1 μm). Conversely, the 9.2 μm particles resulted in higher Der p2-specific IgE titers. Therefore, it was concluded that the prophylactic effect on house dust allergy is dependent on the PLGA particles' size⁷¹².

When PLGA particles were encapsulated with both OVA and CpG ODNs, higher levels of OVA-specific IgG2a/IgG1 were observed with particles of 1 μm or less⁷¹³. The presence of antigens or allergens either on the surface or inside the PLGA particles did not affect the level of activation of Th1-biased responses.

PLGA particles with a mean diameter of ~ 500 nm were encapsulating OVA and CpG-B ODN and investigated for their preventative and therapeutic effects on E.G7-OVA tumors in mice⁷¹⁴. These PLGA particles were shown to have a preventative effect on tumor formation and a marked inhibition of tumor growth with high survival rate in intratumoral injection into tumor mice models. In the context of HBV vaccination, CpG ODNs were encapsulated in nanoparticles with a mean diameter of 120 nm, using PEG and polylactic acid (PLA) diblock copolymer with a cationic lipid named N,N-bis(2-hydroxyethyl)-N-methyl-N-(2-cholesteryloxycarbonyl aminoethyl) ammonium bromide⁷¹⁵. Administration of this DDS to

mice with the hepatitis B surface antigen led to significant induction of antigen-specific IgG2a, activation of the Th1-biased antibody response and upregulation of CTL CD69 expression. Additionally, they induced clearance of HBV in HBV-carrier mice.

Virus-like nanoparticles were also utilized to deliver CpG ODNs to investigate their effect on different diseases such as allergic asthma and melanoma. The bacteriophage Q β capsid protein was used to either encapsulate the CpG ODN and the antigen, or to bind the antigen to the surface of the particle *via* a crosslinker. Specific CTL response was demonstrated for this DDS in patients with stage I–IV melanomas⁷¹⁶. When the DDS was administered to patients with a house dust allergy, improvement in the symptoms of allergic rhinitis and asthma was observed and the allergen-specific IgG increased in these patients, while allergen-specific IgE decreased⁷¹⁷. Furthermore, the administration of the DDS without the allergen has shown a clear therapeutic effect on patients with house dust allergy⁷¹⁸, but not in patients with persistent moderate-to-severe allergic asthma⁷¹⁹.

In the prophylaxis and treatment of cancer, pH-sensitive liposomes that coencapsulate CpG ODN and TAA were investigated. This DDS was administered to mice three times at 10-day intervals, with inoculation of mouse hepatoma BNL-HCC cells 90 days after the initial dose⁷²⁰. Marked inhibition of the growth of the tumor cells and higher survival rates were demonstrated with the advanced administration of the vaccine. Similarly, tumor growth was inhibited and survival rate was improved in mice inoculated with colon cancer CT-26 cells after the administration of the liposomes three times at 7-day intervals⁷²¹. These findings suggest that this system induced long-term memory B cells and activated the production of antigen-specific antibodies. Furthermore, DC-based cancer vaccines were designed by assembling liposomes modified with mannose and CpG ODNs on their surface and melanoma-specific peptide in their core. This DDS was demonstrated to effectively inhibit tumor growth and improve survival rates in mice⁷²².

The surface charge of the liposomes influenced the adjuvant effect of CpG ODN⁷²³. Anionic liposomes induced the highest expression of CD86 in DCs present in the spleen of mice, compared to that obtained with cationic and neutral liposomes. Furthermore, the route of administration might play a role in the effects of liposomes encapsulating CpG ODNs⁷²⁴. The liposomes were administered using intranodal, intradermal, transcutaneous and nasal routes. The highest production of OVA-specific IgG2a was demonstrated with the intranodal and intradermal routes, while the lowest was produced with transcutaneous route.

Another liposomal DDS was developed to encapsulate and deliver CpG ODNs as monotherapy. Tumor growth was suppressed and survival rate was improved in mice *in vivo*. Biodistribution studies showed the accumulation of the DDS in tumor sites, indicating enhanced delivery and uptake⁷²⁵. On the other hand, a combination therapeutic approach was investigated by the co-administration of anti-PD-1 antibody with liposomes formulated with low doses of CpG ODNs and gp100 antigen. In treated mice, a significant four-fold increase of IFN- γ expression was observed in the tumor site. This resulted in the reversal of resistance to PD-1 blockade in a CD8⁺ T cell-dependent manner. The treatment group had remarkable tumor remission and prolonged survival rate⁷²⁶.

When CpG ODNs are bound to the surface of nanomaterials, the binding method affects the production of cytokines⁷²⁷. Silicon nanoparticles were used to bind either electrostatically or covalently to CpG ODNs. The electrostatic binding between positively charged modified nanoparticles and CpG ODNs induced IFN- α production from PBMCs. On the other hand, covalent binding of maleimide-modified nanoparticles with thiol-modified CpG ODNs resulted in pro-inflammatory cytokine induction, such as IL-6 and TNF.

Polyethyleneimine (PEI) was used to complex with CpG ODNs and then further lyophilized and adsorbed onto cryogel. This in turn was triggered with 20 kHz US to release CpG ODNs. The optimal timing of delivering CpG ODNs was demonstrated to be 4 days after injection, which aligned with the peak DC accumulation in the cryogel *in vivo*. This resulted in significantly higher antigen-specific CTL response and a significantly higher IgG2a/c antibody titer⁷²⁸.

Furthermore, flake-shell silica nanoparticles coated with PEI and CpG ODNs bound through electrostatic interaction induced IFN- α production from PBMCs⁷²⁹. Similarly, boron nitride nanoparticles coated with PEI and CpG ODN simulated IFN- α production⁷³⁰. On the other hand, boron nitride nanoparticles coated with chitosan and CpG ODN did not induce IFN- α production⁷³¹. Conversely, chitosan nanoparticles produced by ionic gelation method and loaded with CpG ODNs triggered IFN- α and IL-6 production in PBMCs⁷³². Further investigation of chitosan nanoparticles revealed that nanoparticles of larger molecular weight and higher degree of deacetylation exhibited a better CpG ODN binding and cellular uptake. Mannosylated chitosans with a substitution degree of 15% promoted material uptake through receptor-mediated recognition by macrophages and enhanced their secretion of IL-6⁷³³. On the other hand, proinflammatory cytokines such as TNF and IL-6 were induced when CpG ODNs were bound covalently to the surface of gold nanoparticles or boron nitride nanoparticles^{734,735}.

Binding of one end of the CpG ODN to the surface of the nanomaterial with disulfide bond that can be cleaved by the reductive condition within the endosome was investigated⁷³⁶. Thiol-modified CpG ODN was bound to pyridyl disulfide-functionalized poly(propylene sulfide) nanoparticles and administered to mice three times every two days. IgE production in the lungs and the eosinophil count were reduced after subjecting the mice to house dust mite challenge and a prophylactic effect was further reported

Single-walled carbon nanotube (SWCNT) were modified with phospholipid-PEG and sulfo-LC-N-succinimidyl-3-(2-pyridyldithio) propionate and further bound to one terminus of CpG ODNs *via* disulfide bonds. This system was investigated for its therapeutic effect on brain tumors⁷³⁷ in mouse models implanted with glioma cells. A greater inhibition of tumor growth and improved survival rate were demonstrated compared with the administration of free CpG ODN.

1.5 GENE REGULATION AND KNOCKDOWN

1.5.1 A BRIEF INTRODUCTION TO CELLULAR MECHANISMS FOR GENE REGULATION

The eukaryotic gene expression is a highly regulated process, demonstrated by the dramatic variation in organisms' different organs, tissues and cells, both in terms of structure and function. Given that all this diversity arose from a single cell (*i.e.*, the fertilized egg), gene regulation plays a critical role as an orchestrator that governs the precise functioning of cells in all living organisms. A spectrum of cellular mechanisms is meticulously designed to control gene expression at various levels, to ensure the accurate and timely synthesis of proteins needed for cellular activities. Scientists have been investigating these mechanisms, as crucial tools to unravel complexities of life and deciphering the potential underlying causes behind different diseases, as well as devising targeted therapeutic interventions at various levels.

Gene regulation operates at multiple levels of modulation (Fig. 1.19) that work together and form gene regulatory networks,⁷³⁸ which fine-tune gene expression, enabling cells to adapt and function appropriately under different conditions^{739,740}. These levels include transcriptional⁷⁴¹,

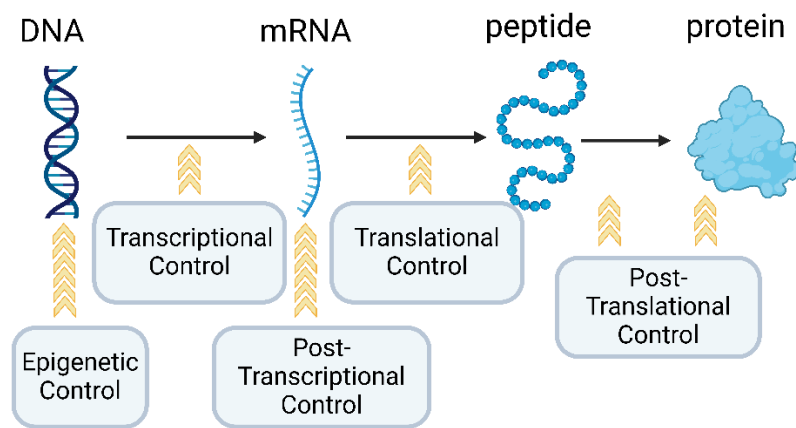


Fig. 1.19. Schematic summary of five different levels of gene regulation mechanisms. Created with BioRender.com

post-transcriptional^{742,743}, translational^{744,745}, post-translational⁷⁴⁶⁻⁷⁴⁸ and epigenetic^{749,750} regulations. All involved mechanisms work in a coordinated manner to ensure the precise regulation of gene expression in response to developmental cues, environmental stimuli and physiological needs.

At the level of transcriptional control, regulatory proteins⁷⁵¹ and non-coding RNAs⁷⁵² regulate the initiation, elongation and termination of RNA production from the genetic code. Post-transcription, several mechanisms contribute to regulating RNA molecules, *via* specific RNA-protein interactions that result in either targeted degradation of mRNA or preventing the access of ribosomes to the translation start codon, thereby inhibiting its translation⁷⁵³. A single gene can produce a diverse set of proteins, due to gene regulation at the post-transcription level through cellular mechanisms including alternative splicing⁷⁵⁴, RNA editing⁷⁵⁵ and RNA stability regulation⁷⁵⁶.

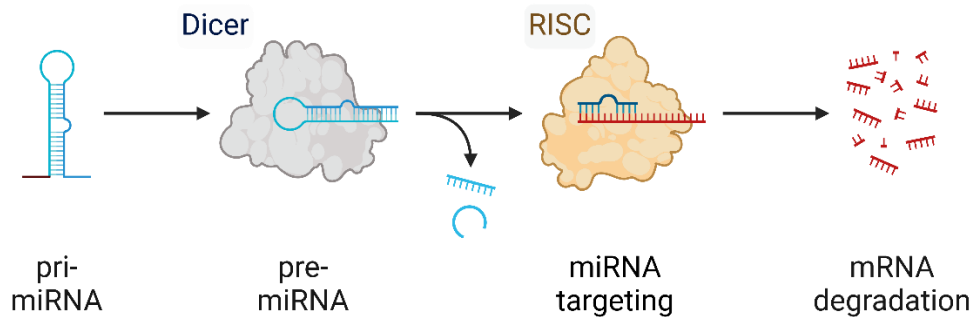
Translational gene regulation happens during protein synthesis and may occur at different steps along the process, with most frequent regulation taking place at the initiation phase⁷⁵⁷. Furthermore, different translation regulation mechanisms could affect either a single mRNA, a subset of mRNAs or most mRNAs translation. Another characteristic of translational control is that the alteration in physiological conditions could trigger multiple and redundant pathways that affect protein synthesis rate eventually. Moreover, single mechanisms might lead to limited minor changes in protein synthesis rate, but collectively influence the translation process significantly⁷⁴⁵.

Afterwards, gene regulation can still occur at the post-translational level⁷⁵⁸, either reversibly *via* post-translational modification (PTMs) of proteins, or irreversibly by proteolysis. These modifications control several core biological processes by modulating protein activity, structure, location and interactions. Finally, at the epigenetic and epitranscriptomic levels, modifications of DNA (usually methylation) or histone proteins affect the transcriptional output of RNA polymerase II⁷⁵⁹. Moreover, RNA chemical modifications (such as *N*⁶-methyladenosine (m⁶A)) are executed co-transcriptionally. These m⁶A sites can be recognized by nuclear or cytoplasmic reader proteins leading ultimately to changes in the localization, degradation or translation of mRNA⁷⁶⁰. The interplay between genetic and epigenetic factors convolutedly shapes the developmental trajectories of cells and tissues. The understanding and elucidation of the cellular mechanisms for gene regulation lead to invaluable insights into fundamental principles of life, paving the way for breakthroughs in genetics, molecular biology and medicine. We will highlight further RNAi as a gene regulatory mechanism in the following section.

1.5.2 RNA INTERFERENCE TO SILENCE GENE EXPRESSION

RNAi is a natural and evolutionary conserved biological process that enables selective gene silencing at the mRNA level⁷⁶¹. It was established in the late 20th century by Andrew Fire and Craig C. Mello through their work on the worm *C. elegans*⁷⁶², which earned them the Nobel Prize in Physiology or Medicine in 2006⁷⁶³. RNAi has emerged since then as a pivotal mechanism in the landscape of cellular genetic regulation processes.

A. miRNA Processing



B. siRNA Processing

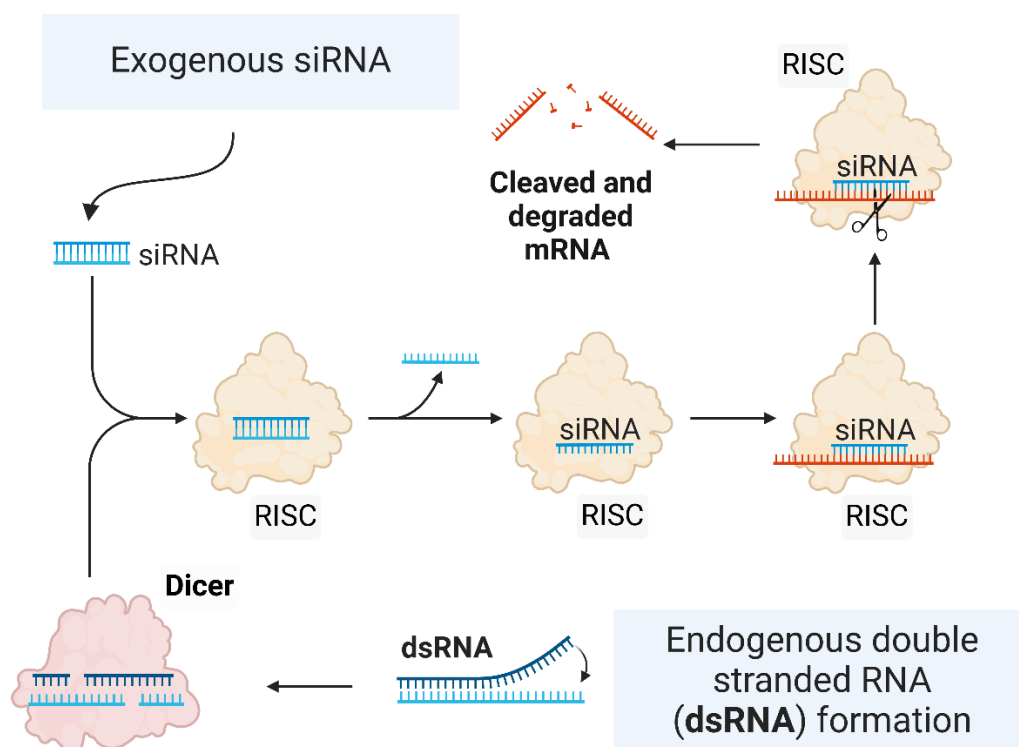


Fig. 1.20. Schematic presentation of the RNAi mechanism for the regulation of gene expression. **A.** miRNA processing pathway starts with endogenously transcribed primary miRNA (pri-miRNA) which is then processed into precursor miRNA (pre-miRNA) through microprocessor complex and is further processed into mature miRNA *via* Dicer. Mature miRNA binds to RISC complex and target mRNA with partial complementarity. **B.** siRNA source can be either endogenous or exogenous. Endogenous siRNA results from processing of dsRNA by Dicer. siRNA is recruited by the RISC complex, which then bind to the complementary mRNA sequence and result in its cleavage. Created with BioRender.com

This cellular mechanism not only regulates developmental and physiological processes, but also serves in defending cells against viral infections⁷⁶⁴. RNAi is initiated by short double-stranded RNA (dsRNA), approximately 22-nt long, named siRNA and miRNA. These dsRNA sequences have either exogenous or endogenous origin. Both pathways converge at the RNA-induced silencing complex (RISC) leading ultimately to prevention of protein translation⁷⁶⁵ (Fig. 1.20).

Differences between miRNA and siRNA can be found in several aspects⁷⁶⁶. For instance, they differ in their biogenesis. miRNA are endogenously encoded in the genome and they are first transcribed as long primary miRNA (pri-miRNA), then processed in the nucleus into precursor miRNA (pre-miRNA) by the Drosha-DGCR8 complex. After that, they are transported to the cytoplasm where they are further cleaved by Dicer, producing the mature miRNA. On the other hand, siRNA can have either an exogenous source, such as viral or synthetic siRNA, or an endogenous source, such as transposons and long dsRNA. In the latter, these sequences are also cleaved in the cytoplasm by Dicer into functional siRNA.

Additionally, siRNAs bind to their target mRNA sequences with perfect base-pairing, resulting in highly specific gene regulation. siRNA-RISC complex lead to target mRNA degradation⁷⁶⁷. On the other hand, miRNA-RISC complex scans the mRNA sequence for potential complementarity and binds to the 3'-untranslated region (UTR) typically with imperfect base-pairing, blocking ribosomes' access to mRNA rather than cleaving it. As a result, miRNA often regulates multiple target genes⁷⁶⁸.

1.5.3 siRNA AS THERAPEUTIC AGENTS

The specificity of siRNA-gene interaction enables it theoretically to silence any disease-related gene, thereby rendering it as a promising therapeutic modality⁷⁶⁹. It is often less complicated and invasive than targeting the genetic code, and can be used for undruggable targets. Furthermore, by targeting mRNA rather than proteins, siRNA therapy circumvents the challenges of cross-reactivity and resistance often encountered in different treatments for various conditions⁷⁷⁰. Besides its therapeutic potential, scientists have used siRNA in functional genomic studies, in which specific manipulation of gene expression enables the elucidation of the roles of specific genes in biological pathways and the underlying disease mechanisms.

The field of siRNA therapeutics has experienced many ups and downs. After a 20-year journey of research and development⁷⁷¹, Onpatro™ (patisiran) became the first commercially available RNAi-based therapeutic to be approved by US FDA and the European Commission (EC) in 2018, for the treatment hATTR amyloidosis with polyneuropathy in adults. More recently, Givlaari™ (givosiran) was approved by the US FDA in 2019⁸¹ and by the EC in 2020⁷⁷², for the treatment of adults with acute hepatic porphyria (AHP). Three more siRNA therapeutics followed: Oxlumo™ (lumasiran) in 2020⁷⁷³, Leqvio™ (inclisiran) in 2021⁷⁷⁴, and Amvut™ (vutrisiran) in 2022⁷⁷⁵.

Although siRNA is promising in drug development, various intra- and extracellular barriers limit its widespread clinical application. Unmodified siRNA suffers from high instability and a poor PK profile, due to its rapid degradation by RNases, nucleases and phosphatases⁷⁷⁶. It can also induce off-target effects by the activation of toll-like receptor 3 (TLR3)⁷⁷⁷. To overcome these challenges, researchers have investigated different chemical modifications and developed several delivery systems, aiming to improve siRNA stability, efficacy, specificity and safety.

1.5.3.1 Chemical Modifications

Unmodified and slightly modified siRNA that were developed at the early stages of siRNA therapeutics could successfully knockdown target genes when administered locally to the target tissue⁷⁷⁸. However, serious side effects were observed, such as induction of IL-12 and IFN-γ

secretion⁷⁷⁹. Subsequently, a wide range of chemical modifications have been established and tested to enhance potency and minimize side effects of siRNA therapeutics. These modifications can be classified into three classes, phosphonate modifications, ribose modifications and base modifications, based on their site in the nucleotide. Typically, these modifications are used in combinations in siRNA.

At the backbone level, one non-bridging oxygen of the phosphodiester was replaced with a sulfur atom to introduce a PS linkage has been heavily investigated in other TNA classes⁷⁸⁰. This renders the molecule more hydrophobic and the bond more resistant to nucleases and can elongate circulation time by binding of the TNA to plasma proteins such as albumin⁷⁸¹. Nevertheless, some studies have shown that the affinity of the TNA towards its target might be reduced and high PS content could aggravate chemistry-related toxicity⁷⁸². Therefore, the number of PS bonds and their positions along the backbone is critical for safe application. Since the PS bond has a chiral sulfur, two configurations are possible, namely the R and S isomers. The stereochemistry of the PS was shown to significantly influence TNA's stability with S configuration being more stable⁷⁸³. Although controlling PS chirality could markedly affect their interaction with RNaseH1, it failed to significantly change the overall therapeutic effect of TNA⁷⁸⁴.

Replacing the phosphodiester group with other residues was also investigated. These include phosphorodithioate (PS2)⁷⁸⁵, methylphosphonate (MP), methoxypropylphosphonate (MOP) and PNA⁷⁸⁶. PS2 could increase the affinity of siRNA towards RISC, while MP and MOP could reduce excessive protein binding and therefore ameliorated hepatotoxicity⁷⁸⁷. Moreover, modifications of the 5'-phosphate which is needed for RISC binding was investigated aiming to find analogues that have similar conformation and electronic properties but are resistant to dephosphorylases that can remove the natural 5'-phosphate in cells. The most potent and metabolically stable analogue was found to be the 5'-(*E*)-vinyl phosphonate (5'-(*E*)-VP) decoration⁷⁸⁸. Using this modification, ideal PD and PK profiles were demonstrated for ss-siRNA, and improved accumulation and potency of ds-siRNA was also revealed^{789,790}.

At the ribose site, modifications at the 2'-position have been extensively used to remove the 2'-OH group that is otherwise attacked by ribonucleases leading to siRNA hydrolysis. The most frequent modification in this context is the use of 2'-OMe⁷⁹¹ that was demonstrated to enhance both stability and affinity of siRNA. The 2'-O-MOE is an analogue of 2'-OMe and has shown even a higher binding affinity of siRNA³⁹.

Fluorine substitutions are also among popular ribose modifications. They include 2'-deoxy-2'-fluoro (2'-F)⁷⁹² and 2'-arabino-fluoro (2'-Ara-F)⁷⁹³. siRNA modified with the highly electronegative fluorine adopts a C3'-endo conformation that positively impacts its binding affinity⁷⁹⁴. Other 2'-position substitutions involve 2'-O-benzyl and 2'-O-methyl-4-pyridine.

Modifications to the whole sugar ring resulted in a series of molecules including unlocked nucleic acids (UNAs)⁷⁹⁵, LNAs⁷⁹⁶, glycol nucleic acids (GNAs)⁷⁹⁷ and PMOs. While LNAs offer structurally rigid strands, UNAs have unconnected 2' and 3' carbons, resulting in higher flexibility and asymmetry of the duplex siRNA that leads to better loading of RISC. GNA and LNA siRNAs were shown to have less hepatotoxicity and more binding affinity, respectively. PMOs have their ribose unit replaced entirely with a morpholine structure that is uncharged at physiological pH and resistant to RNase H⁷⁹⁸.

Finally, at the nitrogen base site, several modifications have been investigated and shown to be beneficial in the context of drug development. Higher resistance to nucleases and less immunogenicity was shown by replacing uridine and cytidine with pseudouridine⁷⁸, 2-thiouridine⁷⁹⁹, N6-methyladenosine, 5-methylcytidine⁴³ or other base analogues.

Alternatively, the natural bases can be modified with methyl groups such as 5 methylcytosine and 6 methyladenosine.

N-ethylpiperidine triazole-modified adenosine analogues⁸⁰⁰ can pair well with uridine and were shown to disrupt siRNA interaction with toll-like receptor 8 (TLR8) and reduced its immunogenicity⁸⁰¹. Analogues of cytosine such as 6'-phenylpyrrolocytosine (PhpC)⁸⁰² demonstrated excellent base pairing fidelity and thermal stability. It has fluorescent properties that enable its use for fluorescence-based detection methods or for studying the cellular uptake and trafficking of siRNA in cells.

After extensive research aimed at comprehending the impact of diverse modifications on siRNA activity, specificity and toxicity, scientists have concluded that substantial alterations, including full-length modifications, are unlikely to impair siRNA activity. Meanwhile, these carefully designed modification approaches have been found to markedly improve siRNA stability and biocompatibility. Consequently, a variety of interconnected modification patterns have been proposed and validated in preclinical and clinical settings, paving the way for advanced therapeutic applications of siRNA. Examples include a universal modification pattern known as standard template chemistry (STC). This STC design was used in revusiran, which was Alnylam's second candidate against ATTR, and showed to maintain siRNA activity while remarkably enhancing its stability and affinity⁸⁰³. However, the clinical investigation of revusiran was discontinued due to unbalanced deaths between the treatment and placebo groups. Although the investigation report demonstrated that deaths in the revusiran treatment group were not correlated to the drug, strong concerns remain regarding STC-modified siRNA⁸⁰⁴.

The following generation of modifications patterns was called enhanced stabilization chemistry (ESC)⁸⁰⁵. This design includes four more PS bonds at the 5'-end of the antisense strand and the 3'-end of the sense strand, as well as reduced 2'-F modifications. This design enabled significant dose reduction combined with potent gene silencing that could last more than one year after single dose administration in phase 1/2 clinical study, marking an unprecedented milestone in the history of pharmaceutical therapy⁸⁰⁶. In addition, the approved drug Givlaari™ was designed based on the ESC pattern⁸⁰⁷. Furthermore, the design variants (DVs) of the ESC strategy were optimized across multiple siRNA using an iterative screening approach⁸⁰⁸. As a result, advanced ESC designs such as DV18 and DV22 have emerged. In these patterns, six PS bonds are maintained at three terminals, while 2'-F content is significantly reduced. These changes demonstrated higher efficacy and specificity of siRNA.

Conjugation of siRNA to *N*-acetylgalactosamine (GalNAc) is another popular modification. However, it can lead to off-target gene silencing in the liver⁸⁰⁹. Attempting to overcome this side effect, a GNA could be placed at the position 7 of the antisense strand⁸¹⁰. This strategy is the main characteristic of ESC+ design, which is currently being used for several investigational siRNA therapeutics.

1.5.3.2 Efficient and targeted delivery systems for siRNA therapies

Although the challenges of siRNA stability against nucleases and its immune recognition in the blood circulation have been substantially improved through chemical modifications, other barriers against successful gene silencing *via* siRNA need to be circumvented⁸¹¹. These are related to siRNA accumulation in the desired tissue, effective transmembrane trafficking and reaching its target at the cytoplasm while escaping endosomes and lysosomes⁴¹.

The molecular size of siRNA ranges between 7 to 8 nm in length, and 2 to 3 nm in diameter, rendering it small enough for glomerular filtration but too large for crossing cellular membranes⁸¹². This results in short life span in the systemic blood circulation and low levels of accumulation in target tissue⁸¹³. Furthermore, siRNA molecular weight ranges between 13 to

16 kD approximately, which is relatively high. Along with its net negative charge, these properties prevent artificial siRNA from crossing cell membranes efficiently. Therefore, DDSs can help siRNA to overcome these barriers and exert its effect^{814,815}.

Strategies for drug delivery of the siRNA drugs approved by the US FDA include using lipid nanoparticles (LNPs) in the case of patisiran, and GalNAc–siRNA conjugates in the other four commercial drugs (*i.e.*, givosiran, lumasiran, inclisiran and vutrisiran).

Several siRNA therapeutics are investigated in clinical trials, and much more are under research and development. For those, various delivery systems have been reported including DPCTM, TRiMTM, LODERTM polymers, exosomes and polypeptide nanoparticles (PNPs). Naked siRNA (*i.e.*, siRNA not associated with any delivery system covalently or noncovalently) can be used for targeting kidney-expressed genes, as they will be naturally filtered to the kidneys⁸¹⁶. Alternatively, naked siRNA can be locally injected in certain target organs such as the eye⁸¹⁷.

Lipid-based delivery platforms

The use of LNPs for siRNA has been on the rise, as they can protect their siRNA cargo and transport them to target tissues and cells⁸¹⁸. Common components are found across different LNPs, such as cationic or ionizable lipids, 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), cholesterol and PEG-lipids. After IV injection, siRNA-LNPs predominantly accumulate in the liver, spleen and kidneys.

LNPs are classified according to their surface charge into ionizable, cationic and neutral LNPs. Furthermore, LNPs use the EPR effect to their advantage to deliver siRNA into tumors⁸¹⁹. Additional active targeting can be achieved by modifying LNPs surfaces to target either the tumor cell surface or their ECM. These tumor-targeting moieties can be antibodies, proteins⁸²⁰, peptides⁸²¹, aptamers⁸²² or small molecules⁸²³.

Patisiran, a commercially available siRNA therapeutic, is formulated in liposomes. The lipid components involved in the formulation are DLin-MC3-DMA, DSPC, cholesterol and R-2,3-bis(octadecyloxy)propyl-1-(methoxy PEG 2000) carbamate (PEG-DMG) at a molar ratio of 50:10:38.5:1.5⁸²⁴. Other siRNA therapeutics with LNP formulation under clinical investigation include TKM-080301, which targets polo-like kinase 1 (PLK1) for the treatment of solid tumors. Eighteen cycles of treatment, four weeks each, have demonstrated tumor reduction, but also some serious adverse effects⁸²⁵.

Polymer-based delivery platforms

Another widely investigated siRNA delivery platform is based on polymer backbones⁸²⁶. For instance, DPCTM delivery system contains a polymer composed of butyl and amino vinyl ether attached reversibly to siRNA by disulfide linkages that can be degraded by glutathione. Furthermore, PEG and a targeting ligand are linked to the polymer alternatively *via* carboxylated dimethyl maleic acid bonds. These bonds would be cleaved in acidic pH in endosomes and lysosomes, exposing the positively charged polymer backbone that triggers H⁺ and Cl⁻ influx to elevate the endosomal osmotic pressure. This in turn drives water inflow to the endosome causing its swelling and destabilization, in a phenomenon known as proton sponge effect⁸²⁷. In the second generation DPC delivery platforms, siRNA is conjugated with cholesterol⁸²⁸. However, clinical trials of siRNA developed using this technology were stopped. One candidate that was designed for the treatment of chronic HBV showed high efficacy but resulted in the death of one animal during the long-term toxicity studies⁸²⁹. Other candidates with similar technology were transferred to a different delivery platform, namely the targeted RNAi molecule (TRiMTM).

In TRiMTM, a targeting ligand is conjugated to siRNA directly *via* covalent bonds⁸³⁰. These targeting moieties could be GalNAc, arginylglycylaspartic (RGD) motifs (which are ligands of

integrin $\alpha\beta3$ and $\alpha\beta5$) and the $\alpha\beta6$ ligand, which aim to transport siRNA to hepatocytes, cancer cells and lung epithelial cells, respectively. This approach aims to achieve high levels of accumulation in target tissue and reduced *in vivo* toxicity. Candidates formulated using this platform are under clinical investigation, for the treatment of alpha-1 antitrypsin (AAT) deficiency⁸³¹ and HBV⁸³². Early results have reported good tolerance and high gene knockdown efficacy.

GalNAc-siRNA conjugates

Although liposome-based siRNA formulation became available in the market, GalNAc-siRNA conjugation became the main delivery platform that was used for subsequent siRNA therapeutic development⁸³³, due to its higher potency and safety profile for liver-targeted siRNA delivery⁸⁰⁸. GalNAc is the ligand of a highly specific receptor on the cell membrane of hepatocytes called asialoglycoprotein receptor (ASGPR)⁸³⁴. It is expressed at high levels by hepatocytes (approximately 500,000 copies per cell) and barely expressed by other cells⁸³⁵. Binding to this surface protein leads to mediated endocytosis that delivers the conjugated ligand to the cytoplasm⁸³⁴. Studies showed that tri- and tetravalent ligands spaced at about 20 Å exhibit higher affinity towards ASGPR. Thus, siRNA was conjugated to tri- or tetravalent GalNAc moieties by covalent linker⁸⁰. This route of cellular uptake is hypothesized to be safer than the interaction of liposomes with cellular membrane. SC administration of GalNAc-siRNA showed better performance than IV route⁸⁰. Furthermore, combining this delivery platform with ESC or ESC+ pattern of chemical modifications of siRNA resulted in an extremely long duration of action, leading to less frequent dosing which could be twice or four times per year⁸⁰⁸. Alternative analogues that can bind to ASGPR have been developed as well⁸³⁶. Furthermore, other propriety RNAi delivery platforms are utilized in a variety of therapeutic pipelines⁸³⁷.

In conclusion, the diverse landscape of siRNA delivery systems highlights the progress made in addressing the challenges associated with its efficient use. While substantial advancements have been achieved, it is evident that there remains considerable room for exploration and improvement. In the context of this thesis research project, we will contribute to this ongoing dialogue by discussing the use of our developed drug delivery system for siRNA delivery. The quest for enhanced specificity, reduced toxicity, and improved delivery efficiency necessitates sustained efforts in unravelling the complexities of siRNA delivery. Continued investigations and innovations in this field will undoubtedly contribute to the development of safer and more effective siRNA delivery platforms for therapeutic applications, paving the way for transformative advances in RNAi-based therapeutics.

2. Mechanoresponsive DDS for controlled Immunostimulation

2.1 INTRODUCTION

US is a superb external trigger to be exploited in combination with active pharmaceutical ingredients (APIs) and DDSs or to control cellular behaviour. It has a spatial and temporal resolution of several hundred micrometres in the millisecond range with high penetration depth into tissue of more than 10 centimetres. Furthermore, it has been used in the clinic for decades for diagnostics and therapy with an excellent safety record³³⁹. The previous approaches towards using US as a trigger to control drug delivery either utilized the thermal or the mechanical effects of US. In the latter, either high-energy US is needed to induce mechanochemical transformations leading to activation of drugs, or microbubbles were used to sensitize the DDS towards low-energy US devices. To overcome these limitations, we aimed to develop a mechanoresponsive DDS, independent of local heating of tissue, that can be activated by low-intensity ($I_{spta,3} \leq 720 \text{ mW/cm}^2$)⁸³⁸⁻⁸⁴⁰ medical US at high frequency (7 MHz) commonly used for clinical imaging, without the administration of sono-adjuvants such as exogenous microbubbles.

The carrier consists of a DNA-inorganic hybrid nanoparticle system composed of high molar mass deoxy polynucleic acids and magnesium pyrophosphate ($\text{Mg}_2\text{P}_2\text{O}_7$), generated through RCA^{176,178,841,842}. The DDS is tailored to protect and deactivate its cargo and release it in the active form upon ultrasonication. We characterized the system to understand its mechanoresponsive properties and investigated its potential applications in both sonopharmacology and sonogenetics, which will be discussed in this chapter and the next one, respectively.

For sonopharmacology, we utilized US-controlled DDS to deliver CpG ODNs as a cargo, which activate TLR9^{631,635,843} and trigger the innate immune response. Type B CpG ODNs have shown TLR9 agonist activity across different species and antitumor activity in breast cancer mouse models⁸⁴⁴. CpG ODNs stimulate TLR9 in DCs, leading to their maturation and the expression of chemokine receptors and immunostimulatory cytokines. These in turn can activate T cells, inducing their proliferation and subsequent production of chemokines such as granzymes, perforins, IFN- γ and TNF. The resulting CTLs exert antitumor activity leading to cancer cells apoptosis⁶²⁰. We demonstrated our DDS's function *in vitro* utilizing both conventional cell culture and a sophisticated triculture model based on human primary immune cells and a melanoma cancer cell line. Furthermore, we successfully demonstrated on-demand US-mediated delivery of CpG ODN *in vivo* in mice model.

2.2 METHODS

Materials and reagents

All oligonucleotides were purchased from biomers.net GmbH (Germany); the sequences are provided in Supplementary Table 1. Diethyl pyrocarbonate (DEPC) (CAS 1609-47-8, Acros Organics) was used to treat water to deactivate DNase and RNase. T4 ligase (EL0011) and dNTPs (100 mM, R0182) were purchased from Thermo Scientific. NxGen® Phi29 DNA polymerase was purchased from Lucigen (30221-2). Emprove® essential Ph Eur, BP, NF Gelatin (Merck, 1.04078.1000) was used to prepare gelatin casts. Agarose standard (Roth, 3810.4) was used to prepare agarose gels in TBE buffer. The latter was prepared from Tris base (CAS 77-86-1, Fisher Bioreagents), boric acid (CAS 10043-35-3, Fluka) and ethylenediaminetetraacetic acid disodium salt dihydrate (CAS 60-00-4, Sigma). Roti-GelStain

Red (Carl Roth, 0984.1) was used to stain nucleic acids in the agarose gels. TriTrack DNA Loading Dye (6X) was used to load samples into agarose gels (Thermo Scientific, R1161) and GeneRuler 1 kb Plus DNA Ladder (Thermo Scientific, SM1331) was used as a reference. TrypLE™ Express Enzyme (1X) (Gibco, 12604013) and 1X PBS (1.7 mM KH₂PO₄, 5mM Na₂HPO₄, 150 nM NaCl, pH 7.4, Lonza) were used in maintenance of cell cultures.

DNA nanocarrier design and production

RCA was used to produce long ssDNA polymers. The resulting strand has tandem repeats of the complementary sequence of the used circular ssDNA template. The template was designed to have binding sites in the final RCA polymer, that hybridize to the loaded TNA cargo. All sequences are provided in Supplementary Table 1. Circular ssDNA templates needed for RCA were obtained by pad-lock ligation of linear ssDNA with a primer as described before¹⁷³. DEPC-treated water was prepared by incubating 0.1% DEPC in water at 37°C overnight followed by autoclavation at 120°C for 20 min and used for all reactions. Ligation reaction components are 20 µM ssDNA, 10X T4 ligase reaction buffer (400 mM Tris-HCl, 100 mM MgCl₂, 100 mM DTT, 5 mM ATP, pH 7.8) and 30 µM primer in a final volume of 100 µL. The mixture was heated to 95°C for 3 min, followed by gradual cooling to 16°C. 5 µL T4 ligase were added and incubated overnight. The enzyme was deactivated by heating at 65°C for 10 min. For the RCA reaction, 20 µL of the ligated template were added to phi29 DNA polymerase reaction buffer (10X: 500 mM Tris-HCl, 100 mM (NH₄)₂SO₄, 40 mM dithiothreitol, 100 mM MgCl₂), 20 µL of dNTPs (100 mM) and 2 µL phi29 DNA polymerase enzyme. The reaction was incubated at 37°C for 1.5 h. Then CpG ODN was added at a final concentration of either 2 µM or 20 µM and the incubation continued overnight.

Loading efficiency of CpG ODN

FAM-labelled CpG ODNs were fed into the RCA reaction. Fluorescent hydrogel was observed under the UV lamp at $\lambda = 365$ nm. The gel was precipitated by centrifugation at 12,000 rpm for 15 min, washed three times to remove any excess oligonucleotides. The excess labelled oligomer was quantified using absorbance spectrophotometer (SpectraMax iD3, Molecular Devices, USA). The loading efficiency was calculated based on the known starting amount of CpG ODN using the best-fit linear regression model of calibration curve of the pure corresponding oligomer.

Pulse-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) is a technique used for the separation of large DNA molecules by applying an electric field that periodically changes direction to a gel matrix containing the DNA samples. The agarose gel (1% w/v) was prepared and run in 0.5X TBE buffer at 6 V/cm, switch time of 60-120 sec and an angle of 120°. The running buffer was maintained temperature of 14 °C using the cooling unit and run time was varied between 24 to 72 h. Post-staining was performed using SYBR™ Gold (Thermofisher Scientific, S11494) following the manufacturer's instructions.

Ultrasound-triggered mechanochemical cargo release

For ultrasonication experiments at $f = 20$ kHz, a Qsonica Q125 sonicator (USA) equipped with a 3 mm diameter microtip probe (A12628PRB20) was used (Fig. 2.1a). Sonication was performed using pulsed ultrasound (2.0 s on, 1.0 s off at 50% Amplitude) and an ice bath was utilized to prevent heating of the samples. The input power level was adjusted around 10 W/cm².

For ultrasonication with imaging US, a 20% gelatin cast with appropriate mould was prepared. The samples to be sonicated were transferred into the wells of the gelatin cast. US was applied on the top of the liquid sample using Lumify Ultrasound System with a L12-4 broadband linear array transducer (12 to 4 MHz extended operating frequency range, aperture size 34mm,

Philips), which is used by healthcare providers as an ergonomic and mobile US imaging device for especially time sensitive situations (Fig. 2.1b). US device was used in a predefined vascular mode (MI=0.9, 5 frames per second) for the duration of 15 min. The sample was transferred for downstream protocols.

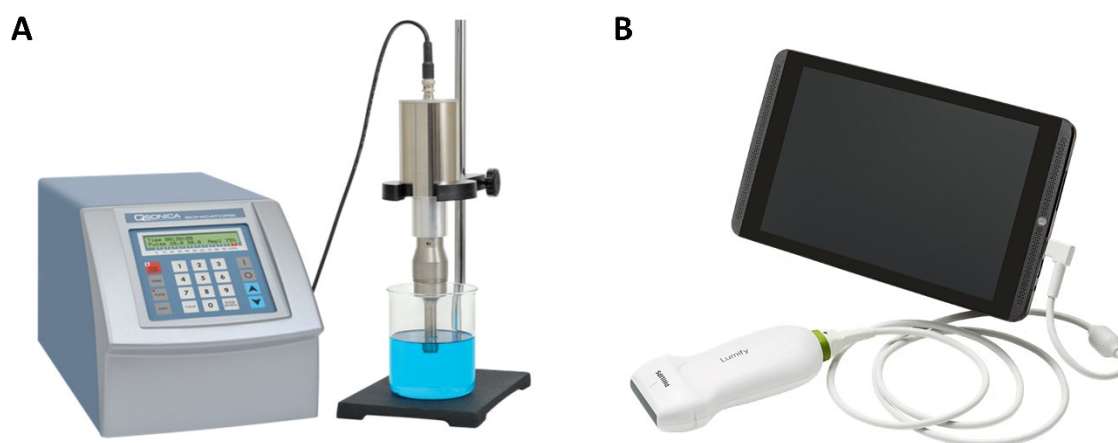


Fig. 2.1. Ultrasound devices used in this work. **A.** One-tip horn Q500 Sonicator® (Qsonica, USA) that operates at US frequency of 20 kHz. **B.** Lumify L12-4 (Philips, USA) broadband linear transducer used for clinical imaging at point-of-care. Ultrasound application (Philips Lumify Ultrasound App) is needed for the operation of the portable L12-4 broadband linear transducer.

Scanning electron microscopy

Samples were fixed in 3% glutaraldehyde in 0.1 M Sorenson's buffer (pH 7.4) at room temperature for 1 h. They were rinsed with 0.2 M sodium phosphate buffer (pH 7.39, Merk) and dehydrated in 30, 50, 70, and 90% acetone, and then three times in 100% acetone, for 10 min each. After critical point drying in CO₂ (E3100, Quorum Technologies), the samples were sputter-coated (Leica EM ACE600) with a 5 nm gold–palladium layer. Images were captured using a high-resolution SEM (Hitachi SU9000) with an acceleration voltage of 10 kV. Particle size analysis was done by measuring particles' diameter using ImageJ 1.50i software.

Polarized light microscopy

The samples were placed on a silicon wafer with a cover slip. The textures and birefringence of the mesophase were observed in a reflection mode using a polarized optical microscope Axioplan 2 (Zeiss, Germany) equipped with an AxioCam MRc color camera (1300*1030 pixels resolution).

Rheology

To measure the mechanical properties of the RCA product, Discovery Hybrid Rheometer (DHR)-3 (TA Instruments, USA) was used with steel 1.99° cone 20 mm Peltier plate geometry (p/n 115082) at 25°C. A dynamic frequency sweeping mode was used, with the strain fixed at 0.1% and frequency increasing from 0.01 to 100 Hz. Furthermore, a dynamic oscillation sweeping mode was used, with the frequency fixed at 1 Hz and % strain increasing from 0.1 to 1000%. The same rheometer and geometry were additionally used to measure the viscosity of DNA hydrogels with a shear rate ($\dot{\gamma}$) logarithmic sweep from 1e-3 to 2000 1/s, then plotted against viscosity η (Pa·s) with a best fit model. All data from DHR-3 were processed and analysed using TA Instruments TRIOS software (v5.1.146572).

Measurement of acoustic pulses

Lumify Ultrasound System with a L12-4 transducer was used in a predefined vascular mode (MI=0.9, frame rate 25Hz). The transducer was vertically dipped ~2 cm into DI water of a 150L acoustic test tank. A sensor of Fibre-Optic Hydrophone System (Precision Acoustics Ltd.) was mounted 1cm below the center of transducer face. An oscilloscope DSOX3024A (Keysight) recorded the output signal of Hydrophone System. The lateral position of sensor and the trigger settings of oscilloscope were optimized to detect the maximum amplitude of acoustic pulses.

Cell culture

The mouse melanoma B16F10, macrophages J774A.1 and human adenocarcinoma HeLa cells were purchased from the American Type Culture Collection (ATCC). All three cell lines were maintained in DMEM medium (Gibco, 41966052) supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 µg/mL) at 37 °C in 5% CO₂. HEK-Blue™ mTLR9, HEK293 null, and HEK-Blue™ TNF cells were purchased from Invivogen. HEK-Blue™ mTLR9 cells overexpress the mouse TLR9 (mTLR9) gene. In addition, they express a secreted embryonic alkaline phosphatase (SEAP) reporter gene under the control of an NF-κB inducible promoter comprised of an IFN-β minimal promoter fused to five NF-κB and AP-1 binding sites. They were maintained in DMEM medium supplemented with glucose (4.5 g/L), L-glutamine (2 mM), 10% (v/v) FBS, penicillin (100 U/mL), streptomycin (100 µg/mL), Normocin™ (100 µg/mL, Invivogen, ant-nr-1), Blasticidin™ (30 µg/mL, Invivogen, ant-bl-05), and Zeocin™ (100 µg/mL, Invivogen, ant-zn-05) at 37 °C in 5% CO₂. HEK-Blue™ Null1 is the parental cell line for the HEK-Blue™ mTLR9 and HEK-Blue™ TNF. These cells very weakly express mTLR9 but express the SEAP reporter gene under the control of the IFN-β minimal promoter fused to five NF-κB and AP-1 binding sites. HEK-Blue™ Null1 cells were maintained in DMEM medium supplemented with glucose (4.5 g/L), L-glutamine (2 mM), 10% (v/v) FBS, penicillin (100 U/mL), streptomycin (100 µg/mL), Normocin™ (100 µg/mL) and Zeocin™ (100 µg/mL) at 37 °C in 5% CO₂. HEK-Blue™ TNF cells express the two receptors that bind to TNF, TNFR1, and TNFR2. They are also transfected with the SEAP reporter gene under the control of the IFN-β minimal promoter fused to five NF-κB and five AP-1 binding sites. The cells were rendered unresponsive to IL-1β by knocking-out the MyD88 gene. They were maintained in DMEM medium supplemented with glucose (4.5 g/L), L-glutamine (2 mM), 10% (v/v) FBS, penicillin (100 U/mL), streptomycin (100 µg/mL), Normocin™ (100 µg/mL), Zeocin™ (100 µg/mL), and puromycin (1 µg/mL, Invivogen, ant-pr-1) at 37 °C in 5% CO₂.

SEAP assay for quantification of cellular response

The stimulation of mTLR9 in HEK-Blue™ mTLR9 cells leads to the production of NF-κB, which in turn promotes the expression of SEAP (Fig. 2.2a). The latter could be detected using HEK-Blue™ Detection Medium (Invivogen, hb-det2) which was prepared according to the manufacturer's instructions and used to culture the cells with different controls and experimental conditions (n ≥ 6, measurements were done for different distinct samples of each condition). HEK-Blue™ mTLR9 cell suspension in HEK-Blue™ Detection Medium (180 µL), aiming for seeding density of 5*10³ cells per well, was incubated with sample to be tested (20 µL) in a 96-well plate. The samples contained either pristine CpG ODNs, RCA-CpG ODN, RCA polymer, or PBS. Each group was tested both with and without the treatment using imaging US. For groups to be treated with imaging US, 6-well plates were used. Control experiments were done using the parent HEK-Blue™ Null1 cell line. After incubation overnight at 37 °C and 5% CO₂, SEAP in the supernatant hydrolyzed the substrate in the medium into purple/blue product that could be quantified by measuring absorbance at λ= 620-655 nm using a plate reader (SpectraMax iD3, Molecular Devices, USA).

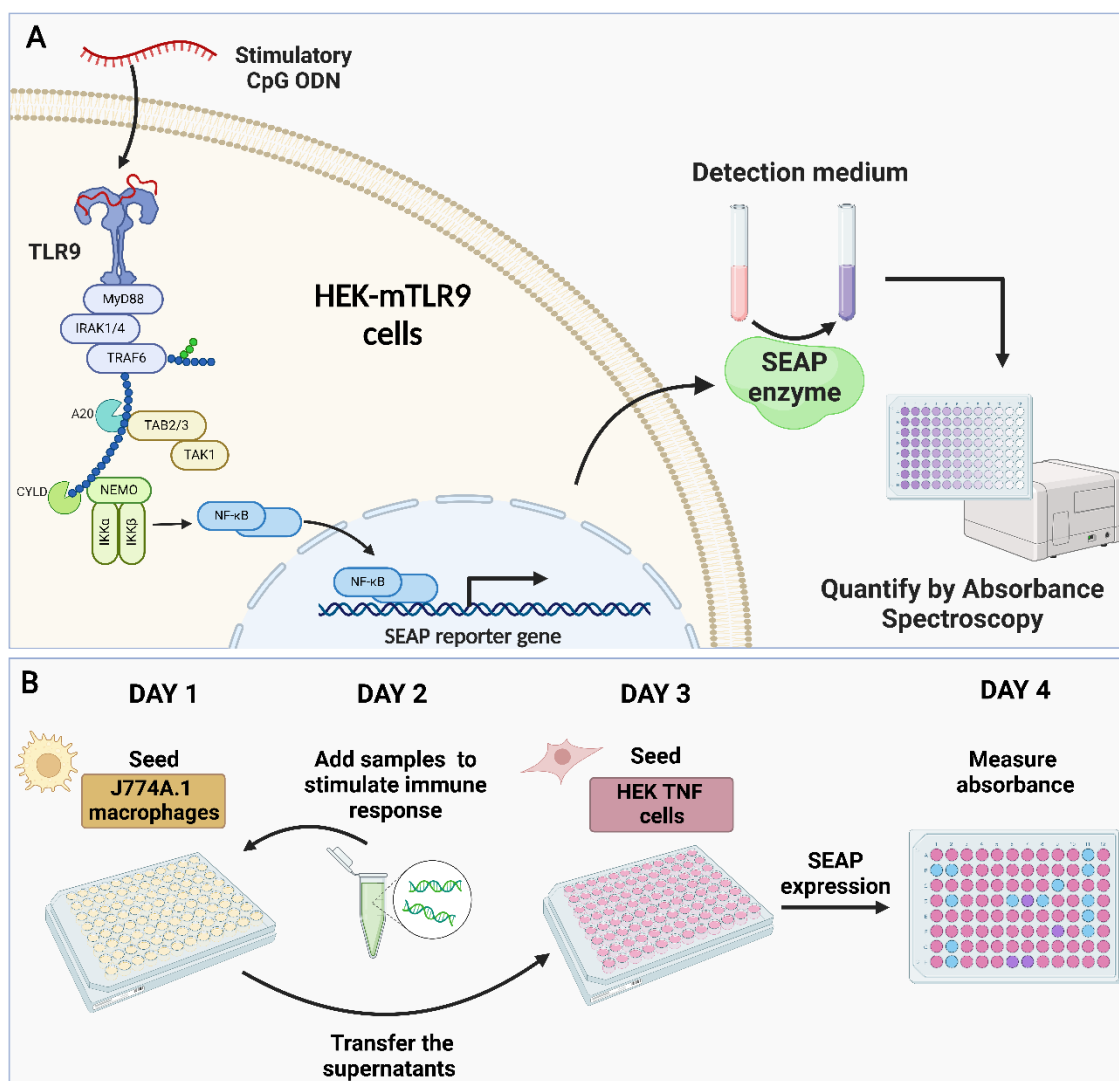


Fig. 2.2. **A.** Schematic presentation of the SEAP reporter assay using HEK-Blue mTLR9 cells, which are transfected to express TLR9 exclusively among the TLR family. They are co-transfected with the SEAP reporter gene that operates under the control of the NF-κB-dependent TLR9 signalling pathway. mTLR9 stimulation by CpG ODNs initiates the signalling pathway resulting in NF-κB production and thereby inducing the expression of the SEAP reporter gene. Using HEK-Blue detection medium, SEAP levels in the supernatant can be detected by measuring absorbance at $\lambda = 620$ nm, as SEAP converts its substrate to blue/purple product **B.** Schematic presentation of the experimental setup used to quantify TNF release stimulated by CpG ODN activation of TLR9 in J774A.1 macrophage. TNF is detected in the macrophages' supernatant by co-incubation with an engineered HEK cell line transfected to express the TNF receptor, which upon stimulation triggers a cellular pathway that ends up with NF-κB production. The latter induces the expression of the SEAP reporter gene which can be quantified by spectrophotometry using suitable detection medium. Created with BioRender.com

The stimulation of J774A.1 macrophages resulted in the production of TNF in the cells' supernatant. J774A.1 macrophages were incubated with different samples (20 μ L) following a similar protocol ($n \geq 6$, measurements were done for different distinct samples of each condition). Then, the supernatant (20 μ L) was removed from the wells and transferred into a new 96-well plate to be incubated with HEK-Blue™ TNF cells suspension in cell culture medium (180 μ L). Upon stimulation by TNF, these cells produce SEAP into their supernatant, which can be quantified by using QUANTI-Blue™ Solution (Invivogen, rep-qbs) following the manufacturer's instructions (Fig. 2.2b). This represents a colorimetric enzyme assay in which

the solution changes from pink to purple/blue after exposure to alkaline phosphatase, which can be quantified by measuring absorbance at 620-655 nm using a plate reader (SpectraMax iD3, Molecular Devices, USA). Recombinant human TNF (Invivogen, rcyc-htnfa) and recombinant human IL-6 (Invivogen, rcyec-hil6) were used as positive and negative controls, respectively.

Primary cells- melanoma triculture

Primary monocytes were isolated from PBMCs from whole blood donated voluntarily by human blood donors (n = 4 distinct donors) in compliance with all relevant ethical regulations. The protocol was approved by the ethics committee of the RWTH Aachen University Hospital (number 355/19), and informed consents were collected from participants. The Ficoll gradient centrifugation method was used to isolate PBMCs, followed by negative isolation of untouched monocytes using Pan Monocyte Isolation Kit, human (Miltenyi, 130-096-537) following the manufacturer's instructions. PerCP Cy5.5-CD14 Antibody, antihuman (eBioscience, 45-0149-41) was used (0.1 µg per 100 µL) to verify the isolated cells' phenotype. Monocytes (1×10^6 per mL) were cultured in 6-well plate in a volume of 3 mL per well in RPMI 1640 medium (Gibco, 11875093) supplemented with 2% autologous serum, IL4 (1000 IU/mL; Miltenyi, 130-093-917) and GM-CSF (800 IU/mL; Miltenyi, 130-095-372). Every two days, 1 mL was removed and replaced with 1 mL complete media supplemented with IL4 (1000 IU/mL) and GM-CSF (1600 IU/mL) to allow for monocytes differentiation into iDCs. On day 7, iDCs were harvested and cultured in fresh complete media supplemented with IL4 (500 IU/mL) and GM-CSF (800 IU/mL). For the positive control of iDCs' maturation into mDCs, the medium was further supplemented with IL4 (500 IU/mL), GM-CSF (800 IU/mL), IL6 (1000 IU/mL; Miltenyi, 130-093-929), IL1-β (10 ng/mL; Miltenyi, 130-095-374), TNF (10 ng/mL; Miltenyi, 130-094-018), and PGE2 (50 µg/mL; CAS 363-24-6, Sigma-Aldrich). For studying the effect of different experimental samples, the materials were added to iDCs on the same day. This included RCA-CpG ODN with and without the application of imaging US. Maturation process took place over 48 h and APC-CD80 Antibody, antihuman (Miltenyi, 130-122-928), FITC-CD86 Antibody, antihuman (Miltenyi, 130-114-097), PE-CD83 Antibody, antihuman (Miltenyi, 130-094-181) were used (0.1 µg per 100 µL) to track the surface biomarkers by flow cytometry (BD FACSCanto™ II, BD Biosciences, USA).

Primary CD8⁺ T cells were isolated from PBMCs using CD8⁺ T cell Isolation Kit, human (Miltenyi, 130-094-156) by negative indirect isolation. PerCP Cy5.5-CD4 Antibody, antihuman (eBioscience 45-0048-41) and FITC-CD8 Antibody, antihuman (Miltenyi 130-113-157) were used (0.1 µg per 100 µL) to verify the isolated cells' phenotype. T cells were cultured in RPMI 1640-GlutaMAX media (Gibco, 61870036) supplemented with penicillin (100 U/mL), streptomycin (100 µg/mL) and 8% human serum. T cells were labelled with CellTrace Violet (ThermoFisher, C34557) following manufacturer's instructions to allow tracking of their proliferation. On day 9, a co-culture of DCs and T cells was established in round-bottom 96-well plates in 1:1 ratio. Media was supplemented with IL2 (10 ng/mL; Miltenyi, 130-097-743) every two days for the duration of 7 days. On day 16, DC: T cells mixtures were transferred to 24-well plates seeded with B16F10 melanoma cells. Pre-seeding of B16F10 cells was done on day 13, in 24-well plates at density of 35×10^3 cells per well. They were also labelled with CellTrace CFSE (ThermoFisher, C34554) following the manufacturer's instructions. The triculture was incubated for 24 h at 37 °C and 5% CO₂. On day 17, the supernatant was collected to quantify TNF production, and B16F10 cells were harvested and analyzed by flow cytometry.

In vivo mechanochemical activation of CpG ODNs

C57Bl/6J wild type (WT) mice were housed under specific pathogen free conditions at the animal facility of the University Hospital Aachen, as approved by the LANUV NRW under the animal grant number 81-02.04.2020.A302. The experiments were done in accordance with the EU Directive 2010/63/EU for animal experiments and the ARRIVE guidelines

(<https://arriveguidelines.org/arrive-guidelines>). Animal group sizes were not increased once significance was reached compared to the respective control group. For imaging and all other processes, animals received 2% inhalative isoflurane anesthesia and protective eye cream (Bepanthen). *In vivo* imaging was done with anaesthetized mice using an IVIS Lumina XR High Resolution (Caliper LifeSciences). Data were analyzed using Digital Living Image Software. The experiment was done in six groups of mice, each has $n = 6$ distinct animals assigned randomly. Control group received 30 μg (1.2 mg/kg) of pristine ODN D-SL01. In another two groups, animals received RCA-CpG ODN (containing 90 μg of ODN D-SL01) labelled with about 1% of cyanine 5-nucleotide in a volume of 125 μL of PBS intravenously. The sample was homogenized in the syringe before being injected through the needle. For ultrasound treatment, anaesthetized mice were shaved using an animal shaver EXACTA (Aesculab) and lied on a heated plate creamed with hypoallergenic ultrasound cream Aquasonic 100 (Parker Laboratories). Imaging US was performed to identify the liver using a Lumify L12-4 linear array transducer in the vascular mode (MI = 0.9, frame rate 5 Hz) for 15 min. As negative controls, a group received US treatment without any prior material injection, and one group was left untreated. Mice were sacrificed 24 h after DDS injection using 10% isoflurane.

Analysis of gene expression in mice liver

Total RNA was isolated from mouse liver tissue using a Retsch Mill with metal beads at the setting 30/sec for 3 min. QIAshredder columns (Qiagen, 79656) were used for further processing. The RNeasy Mini Kit (Qiagen, 74106) was used to isolate total RNA, using RNase inhibitor (Thermo Fisher Scientific, N8080119). Complementary DNA (cDNA) was generated from 500 ng for each sample using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, 4368814). Quantitative real-time PCR was done using a QuantStudio® 5 Real-Time PCR System (Applied Biosystems). Applied Biosystems™ PowerUp™ SYBR™ Green Master Mix was purchased from Thermo Fisher Scientific (A25742) and reactions were performed in duplicates. Thermocycler parameters: UDG activation for 2 min at 50 °C, Dual-Lock DNA polymerase for 2 min at 95 °C, then 40 cycles and denaturation at 95 °C for 1 sec followed by annealing and extending at 60 °C for 30 sec. *Gapdh* was selected as housekeeping gene and the $\Delta\Delta\text{Ct}$ method was used to calculate relative gene expression. Primer sequences were designed using NCBI-BLAST software (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and are provided in Supplementary Table 2.

Immunohistochemistry staining

Liver specimens were perfused with sterile PBS and then preserved in 4% buffered formalin. The sections were deparaffinized and destained. Liver sections were stained with hematoxylin (Merck, MHS128-4L) followed by bluing in tap water. Staining with eosin Y Solution (Sigma Aldrich, HT110216-500ML) was then performed, and the sections were rinsed in absolute ethanol. The sections were further processed in an ascending alcohol series up to xylene. Finally, the slides were covered with Vitro-Clud (R. LANGENBRINCK GMBH, 04-0001). Samples were visualized under ZEISS Axio Imager 2-AX10 microscope, using AxioVison SE64 Rel, 4.9 software.

Statistical Analysis

Statistical significance was calculated by one-way ANOVA analysis with Tukey post hoc test for means comparison. All values are reported as means $\pm$ s.d. P values less than 0.05 were considered statistically significant. Asterisks are shown above brackets linking the pairs of data points being compared in each figure with quantitative data (*: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$, ****: $P \leq 0.0001$). Statistical analysis and data plots were done using Origin Pro 2022b SR1.

2.3 RESULTS AND DISCUSSION

2.3.1 DDS DESIGN AND OPTIMIZATION

We fabricated our delivery platform employing RCA preceded by padlock ligation (Fig. 2.3). In the padlock ligation process, a primer (depicted in yellow) is strategically designed to possess a complementary sequence to both ends of a linear ssDNA strand (depicted in green). Hybridization of the primer to the linear ssDNA prompts its cyclization by bringing its two ends together. The ensuing nick is sealed through ligation, facilitated by the T4 ligase enzyme, establishing a covalent bond and resulting in the creation of a circle ssDNA template. This template in turn is the starting material for the RCA reaction, in which the enzyme Phi29 DNA polymerase synthesizes a complementary ssDNA sequence by continuously adding single nucleotides resulting in a long concatemer ssDNA (depicted in blue) that is composed of tens to hundreds of tandem repeats that are complementary to the circular template.

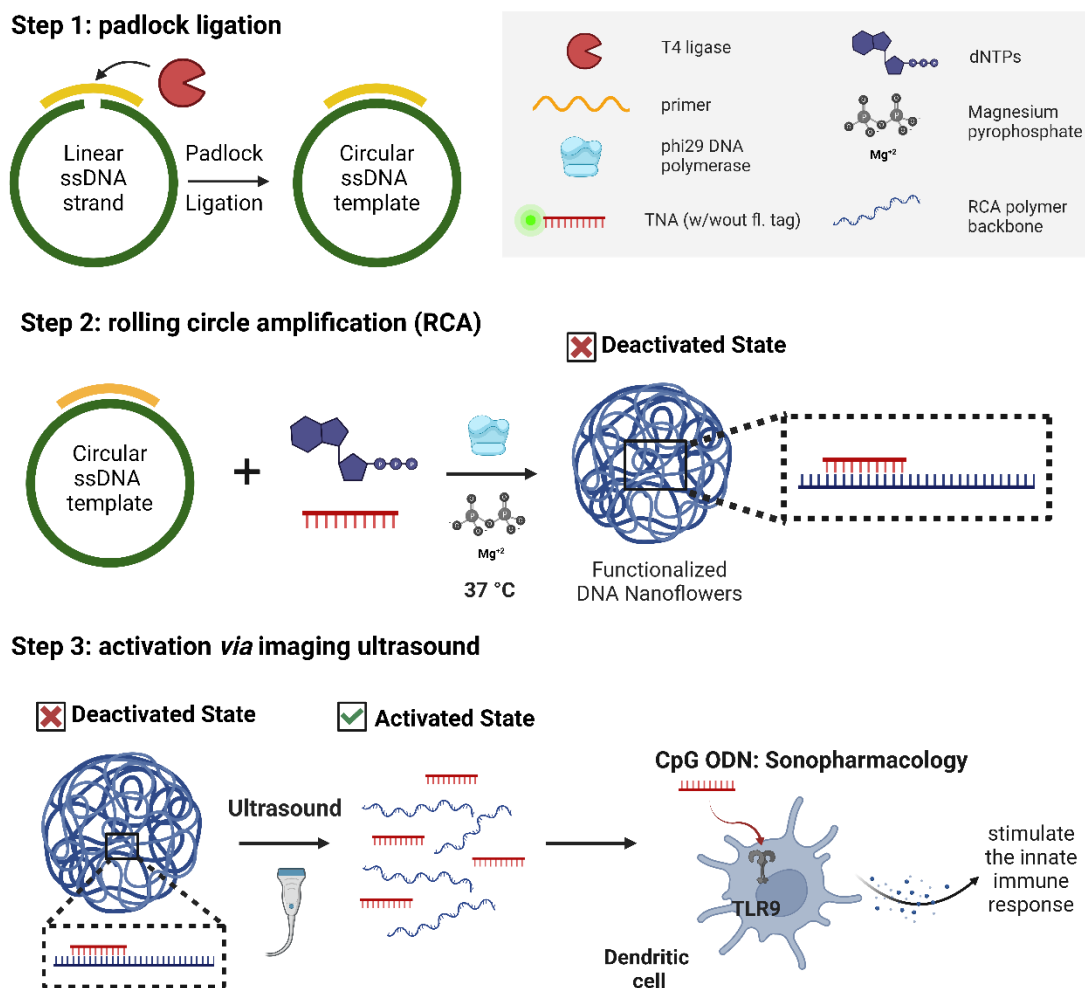


Fig. 2.3. Schematic presentation of the DDS production. First, padlock ligation in which a linear ssDNA template is mixed with a primer that has complementary sequences to the template on both 5'- and 3'- ends. T4 ligase is used to seal the bond and convert the linear template into a circular ssDNA, which serves as the starting material for the RCA. In the second step, the RCA polymer is produced from the circular template that is designed to have a hybridization site for the desired TNA cargo. The resulting RCA NFs accommodate and deactivate CpG ODN cargo. Finally, mechanochemical activation of the DDS by imaging US that leads to the release of the CpG ODNs in their active form, to exert their controlled biological activity in cells, enabling on-demand sonopharmacology achieved *via* polynucleotide mechanochemistry. Created with BioRender.com

We exploited the RCA backbone as an US-responsive material to deliver TNAs, using templates that result in RCA polymers with sequences complementary to the TNA cargo sequence. By loading CpG ODNs onto the RCA carrier, they hybridize to the backbone and become deactivated until exposed to imaging US, which partially degrades the RCA carrier and releases the active CpG ODN (Fig. 2.3).

The first ODN we started with was ODN1826. We designed RCA polymers to have hybridization sites for the ODN at various degrees of complementarity (*i.e.*, 25, 50, 75 and 100%). RCA polymers formed successfully using all the designed templates (Fig. 2.4a) as confirmed by GE. Time-tracking of the formation of the RCA polymer was done by sampling the reaction at given time points and immediately deactivating the enzymatic activity of Phi29 DNA polymerase by heating at 65°C for 10 min. Samples were taken as early as 5 min of reaction time, up to 24 h and then analysed *via* agarose GE. Results show that the RCA process is rapid and efficient, and long DNA polymers can be seen at the top of the gel as early as 5 min of reaction time (Fig. 2.4b).

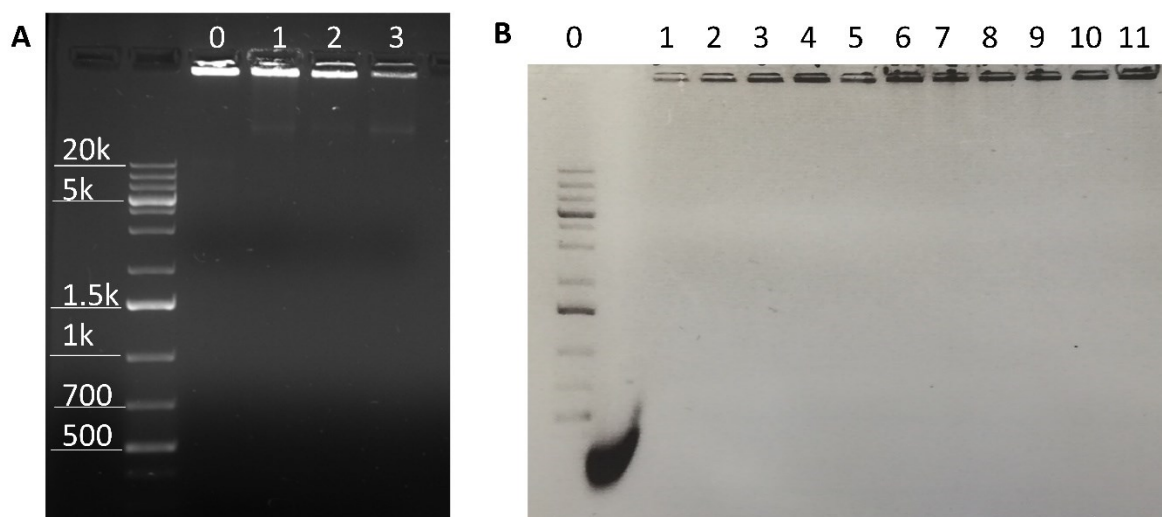


Fig. 2.4 A. Agarose GE demonstrating the successful formation of RCA polymers. Lane 0: kb plus DNA ladder; lanes 1 to 4: RCA polymers using templates that have 100%, 75%, 50% and 25% complementarity to CpG ODN1826, respectively. **B.** Agarose GE showing samples taken from RCA reaction at different time points. Lane 0: kb plus DNA ladder; lanes 1 to 11: RCA samples taken at 5 min, 10 min, 20 min, 40 min, 1 h, 2 h, 4 h, 6 h, 8 h, 24h and 48 h, respectively.

Since the resulting products were too big to migrate through agarose gels, we opted to use PFGE to separate the polymer strands and quantify their length. PFGE is a technique used for the separation of large DNA molecules using an electric field that periodically changes direction (Fig. 2.5). This allows for longer times of running the gel, up to 72 h. A cooling unit is used to prevent heating of the buffer and thereby the melting of the gel. After 24 h of running PFGE, the RCA polymers still did not move from the top of the gel (Fig. 2.6a). We further run the PFGE for 72 h and used samples taken from RCA reaction as early as 5 min., up to 6 h reaction time. The results showed that RCA polymers still did not move through the gel, although a marker with a band of 5.7 million bases (mb) migrated through the agarose gel (Fig. 2.6b). This indicated that the RCA polymers are extremely long and might be potentially physically entangled in the gel in a way that prevents them from migration.

To quantify the DDS loading efficiency using RCA polymers as carriers, we utilized a dye-labelling approach to quantify excess CpG ODNs in the reaction supernatant that did not bind to the RCA polymers. Subsequently, we could calculate how much CpG ODNs were loaded accordingly. Results showed that RCA polymers with 75% complementarity to CpG ODN 1826 (RCA-Ra75) were the most efficient in their binding (80% loading efficiency), followed by RCA polymers with 100% complementarity (RCA-Ra100) with 69% loading efficiency. This could be attributed to the formation of secondary structures in the CpG ODN hybridization sites on backbone of the RCA polymer with 100% complementarity (Fig. 2.7), which might lead to less efficient hybridization to the CpG ODN.

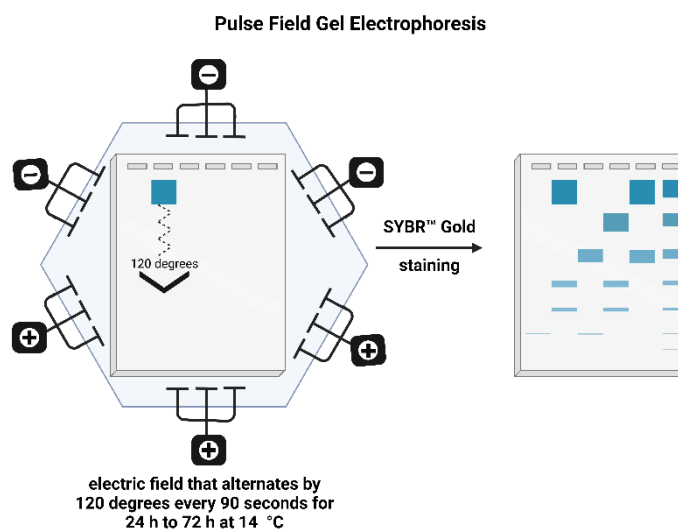


Fig. 2.5. PFGE technique that uses an electric field with changing direction, followed by SYBR Gold staining to visualize the bands. Created with BioRender.com

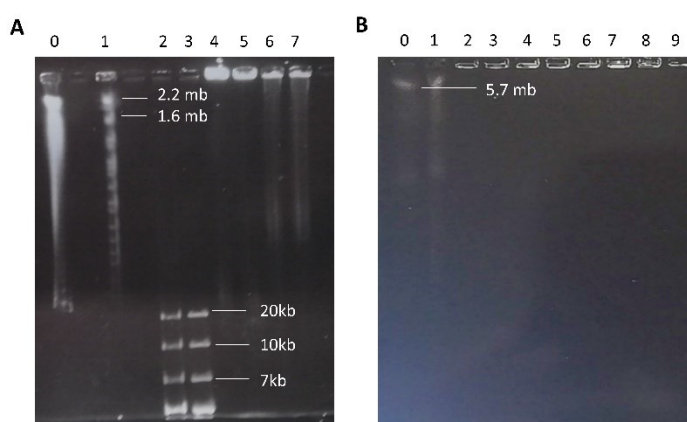


Fig. 2.6. A. Agarose PFGE demonstrating RCA products at the top of the gel after 24 h gel running time. Lane 0: Lambda ladder, Lane 1: *Saccharomyces cerevisiae* ladder; lanes 2,3: Kb plus ladder; lanes 4 to 7: RCA polymers using templates that have 100%, 75%, 50% and 25% complementarity to CpG ODN1826, respectively. **B.** Agarose PFGE demonstrating samples taken from RCA reaction at different time points at the top of the gel after 72 h of gel running time. Lanes 0 and 1: marker with 5.7 mb band; lanes 2 to 9: RCA samples taken at 5 min, 10 min, 20 min, 40 min, 1 h, 2 h, 4 h and 6 h, respectively.

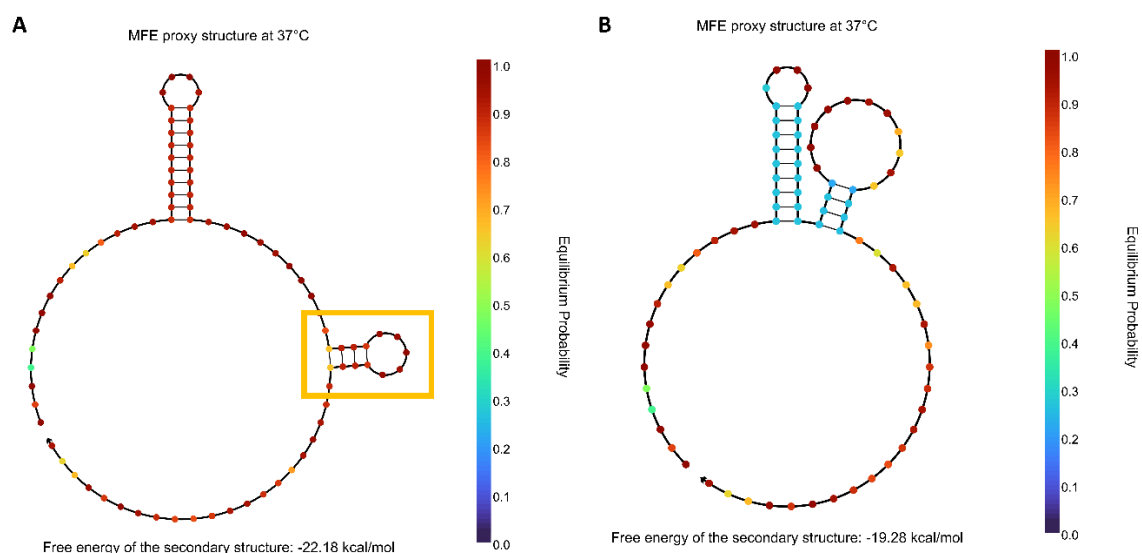


Fig. 2.7. Secondary structures obtained by NUPACK simulations for one unit of the RCA polymer produced using templates that have 100% (A) and 75% (B) complementarity to CpG ODN1826. Secondary structure formation with high probability at the ODN hybridization site in A is highlighted with the yellow box.

To test the system's mechanochemical response, *ex situ* sonication using high-power 20 kHz US was performed. Results showed a rapid release of CpG ODN as seen in GE, starting after 2 min of ultrasonication (Fig. 2.8). Further US exposure for up to 8 min indicated that CpG ODN was stable and did not undergo further fragmentation. This could be attributed to the short length of CpG ODNs that renders them insensitive to the mechanical effects of US.

Incubating HEK mTLR9 cells with RCA-Ra100 and RCA-Ra75 loaded with 5'-Atto488-ODN1826, both before and after *ex situ* treatment with 20 kHz US showed that the released CpG ODN1826 was biologically active and could stimulate its target receptor leading

to a significant increase in mTLR9 stimulation (Fig. 2.9a). RCA polymer on its own did not lead to mTLR9 stimulation. Nevertheless, when RCA-Ra100 and RCA-Ra75 were loaded with PS-modified CpG ODN1826 without the fluorescence tag, no significant difference in mTLR9 stimulation could be observed between samples that were exposed to 20 kHz US and samples that were non-sonicated (Fig. 2.9b).

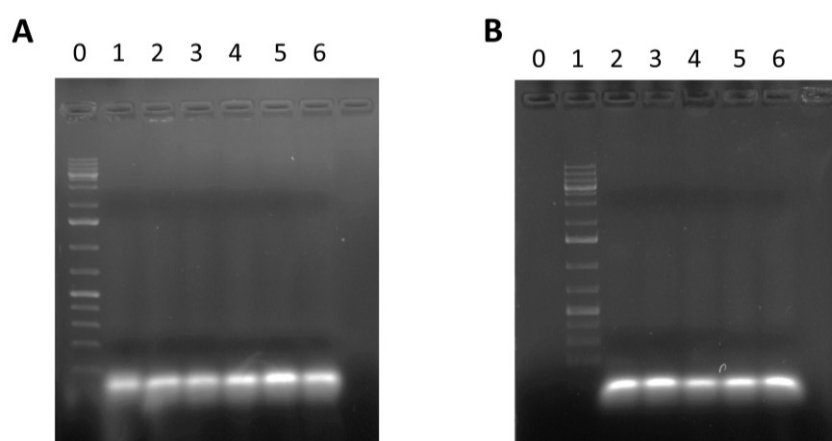


Fig. 2.8. A. Agarose GE demonstrating the effect of 20 kHz US on RCA-ODN complexes. Lane 0: Kb plus ladder; lanes 1 to 5: RCA₁₀₀-ODN1826 after exposure to US for 2 min, 4 min, 6 min, 8 min and 10 min, respectively; lane 6: pristine CpG ODN1826 control. B. Lane 0: Kb plus ladder; lanes 1 to 5: RCA₇₅-ODN1826 after exposure to US for 2 min, 4 min, 6 min, 8 min and 10 min, respectively; lane 6: pristine CpG ODN1826 control.

This observation led us to explore the dose-response relationship of CpG ODNs under investigation (Fig. 2.10). Our results showed that adding a fluorescent tag reduced potency and efficacy of the CpG ODN, probably due to steric effects that decreased its binding affinity to the target receptor. Furthermore, PS modification of the CpG ODN backbone either fully (*i.e.*, PS-ODN) or partially (*i.e.*, mixed ODN) enhanced its potency and efficacy, due to the protection of the CpG ODN backbone against hydrolysis and degradation by nucleases. Maximum stimulation of mTLR9 was achieved with PS-CpG ODN1826 at concentrations as low as 0.05 μM .

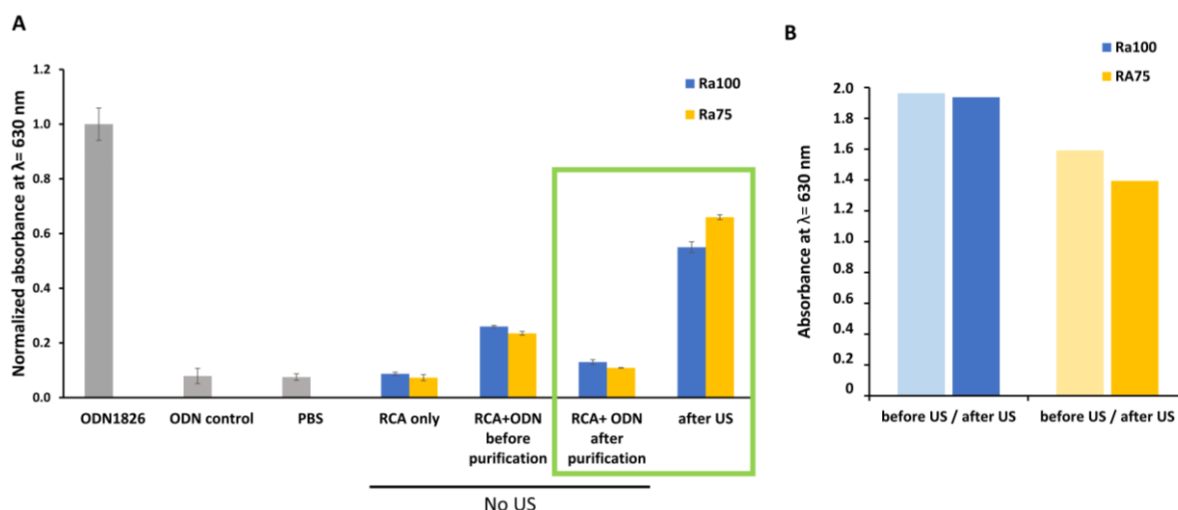


Fig. 2.9. mTLR9 stimulation after incubation of HEK mTLR9 cells with different samples. RCA polymers were synthesized using backbones with 100% (Ra100) and 75% (Ra75) complementarity to CpG ODN1826. **A.** The loaded cargo was 5'-Atto488-ODN1826 and *ex situ* sonication resulted in significant activation of the biological response. **B.** The loaded cargo was PS-ODN1826 and no significant difference in biological response was observed after *ex situ* sonication for either system.

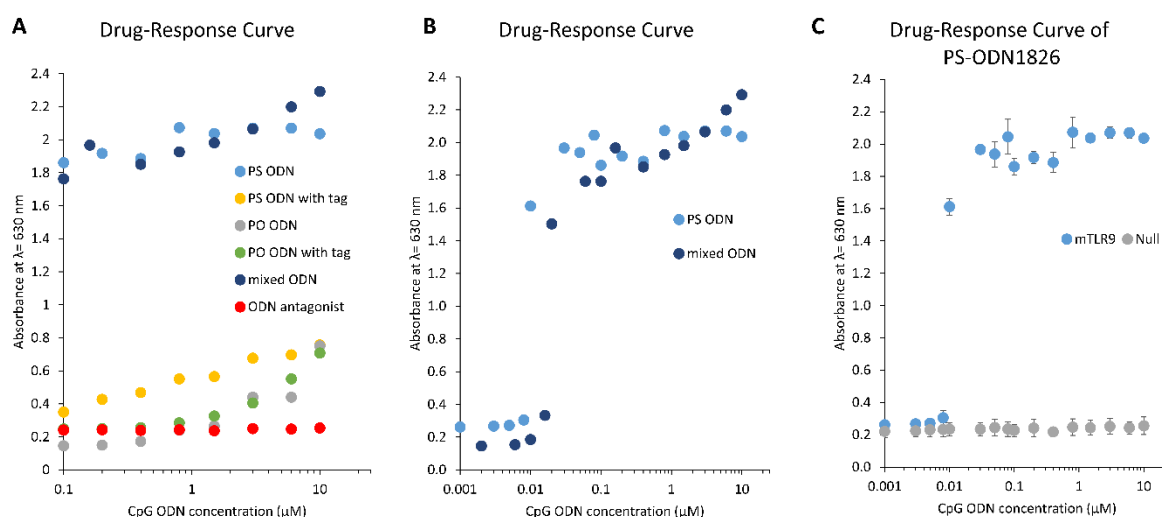


Fig. 2.10. Drug dose-response curve for different CpG ODNs, demonstrating different potency upon using different modifications. **A.** CpG ODN1826 was tested either in PO or PS backbone, either with or without fluorescent tag on the 5'-end. Mixed ODN is PS-modified ODN that has natural PO bonds only at the CG pairs. ODN antagonist is inverted GC sequence. **B.** For PS-ODN and mixed ODN, drug-response curve was further investigated over wider concentration range, as low as 0.001 μM . **C.** Drug-response curve focusing on PS-ODN performance in both mTLR9 and null cells.

This means that the unbound fraction or leaking of PS-CpG ODN1826 in the previous DDS which amounted to approximately 1.3 μ M could already stimulate mTLR9 to the maximum level. Any further release of PS-CpG ODN1826 *via* US would not result in further stimulation of mTLR9, as it has already reached its saturation point. Therefore, this could explain the non-significant difference that was observed for results in Fig. 2.9b.

To further strengthen the binding of CpG ODNs to their RCA carrier and minimize leaking as much as possible, we hypothesized that adding the CpG ODNs during the RCA reaction would lead to higher binding efficiency than their addition post-RCA. We speculated that not all of the hybridization sites would be easily accessible after the RCA polymer is formed and folded. Towards that end, we investigated the timing of the addition of the CpG ODN to the RCA reaction. The addition of the ODN from the beginning of the reaction prevented the RCA process and the enzyme failed to produce any DNA polymers. The optimum addition time point was found to be 1.5 h after starting the reaction (Fig. 2.11a).

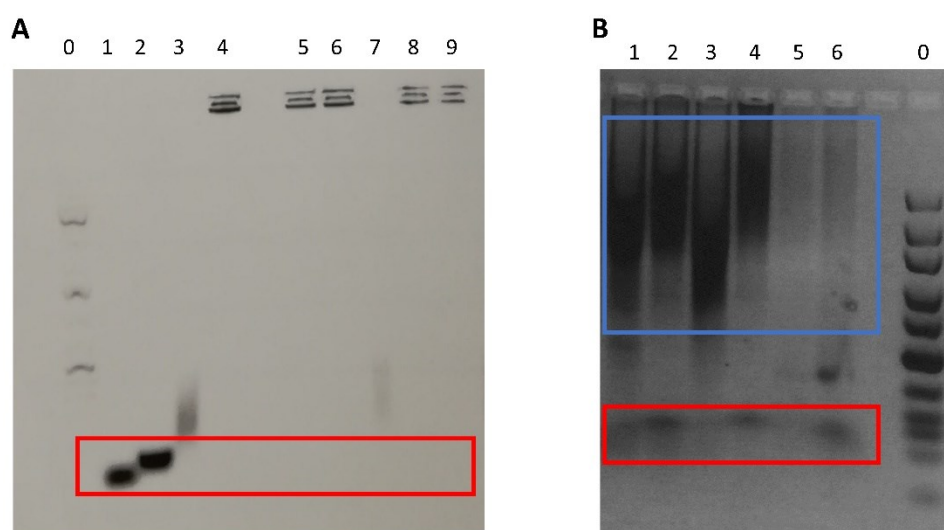


Fig. 2.11. A. Agarose GE demonstrating successful production and loading of RCA-PS-ODN1826. Lane 0: kb plus ladder, lanes 1 and 2: reference PS-ODN1826 and double ODN, respectively; lane 3: RCA100 ligated template; lanes 4 and 5: RCA100 polymer; lane 6: RCA100-PS-ODN1826 complex; lane 7: RCA75 ligated template; lane 8: RCA75 polymer; lane 9: RCA75-PS-ODN1826 complex. B. Agarose GE demonstrating the effects of 20 kHz US on RCA75-PS-ODN1826. Lanes 1 to 5: RCA75-PS-ODN1826 after US treatment; lane 0: kb plus ladder.

This timing gives the enzyme the possibility of starting the polymerization process, and upon the addition of the CpG ODNs, the hybridization process can take place as the hybridization sites are readily synthesized but not yet blocked by polymer folding. After washing the RCA-ODN complex three times with PBS, loading efficiency of PS-CpG ODN1826 was calculated to be approximately 65%. Ultrasonication with 20 kHz US resulted in RCA backbone fragmentation and CpG ODN release (Fig. 2.11b). Significant difference in mTLR9 stimulation could be demonstrated for DDS before and after ultrasonication, with 9.59-fold increase in the response when the DDS is treated with 20 kHz US (Fig. 2.12).

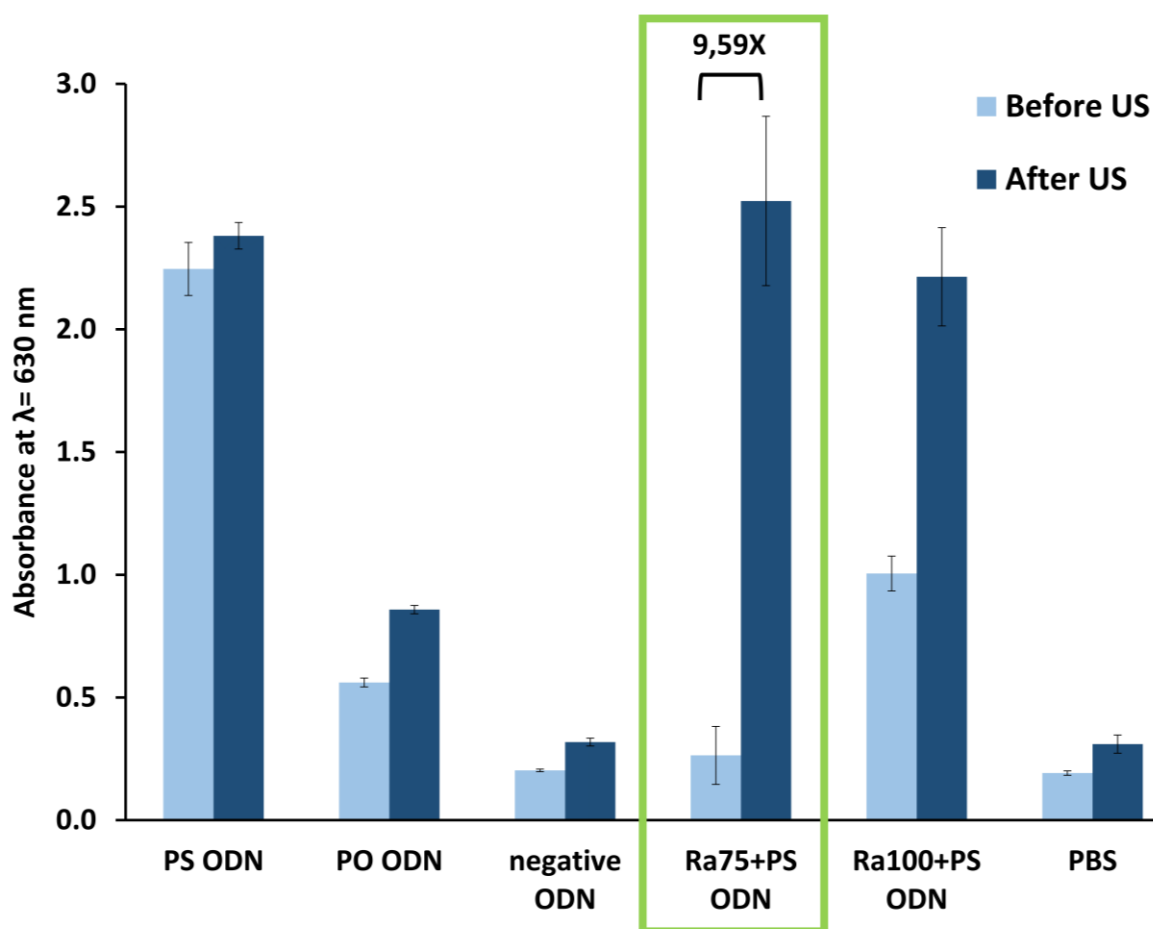


Fig. 2.12. mTLR9 stimulation in HEK-mTLR9 cells after incubation with different samples. *Ex situ* sonication using 20 kHz US was performed.

Up to this point, engineered HEK cells were used as a proof-of-concept, since they are exclusively expressing the target receptor. This enables the detection of biological response that is exhibited solely *via* the activation of mTLR9. Nevertheless, HEK cells are not immune cells and therefore the next experiments were planned to be performed utilizing primary immune cells. In comparison with cell lines, primary cells offer a more biologically relevant and accurate model for studying diseases and testing potential therapies. One potential source for primary immune cells is the mice bone marrow. However, the process is more complicated and depends on the availability of the bones as a side-products from other animal studies. An alternative source of primary immune cells would be from human blood that is donated voluntarily by healthy donors. This source is readily available and less complicated protocols can be used to isolate several types of immune cells.

Nevertheless, to be able to stimulate the human TLR9 (hTLR9) as a target, the CpG ODN must be changed. CpG ODN1826 that was used so far did not show significant activity towards human primary macrophages (Fig. 2.13). Thus, we searched for another CpG ODN that could simulate both hTLR9 and mTLR9, as we plan to perform *in vivo* experiments in mice model which would take place after confirming results in primary immune cells from humans.

CpG ODN D-SL01 fit these criteria as it is considered to be a multispecies TLR9 ligand in a diverse vertebrate species, namely humans, mice, rats, rabbits, pigs and dogs⁸⁴⁴. The PS-ODN D-SL01 was extremely potent, therefore we decided to utilize the natural PO backbone of the ODN D-SL01. Usually, the PO backbone is prone to rapid hydrolysis and degradation, but we hypothesized that our DDS would protect its cargo from the environment until being triggered by US. Thus, we anticipated that the RCA polymer would protect the PO-ODN D-SL01 until being delivered. The RCA polymers with 100% complementarity were the most efficient ones, in terms of both binding and release of their CpG ODN cargo. Therefore, this combination of carrier (*i.e.*, RCA polymer with 100% complementarity) and drug (*i.e.*, PO-D-SL01 ODN) was used for the rest of the experiments. We demonstrated successful deactivation and activation of the ODN, indicating that it was well-protected by the RCA polymer. An activation ratio of 8.25 and 10.08 could be observed after ultrasonication of RCA polymers that were loaded with PO-D-SL01 ODN, using an unlabelled and FAM-labelled primer, respectively (Fig. 2.14).

The response after 20 kHz US exposure was even better than that of the pristine PO ODN D-SL01 and almost 75% of that of the corresponding ODN with PS backbone. After this design optimization process, a medically relevant imaging US device was used for *in situ* sonication in all the following characterization, *in vitro* and *in vivo* experiments

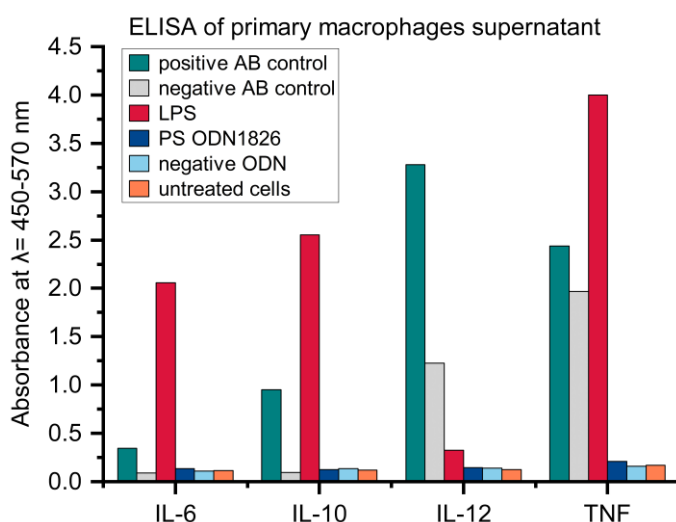


Fig. 2.13. Quantification of different cytokines and interleukins in the supernatants of primary macrophages that were stimulated by different samples.

Table 2.1. DDSs tested in cell culture experiments in Fig. 2.14

DDS #	ODN percentage complementarity	FAM-primer	D-SL01 Backbone
100-	100%	No	PO
100-	100%	Yes	PO
100-	100%	No	PS
100-	100%	Yes	PS
75-	75%	No	PO
75-B	75%	Yes	PO
75-	75%	No	PS
75-	75%	Yes	PS

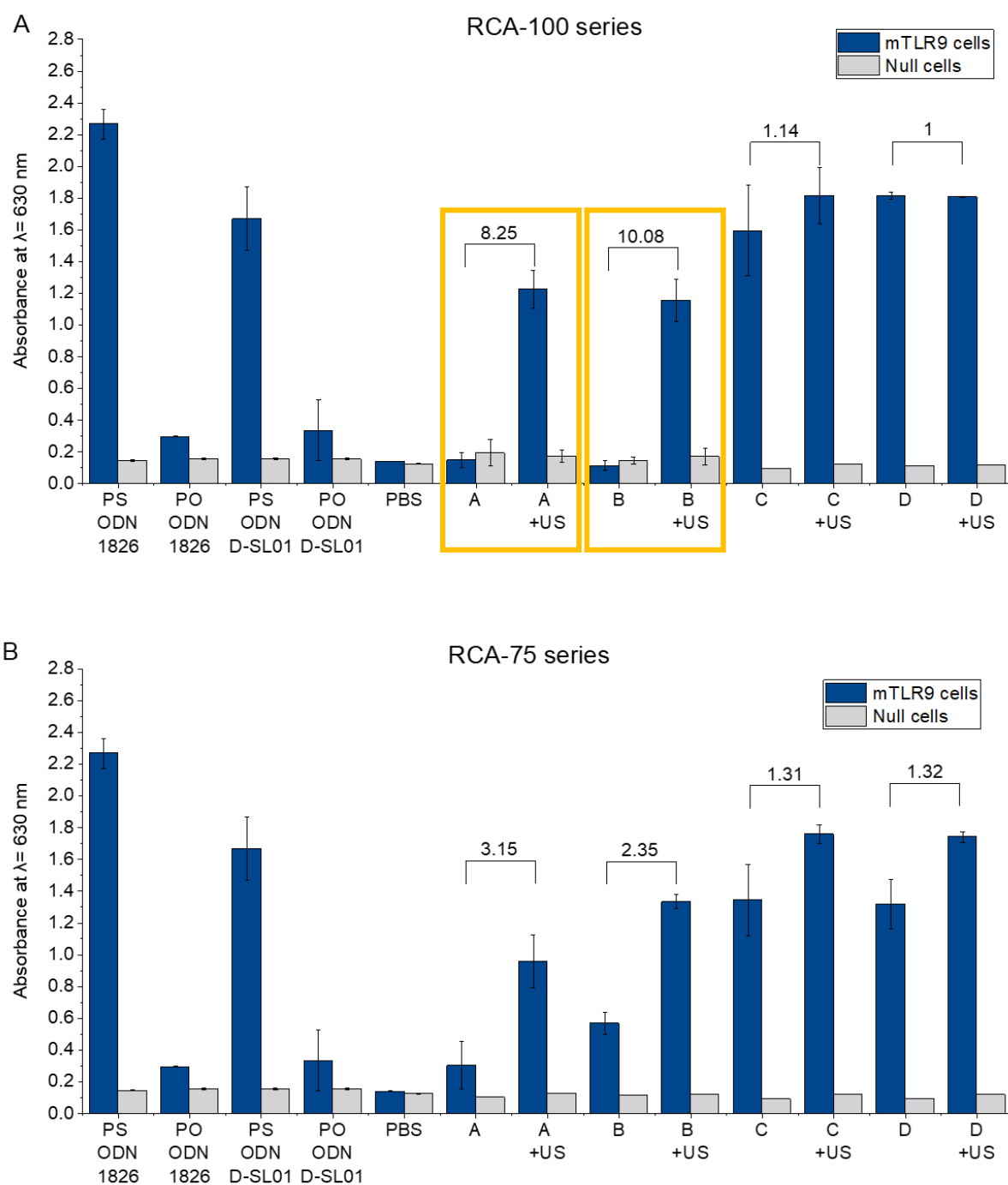


Fig. 2.14. mTLR9 stimulation in HEK-mTLR9 cells after incubation with different RCA-ODN-DSL01 complexes, either before or after sonication with 20 kHz US. RCA polymers that exhibit 100% or 75% complementarity are used to fabricate **A.** RCA-100 series and **B.** RCA-75 series, respectively. A detailed key to type of RCA-ODN complex is given in Table 2.1.

2.3.2 IMAGING US-MEDIATED ACTIVATION OF THE DDS

Using CpG ODN D-SL01 as cargo, agarose GE confirmed RCA-ODN complex production, observed at the top of the gel (Fig. 2.15). After 15 min of sonication with imaging US device (*i.e.*, Lumify L12-4 broadband linear transducer, vascular mode, MI=0.9), the RCA carrier backbone was partially degraded and the active CpG ODN D-SL01 was released (Fig. 2.15).

Loading efficiency was quantified using a dye-labelling approach (Fig. 2.16a,b), with loading increasing as reaction time increased as expected (Fig. 2.16c). At 2 μM and 20 μM ODN, the final loading efficiencies were $67\% \pm 1\%$ and $79\% \pm 15\%$, respectively. The same approach was used to quantify released CpG ODN after sonication with imaging US (Fig. 2.16d), which exceeded 30% for samples taken from full-time reaction (*i.e.*, 24 h).

The RCA-ODN complex formed a viscous gel material that can be observed directly in the reaction tube (Fig. 2.17a) and the measured viscosity of the materials increased over reaction time (Fig. 2.17b). Therefore, we characterized rheological properties of the material, which exhibited hydrogel properties (*i.e.*, $G' > G''$). The frequency dependence of the elastic $G'(\omega)$ and loss $G''(\omega)$ moduli in the linear regime (strain $\gamma_0 = 0.1\%$, Fig. 2.18a) was measured before and after sonication (Fig. 2.18b). Prior to US treatment, the plateau modulus remained constant at $\omega/2\pi < 1$ Hz, with terminal behaviour falling outside the probed frequency window. Remarkably, at $\omega/2\pi > 1$ Hz, corresponding to shorter timescales, G' became dominant (*i.e.*, the particles became largely immobile indicating a transition to a glassy state). This behaviour was consistent with theoretical predictions for concentrated soft particle suspensions^{845,846}. Surprisingly, after US both G' and G'' increased significantly (Fig. 2.18b), indicating the formation of new additional intermolecular hydrogen bonds between ssDNA polymers, leading to crosslinking and stiffening of the resulting network.

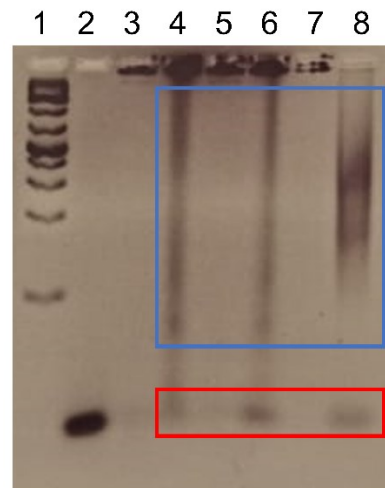


Fig. 2.15. Agarose GE demonstrating the effect of imaging US on RCA-ODN complex. Lane 0: Kb plus ladder; lane 2; reference ODN-DSL-01; lanes 3,5 and 7: RCA-ODN-DSL-01; lanes 4 and 6: RCA-ODN-DSL-01 after sonication with imaging US; lane 8: RCA-ODN-DSL-01 after sonication with 20 kHz US.

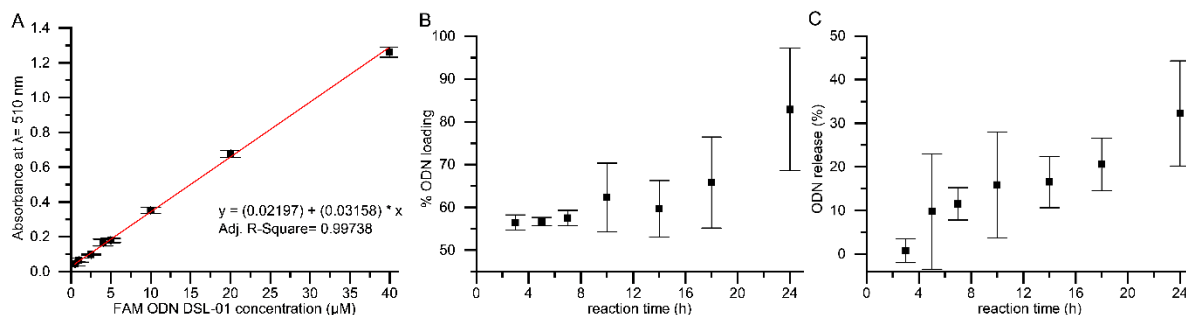


Fig. 2.16. **A.** Calibration curve of FAM-ODN D-SL01, which was used to calculate the concentration of bound and free ODN using the best fit linear regression model. **B.** RCA-CpG ODN loading efficiency increases with longer reaction time. **C.** Quantification of CpG ODN release efficiency after sonication, by calculating the percentage of released CpG ODN that can be detected in the supernatant. Different RCA reactions were performed for different durations followed by sample sonication and analysis.

Furthermore, higher CpG ODN release was speculated to have a correlation with the RCA-ODN complex viscosity before sonication. Higher CpG ODN release was observed when samples had higher viscosity (Fig. 2.19a). We further investigated the potential effect of varying the degree of complementarity between the RCA backbone and the CpG ODN cargo on the material's rheological properties. Thus, we used samples from the previous ODN cargo (*i.e.*, ODN1826) and its corresponding different RCA polymers that have varying degree of complementarity towards the CpG ODN (*i.e.*, from 25 to 100%). Results showed no correlation between the degree of complementarity and the rheological properties (Fig. 2.19b). However, the most intense gel stiffening behaviour was demonstrated for the samples in which the RCA polymer had 75% complementarity to the CpG ODN. This corresponds to the complex that achieved the most efficient binding in our previous experiments, indicating that the quantity of loaded CpG ODN might play a role in shaping the physicochemical properties of the resulting RCA-ODN complex.

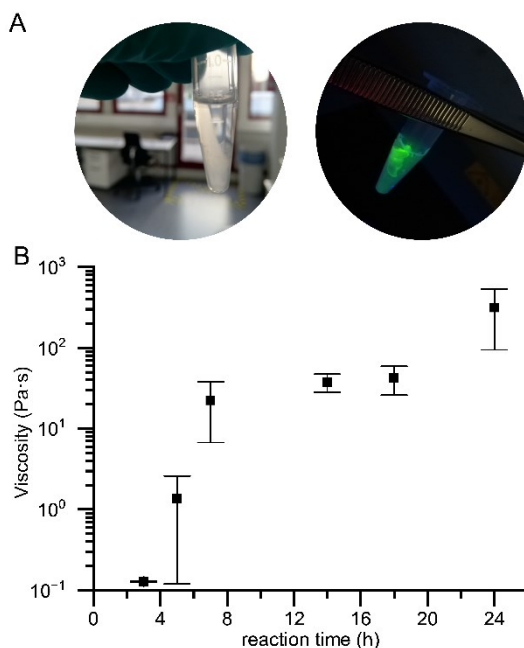


Fig. 2.17. **A.** Visualization of the phase separation of a gel containing RCA-CpG ODN. Fluorescently labelled CpG ODN is concentrated in the gel phase, as observed under UV light. **B.** Viscosity of RCA-CpG ODN increases with increasing reaction time.

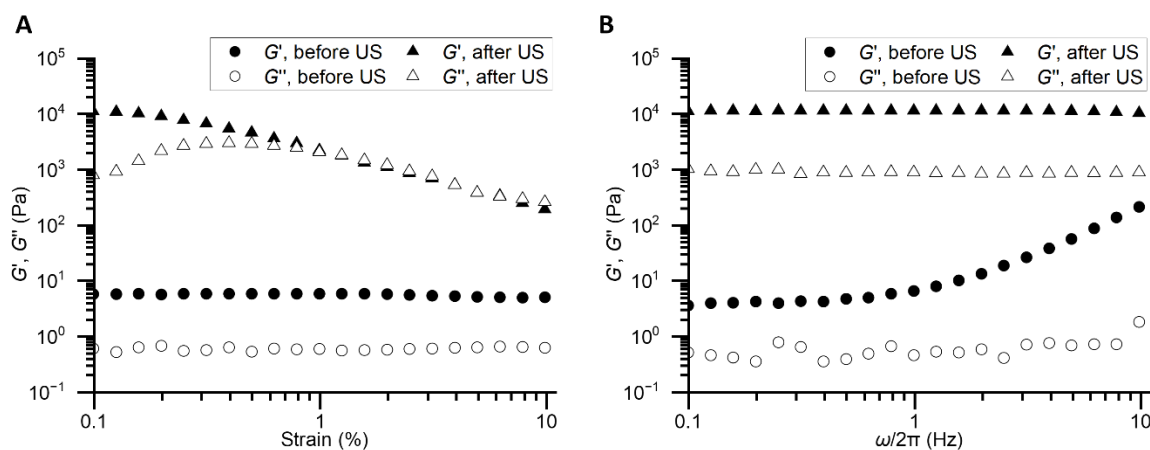


Fig.2.18. **A.** Oscillation sweep (at frequency of 1 Hz) of RCA-CpG ODN before and after sonication with imaging US. **B.** Rheology of RCA-CpG ODN showing the frequency dependence of G' and G'' in the linear regime (strain amplitude $\gamma_0 = 0.1\%$) before and after sonication with imaging US.

To acquire a better insight on the mechanism behind CpG ODN release, we investigated the structural changes resulting from sonication. SEM images showed distinct architectures of NFs (Fig. 2.20a), which exhibited birefringence under PLM, indicating an ordered assembly of material inside the NFs (Fig. 2.21). To gain better insights on the molecular arrangement of the RCA backbone within NFs, its secondary structure and the thermodynamic parameters were calculated using the NUPACK software suite⁸⁴⁷. The minimum free energy (MFE) for secondary structures of RCA used to bind CpG ODN is shown in Fig. 2.22. The results indicate that DNA chains have a high tendency (equilibrium probabilities > 0.9) to form periodic stem-loops within their structures. The probabilities for constructs with an odd number of repeats are lower than those with an even number. Since one region of the template sequence forms one stem-loop with a complementary domain in the next repeat, one stem-loop forming domain remains unpaired for an odd number of repeats. This leads to an increased number of stem-loop forming combinations, hence more energetically similar MFE structures exist. The circular conformation shown in Fig. 2.22 arises from the polymer graph representation of the structure; however, unpaired regions may also yield an elongated linear conformation in reality. It has been demonstrated that linear DNA chains with stem-loops self-assemble into structures with lamellar ordering. Similarly, the strong birefringence patterns observed in the NFs in this study may be attributed to the formation of liquid crystalline phases as shown in Fig. 2.21. The formation of hairpin secondary structures in the RCA backbone as demonstrated by NUPACK simulations (under thermodynamic equilibrium) is consistent with previous reports of hairpin-driven assembly of liquid crystalline RCA products^{176,848} and *in situ* nucleic acid-driven crystallization of metal salts (*i.e.*, $\text{Mg}_2\text{P}_2\text{O}_7$) generated during the reaction^{842,849}.

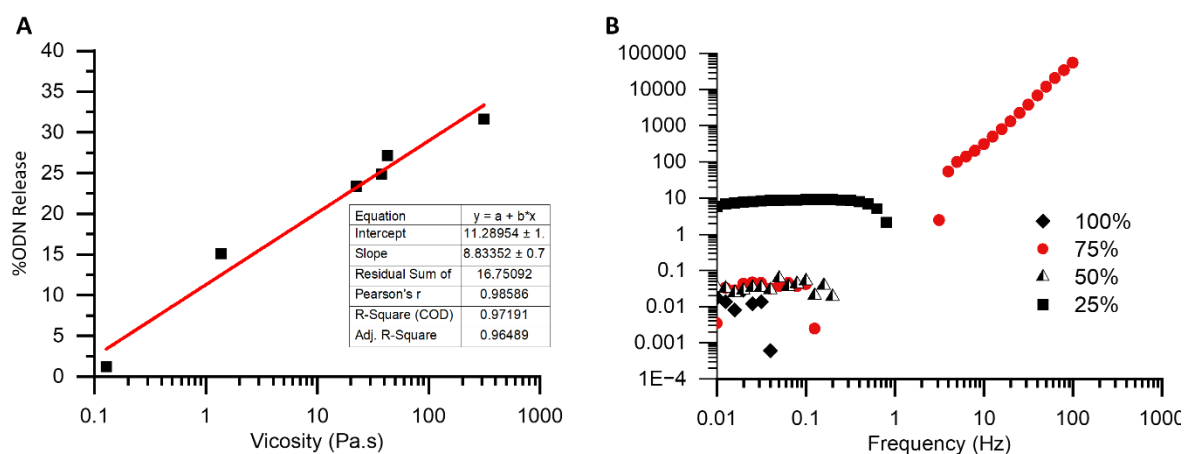


Fig. 2.19. **A.** US-response of RCA-ODN-DSL-01 is related to its viscosity, as evidenced by increased release efficiency. **B.** Rheology of RCA-ODN showing the frequency dependence of G' and G'' in the linear regime (strain amplitude $\gamma_0 = 0.1\%$) for RCA polymers using templates that have 100%, 75%, 50% and 25% complementarity to CpG ODN1826.

After 24 h reaction time, SEM analysis showed peak particle size distribution of $2.1 \pm 0.6 \mu\text{m}$ (Fig. 2.23). After sonication, SEM images showed large microparticles with a significantly altered surface morphology, as well as small particles, some in rod-like shapes (Fig. 2.20b). The particle size distribution shifted towards smaller sizes, with a predominant population ranging between 100-700 nm, alongside a minor portion of aggregated large microparticles (Fig. 2.23). Correspondingly, the birefringence disappeared, indicating the disassembly of the NFs ordered structure (Fig. 2.21).

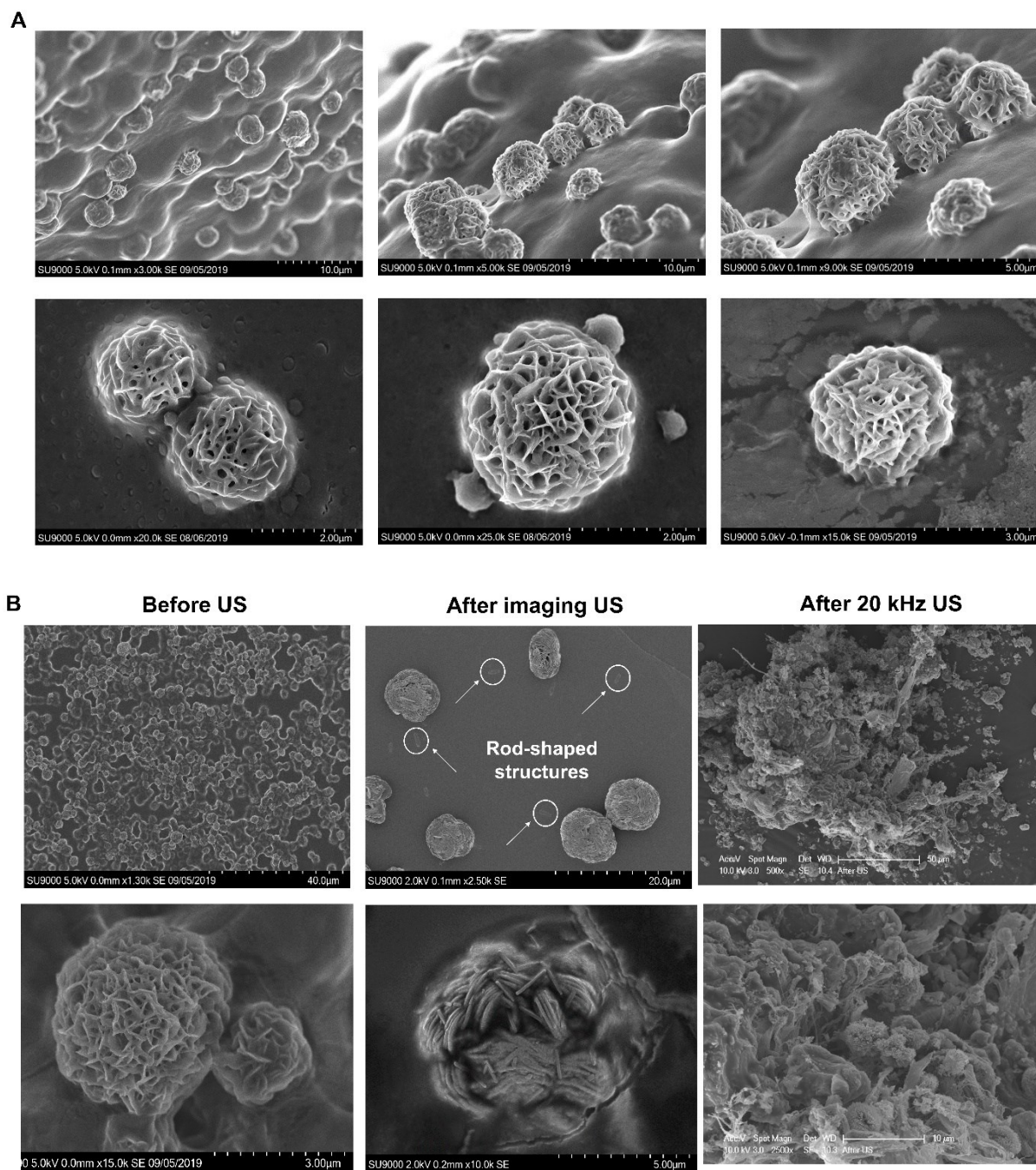


Fig. 2.20 **A.** SEM images showing RCA-ODN forming NFs at different magnification powers. **B.** SEM images showing RCA-CpG ODN before and after sonication with either imaging US or 20 kHz US. Characteristic petal-shaped NFs were formed, then fragmented and partially destroyed after exposure to imaging US while being completely destroyed after exposure to 20 kHz US.

Furthermore, we tracked the birefringence of RCA-ODN complex over reaction time (Fig. 2.24). At the beginning, particles were smaller, and no liquid crystals were observed. After about 5 h reaction time, particles started showing birefringence. To investigate whether these liquid crystals were thermotropic, we exposed the RCA-ODN complex to cycle of heating and cooling down. PLM images showed that big particles demonstrated a stable birefringence, while small particles exhibited a transient birefringence at 80-90 degrees, which disappeared after cooling down (Fig. 2.25a). Dilution of material did not affect the birefringence signal either, indicating that these liquid crystals are not lyotropic either (Fig. 2.25b).

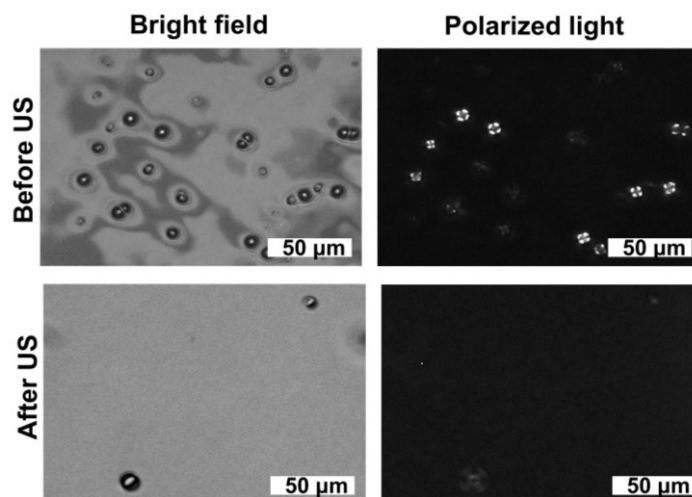


Fig. 2.21. Optical microscopy images of RCA-CpG ODN demonstrating liquid crystals formation that vanishes after US.

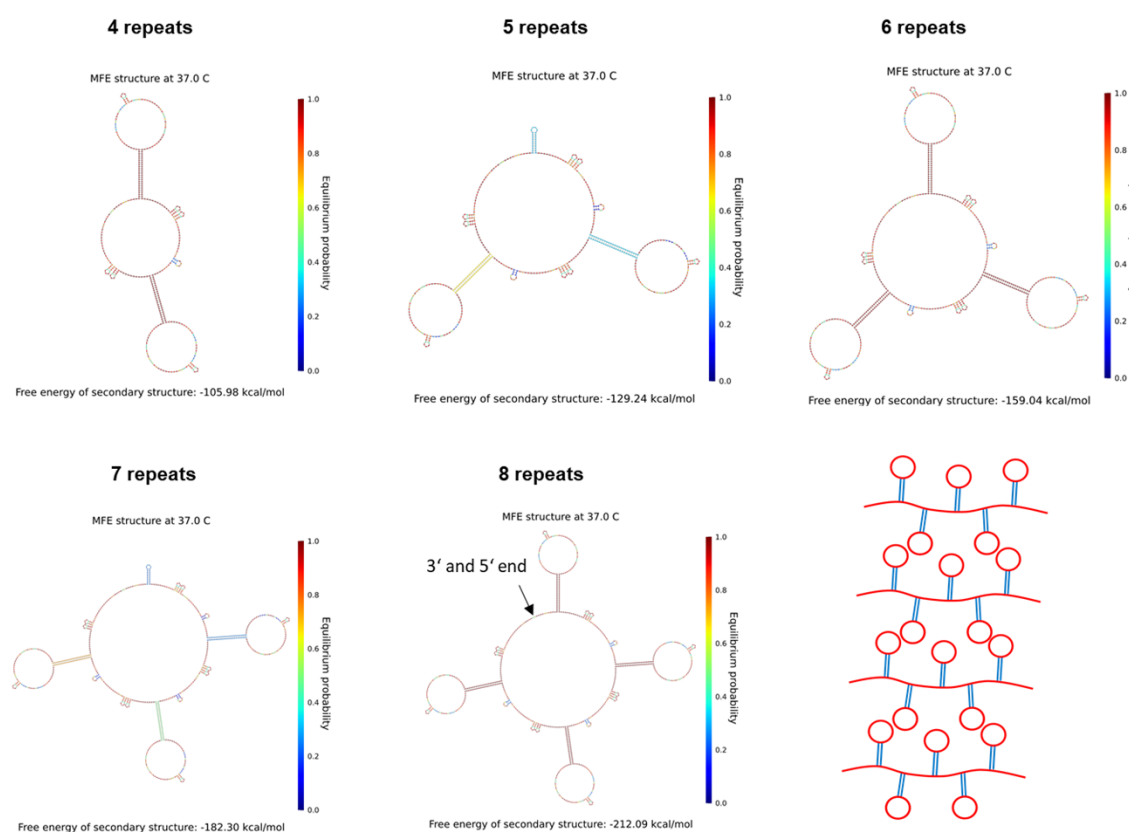


Fig. 2.22 MFE structures and their corresponding Gibbs energies obtained from the NUPACK software, for sequences containing 4 to 8 repeats of the template's complementary sequence (www.nupack.org). The schematic illustration shows the elongated conformation, which induces the lamellar ordering.

The size distribution of NFs through the RCA reaction time was tracked *in situ* using NanoTrac (Fig. 2.26). After 30 min of reaction time, a particle peak could be seen with an average of 400 nm in diameter. After another 30 min., a smaller peak with an average diameter of 100 nm, indicating the formation of a new population, in addition to the existing one. Furthermore, a small portion of the particles had a big diameter of about 7 microns, which could be attributed to potential particles aggregation to a certain extent. Over the next few hours, the same trend could be observed: a population that has grown to around 1 μm in diameter, a second population of fresh particles with smaller size in the range of 50-200 nm in diameter and a small population of aggregated particles with diameter exceeding 5 microns. The change in the relative abundance of each population throughout the reaction could give insights into the RCA dynamics.

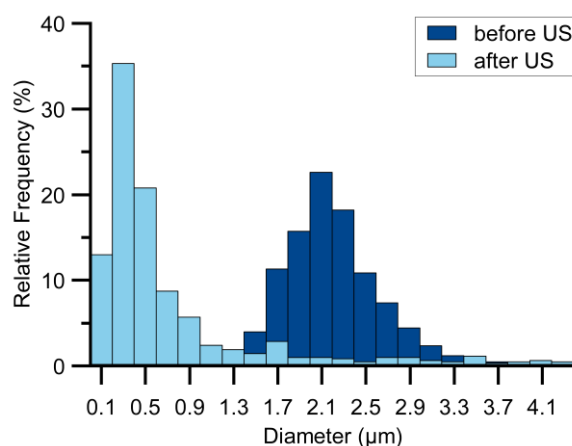


Fig. 2.23. Particle size distribution of RCA-CpG ODN before and after exposure to imaging US, showing particles' partial destruction and shifting towards a smaller diameter range after US.

The induction of this transition and the mechanochemical activation of RCA-CpG ODN by US could be associated with several mechanisms including inertial cavitation, thermal- and viscous energy transfer, as well as electrokinetic effects⁸⁵⁰. Thermal mechanisms can be potentially excluded since the thermal index (TI) was kept within the approved limits⁸⁵¹ and the measured change in temperature during sonication was ~ 1.6 $^{\circ}\text{C}$ (Fig. 2.27)). On the other hand, cavitation can potentially play a role in our system and might still be possible at the used MI of 0.9^{851,852}.

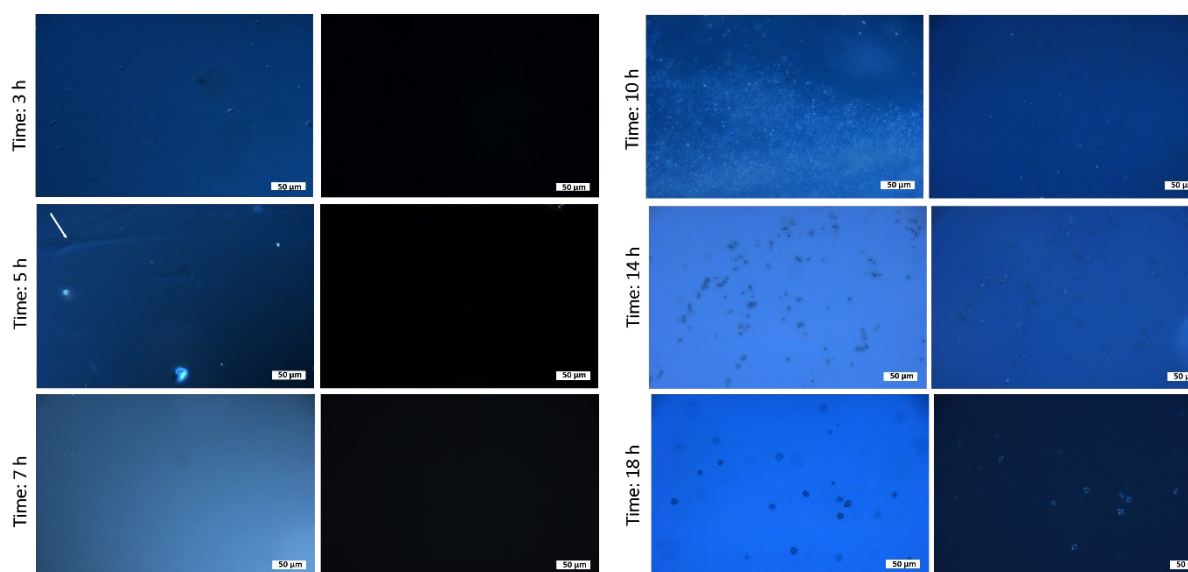


Fig. 2.24. Optical imaging microscopy images for time tracking of the formation of liquid crystals during the RCA-ODN complex formation by examining samples taken at different timepoints during the reaction, demonstrating birefringence starting at 14 h reaction time.

NFs are fixed in space within the network, allowing fluid flow relative to NFs even if the density mismatch between NFs and the fluid is low or absent. This creates viscous frictional forces between NFs and the surrounding fluid, whose molecules move reciprocally in the US field. Although according to our estimations, the total US energy delivered to the sample is more than enough to destroy the hydrogen bonds between ODN and the RCA polymer, the absence of US image contrast of the sample is evidencing its low US absorption and leads us to the consideration of electrokinetic effects (Fig. 2.28)⁸⁵⁰.

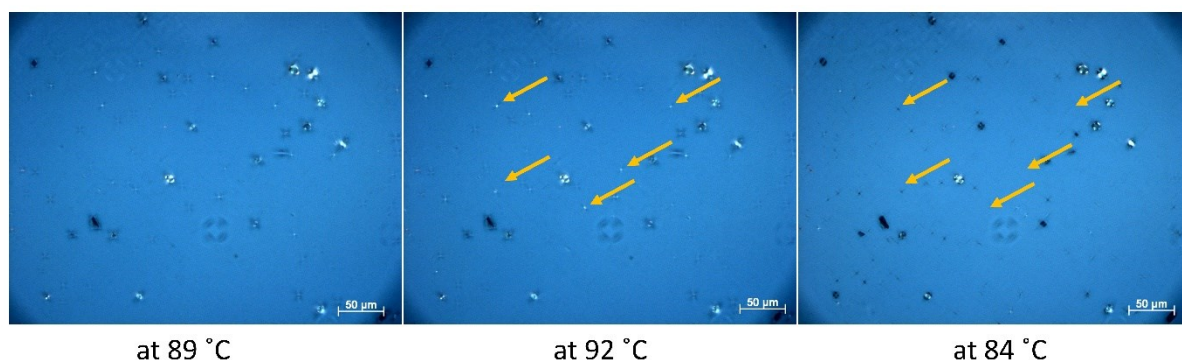
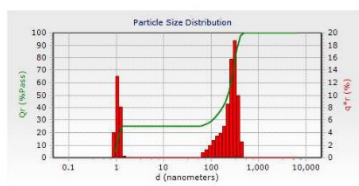


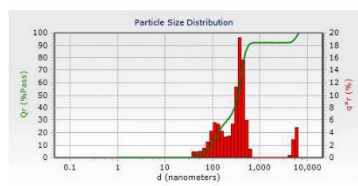
Fig. 2.25. Optical imaging using polarized light microscope for observing the effects of heating on the liquid crystals formed by the RCA-ODN complex. The large particles maintained their birefringence while small particles that did not exhibit birefringence at room temperature showed liquid crystals when heated to 92 °C and then disappeared after cooling down to 84 °C, as indicated by the yellow arrows.

RCA-CpG ODN includes hybridized sequences that are formed through specific hydrogen bonding, and the resulting negatively charged double stranded structure is stabilized by positive counterions. The fluid flows may drag away these counterions, which leads to dissociation due to the Coulomb repulsion between negatively charged phosphodiester backbones of the RCA and the ODN. Thus, as an additional possible explanation, we propose that the application of US triggers electrostatic repulsion forces, which are responsible for the observed ODN release. After this ODN release, additional internal hydrogen bonds within the RCA polymers could form, leading to additional cross-linking of the ssDNA network as confirmed by the rheology experiments (stiffening of the DNA gels). Further investigation is required to fully understand the mechanism of ODN release, which is outside the scope of this project and will be addressed in future research.

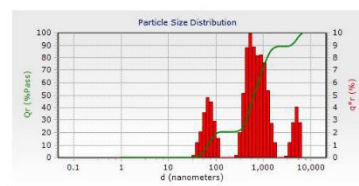
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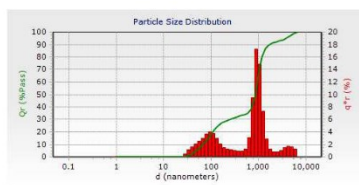
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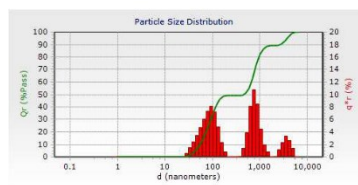
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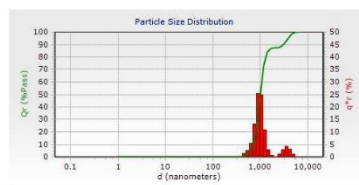
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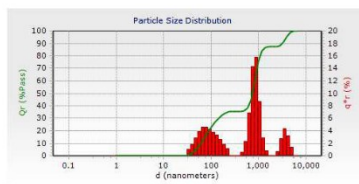
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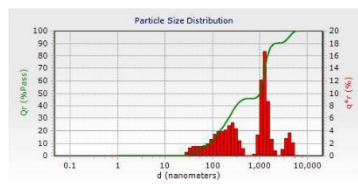
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Time 18 hr



Time 24 hr



Time 41 hr

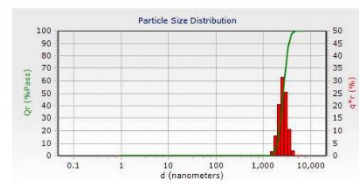


Fig. 2.26. Time tracking of particles' size distribution of RCA-ODN reaction by taking *in situ* measurements at different time points, starting from 30 min and ending with 41 h.

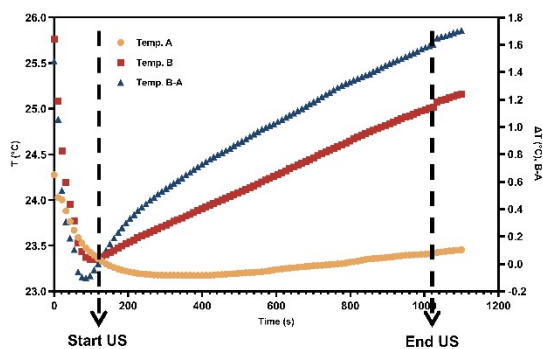
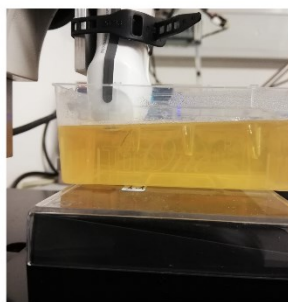
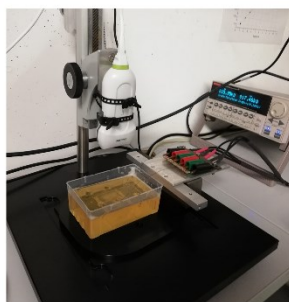


Fig. 2.27. Setup used for temperature measurement *via* thermal sensor during sonication with imaging US. Curve A is the reference well and curveB is the sonication well.

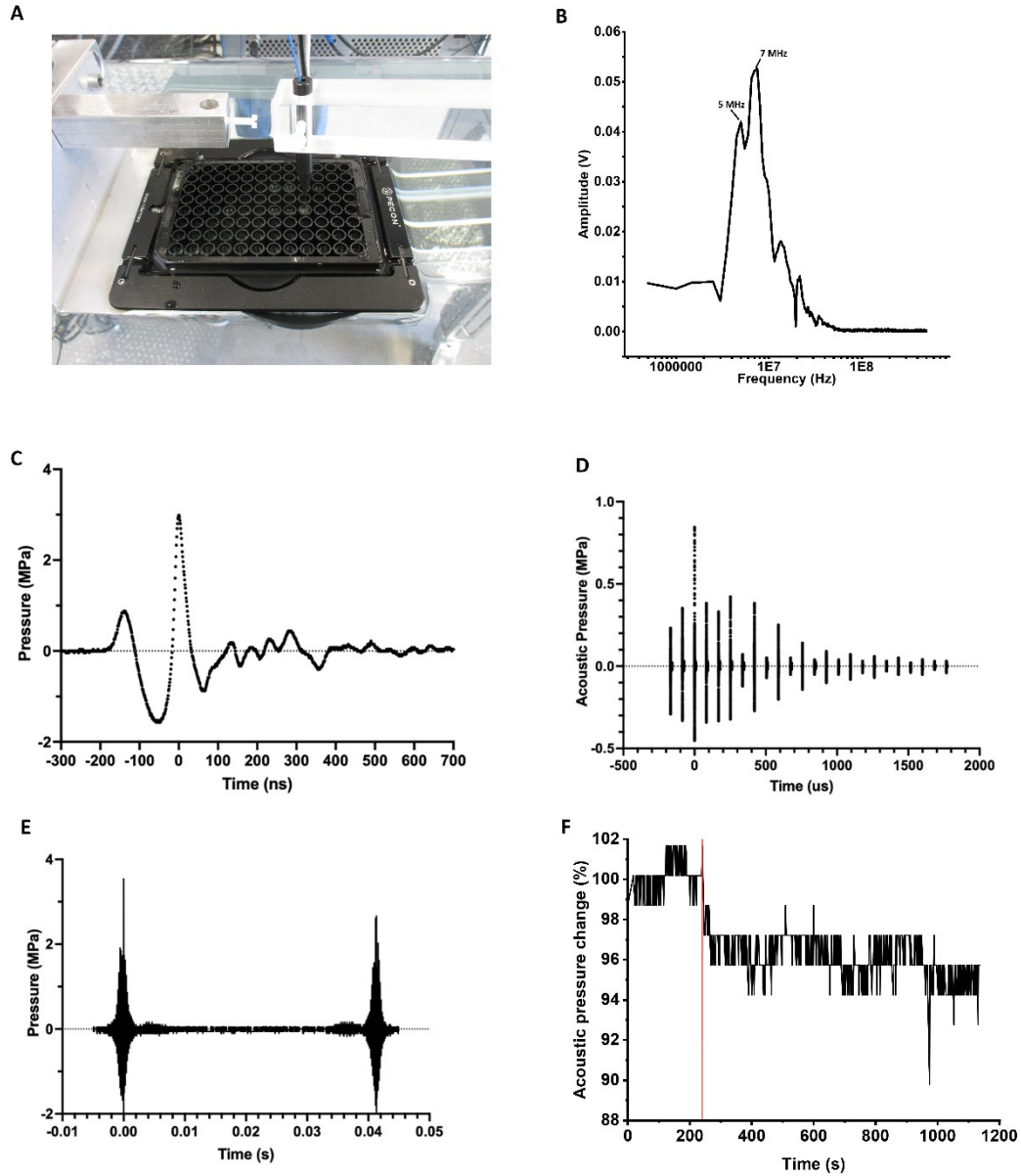


Fig. 2.28. Acoustic measurements. **A.** Experimental setup using Lumify transducer that was fixed ~1 mm above sonographic 96-well plate, while the hydrophone sensor was positioned ~5 mm below the well plate. The plate was mounted on a movable frame of motorized 3D positioning system. **B.** Acoustic pressure values were calculated using hydrophone sensitivity at 7 MHz the burst duration of approximately 200 ns was estimated. The MI of 0.6 was calculated as $p/\sqrt{f}$, where p is the peak negative pressure (MPa) and f is the frequency (MHz). **C.** The acoustic pressure pulses from the acquisition of neighbouring spatial points. Excess data points between pulses were removed for clarity. **D.** Acoustic pressure pulses at a frame rate of 25 frames per sec (fps) are illustrated. Excess data points between pulses were also removed. The peak sound intensity of ~300 W/cm² can be roughly estimated using formula for harmonic waves $I = p^2/2\rho c$, where p - maximum pressure, ρ - water density, c - speed of sound. The spatial-peak temporal-average intensity was calculated by considering pulses duty cycle (frame rate used, ignoring lower intensity neighbouring pulses) $I_{spta} \approx 1.5$ mW/cm². Assuming a sonication area of ~0.5 cm² and a total processing time of 15 min, the total delivered acoustic energy can be estimated to be ~700 mJ. **F.** Acoustic pressure change over sonication time. The first 4 min were recorded in reference well and the last 15 min were recorded in gel-containing well. The red line indicates the transition between the two wells.

2.3.3 CONTROLLED IMMUNOSTIMULATION VIA LOW INTENSITY IMAGING ULTRASOUND

2.3.3.1 US-induced immunostimulation *in vitro*

To explore the biological function of the DDS, a series of cell culture experiments was performed *in vitro*. As a proof-of-concept, we used the engineered HEK mTLR9 cell line that we described earlier. SEAP was utilized as a reporter gene which is expressed upon mTLR9 stimulation. The levels of SEAP produced can be determined using appropriate detection kits that provide a substrate for the SEAP enzymatic activity. Conversion of the substrate into a chromophore allows its quantification using spectrophotometry.

We tested our DDS in both inactive (*i.e.*, before US) and activated (*i.e.*, after US) states (Fig. 2.29a). Ultrasonication led to a 255% increase in activity compared to the untreated DDS and a 3.5-fold increase compared to pristine ODN D-SL01. This can be attributed to the RCA-ODN complex protecting the ODN cargo, which is otherwise readily degraded in cellular environment leading to poor activity. US and/or inert NFs did not cause any significant mTLR9 stimulation. Furthermore, sulforhodamine B (SRB) viability assay demonstrated the safety of using US in cell culture (Fig. 2.29b).

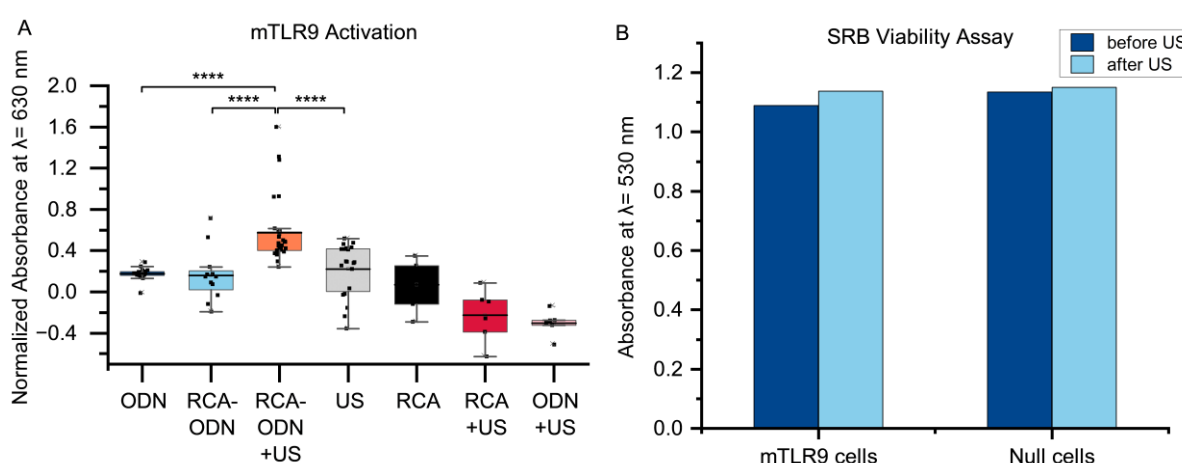


Fig. 2.29. Mechanochemical control of immune response *via* CpG ODN. **A.** Stimulation of mTLR9 in HEK-Blue™ mTLR9 cells. RCA-CpG ODN activated by imaging US exhibited a significant stimulatory effect on mTLR9. Pristine DSL-01 ODN (0.2 μ M) and inert RCA, with and without sonication, were used as controls. Imaging US alone did not elicit a significant response in the cells. **B.** SRB assay to demonstrate cell viability after exposure to imaging US.

Next, we validated the results in J774A.1 macrophages cell line which are immune cells that naturally express TLR9 without any genetic engineering. Upon TLR9 activation, mouse macrophages secrete TNF into the medium, which can be quantified using with an engineered HEK-Blue™ TNF reporter cell line. This reporter cell line utilizes the same SEAP reporter gene and measuring its activity reflects how much TNF reporter cells were exposed to. To validate the function of the reporter cells and their sensitivity and selectivity towards activation *via* TNF, we incubated the cells with different concentrations of pure TNF ranging between 0.1 to 10^4 pg/mL (Fig. 2.30a). Afterwards, J774A.1 macrophages were incubated with different concentrations of pristine ODN D-SL01 to determine their response. Subsequently, different samples including RCA-ODN with and without sonication were incubated with macrophages. After 24 h, supernatants were collected and incubated with HEK-Blue™ TNF reporter cells for

another 24 h, and a detection kit was used to quantify SEAP in their supernatants. The results showed a 288% increase in TNF production after activation of the DDS *via* imaging US (Fig. 2.30b).

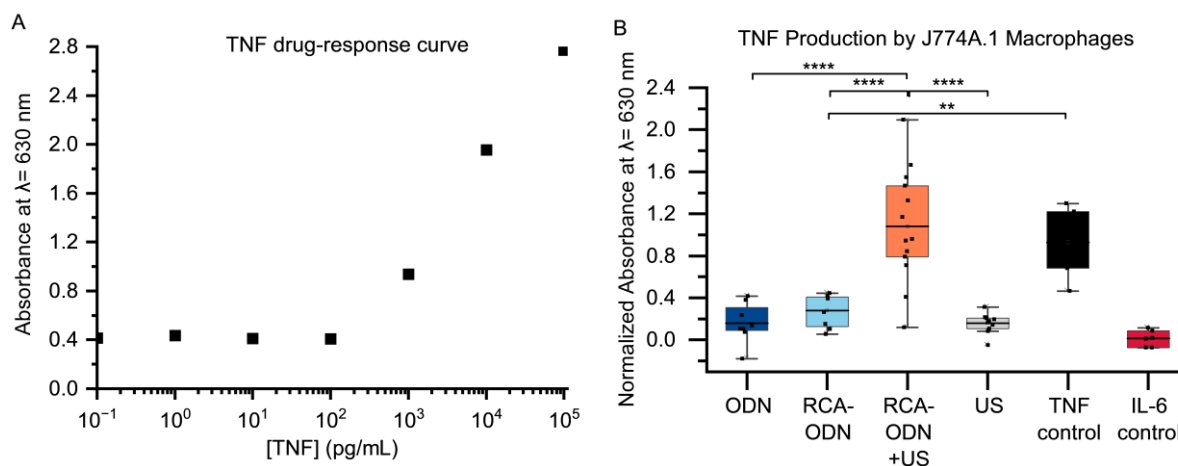


Fig. 2.30. **A.** Dose-response curve of the HEK-TNF reporter cell line showing the stimulation of the TNF receptor by incubation with increasing concentration of pristine TNF as a positive control. **B.** Production of TNF following stimulation of J774A.1 macrophages. RCA-CpG ODN in combination with imaging US resulted in a significant increase in TNF levels, comparable to that of the pure TNF positive control ($1 \text{ ng} \cdot \text{mL}^{-1}$). Imaging US alone, pristine ODN ($0.2 \text{ } \mu\text{M}$), and RCA-CpG ODN prior to US did not elicit a significant production of TNF.

Primary cells offer an *in vitro* model with high biological and clinical relevance for studying drug efficacy, toxicity and safety. In addition, they provide more reliable and translational results when compared to cell lines, since they retain characteristics from the donor. Therefore, after verifying our DDS's ability to efficiently deliver ODN D-SL01 and activate its target receptor, we evaluated its efficacy for cancer immunotherapy by investigating the interaction between APCs, T cells and cancer cells.

To create an experimental model that is highly relevant towards *in vivo* applications and captures the true diversity and complexity of living tissue, we isolated primary immune cells from healthy human donors' blood. The first APC to be investigated was macrophages. Our results showed low response of macrophages towards stimulation with pristine D-SL01 (Fig. 2.31). We further test PS-modified D-SL01 which was expected to exert more potent effects. However, this did not result in macrophages' stimulation. Hence, the quantity of ODN loaded to the DDS would need to be increased dramatically, which is impractical and causes higher incidence of side effects. Alternatively, we aimed to stimulate DCs, which have higher levels of TLR9 expression. Thus, they are expected to be more sensitive to CpG ODNs stimulation.

While DCs play a crucial role in the immune system, they are scarce in the bloodstream, constituting only about 0.1 - 1% of total blood mononuclear cells⁸⁵³. Consequently, isolation methods often resort to differentiating DCs from monocytes extracted from PBMCs. In our study, we adopted this approach and devised a 17-day experimental model, involving the co-cultivation of DCs, T cells, and B16F10 melanoma cells. The experiment was divided into three stages (Fig. 2.32).

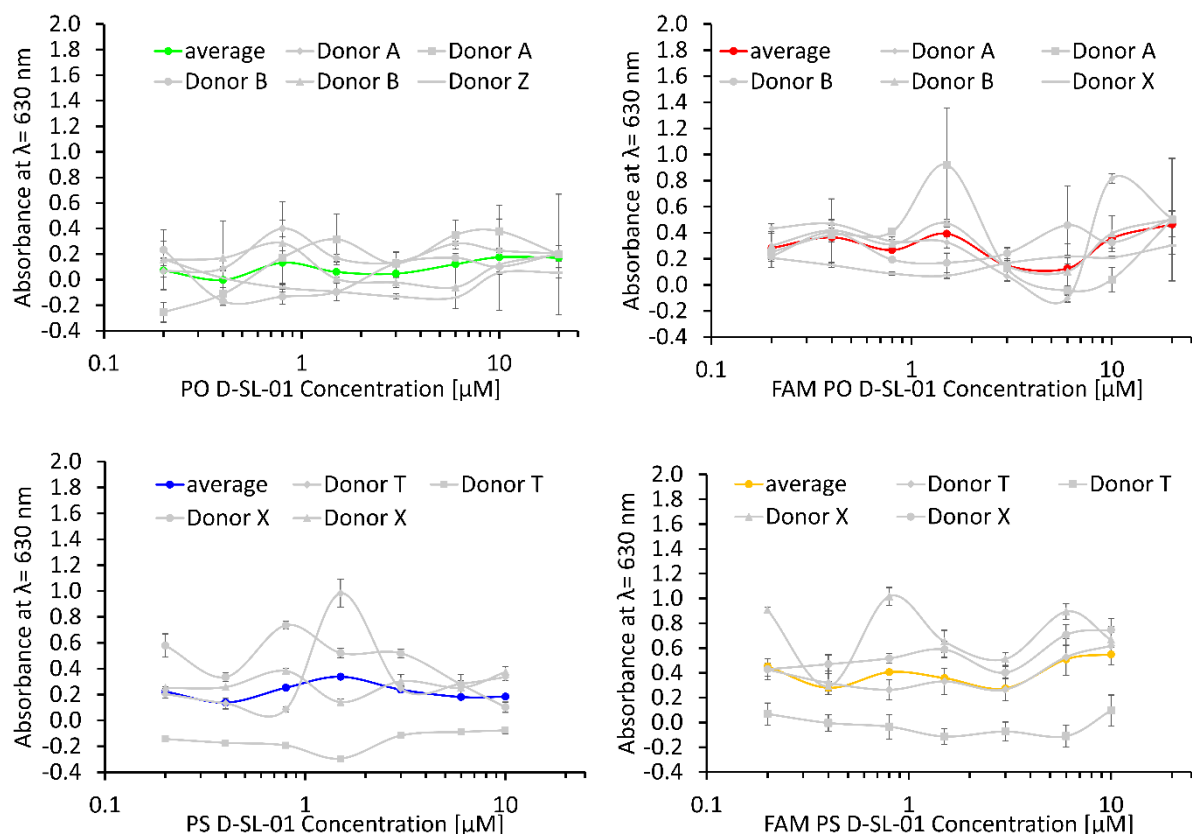
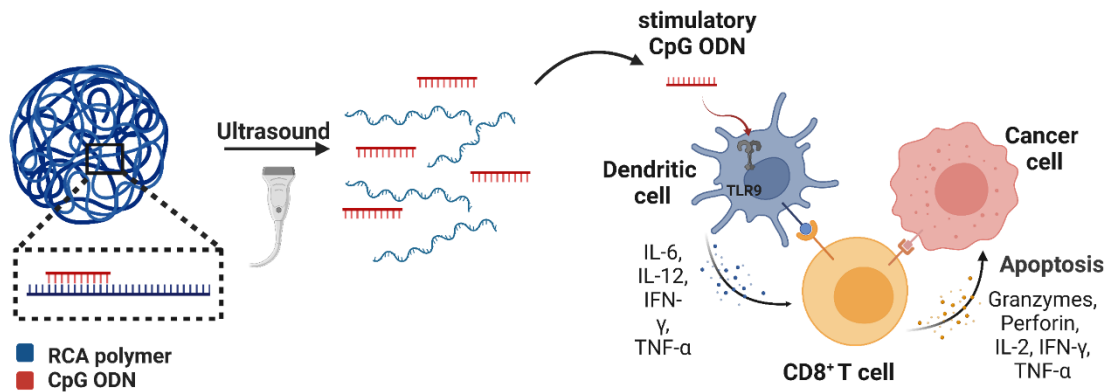


Fig. 2.31. TNF quantification in the supernatants of primary macrophages in response to stimulation by increasing concentrations of ODN DSL-01, either in the natural PO or modified PS backbone.

First, PBMCs were extracted from human whole blood using Ficoll gradient method, and monocytes were negatively isolated followed by assessment of their purity and phenotype using CD14-biomarker (Fig. 2.33). Monocytes' differentiation into immature DCs (iDCs) was stimulated with growth factors, followed by maturation into mature DCs (mDCs). A cytokines cocktail was used as a positive control for the maturation step, while the DDS was tested with or without the application of US. The process was accompanied by an increased expression of surface biomarkers, such as CD80, CD83 and CD86 (Fig. 2.34). CD80 and CD86's relative expression levels play important roles in balancing T cells' activation and attenuation by interacting with target receptors CD28 and CTLA-4 leading to enhancement or offsetting T cell activation, respectively⁸⁵⁴.

A Tri-culture model for investigating CpG ODN role in cancer immunotherapy



B

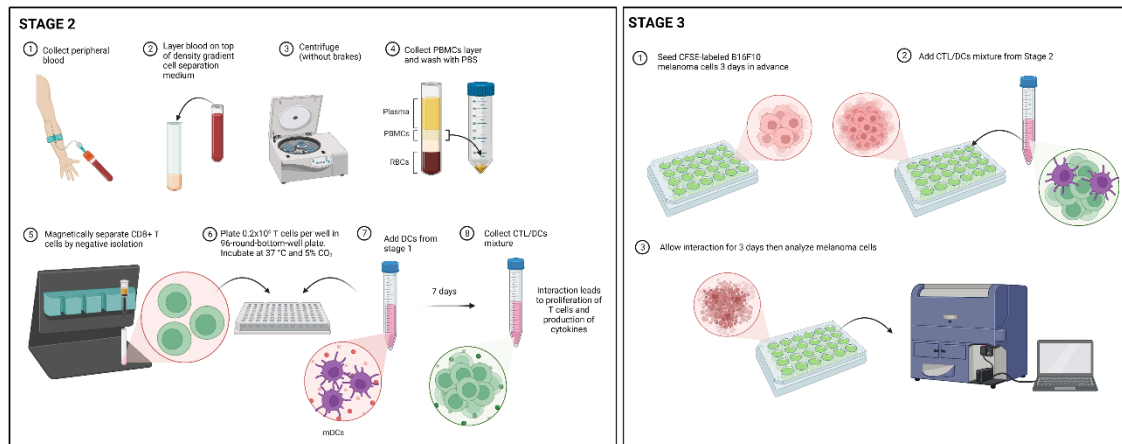
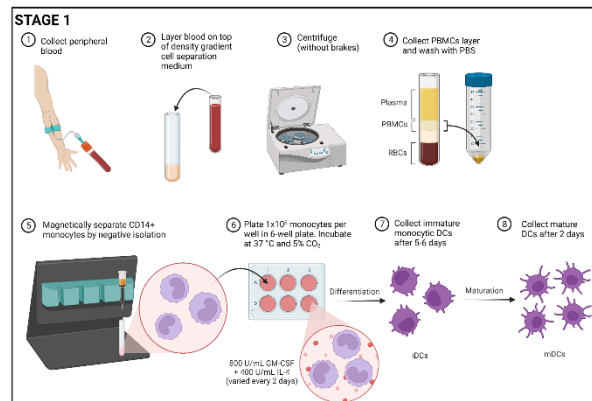
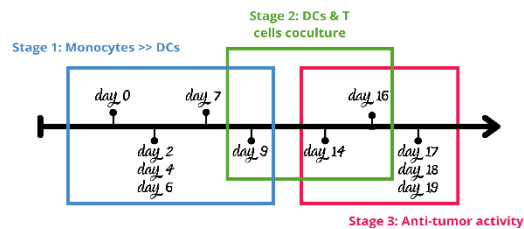


Fig. 2.32. A. US-mediated activation of CpG ODN and its role in cancer immunotherapy. **B.** Experimental design and timeline of *in vitro* triculture cell experiment with three different stages. In stage 1, primary monocytes are isolated from primary blood mononuclear cells (PBMCs) and differentiated into immature dendritic cells (iDCs), which are then activated to become mature dendritic cells (mDCs). In stage 2, primary CD8⁺ T cells are isolated from PBMCs and co-cultured to interact with DCs resulting in T cell proliferation. In stage 3, the DC:T cell mixture is added to pre-seeded B16f10 melanoma cells, and their interaction is analysed after 24 h. Created with BioRender.com

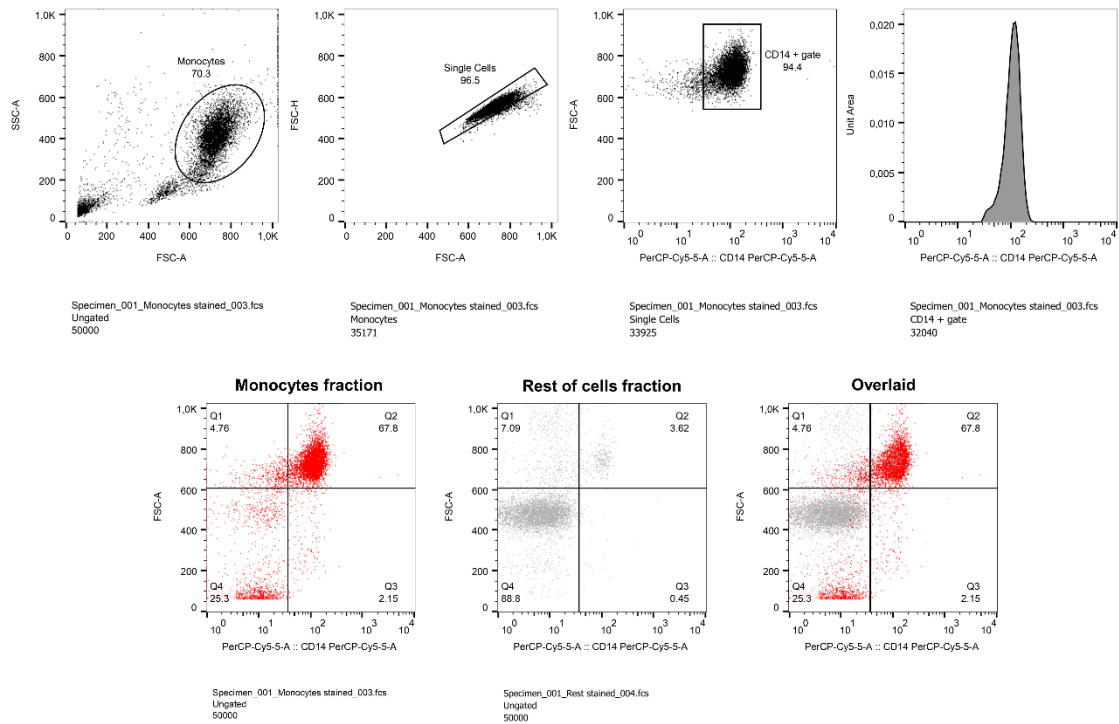


Fig. 2.33. Primary cells isolation from human whole blood. Isolation of monocytes from whole blood using CD14 as surface biomarker. The positive fraction was enriched with CD14⁺ cells, while a small percentage of the negative fraction contained CD14⁺ cells. The data are representative of three independent experiments.

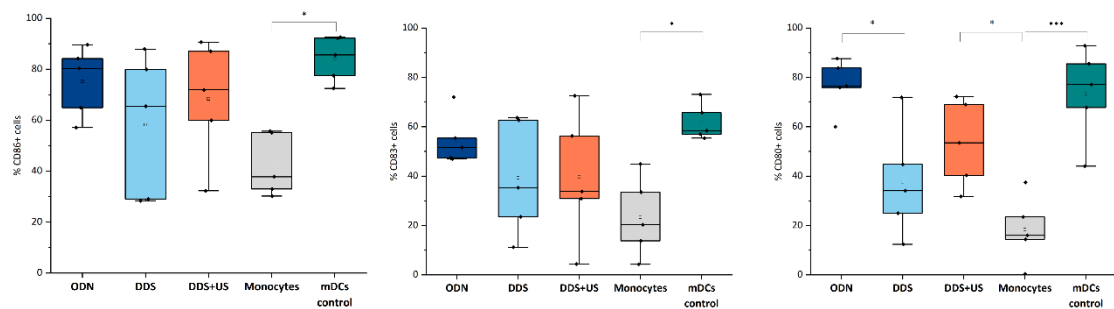


Fig. 2.34. Analysis of expression of surface biomarkers. CD80, CD83 and CD86 surface biomarkers expressed by monocytes and DCs that were exposed to different conditions.

In the second stage, CD8⁺ T cells were isolated from PBMCs and their purity and phenotype were assessed using CD4 and CD8 as biomarkers (Fig. 2.35). CD8⁺ T cells were stained with CellTrace Violet to track their proliferation. This dye permanently binds to intracellular proteins and render cells fluorescent. After cell division, each daughter cell contains half of the parent cell's dye, and therefore half the fluorescence, enabling quantification of proliferation *via* flow cytometry (Fig. 2.36a).

On day 9, mDCs and CD8⁺ T cells were cocultured in 1:1 ratio and allowed to interact for 7 days. As a positive control, T cells were activated using concanavalin A (ConA). T cells proliferation results in multiple cell generations, evident by tracking the dilution of CellTrace Violet dye using flow cytometry (Fig. 2.36b). T cells interaction with mDCs treated with the DDS and US induced their effective proliferation. TNF production by activated T cells was evaluated and showed significant increase in samples treated with DDS and US (Fig. 2.37). The standard deviation observed could be explained by the high inter-donor variability of surface biomarkers expression as well as cytokine release of primary human blood cells, as described previously^{855,856}. In the last stage, a triculture of DCs, T cells and melanoma cells was established. B16F10 melanoma cells were labelled with CellTrace CFSE. After 24 h of incubation of the triculture, viable melanoma cells were significantly reduced when they were incubated with DC: T cell mixture that was treated with DDS plus US (Fig. 2.38).

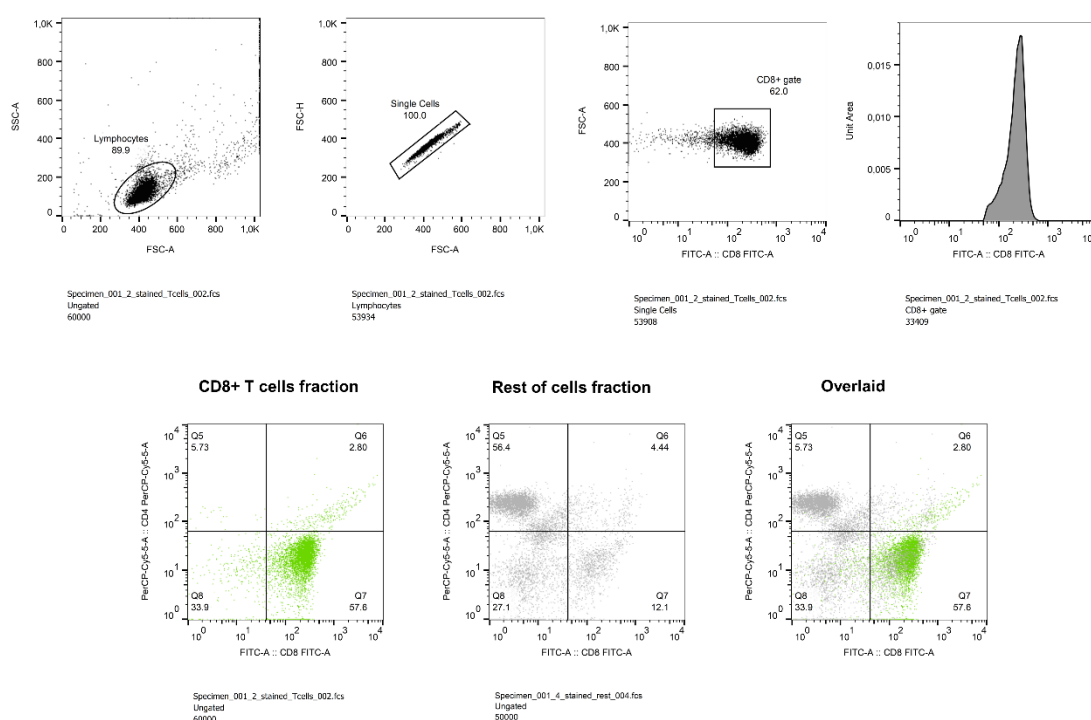


Fig. 2.35. Primary cells isolation from human whole blood. Isolation of CD8⁺ T cells from whole blood using CD8 and CD4 as surface biomarkers. The positive fraction was enriched with CD8⁺/CD4⁻ cells, while a small percentage of the negative fraction were CD8⁺/CD4⁺. The majority of cells in the negative fraction were found to be CD8⁻/CD4⁺. The data are representative of three independent experiments.

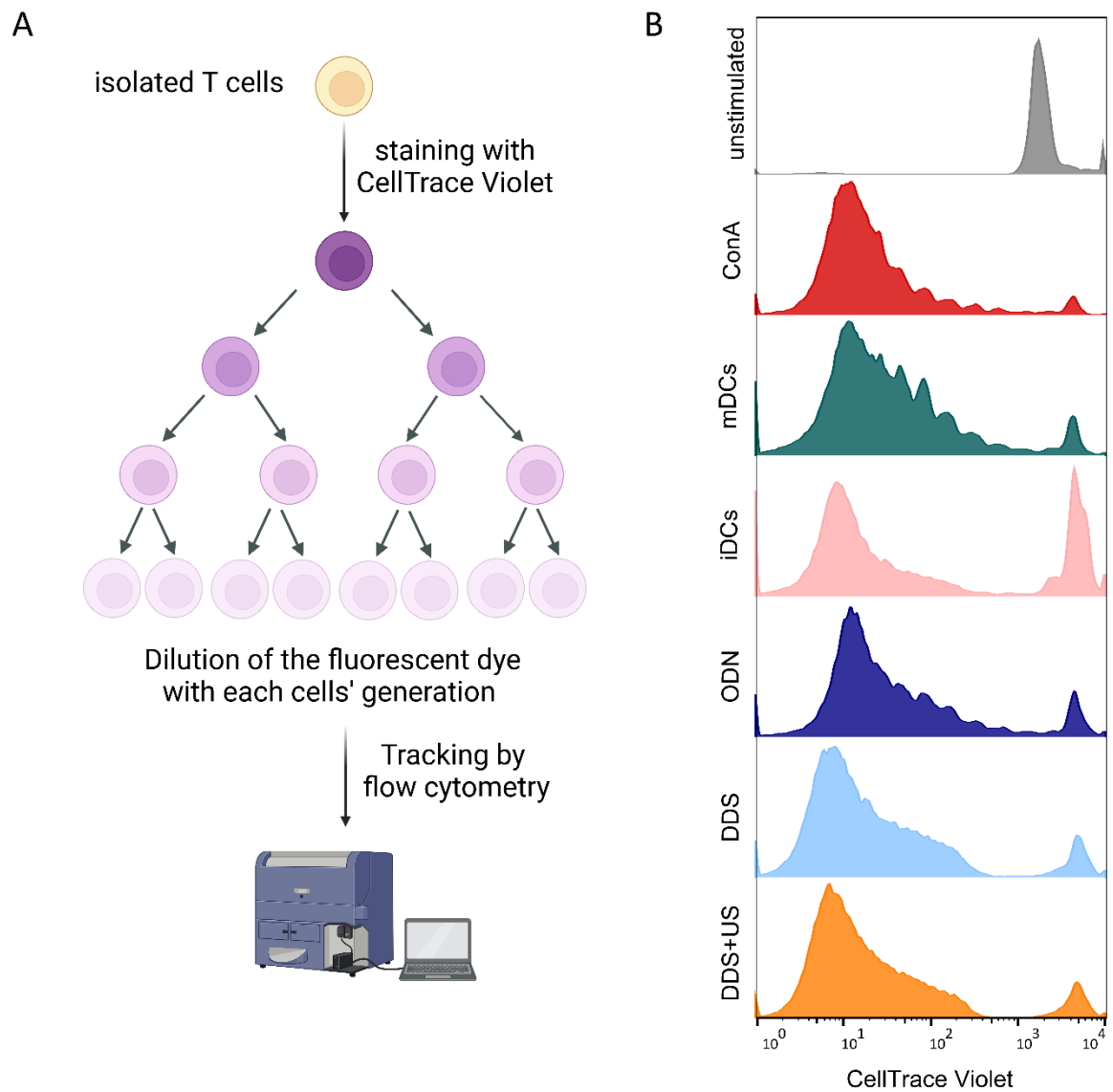


Fig. 2.36. A. Proliferation tracking of T cells labelled with CellTrace Violet utilizing the dye dilution method. Created with BioRender.com **B.** Flow cytometry histograms for T cells tracking. Unstimulated cells exhibit a single peak with high fluorescence intensity, indicative of a lack of proliferation. Multiple peaks with decreasing fluorescence intensity are observed when the cells are stimulated and undergoing proliferation under different experimental conditions.

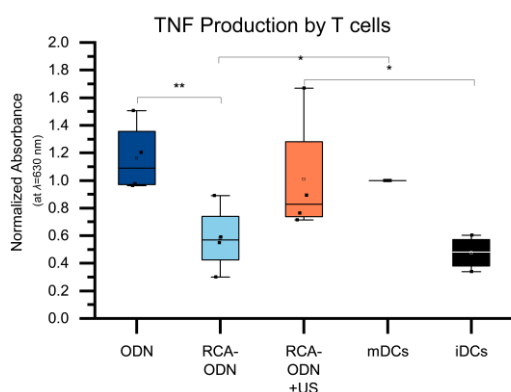


Fig. 2.37. TNF production by T cells after co-culture with DCs treated under different conditions. Increased TNF production is observed when T cells were co-cultured with mDCs control, DCs incubated with pristine DSL01 ODN (2 μ M) and DCs incubated with US-activated RCA-CpG ODN DDS.

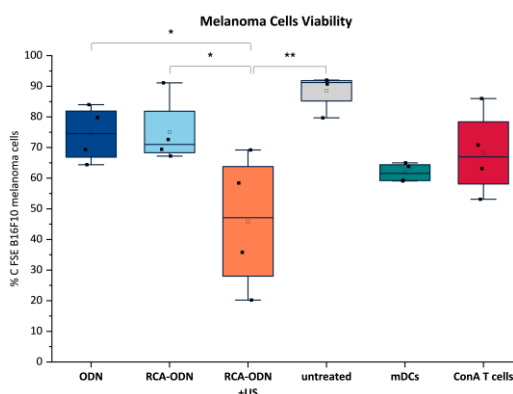


Fig. 2.38. Viability of CFSE-labelled B16F10 melanoma cells after 2 h incubation with DC:T cells co-cultures that were exposed to different types of stimulation. The samples which have DDS activated with imaging US show significant killing of melanoma cells, as demonstrated by a marked decrease in cell viability.

2.3.3.2 US-induced immunostimulation *in vivo*

To evaluate the system's performance *in vivo*, mice were injected intravenously with RCA-CpG ODN containing 90 μ M (90 μ g in 125 μ L) ODN D-SL01, and subjected to either 15 min of imaging US or left without sonication. Additional mice groups either received pristine ODN D-SL01, US only, or left completely untreated (Fig. 2.39). Longitudinal distribution using fluorescence tomography (FLT) revealed that the DDS (labelled with Cy5-modified nucleotides in the RCA backbone) accumulated mostly in the liver, where Kupffer cells are expected to

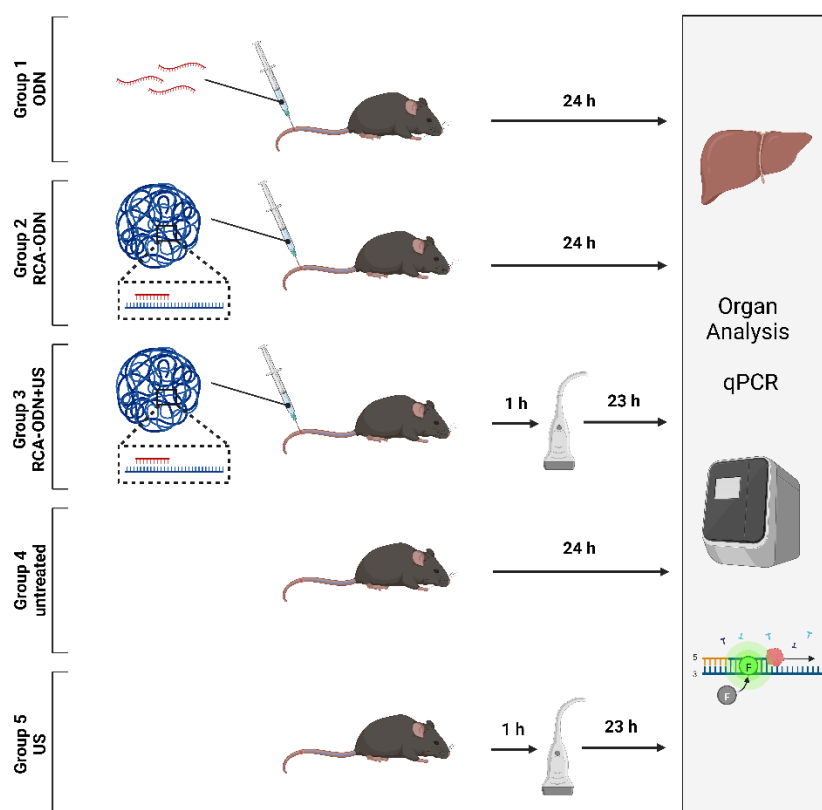


Fig. 2.39. US-controlled immunostimulation *in vivo*. Animal groups used in animal experiments *in vivo*. Group 1 served as a positive control and received pristine CpG ODN. Group 2 and 3 received RCA-CpG ODN with and without imaging US treatment, respectively. Group 4 was left untreated, while group 5 was only exposed to US. Created with BioRender.com

uptake and internalize the material⁸⁵⁷ (Fig. 2.40). The signal declined after 60 min, hence we performed ultrasonication 1 h post injection and mice were sacrificed after another 23 h. Livers were perfused from blood, total RNA was isolated from liver tissues and the expression of selected inflammatory mediators was investigated using quantitative real-time PCR (qPCR).

Our results demonstrated that US-activated DDS induced a profound induction of several cytokines such as Tnf and Ccl-22, whereas DDS alone induced other cytokines such as Il1- β and Il-10 (Fig. 2.41). Without US activation, the DDS remained inactive indicating successful binding of the CpG ODN without detectable non-specific leakage from the carrier. TNF is one of the major pro-inflammatory mediators that has different roles in health and disease. Its stimulation has been associated with a more robust immune response and an increased activity of different types of T cells⁸⁵⁸. Together with IL-10 which can promote anti-tumor effects^{859,860}, this pattern of immunostimulation could be beneficial for promoting anti-tumor immunity. It might turn ‘cold’ tumors into ‘hot’ tumors which respond better to immunotherapeutic treatment⁸⁶¹. On the other hand, induction of immunosuppressive cytokines such as IL-1 β ⁸⁶² and chemokines such as CCL22⁸⁶³ indicates the potential of eliciting a balanced immune response. Importantly, it should be kept in mind that exaggerated inflammation might otherwise lead to side effects such as cytokine release syndrome (CRS).

Meanwhile, the pristine ODN D-SL01 or US alone did not trigger any molecular response. These changes in whole liver tissue are very pronounced and can be attributed to a CpG ODN-driven activation of the hepatic immune cells. It is anticipated that the RCA-ODN complex would exist in the liver macrophages within a complex biological matrix that resembles the dense environment of gels before injection, thereby enabling it to react to US and trigger CpG ODN activation. Moreover, histological analysis of the liver tissue showed no signs of distortion demonstrating the safety of both RCA-CpG ODN and imaging US (Fig. 2.42). These results therefore confirm our DDS’s functionality, safety and successful on-demand mechanochemical activation by imaging US *in vivo* leading to the desired pharmacological response.

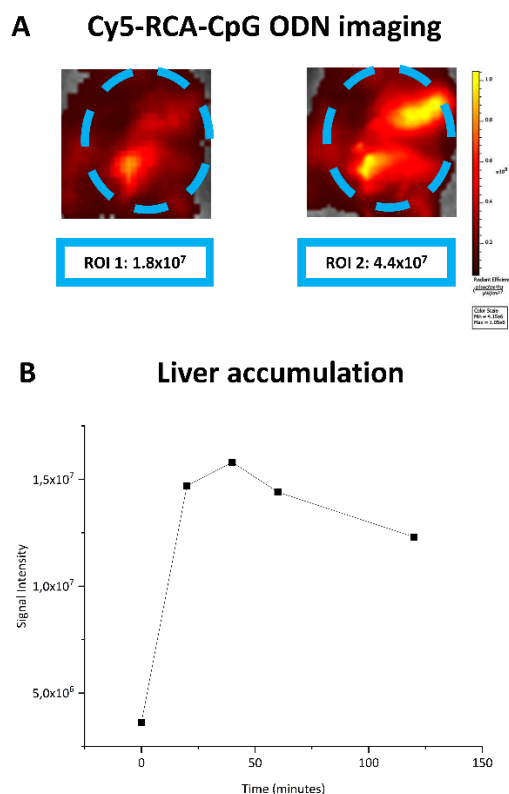


Fig. 2.40. A. *In vivo* imaging of RCA-CpG ODN by incorporating Cy5-labeled nucleotides into RCA polymer backbone. Pre-scan before RCA-CpG ODN administration (left) and 60 min after RCA-CpG ODN administration (right). The region of interest for signal quantification is highlighted by the blue dashed circle. **B.** Quantification RCA-CpG ODN accumulation in the liver region of interest using FLT.

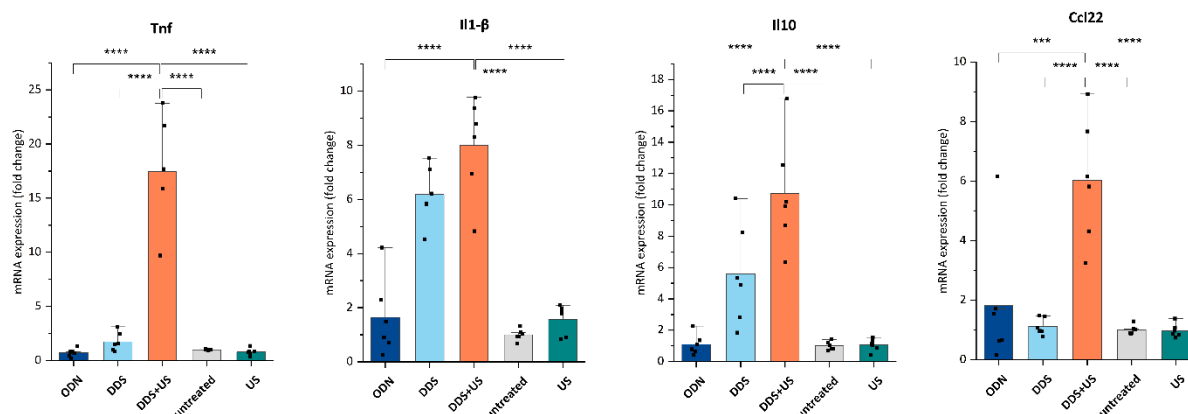


Fig. 2.41. Quantitative real-time PCR analysis of inflammatory mediators' expression in whole liver tissue demonstrates successful immunostimulation following the activation of ODN D-SL01 *in vivo* by imaging US.

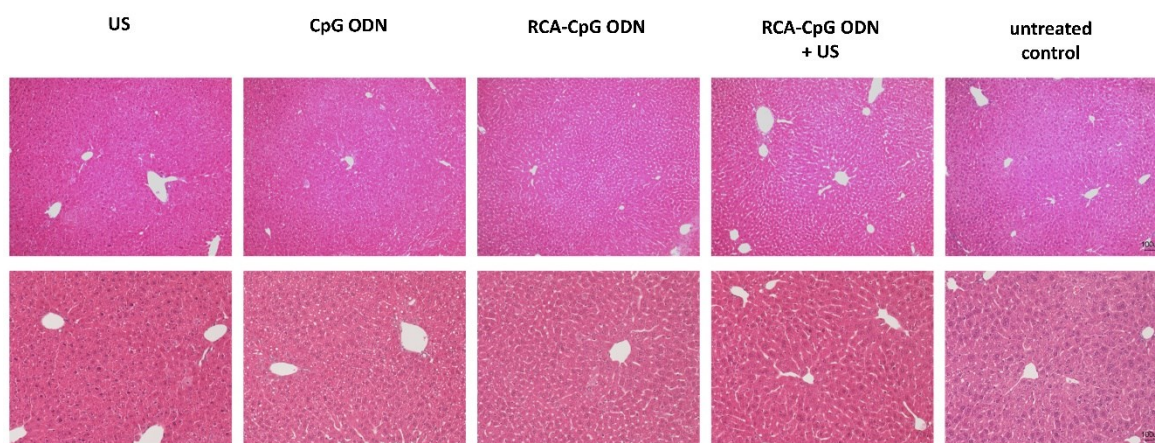


Fig. 2.42. Immunohistochemistry staining of liver specimens demonstrates no change or distortions, indicating the safety of using both RCA-CpG ODN and imaging US *in vivo*.

2.4 CONCLUSION AND OUTLOOK

We transferred the conceptual framework of polymer mechanochemistry into application *in vitro* and *in vivo* to control a biomedically relevant function such as immunostimulation. Towards that goal, we fabricated RCA polymer-based DDS that inherently respond to ultrasonication using low-power medical devices without the addition of external microbubbles.

Different CpG ODNs were successfully deactivated *via* loading to the RCA polymer and subsequently mechanochemically released in their intact and biologically active form. The loading and release efficiencies were optimized and the system was extensively characterized both before and after ultrasonication. Sensitivity to low-intensity US at high-frequency was achieved by fabricating a hierarchically structured gel. At the molecular level, specific non-covalent interactions led to cargo binding to the polymer backbone within NFs. At the macroscopic scale, the NFs were immobilized within a colloidal gel matrix. On-demand cargo release triggered by US was caused most likely by electrokinetic effects.

In vitro and *in vivo* studies demonstrated successful controlled delivery and immune response in different models. Previously, nucleic acid delivery has been augmented with US employing different carrier systems. Hybrid systems consisting of microbubbles and a transfection reagent, targeted MB equipped with a ligand against a specific receptor, as well as co-encapsulation of the TNA with another drug such as Paclitaxel were exploited. However, these systems required

either higher US power or microbubbles as adjuvants that enhance their response towards lower intensity US. In the previous applications, the role of the US was to enable improved tissue penetration, accompanied by off-target effects caused by shock waves, water jetting, and other phenomena. However, direct activation of TNAs from a mechanoresponsive carrier using a clinically approved US device has not been reported. A strong benefit arising from the paradigm change employing our approach was demonstrated. Since RCA carriers are programmable, they can be tailored to accommodate different types of cargo including other types of TNAs (*e.g.*, siRNA, RNA decoys, ribozymes, ASO, aptamers and DNazymes) as well as small molecules and proteins. For the codelivery of multiple drugs, altering the RCA polymer backbone to encode aptamer sequences that have high binding affinity to the designated cargo is needed (Fig. 2.43a,b and d). Alternatively, proteins and ABs can be conjugated with short DNA oligomers that hybridize to the RCA polymer backbone (Fig. 2.43c). All these variations of our DDS open the door for exciting possibilities for combination therapies in different diseases. In the context of cancer immunotherapy, we foresee using our DDS as multifunctional carrier to deliver CpG ODNs, TAAs and/or checkpoint inhibitors. This combination can be investigated in the future in tumor model *in vivo* to achieve targeted cancer immunotherapy.

DDS design for simultaneous delivery of multiple cargo

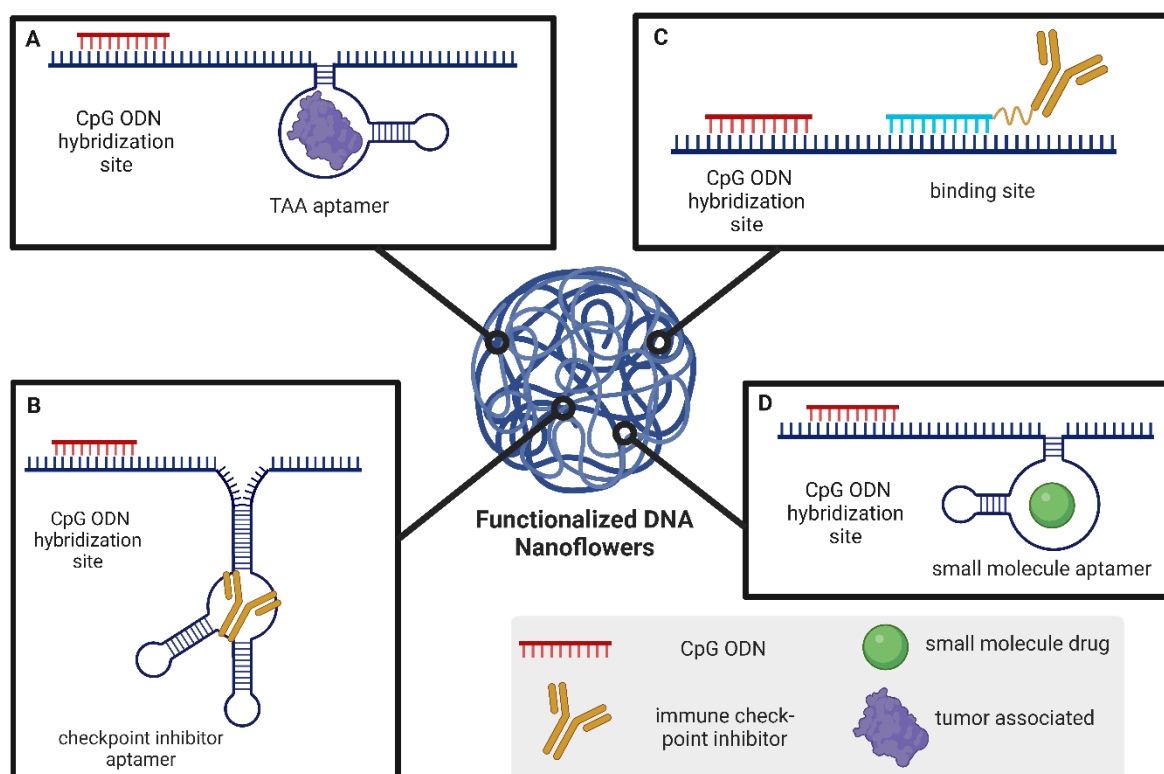


Fig. 2.43. Scheme showing the versatility of the developed mechanoresponsive DDS in which it can be adapted to accommodate different types of cargo. **A.** Delivery of CpG ODN and TAA loaded *via* hybridization site and aptamer, respectively, to increase targeting of cancer immunotherapy. **B.** Delivery of CpG ODN and checkpoint inhibitor, loaded *via* hybridization site and aptamer, respectively, to increase efficiency of cancer immunotherapy. **C.** Alternative loading strategy for checkpoint inhibitors that don't have selective aptamers, by conjugation of the protein with a short oligonucleotide sequence that has a hybridization site on the RCA polymer. **D.** Delivery of CpG ODN and small molecule drug loaded *via* hybridization site and aptamer, respectively, to improve cancer therapy. Created with BioRender.com

3. Mechanoresponsive DDS for controlled gene knockdown

3.1 INTRODUCTION

To demonstrate the activation of other types of TNAs by US, we aimed to deliver siRNA using the same DDS. siRNA are short dsRNA molecules that range from 20 to 24 nt in length. Their distinct structures include 5'-phosphate and 3'-OH endings with two 3'-overhangs on each side of the duplex. Since synthetic siRNA can induce RNAi in mammalian cells, researchers have been extensively using it as a gene silencing tool to explore genes' function and as a therapeutic agent that can theoretically target any disease⁷⁶⁷.

The siRNA natural mechanism of action includes cutting of longer dsRNAs by Dicer (a member of the RNase III family), to isolate an antisense (or guide) strand and a sense (or passenger) strand. Then, siRNA is incorporated into RISC where it interacts with argonaute-2 (AGO-2) protein that degrades the sense strand. The antisense strand guides the RISC through binding to its target complementary mRNA sequence, leading to AGO-2 mediated cleavage⁸⁶⁴.

Despite the immense therapeutics promise of siRNA, its widespread application in the clinical setting faces several challenges related to siRNA stability, delivery and toxicity⁸⁶⁵. A large set of chemical modifications of siRNA have been successfully developed to enhance its performance by prolonging its half-life, improving its stability in the blood, modulating the body's immune response and reducing off-target side effects⁸⁶⁶. Furthermore, the use of delivery platforms was demonstrated to be required for the successful use of siRNA *in vivo*⁸⁶⁷. Various delivery strategies were investigated, including siRNA conjugates and nanoparticles-based DDS using different materials such as nucleotides, lipids and polymers⁸⁶⁸. In this work, we develop our delivery platform based on RCA carriers that can be triggered by medically compatible and low energy imaging US device. This work adds to the sonogenetics toolbox, enabling on-demand gene downregulation *in vitro*. Some experimental components of this chapter were conducted during Ms. Xiaofeng Zhang research internship.

3.2 METHODS

RCA-RNA complex preparation

The same protocol that was described earlier to synthesize RCA-CpG ODN complex was used to prepare RCA-RNA complex. The template was designed with hybridization sites for either sense strand, antisense strand or both.

Cell culture

Human adenocarcinoma HeLa cells were purchased from the American Type Culture Collection (ATCC) and maintained in DMEM medium (Gibco, 41966052) supplemented with 10% (v/v) FBS, 100 U/mL penicillin and 100 µg/mL streptomycin at 37°C in 5% CO₂.

HeLa cells transfection with EGFP gene

HeLa cells were transfected with pSBtet-GP, which was a gift from Eric Kowarz (Addgene Plasmid #60495; <http://n2t.net/addgene:60495>; RRID:Addgene_60495) following the published protocol⁸⁶⁹. The success of EGFP transfection and expression were confirmed using flow cytometry and confocal microscopy.

EGFP knockdown by siRNA

HeLa EGFP cells were seeded in 24-well plates at a seeding density of 2*10⁴ cells/well in a volume of 1 mL complete media (DMEM supplemented with 4.5 g/L glucose,

2 mM L-glutamine, 10% (v/v) FBS, 100 U/mL penicillin and 100 µg/mL streptomycin). After 24 h, 500 µL of media was removed and siRNAs or RCA products in Opti-MEM™ media (Gibco by life technologies, 31985070) were added for a final volume of 600 µL. In control wells, cells were transfected with 20 pmol RNA with or without 1 µL Lipofectamine™ 2000 (Invitrogen™, 11668019) per well. For RCA-RNA mixture, 30 µL of both RCA-sense ssRNA and RCA-antisense ssRNA were mixed in 40 µL Opti-MEM medium and added to the well. After that, cells were sonicated with imaging US (vascular mode, 10 min, MI = 1.0) or left untreated. Cells were harvested after 3 days, and flow cytometry was used to quantify *EGFP* expression ($n \geq 4$, measurements were done for different distinct samples of each condition).

Confocal Microscopy

TCS SP8 confocal scanning laser microscope from Leica Microsystems (Wetzlar, Germany) was used to confirm the successful transfection of HeLa-EGFP cells, by seeding cells in their growth medium in 8-well µslide (Ibidi, 80826) with a seeding density of 8×10^4 cells/well in a volume of 300 µL. Cells were allowed to adhere to the slide before examination under the confocal microscope.

Cytotoxicity assay

MTS assay was used to evaluate the viability of HeLa-EGFP cells after co-culture with different control and experimental conditions (MTS Cell Proliferation Colorimetric Assay Kit, BioVision, K300-500) following the manufacturer's instructions. Briefly, the experiments involving cell culture were done as described in the protocols, then at the last step, MTS reagent was prepared following manufacturer's instructions and then was added to each well in a volume that is adjusted based on the well size. Plates were incubated for 0.5-4 h at 37 °C in standard culture conditions. Absorbance was measured ($n = 6$, measurements were done for different distinct samples of each condition) using a plate reader at $\lambda = 490$ nm (SpectraMax iD3, Molecular Devices, USA).

3.3 RESULTS AND DISCUSSION

3.3.1 DDS DESIGN AND CHARACTERIZATION

In the case of siRNA delivery, the cargo to be delivered is composed of two TNAs, as both the sense and the antisense strands are needed to form the functional dsRNA molecule that exhibits gene silencing at the mRNA level. Loading both sense and antisense strands on the same RCA polymer that has a backbone designed with sequences that offer hybridization sites for both strands was not feasible, due to extremely low loading efficiency. This could be attributed to the high affinity of the short ssRNA strands to hybridize to each other's, rather than hybridizing to the RCA polymer backbone. Moreover, the RCA backbone would have self-complementary sequences at the hybridization sites rendering these binding sites potentially inaccessible. To overcome this issue, two RCA products were designed, where each offers one hybridization site for one ssRNA; either the sense or the antisense strand. Each RCA-RNA complex is synthesized and purified separately following the same protocol that was optimized for loading CpG ODNs. Afterwards, they were mixed in 1:1 ratio and used for downstream applications (Fig. 3.1).

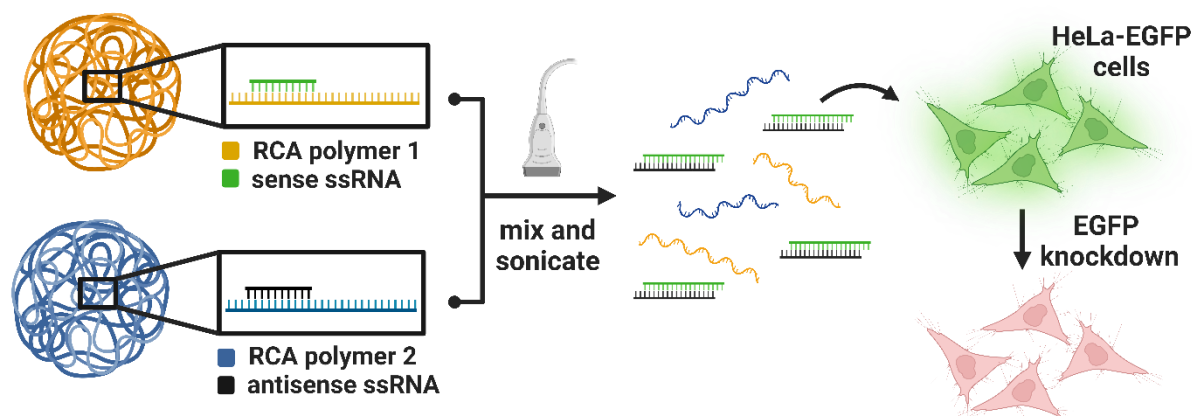


Fig. 3.1. Schematic presentation of gene knockdown using RCA-siRNA. Two RCA polymers are needed, where each accommodates one ssRNA; either the sense or the antisense strand. The RCA-RNA complexes were purified as described earlier and mixed in 1:1 ratio. After US application, both RNA strands were activated and hybridized to form the dsRNA molecule, which effectively interferes with the mRNA of interest (Egfp in this case) leading to gene knockdown. Created with BioRender.com

The RCA-RNA complexes were synthesized successfully as observed in agarose GE (Fig. 3.2). To ensure the stability of the RNA strands and the expected duplex, we sonicated RCA-RNA complexes both separately and after mixing together *via* high-power 20 kHz US device (10 min, 50% amplitude, 2 sec off and 1 sec on). The RCA backbone was completely degraded into a uniform product and the dsRNA band was observed in high intensity, indicating the stability of the TNA against sonication (Fig. 3.2a).

Loading efficiency was calculated as described earlier using Cy5-labelled RNA strands, and found to be $74\% \pm 14\%$ and $67\% \pm 5\%$ for sense and antisense ssRNA, respectively (Fig. 3.2b,c). Rheological studies showed hydrogel formation that exhibited similar behaviour as the one obtained by the RCA-ODN complex (Fig. 3.3). Moreover, SEM analysis of the resulting material revealed the formation of nanoparticles with the anticipated architecture and an average diameter of $1.3 \mu\text{m}$ (Fig. 3.4).

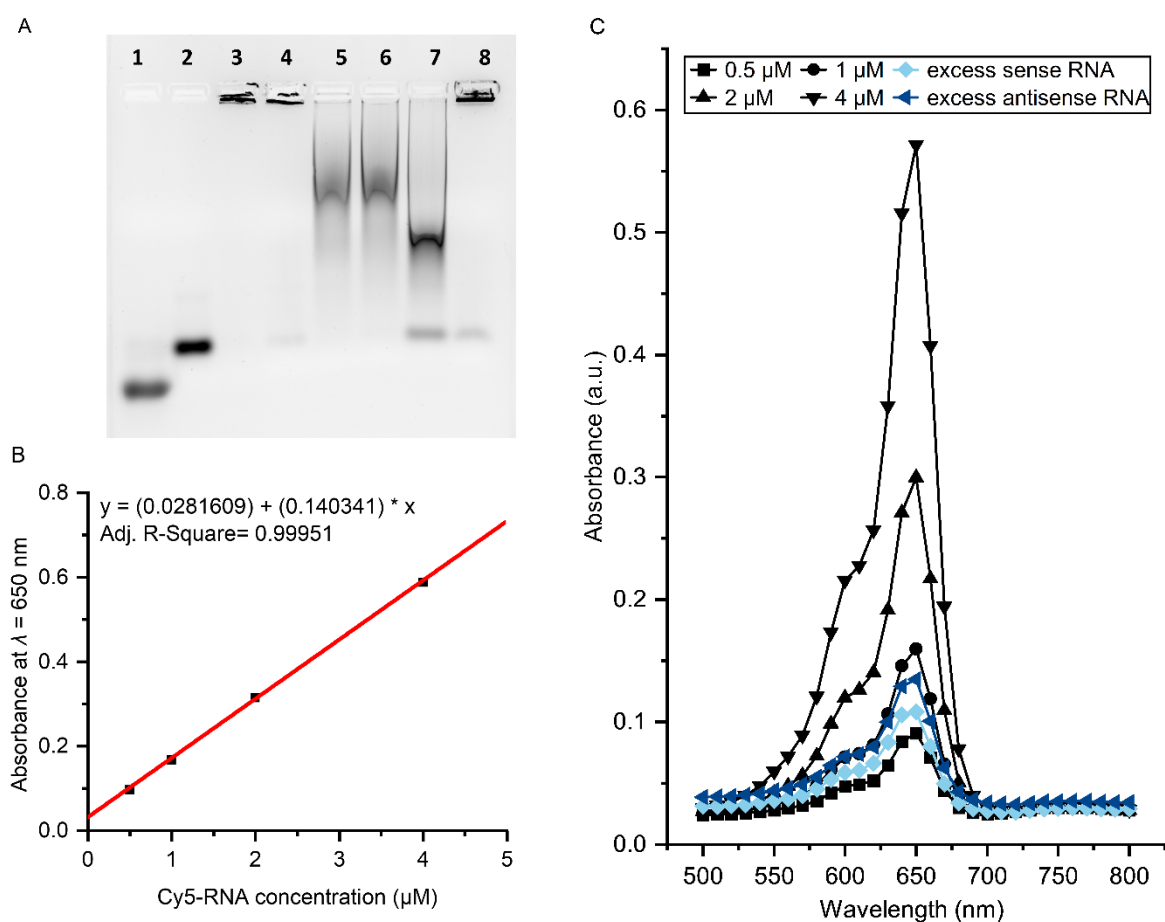


Fig. 3.2. Characterization of RCA-RNA. **A.** Agarose GE demonstrating the formation of RCA-RNA complexes. Lanes 1 and 2: reference sense ssRNA and dsRNA, respectively; lanes 3 and 4: RCA loaded with either sense ssRNA or antisense ssRNA, respectively; lanes 5 and 6: products in lanes 3 and 4 after exposure to 20 kHz US; lane 7: mixed RCA-RNA complexes after exposure to 20 kHz US; lane 8: mixed RCA-RNA complexes. **B.** The calibration curve of Cy5-RNA, which was used to calculate the concentration of both bound and free ssRNA using the best-fit linear regression model. **C.** Absorbance spectra of unbound Cy5-labelled ssRNA strands, with maximum absorption at $\lambda = 650$ nm.

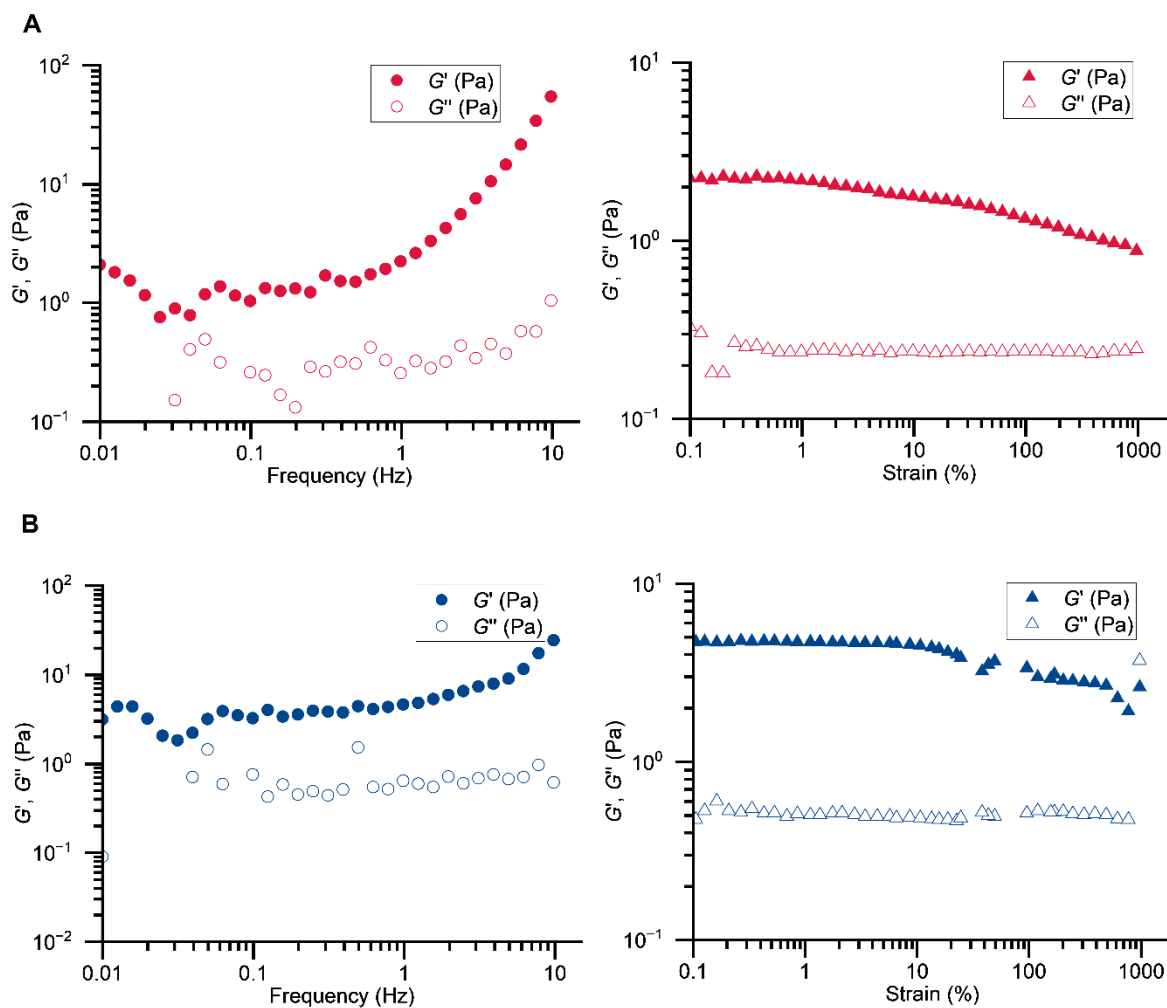


Fig. 3.3. **A.** Rheological analysis of RCA-RNA loaded with antisense ssRNA strand. **B.** Rheological analysis of RCA-RNA loaded with sense ssRNA strand. Both hydrogels exhibit elastic behaviour when subjected to increasing frequency at a fixed strain percentage.

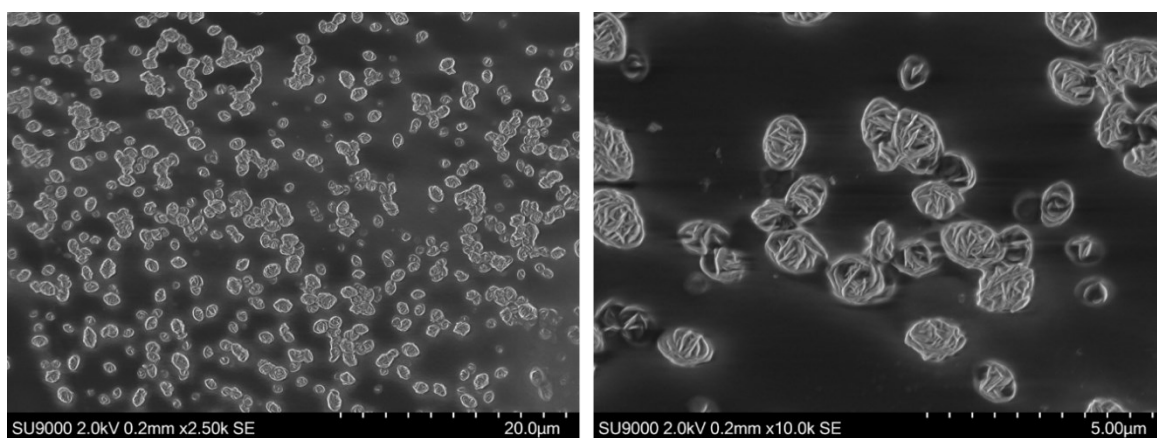


Fig. 3.4. SEM images demonstrating the formation of nanoparticles of RCA-ssRNA complex after loading the sense ssRNA strand as cargo.

3.3.2 CONTROLLED GENE REGULATION VIA LOW INTENSITY ULTRASOUND

To demonstrate the system's function *in vitro*, *EGFP* was used as a proof-of-concept knockdown model. We utilized transfected HeLa-EGFP cells (Fig. 3.5) that were prepared previously in our lab following standard methods in literature⁸⁶⁹.

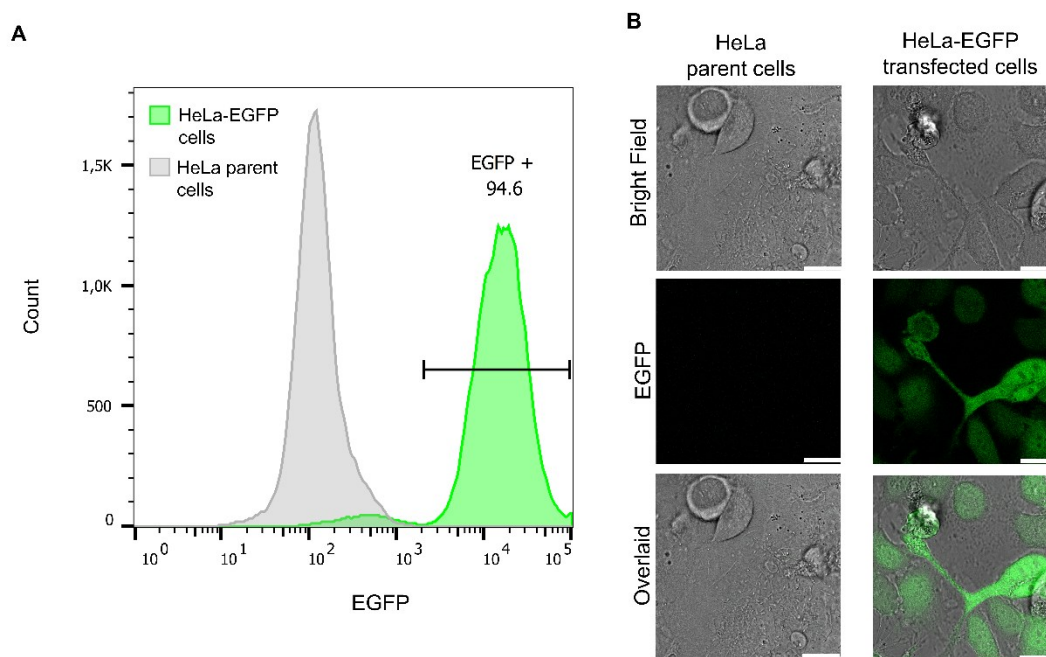


Fig. 3.5. HeLa cells were successfully transfected to stably express *EGFP*, as evidenced by **A.** flow cytometry and **B.** confocal microscopy images.

To further test our siRNA function, we performed control experiments in which HeLa-EGFP cells were incubated with different samples (Fig. 3.6). Cells' incubation with pristine dsRNA, sense ssRNA or antisense ssRNA did not lead to significant decrease in EGFP protein expression. On the other hand, the use of lipofectamine, as a standard and commercially available transfection agent, led to significant *EGFP* knockdown. Lipofectamine is a mixture of 3:1 of 2,3-dioleoyloxy- [2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propaniminium trifluoroacetate (DOSPA) and dioleoyl-phosphatidylethanolamine (DOPE), which can complex with negatively

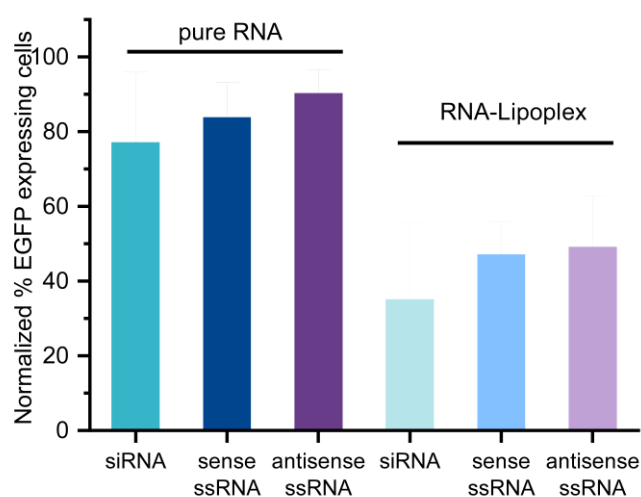


Fig. 3.6. Percentage of HeLa-EGFP cells expressing EGFP after incubation with different RNA molecules either in the absence or the presence of lipofectamine. Readout is normalized to untreated samples.

charged nucleic acids to help them overcome the

electrostatic repulsion of the cell membrane. These results aligned with literature in which naked RNA is not efficiently taken up by cells, while its complexation with cationic compounds enhanced its uptake and therefore its gene regulatory activity.

Pre-seeded adherent cells were treated with either siRNA, siRNA-lipoplex, DDS with or without US. After 3 days of incubation, flow cytometry analysis showed significant reduction in *EGFP* expression, by about 75%, when DDS was activated by US (Fig. 3.7a). The reduction was significantly higher than that of the siRNA-lipoplex control. Cell viability was confirmed through an MTS assay, indicating the safe application of both the DDS and imaging US *in vitro* (Fig. 3.7b). In contrast, siRNA lipoplex significantly decreased cell viability.

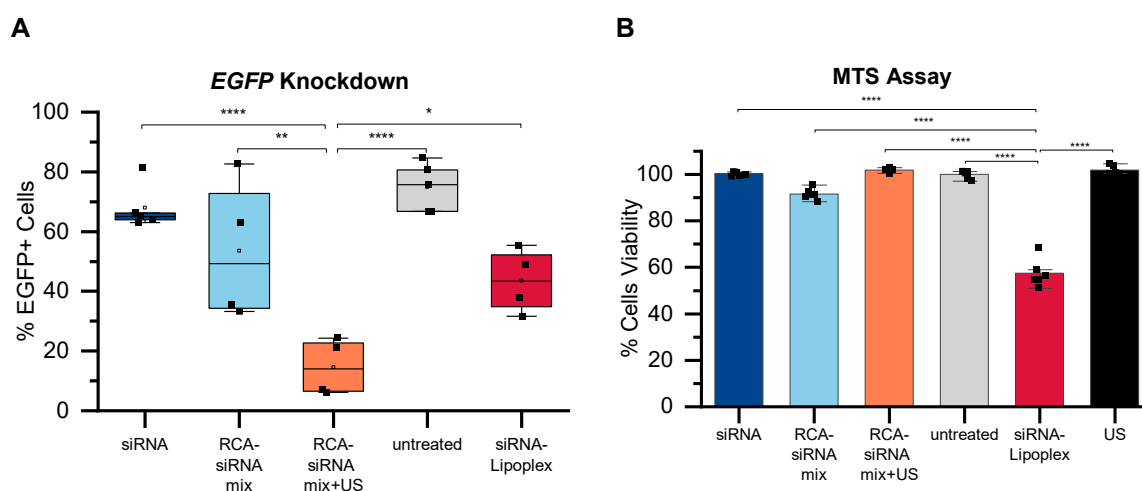


Fig. 3.7. A. Percentage of EGFP expressing HeLa-EGFP cells after treatment with various samples. Treatment with pristine siRNA and RCA-siRNA mixture did not significantly alter EGFP expression. The positive control, siRNA-lipoplex, showed successful gene knockdown as expected. Notably, US-activated RCA-siRNA mixture demonstrated a significantly higher level of gene knockdown compared to the siRNA-lipoplex control. **B.** MTS cell viability assay demonstrating the safety of RCA-siRNA mixture and imaging US for cells.

3.4 CONCLUSION AND OUTLOOK

Gene regulation is one of the most important cellular processes and its modulation with spatiotemporal control is important for both basic research fields aiming to better understand biological processes, as well as therapeutic applications. We utilized our mechanochemically responsive DDS that can be controlled by medically relevant imaging US device to successfully deliver siRNA *in vitro*. Gene knockdown of *EGFP* was achieved in HeLa transfected cells as a first proof-of-concept. Further research in the future aims to use the same platform to deliver siRNA that targets functional genes and have potential therapeutic applications, for example *PKDI* gene that causes polycystic kidney disease⁸⁷⁰. Furthermore, this platform can be used to deliver RNA for the purpose of addressing relevant biological questions in different fields such as aging and diabetes development.

Aging is a complex quantitative trait with contributions from genetics, the environment and their interactions. For several decades, scientists have been investigating the processes that limit lifespan to understand the biology of aging. Recent progress has identified several key hallmarks of aging including genomic instability, telomere attrition, cellular senescence and

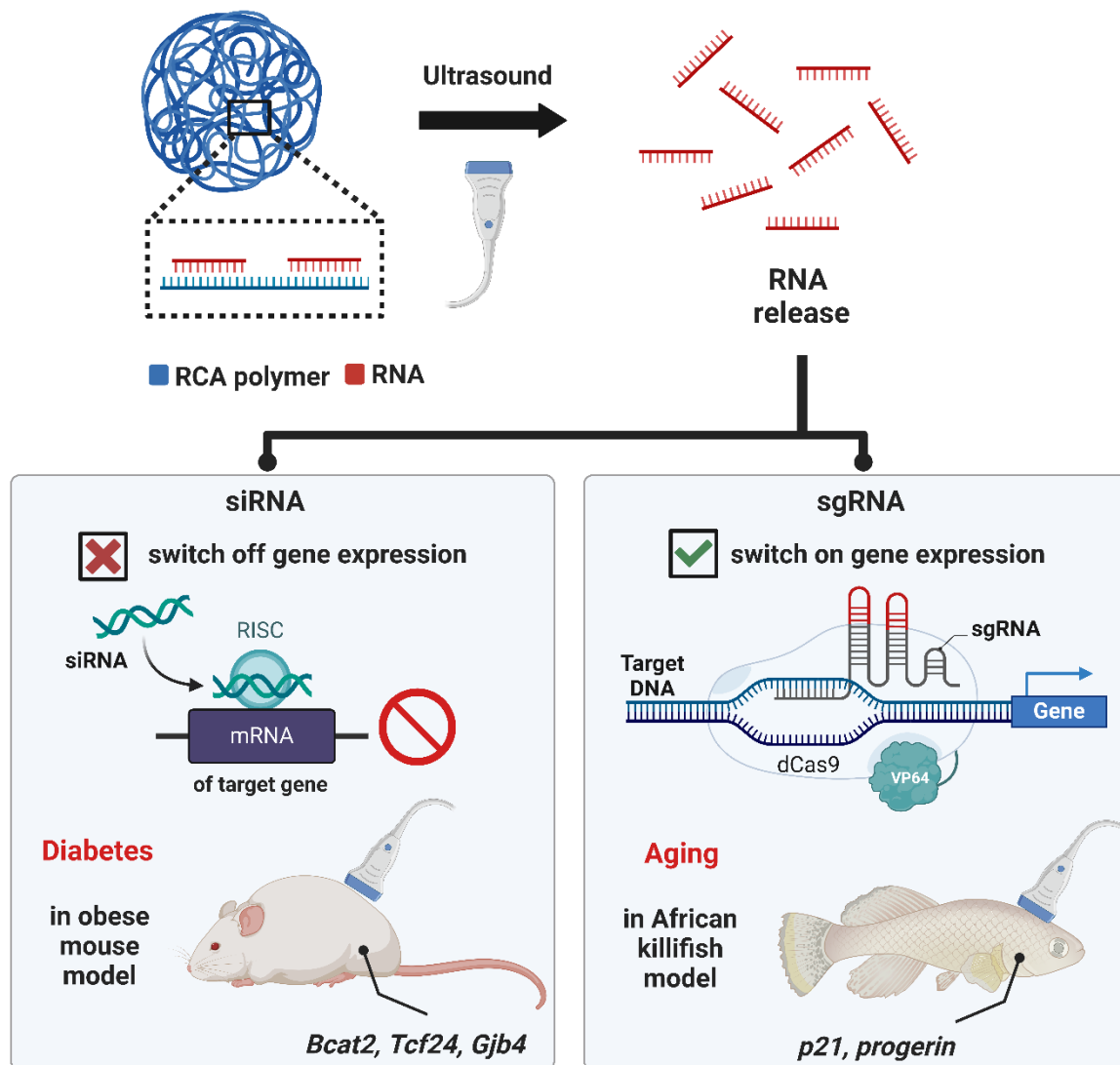


Fig. 3.8. Ultrasound-induced RNA delivery can be used to study biological questions in diabetes and aging. The RNA cargo is loaded onto an RCA carrier and injected into the appropriate animal model. Application of US releases RNA strands in their bioactive form to reduce or enhance gene expression, either *via* the RNAi mechanism or CRISPR/Cas9 system, by the delivery of siRNA and sgRNA, respectively. Created with BioRender.com

stem cell exhaustion⁸⁷¹. However, the identification of vertebrate genes that control aging is hindered by the relatively long lifespans of animal models. Only recently, a species of annual fish with an exceptionally short lifespan was described⁸⁷²; the turquoise killifish (*Nothobranchius furzeri*), which has a maximum life expectancy of only three months in captivity. *N. furzeri* shares many hallmarks of aging with mammals, including telomere shortening, mitochondrial dysfunction, cellular senescence, loss of regenerative capacity and cognitive decline⁸⁷³⁻⁸⁷⁹. In addition, the establishment of transgenesis and genome engineering⁸⁸⁰⁻⁸⁸³, as well as the availability of reference sequences for the *N. furzeri* genome^{884,885} makes the killifish an attractive aging research model. Our system can be used to deliver single guide RNA (sgRNA), a part of the CRISPR-Cas gene editing system, as a targeting mechanism for the Cas9 enzyme. When the sgRNA binds to the Cas9 enzyme, it forms a complex that can recognize and cleave the target DNA sequence. This can be exploited to induce the transcription of target genes in aging models such as *p21* and *progerin* (Fig. 3.8). A second field of application would be diabetes. In healthy individuals, whole-body glucose

homeostasis is achieved through a variety of hormonal signals (*e.g.*, insulin, glucagon) and interorgan crosstalk, with liver and endocrine pancreatic islet cells playing key roles. During type 2 diabetes, this process becomes dysfunctional due to insulin resistance, leading to chronic hyperglycemia, severe secondary complications and premature death⁸⁸⁶. Obesity, driven by overnutrition and sedentary lifestyle, is a major type 2 diabetes risk factor. In the obese state, excess free fatty acid influx from insulin-resistant adipose tissue into the liver may lead to non-alcoholic fatty liver disease⁸⁸⁷, a known risk factor affecting more than 70% of people living with type 2 diabetes⁸⁸⁸. Another defining hallmark of this disease is progressive islet cell dysfunction with eventual loss of insulin-producing β -cells. Loss of functional islet mass is caused by insulin resistance and genetic predisposition, and driven by nutrient toxicity, oxidative and endoplasmic reticulum stress, inflammation, and loss of cellular identity⁸⁸⁹. Currently, no medical treatment can halt or reverse the loss of β -cell mass, and while lifestyle interventions are among the best preventive and curative means in relation to NAFLD, these therapies are not consistently adhered to or do not show success in all patients⁸⁹⁰⁻⁸⁹². In summary, liver- and islet-specific pharmacologic therapies remain vital for the treatment of type 2 diabetes. Using our sonogenetic tools, the expression of risk genes in liver and islets can be modified and thereby their roles in type 2 diabetes pathophysiology can be investigated. Candidate genes include ones that have roles in β -cell proliferation (*Lefty1*, *Apoa2*, *Pcp4l1*)⁸⁹³, cilia function (*Kif3a*)⁸⁹⁴, insulin secretion and β -cell survival (*Gjb4*)⁸⁹⁴. This would enable the investigation of their roles and whether their modulation in both liver and islets could be a viable therapeutic option to halt or reverse type 2 diabetes pathogenesis.

4. DNA Circuits and DNA Dissipative Systems for Drug Delivery

4.1 INTRODUCTION

DNA-based nanomaterials have elicited considerable scientific research interest across various application domains. On one hand, DNA circuits can be programmed to achieve a certain function^{189,190}. On the other hand, further deactivation of DNA circuit followed by multiple cycles of assembly and disassembly results in a dissipative system¹⁹¹. A dissipative system is characterized as an open system that exists far from thermodynamic equilibrium and facilitates the exchange of matter and energy with its external surroundings. In contrast, conservative systems are closed and thereby maintain a steady equilibrium state. Systems found in nature, particularly biological systems, often exist far from equilibrium due to continuous influxes and effluxes of matter and energy from other systems or the environment. In recent years, out-of-equilibrium systems featuring energy-dissipating processes have gained substantial attention in different fields such as chemistry, material science, and synthetic biology⁸⁹⁵.

One significant application of these systems is the generation of self-assembled macromolecules through energy-dissipating processes, a phenomenon explored in recent studies⁸⁹⁶. Dissipative self-assembly involves at least two chemical reactions: monomer activation and deactivation. Precursor monomers are maintained in a steady state, converted into activated monomers in the presence of fuel. Only activated monomers can form high-energy aggregates in a thermodynamically favourable manner. Simultaneously, deactivation reactions occur, reverting building blocks back to precursors, accompanied by the irreversible conversion of part of the consumed energy into waste or heat, while the remaining energy shifts the system's equilibrium state. The kinetic asymmetry, particularly the preferential binding of fuel to monomers rather than aggregates and the subsequent waste conversion primarily occurring on the aggregate state, is crucial in obtaining self-assembled structures in dissipative systems⁸⁹⁷.

Additionally, these dissipative systems have been applied to create programmable dynamic systems for tasks such as cargo delivery, signal detection and molecular motors. These systems undergo multiple transient states synchronized with energy uptake and dissipation, driven by various stimuli like pH, chemicals, enzymes or photons^{898,899}. The key to designing such systems lies in the kinetic control, thereby regulating fuel input and energy dissipation. Unlike many dynamic systems operating under straightforward thermodynamic conditions, dissipative systems autonomously revert back to equilibrium states during energy dissipation, showcasing their unique capabilities and potential applications²⁶¹.

Here we aim to utilize DNA nanodevices for programmed drug delivery with controlled kinetic profile. The overall vision is to design a pre-programmed system that can interact with the surrounding environment and respond accordingly, without the need of exogenous intervention. The parameters of such systems must be pre-defined and then assembled and administered to the biological system. Upon exposure to the trigger, controlled drug release takes place. Further positive and negative feedback loops from the environment would either potentiate the delivery or halt it, according to the temporal cellular needs. Upon change in the circumstances, additional feedback cues trigger a second cycle of releasing the active drug, followed by its regulation by the environment. Such autonomous control would enable multiple cycles of stimulation and inhibition of the desired biological response external interventions. Towards that vision, we designed DNA circuits that can be activated once, and further transformed them into DNA dissipative systems that can be activated multiple times. Some experimental components of this chapter were conducted during Ms. Andreea-Maria Ranea bachelor thesis, and Ms. Xiaofeng

Zhang master thesis. Selected data figures have been sourced from that work and the associated publication [Ishaqat, A., Zhang, X., Liu, Q., Zheng, L. and Herrmann, A., 2023. Programming DNA Circuits for Controlled Immunostimulation through CpG Oligodeoxynucleotide Delivery. *Journal of the American Chemical Society*, 145 (23), 12465–12474].

4.2 METHODS

Materials and reagents

All oligonucleotides were synthesized and HPLC-purified by Biomers Co. Ltd. (Germany). All oligonucleotides' sequences are listed in Supplementary Table 3. T7 exonuclease (10 U/ μ L, M0263L) was purchased from New England Biolabs and exonuclease III (ExoIII) enzyme (200 U/ μ L) was purchased from Thermo Scientific (EN0191). Agarose standard (Roth, 3810.4) was used to prepare agarose gels in 1X TBE buffer. The latter was prepared from Tris base (CAS 77-86-1, Fisher Bioreagents), boric acid (CAS 10043-35-3, Fluka) and ethylenediaminetetraacetic acid disodium salt dihydrate (CAS 60-00-4, Sigma). Roti-GelStain Red (Carl Roth, 0984.1) was used to stain the agarose gels, TriTrack DNA Loading Dye (6X) was used to load samples into agarose gels (Thermo Scientific, R1161) and GeneRuler Ultra Low Range DNA Ladder (Thermo Scientific, SM1213) was used as a reference. DEPC (CAS 1609-47-8, Acros Organics) was used to treat water to deactivate DNase and RNase. TrypLE™ Express Enzyme (1X) (Gibco, 12604013) was used in the maintenance of cell cultures. 10X PBS solution (Lonza, BE17-517Q) was diluted ten times with autoclaved water to be ready for use (1.7 mM KH_2PO_4 , 5mM Na_2HPO_4 , 150 nM NaCl, pH 7.4).

Hybridization and enzymatic release of CpG ODN

DNA duplexes were prepared by the following general procedure: CpG ODN and the corresponding cDNA were mixed in PBS solution in a stoichiometric ratio and the mixture was annealed by heating to 95°C for 5 min followed by cooling down gradually to 4°C. The DNA duplex stock solution was stored at 4°C, with a final concentration of 50 μ M. To investigate the controlled release of CpG ODN, two types of enzymes were used; ExoIII and T7 exonuclease. The enzymatic reaction with T7 exonuclease was typically assembled in a volume of 50 μ L by addition of Milli Q water, 5 μ L of 10X NEB bugger 4 (50 mM Potassium Acetate, 20 mM Tris-acetate, 10 mM Magnesium Acetate and 1 mM DTT, pH 7.9) and 2 μ L enzyme. For ExoIII reaction, 10X Reaction Buffer (660 mM Tris-HCl, 6.6 mM MgCl_2) and 1 μ L ExoIII were used. The reaction was incubated at 37°C, for 30 min and later stopped by adding 11 mM EDTA.

Characterization of the products by gel electrophoresis

GE was performed to analyze the hybridization and release of CpG ODN. Agarose gel (4% w/v) was prepared in 1X TBE buffer (10.8 g tris base, 5.5 g boric acid, 0.75 g EDTA) and 10 μ L gel stain red was added for binding to DNA molecules for visualization under UV light. Samples (20 μ L) were mixed with 6 μ L TriTrack DNA loading dye (4 μ L) and loaded into the well of the solidified gel. Electrophoresis was carried out in 1X TBE buffer at 50 V for 2 h. After that, the gel was visualized using gel imaging system (Bio-Rad).

FRET kinetic tracking of the DNA circuit.

The ps3ODN strands were conjugated to a BMN-Q620 quencher at the 5' end and a 6-FAM at the 3' end. The reaction was assembled in a total volume of 200 μ L, by sequentially adding water, tagged ps3-ODN, 10X NEB buffer 1 (20 μ L), and the cDNA strand with or without 3'-Cy5 modification. T7 exonuclease was added later (after 5 min of starting measurement) to initiate the hydrolysis reaction. The solution was mixed gently by pipetting up and down. Fluorescence intensity was measured over time in a fluorescence spectrometer (Spectramax id3, 140 ms integration time, interval 1 min, 37 °C), with $\lambda_{\text{exc}} = 490$ nm, $\lambda_{\text{em}} = 530$ nm for FAM, and $\lambda_{\text{exc}} = 635$ nm, $\lambda_{\text{em}} = 675$ nm for Cy5.

Cell culture

The J774A.1 macrophages cell line was purchased from the ATCC while HEK-Blue™ mTLR9, HEK293 null, and HEK-Blue™ TNF- α cells were purchased from Invivogen. All cell lines were maintained as described earlier in chapter 2.

Quantification of TLR9 stimulation *in vitro*

To stimulate mTLR9, 180 μ L cell suspension (seeding density of 5×10^3 cells per well) was incubated with 20 μ L sample in a 96-well plate. The samples contained either pristine ODN, DNA duplex, DNA duplex with 2 μ L (0.1 U/ μ L) exonuclease, or PBS. After stimulation of HEK-Blue™ mTLR9 and HEK-Blue™ Null1 cells, NF- κ B promotes the expression of SEAP, which can be detected using HEK-Blue™ Detection Medium (Invivogen, hb-det2) as the substrate is consumed by SEAP and color change from pink to blue can be quantified by optical absorption spectroscopy

The HEK-Blue™ Detection Medium was prepared according to the manufacturer's instructions. After incubation for 18 h at 37°C and 5% CO₂, the SEAP in the supernatant hydrolyzes the substrate in the medium into purple/blue product that can be quantified by measuring absorbance at $\lambda = 630$ nm using a plate reader (SpectraMax iD3, Molecular Devices, USA). To track the time-dependent mTLR9 stimulation, QUANTI-Blue™ Solution (Invivogen, rep-qbs) was used. Samples (20 μ L) from the supernatant of HEK-Blue™ mTLR9 cells incubated with different samples were taken at different time points and incubated with 180 μ L QUANTI-Blue™ Solution according to the manufacturer's instructions in 96-well plates at 37 °C. After 4 h, the absorbance at $\lambda = 630$ nm was measured using a plate reader (SpectraMax iD3, Molecular Devices, USA).

The stimulation of J774A.1 macrophage results in the production of TNF- α in the supernatant. Cells were incubated with 20 μ L of different samples as explained earlier. Then, 20 μ L of the supernatant was removed from the wells and transferred into a new 96-well plate to be incubated with 180 μ L HEK-Blue™ TNF- α cell suspension. Upon stimulation by TNF- α , these cells produce SEAP into their supernatant, which can be quantified by using QUANTI-Blue™ Solution as instructed by the manufacturer. This represents a colorimetric enzyme assay that changes from pink to purple/blue after exposure to alkaline phosphatase activity, which can be quantified by measuring absorbance at $\lambda = 630$ nm using a plate reader (SpectraMax iD3, Molecular Devices, USA). Recombinant human TNF- α (Invivogen, rcyc-htnfa) and recombinant human IL-6 (Invivogen, rcyec-hil6) were used as positive and negative controls, respectively.

4.3 RESULTS AND DISCUSSION

4.3.1 EXONUCLEASE III ACTIVATED DNA CIRCUIT AND DISSIPATIVE SYSTEM

Design of the DNA circuit

The DNA circuit was designed to deactivate the immunostimulatory CpG ODN1826 by hybridization with a complementary DNA sequence (cDNA). Then activation of the DNA circuit and thereby the CpG ODN1826 was achieved utilizing ExoIII enzyme, which exhibits 3'- to 5'-exodeoxyribonuclease activity specific for dsDNA. It degrades dsDNA from blunt ends, 5'-overhangs or nicks. It results in releasing 5'-mononucleotides from the 3'-ends of unprotected DNA strands and leads to stretches of ssDNA. It is considered to be inactive towards 3'-overhang ends of DNA with at least four bases and towards PS-modified DNA backbone.

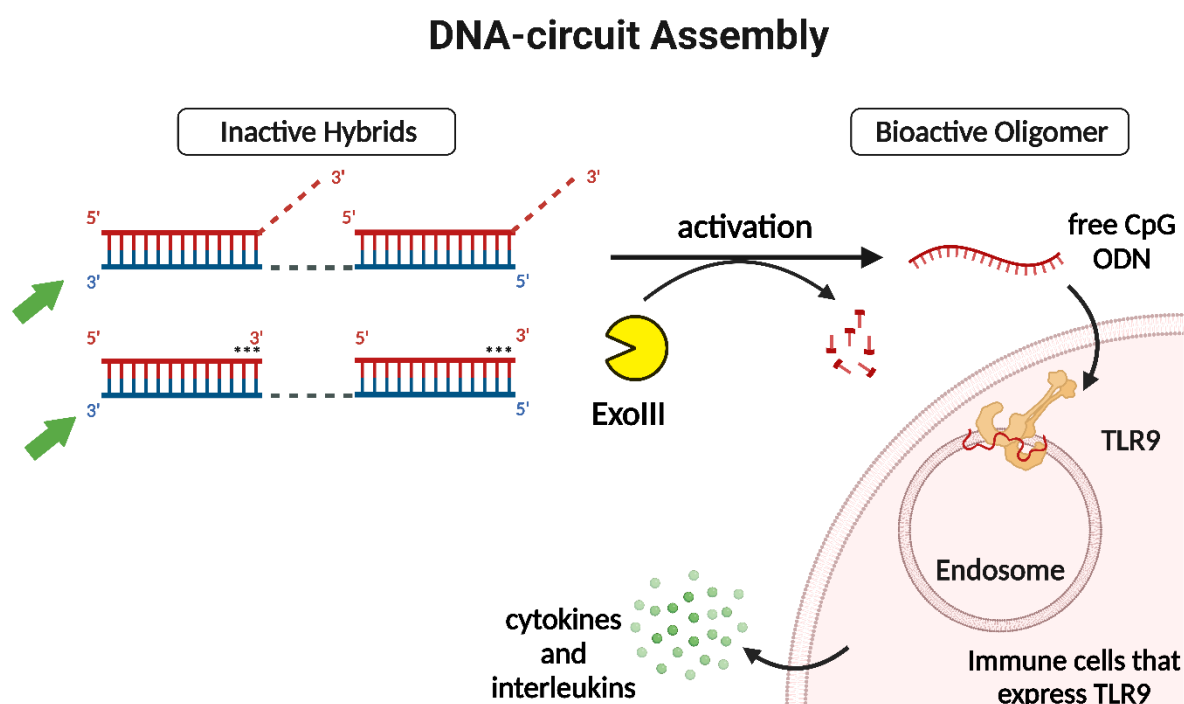


Fig. 4.1. DNA circuit design with bioactive CpG ODN1826 as the output strand. Modification at 3'-end with either 6A overhang or PS (indicated by *) bonds aims to protect CpG ODN (red) against ExoIII (yellow). Linear cDNA strand (blue) is designed with two binding sites complementary to the sequence of CpG ODN1826. A linker is indicated by the dashed segment and has a variable length depending on the design. Assembly of cDNA and CpG ODN deactivates the latter. Activation and release of free CpG ODN are achieved *via* the selective enzymatic activity of ExoIII that starts at the 3'-end of the cDNA strand as indicated by the green arrows. The aim is to achieve control over immunostimulation by utilizing the programmed DNA circuit, where activated CpG ODN stimulates the intracellular TLR9 receptor (orange). Created with BioRender.com

Therefore, to protect the therapeutic ODN1826 from the enzymatic activity of ExoIII, we added six adenine bases as a 3'-overhang to ODN1826 (named ODN1826-6A). To confirm the biological activity of the modified ODN, we established its dose-response curve in HEK mTLR9 cells. Based on the results, we aimed to achieve a final concentration of 5 μ M ODN1826-6A in cell culture experiments.

Linear cDNA sequence was varied in length to investigate the minimum length needed to successfully deactivate ODN1826-6A by preventing its binding to its target receptor, TLR9. The results of stimulating mTLR9 in cell culture experiments are summarized in Table 4.1.

Results demonstrated that 1:1 DNA duplex between ODN1826-6A and cDNA 20-nt long (cDNA₂₀) did not affect the biological stimulation of mTLR9 negatively. Increasing the length of cDNA to 40 nt (cDNA₄₀) resulted in significant lowering of mTLR9 stimulation to just 27.4% of that of the positive control of pristine ODN1826-6A. Further lengthening of the cDNA to either 120 or 200 nt did not result in further significant deactivation of the ODN. Therefore, cDNA₄₀ was used for the subsequent experiments.

We studied the effect of using different ratios of ODN:cDNA on the hybridization process using agarose GE (Fig. 4.3). Although the desired hybridized DNA duplex was formed successfully (green box), another product was observed and predicted to be a half-duplex structure in which one cDNA₄₀ strand was hybridized to only one ODN1826-6A strand rather than two (yellow box). Excess ODN1826-6A and cDNA₄₀ bands were also seen on the gel (blue and red boxes, respectively). Different media were used including PBS, TBE and water, with PBS yielding the best outcome.

Attempting to further enhance the binding, we investigated the 2:1 ratio system in other media, including NEB3 buffer, PBS supplemented with MgCl₂ and cell culture medium (Fig. 4.4). Although still far from ideal hybridization, the best performance was observed when using cell culture medium. Then, we incubated the DNA duplex with ExoIII and its buffer and analysed the reaction outcome. GE showed that the DNA duplex was fully disassembled. The ODN1826-6A band increased in intensity indicating a successful reaction. However, the half-hybrid structure was resistant to the enzyme, which was expected due to the 3'-end ssDNA (Fig. 4.5).

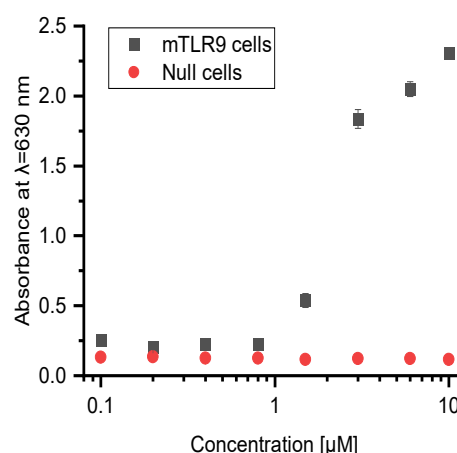


Fig. 4.2. Dose-response curve of ODN1826-6A in mTLR9 and null cells.

Table 4.1. biological activity of DNA duplexes formed by assembling ODN1826-6A and different cDNA strands.

cDNA length (nt)	ODN1826-6A: cDNA ratio	mTLR9 activation (% of pristine ODN6A)
20	1:1	98.7%
26	1:1	53.7%
40	2:1	27.4%
120	6:1	28.1%
200	10:1	25%

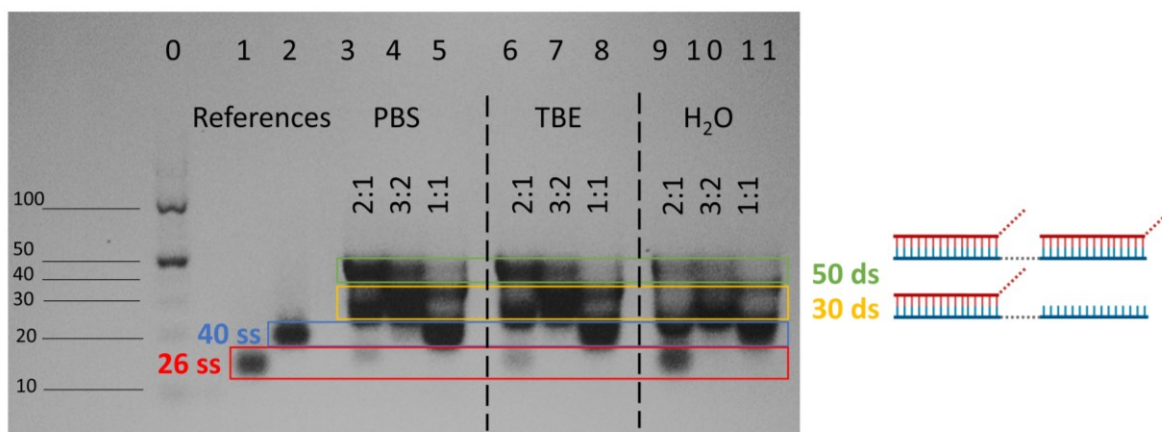


Fig. 4.3. Agarose GE to demonstrate DNA circuit assembly using ODN1826-6A and cDNA₄₀ in different ratios in different media. Lane 0: Orange ruler DNA ladder; lanes 1 and 2: reference ODN1826-6A and cODN₄₀, respectively; lanes 3 to 5: DNA circuits in different ratios in PBS; lanes 6 to 8: DNA circuits in different ratios in TBE; lanes 9 to 11: DNA circuit in different ratios in water. Target DNA duplex, half-hybrid DNA duplex, excess ss ODN1826-6A and cODN₄₀ are all visualized in the gel and indicated on the right side.

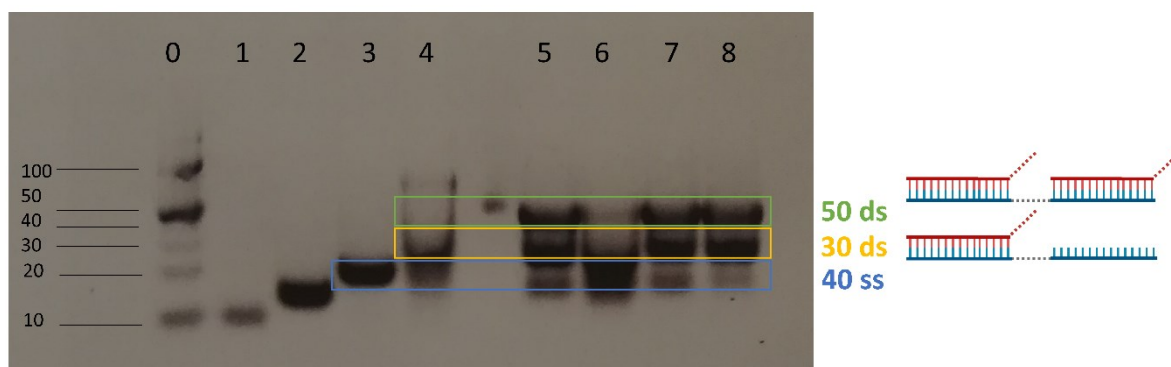


Fig. 4.4. Agarose GE demonstrating DNA circuit assembly in different media at 2:1 ratio of ODN1826-6A:cODN₄₀. Lane 0: Orange ruler DNA ladder; lanes 1 to 4: reference strands of cODN₂₀, ODN1826-6A, cODN₄₀ and cODN₁₂₀, respectively; lanes 5 to 8: DNA circuit in PBS, NEB3 buffer, PBS supplemented with MgCl₂ and cell culture medium, respectively. The excess ODN1826-6A was observed to be minimal when cell culture medium was used.

Aspiring to enhance the hybridization efficiency, we inserted extra bases (from one to four) as a linker between the two hybridization sites of cDNA₄₀ sequence. Our hypothesis is that the 6A-overhang of the CpG ODN might be sterically preventing the hybridization of the second CpG ODN onto the cDNA strand, leading to the formation of half-hybrid structures that were observed earlier. Therefore, a linker that gives an extra 3D space might be helpful in enabling the full hybridization process.

The resulting cDNA varied in length between 40 nt (*i.e.*, when there is no linker) to 44 nt (*i.e.*, when there is 4 nt linker). We could obtain better hybridization and almost no half-hybrid formation when cDNA with a linker is used. After adding ExoIII, all DNA duplexes disappeared completely, and free CpG ODN band was observed. Nevertheless, the band intensity was not as high as expected and the half-hybrid duplex band increased in intensity (Fig. 4.6). These results might indicate an unexpected hydrolysis of the liberated ODN1826-6A.

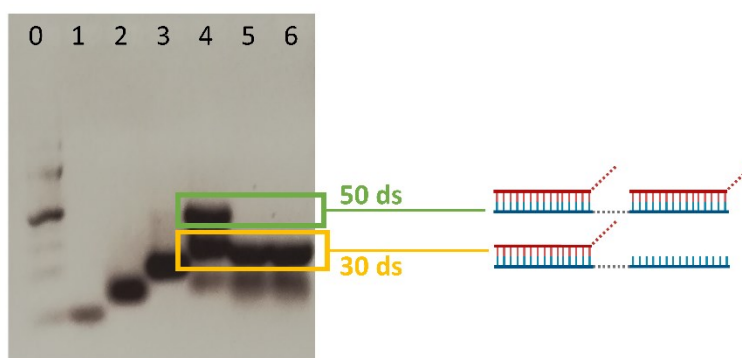


Fig. 4.5. Agarose GE demonstrating the enzymatic activity of ExoIII when incubated with ODN1826-6A:cODN₄₀ DNA duplex assembled in cell culture medium at 2:1 ratio. Lane 0: Orange ruler DNA ladder; lanes 1 to 3: reference cODN₂₀, ODN1826-6A and cODN₄₀, respectively; lane 4: DNA duplex; lane 5 and 6: DNA duplex after incubation with ExoIII. The target DNA duplex was fully consumed leading to the liberation of free ODN1826-6A, which is observed in the relevant band that exhibited higher intensity after the enzymatic reaction. Half-hybrid DNA duplex band remained resistant to ExoIII.

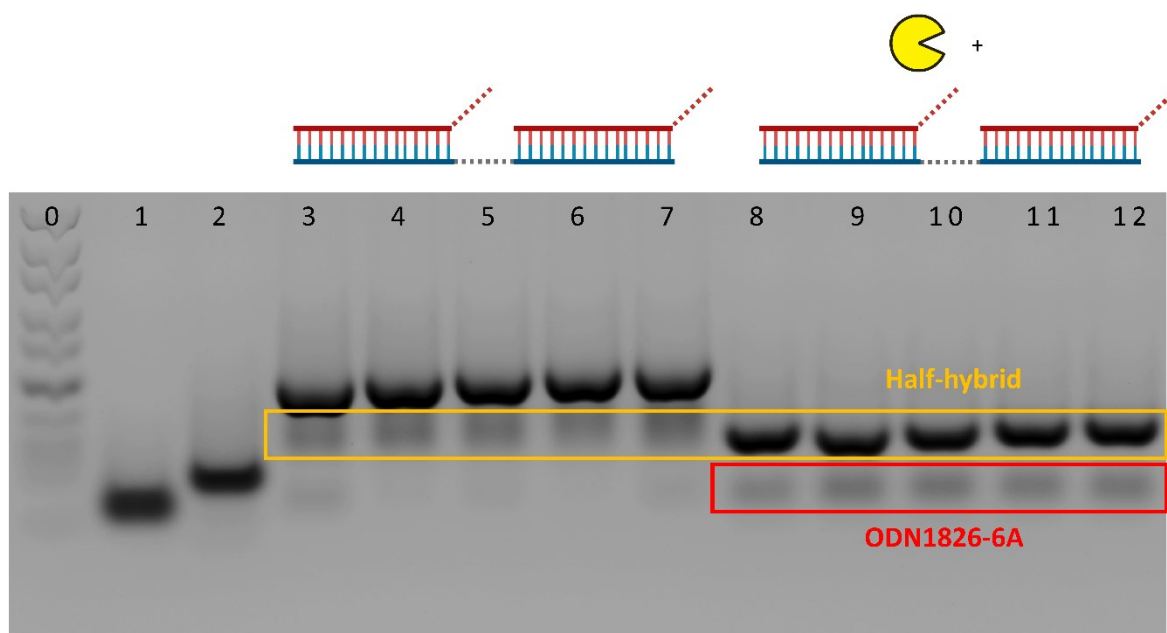


Fig. 4.6. Agarose GE showing DNA circuit assembly and activation by ExoIII, using ODN1826-6A and cODN₄₀₋₄₄. Lane 0: ULR DNA ladder; lanes 1,2: reference ODN1826-6A and cODN₄₀, respectively; lanes 3 to 7: ODN1826-6A hybridized with cODN₄₀, cODN₄₁, cODN₄₂, cODN₄₃ and cODN₄₄, respectively; lanes 8 to 12: same products from lanes 3 to 7 but after the reaction with ExoIII.

Therefore, we investigated another strategy to protect CpG ODN against enzymatic degradation, which is using PS bonds in the backbone (Fig. 4.1) rather than the 3'-overhangs. We either included 1, 2 or 3 bonds at the 3'-end of the CpG ODN (named ODN1826-ps2, -ps3 and -ps4) or used a CpG ODN with a backbone that is entirely made of PS bonds (ps-ODN). After hybridization, the target DNA duplex was the major product, while after ExoIII reaction, free PS-modified ODNs were observed in a relatively high intensity, indicating their stability against any potential enzymatic degradation (Fig. 4.7).

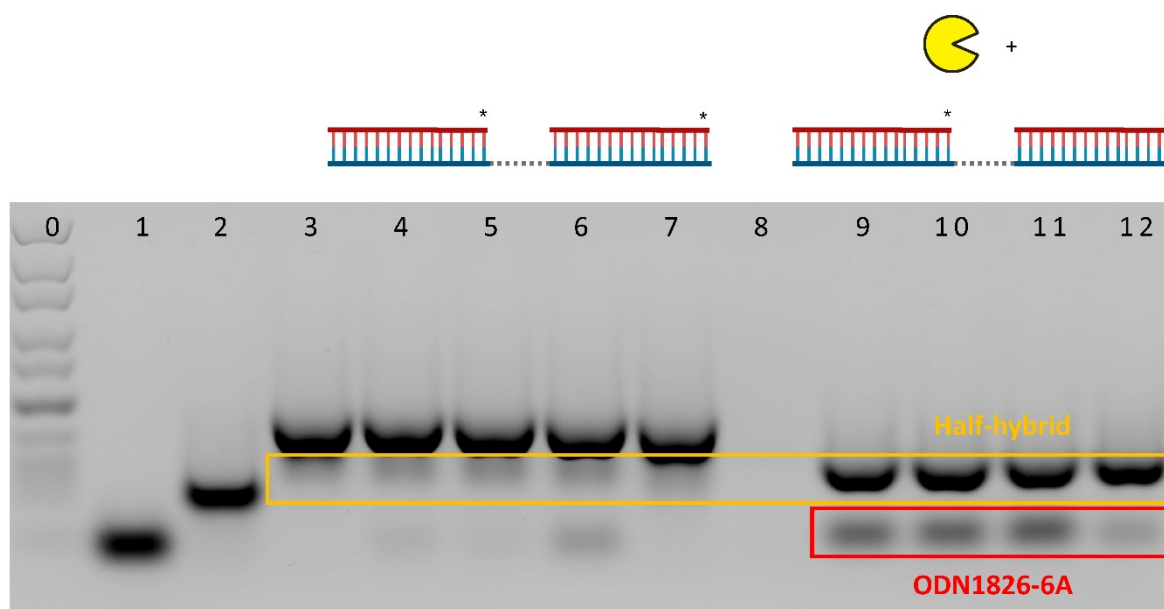


Fig. 4.7. Agarose GE showing DNA circuit assembly and activation by ExoIII, using PS-modified ODN1826 (ps2, ps3, ps4 and ps) and cODN₄₀₋₄₄. Lane 0: ULR DNA ladder; lanes 1,2: reference ODN1826 and cODN₄₀, respectively; lanes 3 to 7: cODN₄₀ hybridized with ODN1826, ODN1826-ps2, ODN1826-ps3, ODN1826-ps4 and ODN1826-ps, respectively; lanes 8 to 12: same products from lanes 3 to 7 but after the reaction with ExoIII.

Before testing the function of the DNA circuit in cell culture, we sought to exclude any effects of ExoIII on the cells' viability or any potential mTLR9 stimulation. Thus, we incubated HEK-mTLR9 cells and null cells with two different concentrations of ExoIII mixed with its buffer. Surprisingly, we could observe SEAP expression in both HEK mTLR9 cells and their parent HEK Null cells, which lack mTLR9 transfection (Fig. 4.8). Repeating the experiment with ExoIII separate from its buffer showed that the enzyme itself did not give positive readout while the presence of buffer with cells of both types gave a high positive readout, indicating a reaction that is independent of mTLR9 stimulation. On the other hand, incubating the detection medium with either ExoIII, its buffer or both did not result in any positive readout. Therefore, we could exclude that the false positive absorbance readout resulted from an interaction between either the enzyme or its buffer with certain components in the detection medium.

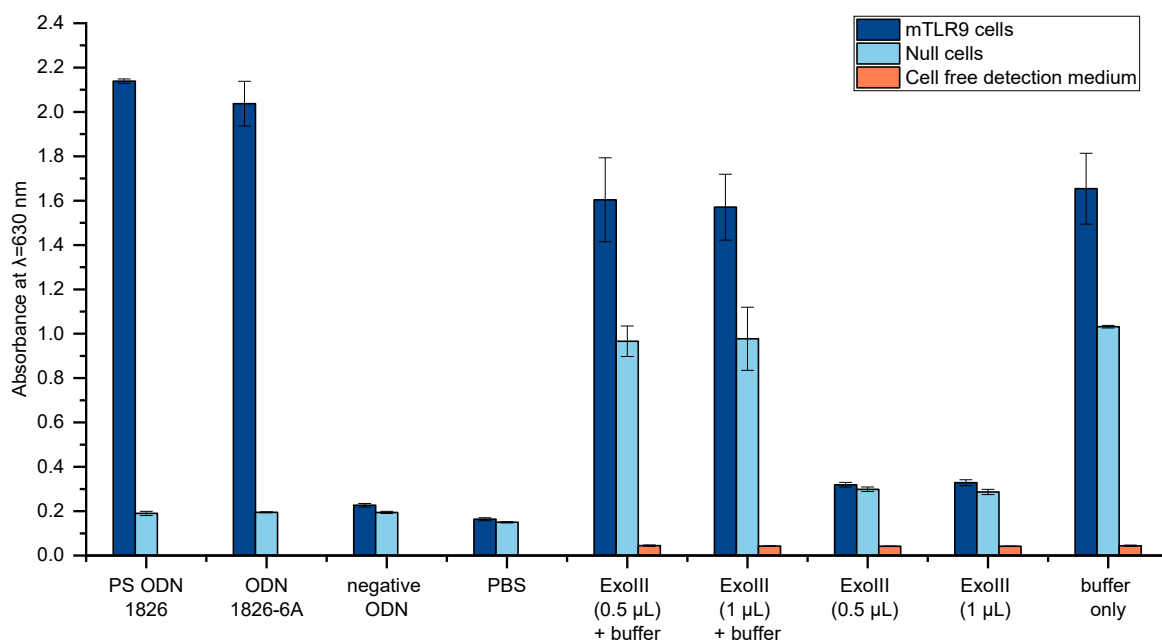


Fig. 4.8. Activation of mTLR9 HEK and Null HEK cells upon incubation with different materials showing high level of false positive read out when the ExoIII buffer is incubated with both cell types; mTLR9 and null cells, but not with the detection medium.

Since we could not establish the reason behind the false positive readouts when we using ExoIII buffer, which is supplied by the manufacturer, we investigated the enzymatic performance of ExoIII in the absence of its buffer. We hybridized the DNA circuit in cell culture medium and compared its reaction with ExoIII either with or without its buffer. Agarose GE results demonstrated no difference in the reaction outcome and that ExoIII could successfully perform its enzymatic activity in cell culture medium alone (Fig. 4.9). Thus, ExoIII buffer was excluded from further cell culture experiments.

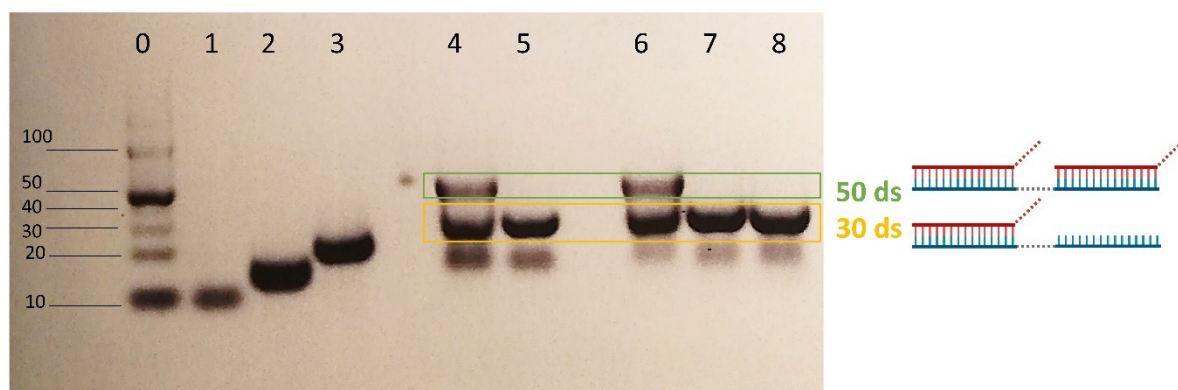


Fig. 4.9. Agarose GE demonstrating the enzymatic activity of ExoIII in cell culture medium, either in the presence or absence of its buffer. Lane 0: Orange ruler DNA ladder; lanes 1 to 3: reference cODN₂₀, ODN1826-6A and cODN₄₀, respectively; lanes 4 and 6: DNA duplexes hybridized in PBS supplemented with MgCl₂ and in cell culture medium, respectively; lane 5: DNA duplex from lane 4 after incubation with ExoIII; lanes 7 and 8: DNA duplex from lane 6 after incubation with ExoIII.

Next, we performed cell culture experiments by incubating the DNA duplex with cells, either with or without the addition of ExoIII (Fig. 4.10). Hybrids were significantly less active than the pristine ODN1826-6A indicating successful deactivation step *via* duplex formation. The presence of ExoIII induced a higher level of mTLR9 stimulation. However, increasing the concentration of ExoIII did not result in enhancement in the biological response. Moreover, the biological response after ExoIII addition was not sufficiently high to recover the system's full potential of stimulating mTLR9. This could be attributed to the lack of complete hybridization, leading to significant portion of the CpG ODN to be sequestered in the half-hybrid structure that does not respond to ExoIII-mediated activation. Therefore, this portion of the CpG ODN dose is ineffective, leading to the observed suboptimal performance of the DNA circuit.

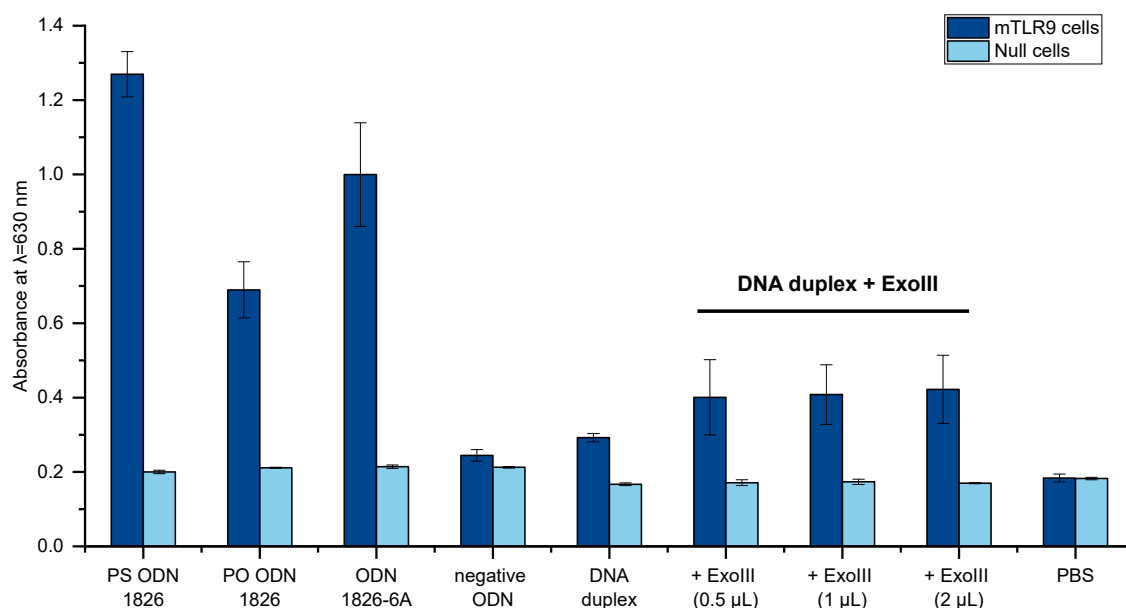


Fig. 4.10. Stimulation of mTLR9 in cell culture in response to different conditions.

Alternative design of cDNA

To overcome this setback, we aimed to manipulate the DNA circuit design to have ODN:cDNA ratio of 1:1 rather than 2:1, which could exclude the formation of half-hybrids and improve the overall efficiency of the hybridization process. Nevertheless, the design of the cDNA must be bulky enough to sterically inhibit the CpG ODN from binding to its target receptor. Towards that goal, we created three potential designs for such a system, employing either G-quadruplex (GQ), hairpin (HP) or tetrahedral (TH) secondary structures (Fig. 4.11). In these designs, one hybridization site is needed to bind one strand of CpG ODN. A linker between this hybridization site and the sequence forming the secondary structures was inserted with a length varying between 4, 10 and 14 nt. The presence of such a linker is expected to allow sufficient 3D space for better hybridization between CpG ODN and cDNA strands.

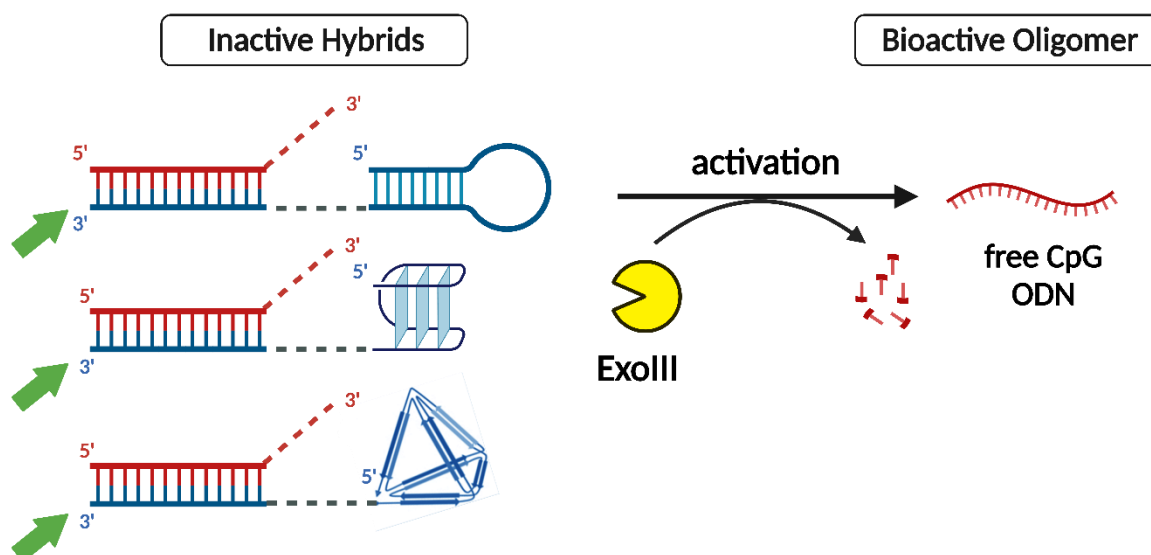


Fig. 4.11. Different designs of the DNA circuit using cODN that has only one binding site to hybridize with CpG ODN1826. The cODN has secondary structures utilizing either HP loop, GQ or TH. Created with BioRender.com

Sequences rich in guanine in nucleic acids can form GQ secondary structures. Four guanine bases form a square planar structure called a guanine tetrad *via* hydrogen bonding that results in a geometry different than that proposed by Watson and Crick. Stacking of two or more guanine tetrads on top of each other's leads to the formation of a GQ. Monovalent cations such as K^+ and Na^+ form coordinate bonds with the carbonyl oxygen atoms of the stacked tetrads leading to the stabilization of the QG structure under physiological pH and salt conditions. GQ structures are also resistant to nuclease degradation, but can be unwound through helicase enzymes. HP structures are stem-loop secondary structures that result from intramolecular base pairing within the same strand. Such structures have been used as an energy source for autonomous DNA devices⁹⁰⁰.

TH DNA nanostructures are characterized by their distinctive shape and high stability. They can be assembled from four short ssDNA strands or from one long ssDNA strand. TH DNA structures have been investigated as nanocarriers for applications in drug delivery and bioimaging⁹⁰¹. We used one ssDNA strand to form our TH secondary structure as described by Li *et al.*⁹⁰² However, due to the extremely low yield of synthesis of DNA oligomers longer than 200 nt by the manufacturer, we split the sequence of the ssDNA strand into three shorter strands that could be synthesized separately with high yields. Then, we ligated them to form the complete sequence needed to form the TH. To provide a hybridization site for the CpG ODN, we extended the third strand with the CpG ODN's complementary sequence. Subsequently, the resulting long ssDNA can self-assemble into the desired tetrahedron upon annealing.

Hybridization of ODN1826-6A with GQ-cDNA series showed the most efficient results with no intermediate side products (Fig. 4.12b). In terms of release, HP-cDNA-based duplexes showed improved CpG ODN release, while GQ-cDNA-based duplexes showed only a small amount of released CpG ODN (Fig. 4.12a). This result further indicated our previous speculation that ODN1826-6A could be potentially degradable by ExoIII. Therefore, we used PS modifications at the 3'-end to protect the ODN. The use of ODN1826 with either 2- or 3-PS bonds at the 3'-end led to efficient hybridization with both HP- and GQ-series of various linker length. The subsequent addition of ExoIII released the CpG ODN efficiently. Intermediate by-products could be observed to different extent in both series (Fig. 4.13).

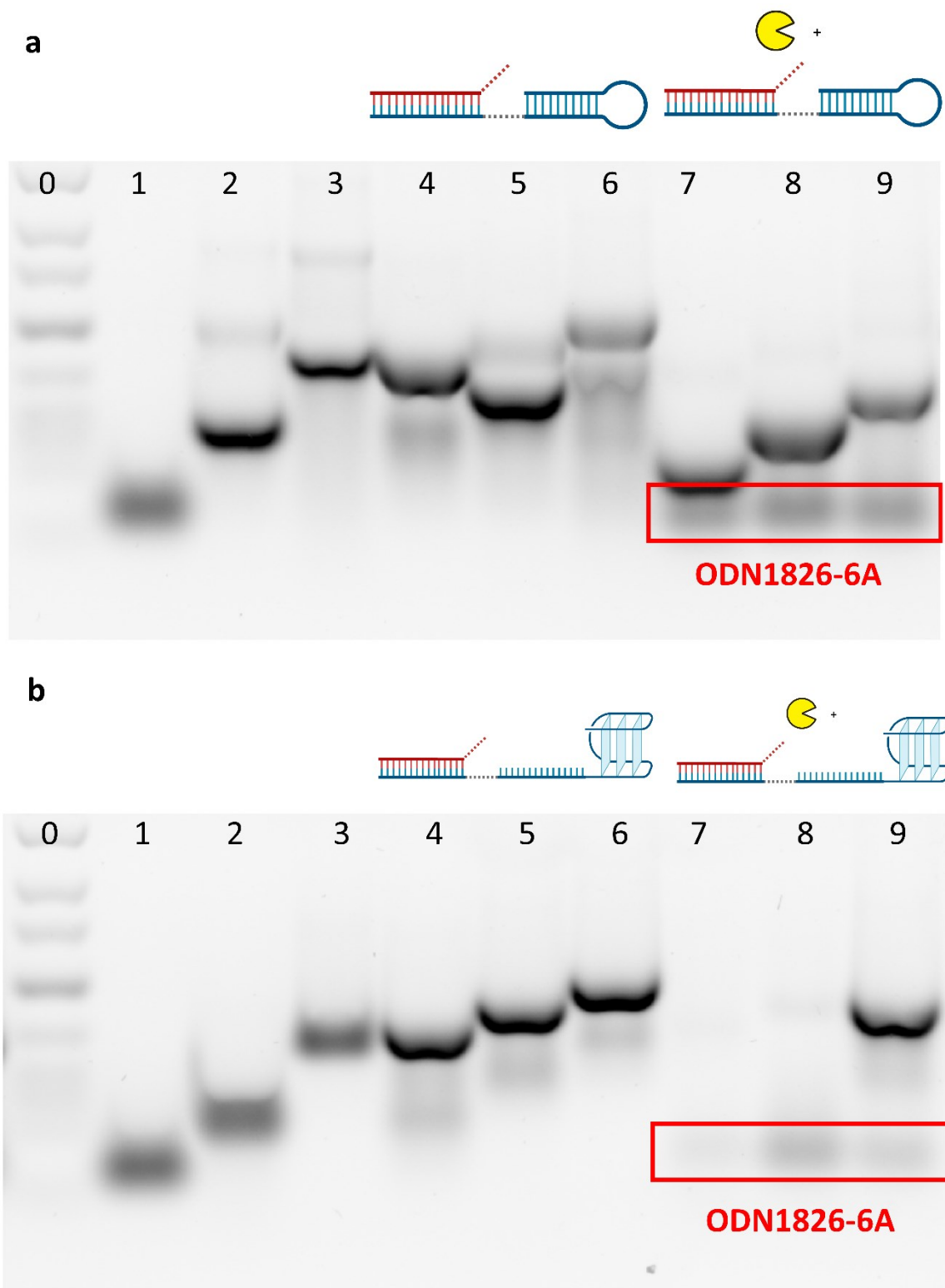


Fig. 4.12. Agarose GE showing DNA circuit assembly and activation by ExoIII, using ODN1826-6A and HP-cODN and GQ-cODN in **A** and **B**, respectively. In gel **A**, lane 0: ULR DNA ladder; lanes 1 to 3: reference ODN1826-6A, HP₄ and HP₂₄, respectively; lanes 4 to 6: ODN1826-6A hybridized with HP-cODN₄, HP-cODN₁₄ and HP-cODN₂₄, respectively; lanes 7 to 9: same products from lanes 4 to 6 but after the reaction with ExoIII. In gel **B**, lane 0: ULR DNA ladder; lanes 1 to 3: reference ODN1826-6A, GQ₄ and GQ₂₄, respectively; lanes 4 to 6: ODN1826-6A hybridized with GQ-cODN₄, GQ-cODN₁₄ and GQ-cODN₂₄, respectively; lanes 7 to 9: same products from lanes 4 to 6 but after the reaction with ExoIII.

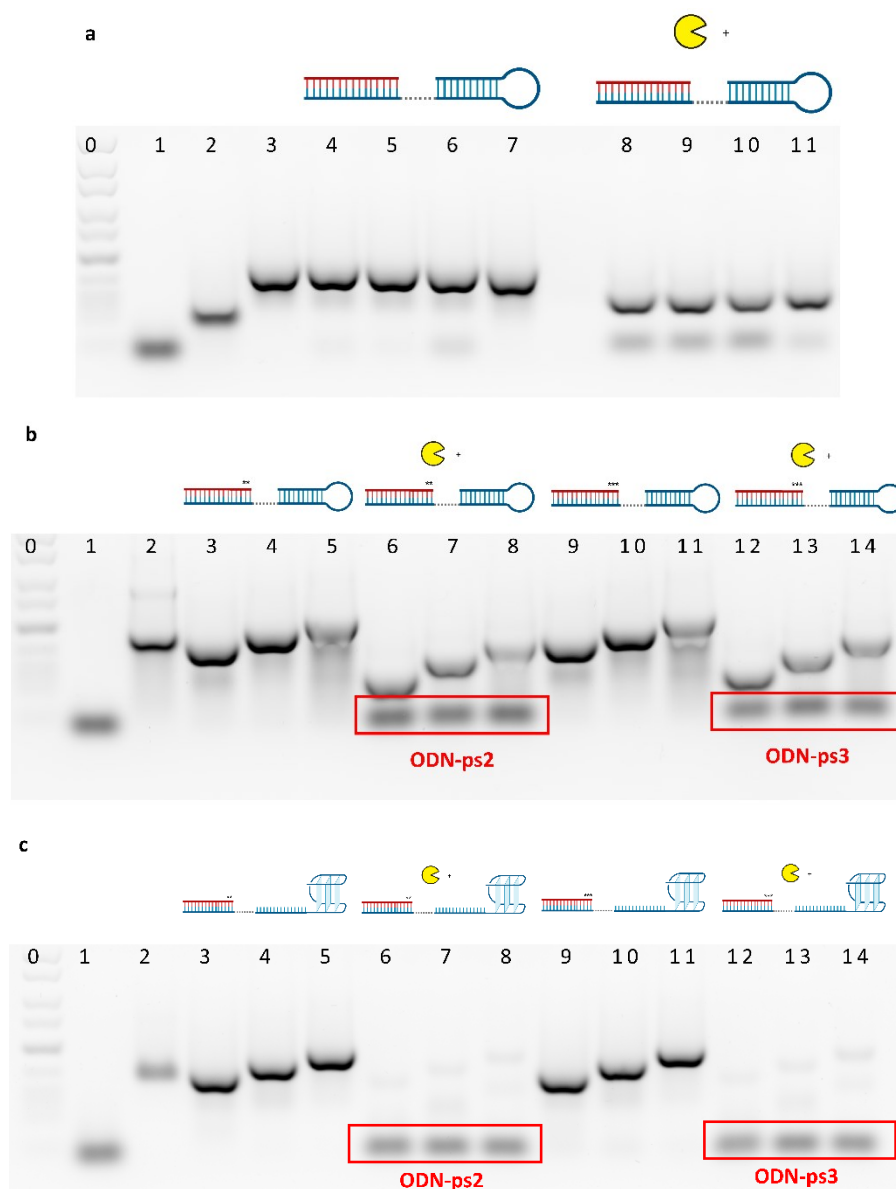


Fig. 4.13. Agarose GE showing DNA circuit assembly and activation by ExoIII, using PS-modified ODN1826-6A and either HP-cODN or GQ-cODN series. In gel **A**, different number of PS bonds was investigated (2,3,4 or full PS backbone) in combination with HP-cODN₄. Lane 0: ULR DNA ladder; lanes 1 and 2: reference ODN1826 and HP-cODN₄, respectively; lanes 3 to 7: HP-cODN₄ hybridized with ODN1826, ODN1826-ps2, ODN1826-ps3, ODN1826-ps4 and full PS backbone ODN1826, respectively; lanes 8 to 12: same products from lanes 3 to 7 but after the reaction with ExoIII. In gel **B**, only ODN1826 modified with either 2- or 3-PS bonds was used, in combination with HP-cODN series. Lane 0: ULR DNA ladder; lanes 1 and 2: reference ODN1826 and HP-cODN₄, respectively; lanes 3 to 5: ODN1826-ps2 hybridized with HP-cODN₄, HP-cODN₁₄ and HP-cODN₂₄, respectively; lanes 6 to 8: same products from lanes 3 to 5 but after the reaction with ExoIII; lanes 9 to 11: ODN1826-ps3 hybridized with HP-cODN₄, HP-cODN₁₄ and HP-cODN₂₄, respectively; lanes 12 to 14: same products from lanes 9 to 11 but after the reaction with ExoIII. In gel **C**, only ODN1826 modified with either 2- or 3-PS bonds was used, in combination with GQ-cODN series. Lane 0: ULR DNA ladder; lanes 1 and 2: reference ODN1826 and GQ-cODN₂₄, respectively; lanes 3 to 5: ODN1826-ps2 hybridized with GQ-cODN₄, GQ-cODN₁₄ and GQ-cODN₂₄, respectively; lanes 6 to 8: same products from lanes 3 to 5 but after the reaction with ExoIII; lanes 9 to 11: ODN1826-ps3 hybridized with GQ-cODN₄, GQ-cODN₁₄ and GQ-cODN₂₄, respectively; lanes 12 to 14: same products from lanes 9 to 11 but after the reaction with ExoIII. One asterisk symbol (*) is used to indicated one PS bond modification.

Tetrahedral DNA formation

We could successfully recreate the tetrahedron previously reported by Li *et al.*, and the extended tetrahedron that we designed to have a hybridization site for the CpG ODN (Fig. 4.14a). Stability of obtained tetrahedral structures was investigated at different temperatures, including heating at 95 °C for 2 min, storing at 4 °C and freezing at 20 °C followed by thawing at room temperature. GE results showed an intact band in all cases (Fig. 4.14b), indicating high stability of the formed TH structures. Hybridization of different ODNs to TH and extended TH structures showed excess unbound CpG ODN. The intensity of the band is less than the starting material, therefore we could conclude that hybridization to a certain extent occurred successfully (Fig. 4.14c).

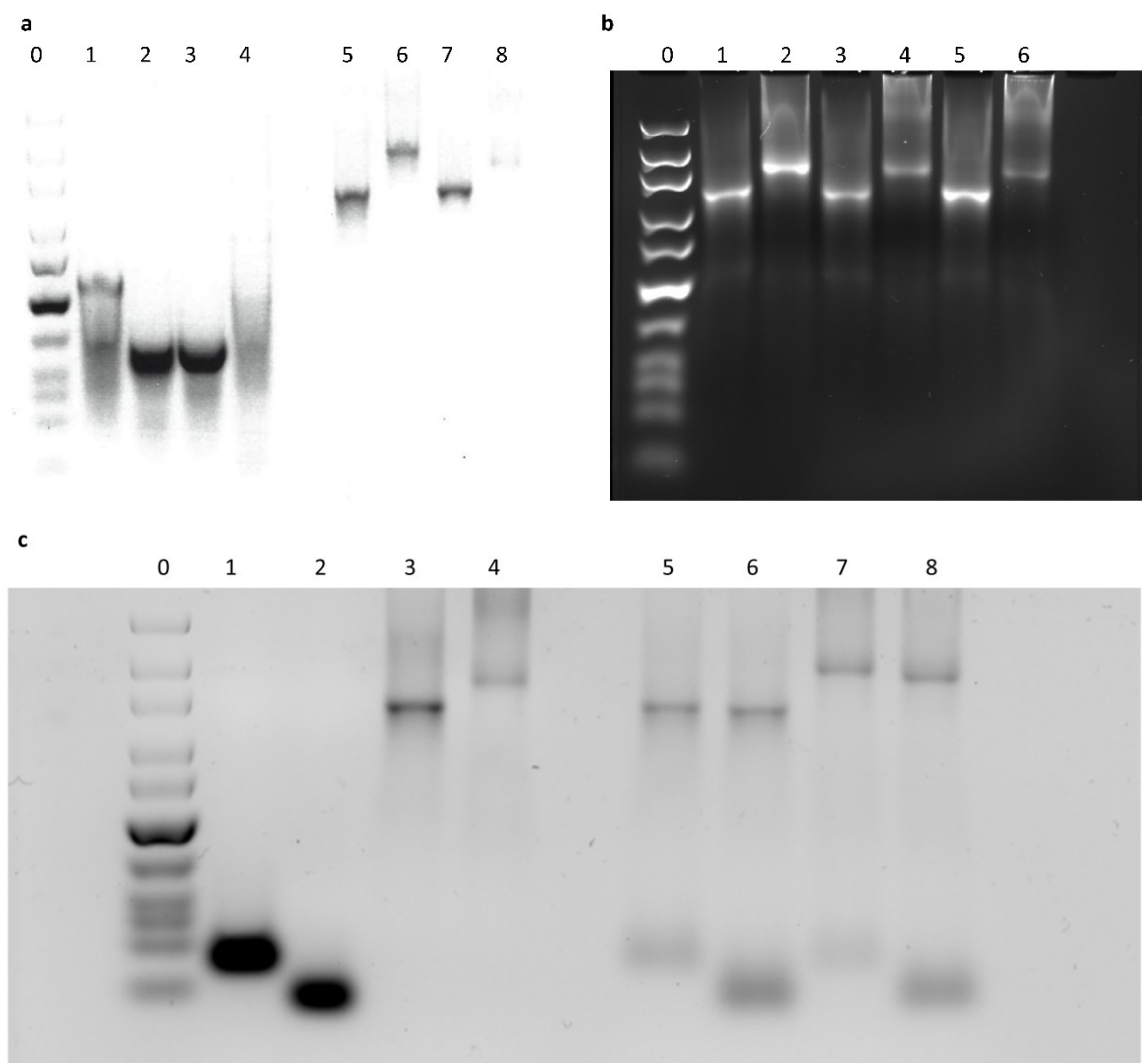


Fig. 4.14 DNA circuit utilizing TH-cODN design **A.** Agarose GE of TH DNA assembly. Lane 0: ULR DNA ladder; lanes 1 to 4: reference strand 1, strand 2, strand 3 and extended strand 3, respectively; lane 5: assembly of strands 1,2 and 3; lane 6: TH after ligation of mixture in lane 5; lane 7: assembly of strands 1,2 and extended strand 3; lane 8: TH after ligation of mixture in lane 7. **B.** Agarose GE of ligated and self-assembled tetrahedral structure and extended tetrahedral structure after exposure to different conditions. Lane 0: ULR DNA ladder; lanes 1 and 2: heating at 95 °C for 2 min; lanes 3 and 4: storing at 4 °C; lanes 5 and 6: freezing at 20 °C followed by thawing at room temperature. **C.** Agarose GE showing the hybridization of tetrahedral DNA with CpG ODNs. Lane 0: ULR DNA ladder; lanes 1 and 2: reference ODN1826-6A and mixed ODN, respectively; lanes 3 and 4: TH and extended TH DNA structures; lanes 5 and 6: TH mixed with ODN1826-6A and mixed ODN, respectively; lanes 7 and 8: extended TH mixed with ODN1826-6A and mixed ODN, respectively.

ExoIII activity on ssDNA

Although inconsistent with the manufacturer specifications for the enzymatic activity of ExoIII, we speculated that ExoIII could induce the degradation of ODN1826-6A based on our previous results. Therefore, we aimed to investigate this unexpected potential degradation further. We incubated ExoIII from two different batches, older and newer ones, with pristine ssDNA of different CpG ODNs using either PO or PS-modified backbones.

Without the addition of its buffer, ExoIII could partially degrade ssDNA as the bands observed on the gel were significantly lower in their intensity. Not only PO ssDNA, but also PS-modified ssDNA seemed to be partially degraded. No difference was observed between different batches of ExoIII, excluding potential contamination or other storage-related changes that could potentially affect the enzyme (Fig. 4.15). In the presence of its buffer, ExoIII degraded PO ssDNA completely (Fig. 4.16). More resistance towards enzymatic activity could be observed for ssDNA that is either PS-modified or has secondary structures like HP or GQ. These findings supported the previous observations where no or minimal amount of CpG ODN could be detected after triggering the DNA circuit with ExoIII. This unexpected activity would prevent the DNA circuit from achieving its goal and delivering intact CpG ODNs to stimulate the immune system in a controlled manner. Therefore, we restructured our DNA circuit and replaced ExoIII with another enzyme as an alternative; T7 exonuclease.

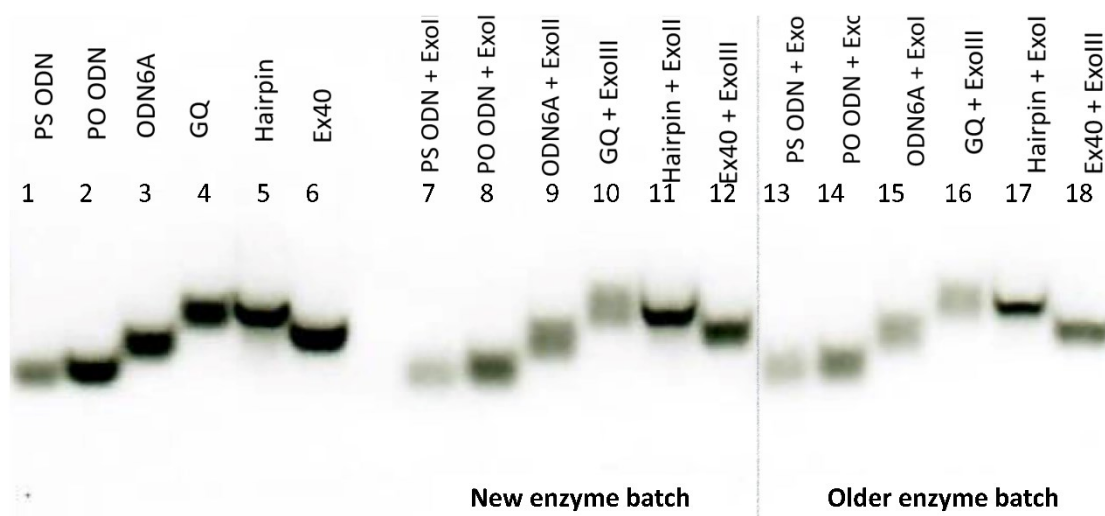


Fig. 4.15. Agarose GE to investigate the enzymatic activity of ExoIII on ssDNA strands in the absence of its buffer. Lane 0: ULR DNA ladder; lanes 1 to 6: reference ps-ODN, ODN1826, ODN1826-6A, GQ-cODN₁₄, HP-cODN₁₄ and cODN₄₀, respectively; lanes 7 to 12: same strands after incubation with ExoIII from a new batch; lanes 13 to 18: same reference strands after incubation with ExoIII from an old batch. Lower intensity of the bands was observed after incubation with ExoIII.

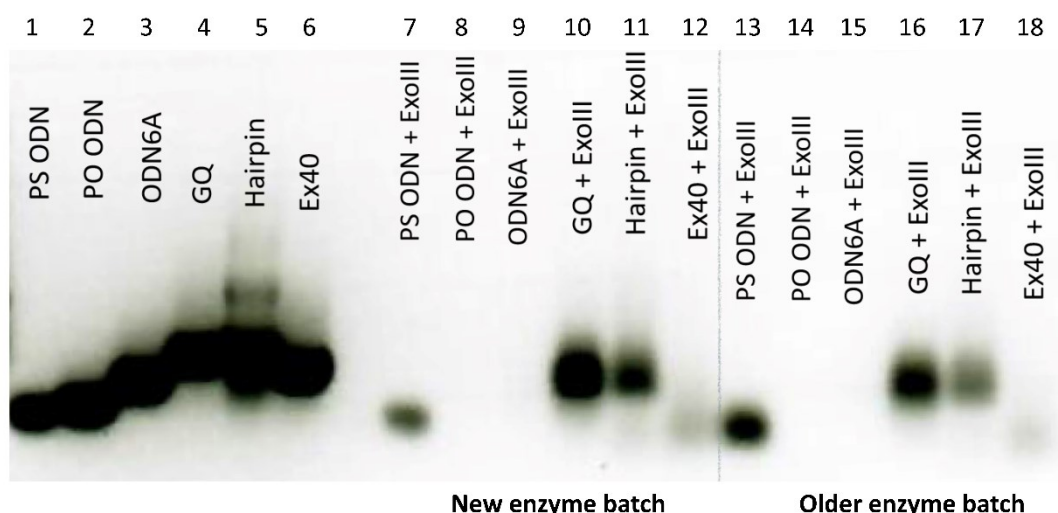


Fig. 4.16. Agarose GE to investigate the enzymatic activity of ExoIII on ssDNA strands. The enzyme buffer was added. Lane 0: ULR DNA ladder; lanes 1 to 6: difference reference ssDNA strands; lanes 7 to 12: same reference strands after incubation with ExoIII from a new batch; lanes 13 to 18: same reference strands after incubation with ExoIII from an old batch. Complete hydrolysis of PO ODN and PO ODN1826-6A was observed. Other ssDNA strands were present in significantly lower intensity after the incubation with ExoIII.

4.3.2 T7 EXONUCLEASE ACTIVATED DNA CIRCUITS AND DISSIPATIVE SYSTEM

Design of the DNA circuit

As explained earlier, the DNA circuit is similarly designed to deactivate the immunostimulatory CpG ODN1826 by hybridization with a cDNA. Then, ODN1826 was activated utilizing T7 exonuclease rather than ExoIII. T7 exonuclease catalyzes the removal of nucleotides in the 5'- to 3'- direction (*i.e.*, opposite to that of ExoIII) from linear or nicked dsDNA with blunt ends or 3'-overhangs. To protect the ODN from the enzymatic activity of T7 exonuclease, two strategies were used, either with 5'-overhangs consisting of six adenine bases (named 6A-ODN1826) or the modification of two or three consecutive PS bonds (named ps2-and ps3-ODN1826, respectively) at the 5'-end (Fig. 4.17).

The cDNA was designed to be either linear, have either a HP loop or a GQ as secondary structures at the 3'-end. It was anticipated that the HP and GQ structures can sterically hinder the binding of the DNA duplex to TLR9, thus achieving efficient deactivation of the CpG ODN. Since our previous data from the ExoIII-based DNA circuits showed that the linear cDNA length must be at least 40 nt for the deactivation of ODN1826 *in vitro*, linear cDNA with two repeating sequences complementary to ODN1826 was used. We varied the length of the linker between the two hybridization sites between 0 to 4, resulting in cDNA of varying length from 40 to 44, termed cDNA₄₀₋₄₄, with the subscript number indicates the total length of the strand. In the HP and GQ cDNA design, one hybridization site is needed to bind one molecule of CpG ODN. A linker between this hybridization site and the sequence forming the HP or GQ secondary structures was inserted with a length varying between 4, 10 and 14 nt. The presence of such a linker is expected to allow sufficient 3D space for better hybridization between CpG ODN and cDNA. When 6A-ODN1826 was used in the DNA circuit with different types of cDNA, the hybridization reaction was successful (Fig. 4.18). However, no band for the free 6A-ODN1826 was observed after the reaction with T7 exonuclease. It seemed that T7 exonuclease could digest the whole DNA duplex and the presence of 6 nt overhang was insufficient to protect the ODN against enzymatic degradation.

DNA-circuit Assembly

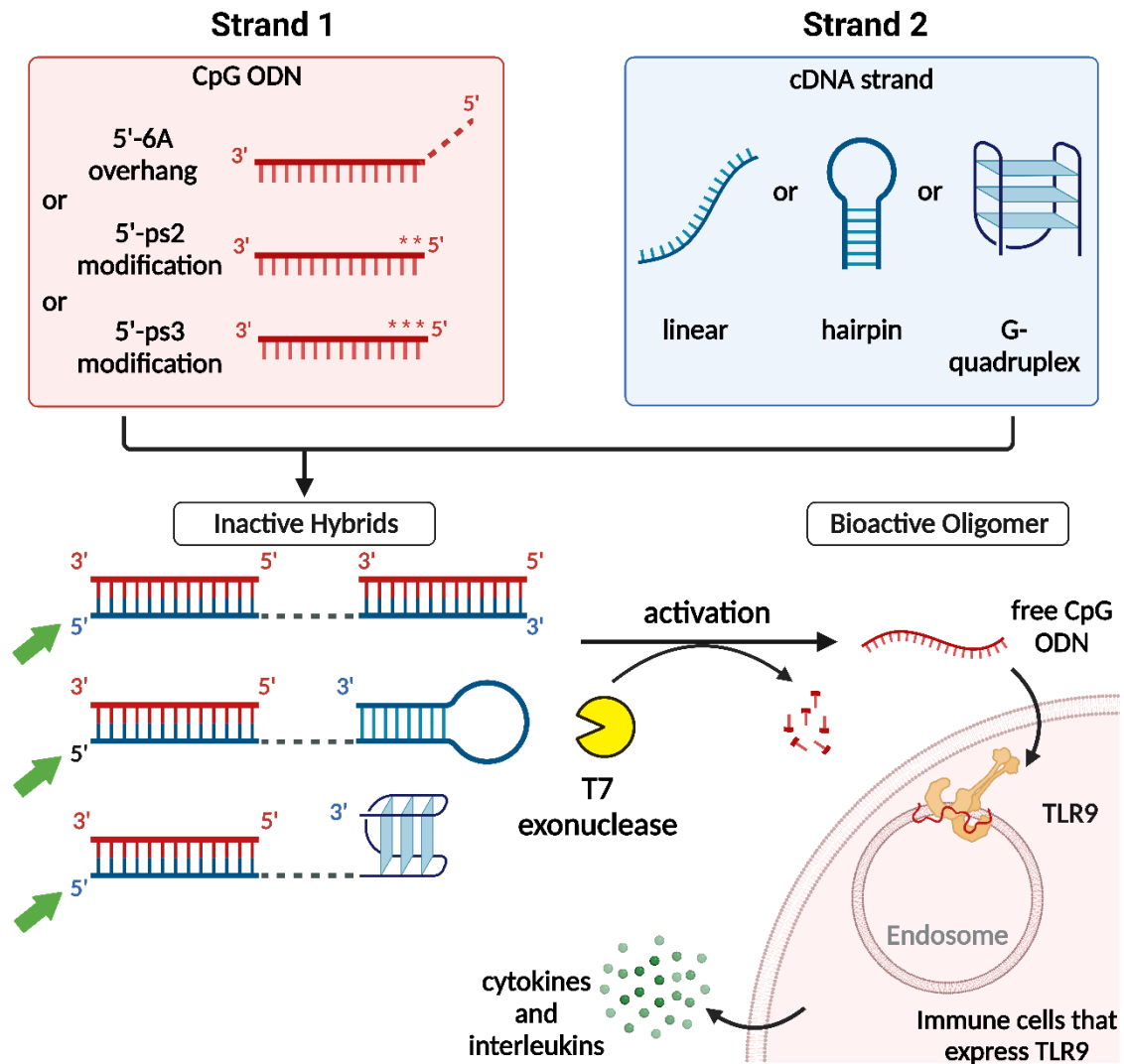


Fig. 4.17. DNA circuit design with bioactive CpG ODN1826 as the output strand. Modification at 5'-end with either 6A overhang or PS bonds aims to protect CpG ODN against T7 exonuclease. cDNA strand is designed in 3 configurations; linear, HP or GQ and contains one or more binding sites complementary to the sequence of ODN1826. A linker is indicated by the dashed segment and has a variable length depending on the design. Assembly of cDNA and CpG ODN deactivates the latter. Activation and release of free CpG ODN are achieved via the selective enzymatic activity of T7 exonuclease that starts at the 5'-end of the cDNA strand as indicated by the green arrows. Control over immunostimulation is realized by utilizing the programmed DNA circuit, where activated CpG ODN stimulates the intracellular TLR9 receptor. Created with BioRender.com

In contrast, CpG ODN sequences with either two PS bonds (ps2-ODN1826) or three PS bonds (ps3-ODN1826) at the 5'-end were stable against T7 exonuclease (Fig. 4.19). Selective hydrolysis of the cDNA strand was achieved leading to the liberation of CpG ODN in its free and intact form. The latter can potentially activate the innate immune system by stimulating TLR9 in immune cells and initiating a cascade of cellular signals that result in the secretion of cytokines and interleukins. As expected, a residual strand is observed as a by-product when using cDNA strands bearing HP or GQ motifs. This is due to the inability of T7 exonuclease to digest the ss linker in these structures. The linker's length in cDNA and the number of PS bonds in ODN1826 did not affect the ODN stability, hybridization process or ODN release significantly.

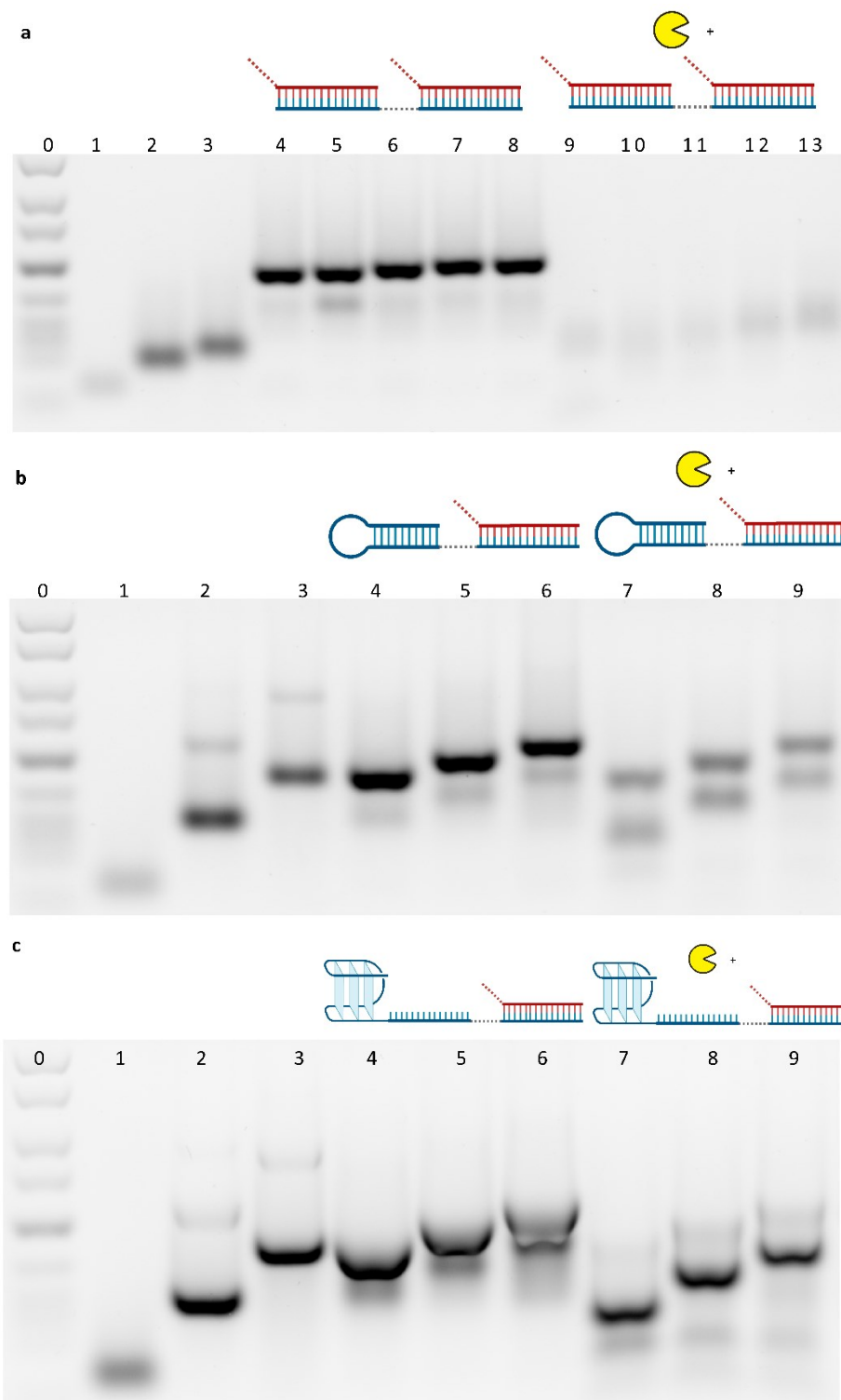


Fig. 4.18. Agarose GE showing **A.** 6A-ODN1826 hybridization with cODN₄₀₋₄₄. Lane 0: ULR DNA ladder; lanes 1 to 3 reference 6A-ODN1826, cODN₄₀ and cODN₄₄, respectively; lanes 4-8: 6A-ODN1826 hybridized with cODN₄₀, cODN₄₁, cODN₄₂, cODN₄₃ and cODN₄₄ respectively; lanes 9-13: same duplexes after the reaction with T7 exonuclease. **B.** 6A-ODN1826 hybridization with HP series. Lane 0: ULR DNA ladder; lanes 1 to 3: references: 6A-ODN1826, HP-cODN₄ and HP-cODN₂₄; lanes 4 to 6: 6A-ODN1826 hybridized with HP-cODN₄, HP-cODN₁₄ and HP-cODN₂₄, respectively; lanes 6-8: same duplexes after the reaction with T7 exonuclease. **C.** 6A-ODN1826 hybridization with GQ series. Lane 0: ULR DNA ladder; lanes 1 to 3: references: 6A-ODN1826, GQ-cODN₄ and GQ-cODN₂₄; lanes 4 to 6: 6A-ODN1826 hybridized with GQ-cODN₄, GQ-cODN₁₄ and GQ-cODN₂₄, respectively; lanes 6-8: same duplexes after the reaction with T7 exonuclease.

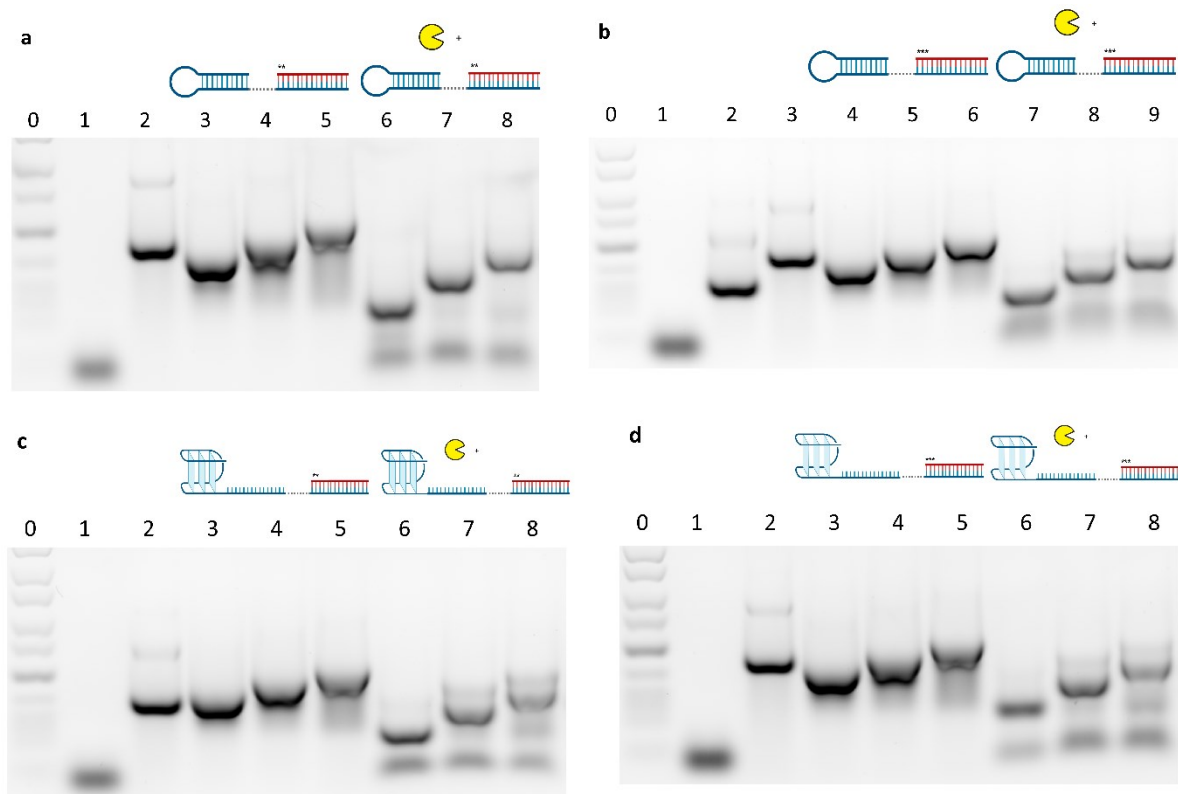


Fig. 4.19. Agarose GE showing DNA circuit assembly and T7 exonuclease activity for HP series with **A.** ps2-ODN1826 and **B.** ps3-ODN1826, and GQ series with **C.** ps2-ODN1826 and **D.** ps3-ODN1826. In gels **A** to **D**, lane 0: ULR DNA ladder; lane 1: reference CpG ODN1826; lane 2: reference cDNA strand (HP-cODN₂₄, HP-cODN₄, GQ-cODN₂₄, GQ-cODN₄, respectively); lanes 3 to 5: the hybridization products; lanes 6 to 8: the DNA duplexes after the reaction with T7 exonuclease. One asterisk symbol (*) is used to indicated one PS bond modification.

Programming DNA circuit kinetics using Förster resonance energy transfer (FRET)

To evaluate the molecular changes and track real-time kinetics of the DNA circuit, we used a FRET-based reporter system (Fig. 4.20a). We utilized ps3-ODN1826 hybridized to either HP-cODN₄ or HP-cODN₁₄. On one hand, the CpG ODN was modified with 6-FAM at the 3'-end and the dark quencher BMN-Q620 at the 5'-end. In its free form, this modified CpG ODN is in a quenched state. On the other hand, the cDNA strand was modified with Cy5 at the 3'-end, rendering it fluorescent in its free state. Upon hybridization, the CpG ODN is forced to be in a linear form that makes the distance between FAM and BMN-Q620 long enough for FAM to emit fluorescent signal without being quenched. Moreover, the BMN-Q620 becomes in a close proximity to the Cy5 label on cDNA, thereby quenching its fluorescence signal. The selection of the fluorescent dyes and the quencher was critical, as we needed two orthogonal dyes with minimal overlap in their absorption and emission spectra, that can be also quenched using the same quencher.

Tracking our system by measuring the fluorescence intensity demonstrated that upon duplex formation, FAM-fluorescence signal increased while Cy5-fluorescence signal was quenched (Fig. 4.20b,c). Upon the addition of T7 exonuclease, the CpG ODN was released and its fluorescence signal declined as FAM got quenched. At the same time, Cy5-fluorescence signal increased, indicating further the release of the ODN carrying the BMN-Q620 quencher. In summary, the FAM-fluorescence signal was used to track the CpG ODN, and the Cy5-fluorescence signal was used to track the cDNA strand and the formation of its by-product.

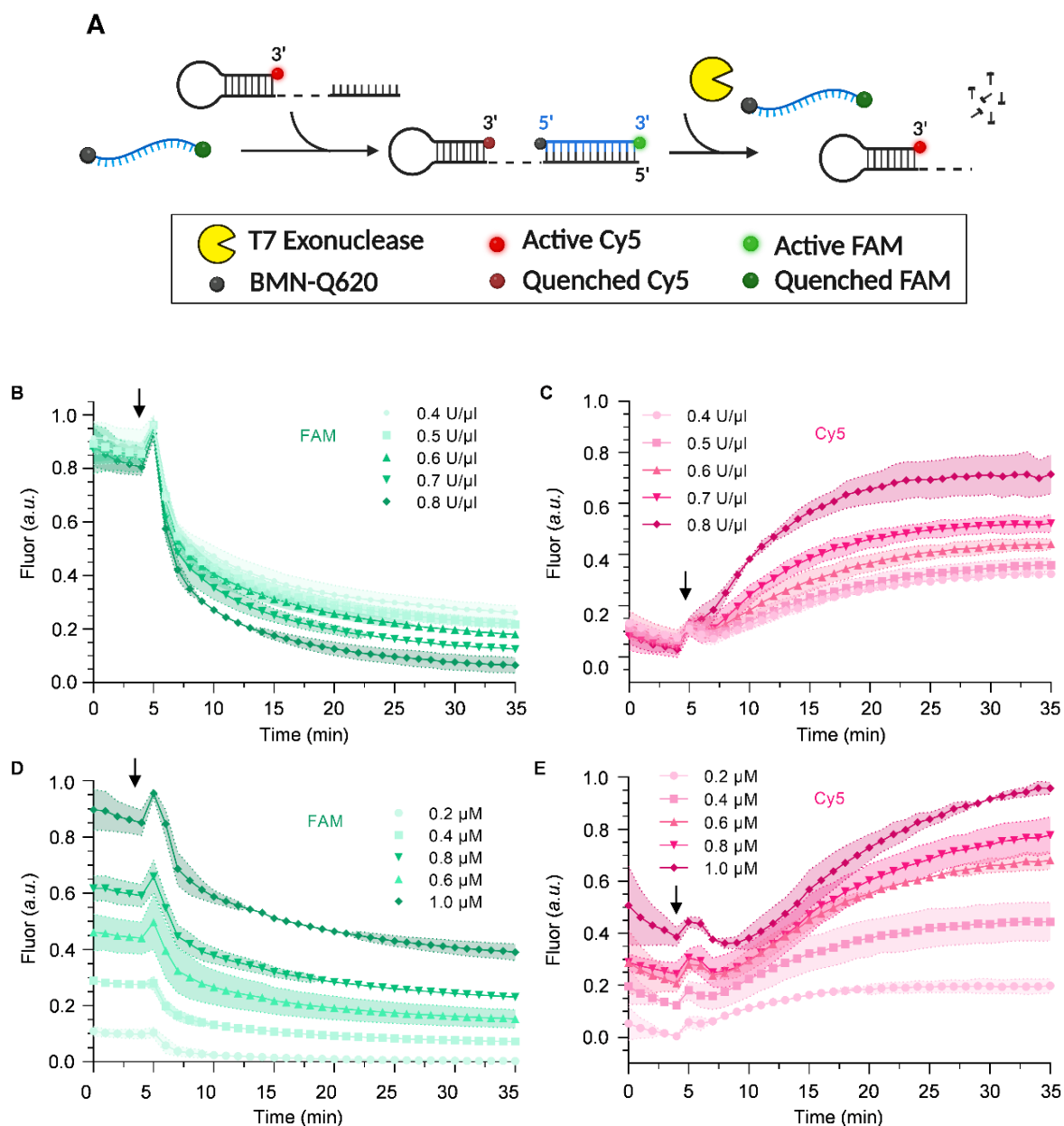


Fig. 4.20. **A.** Schematic demonstration of FRET-pair-based kinetic tracking of the DNA circuit. CpG ODN is conjugated to a BMN/FAM pair (black/green), and HP-cODN₄ is labeled with Cy5 (red). The waste generated by T7 exonuclease digestion contains mononucleotides and the remaining structural fraction of HP-cODN₄. Fluorescence intensity measurement of **B.** FAM and **C.** Cy5 over time for enzyme concentration-dependent kinetic tracking of the DNA circuit. Starting with 0.4 μM DNA duplex (ps3-ODN1826 and hp4) followed by the addition of T7 exonuclease (0.4-0.8 U/μL) indicated by the arrow. Fluorescence intensity measurement of **D.** FAM and **E.** Cy5 over time for DNA duplex concentration-dependent kinetic tracking of the DNA circuit. Starting with (0.2-1 μM) DNA duplex, followed by the addition of 0.6 U/μL T7 exonuclease indicated by the arrow. Reaction conditions: 50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate and 1 mM DTT, 37°C (Reprinted (adapted) with permission from⁹⁰³, Copyright 2023 American Chemical Society).

To study different parameters that affect the DNA circuit kinetics, we prepared mixtures containing 0.4 μM ps3-ODN1826-HP-cODN₄ hybrid and added different concentrations of T7 exonuclease ranging from 0.4 to 0.8 U/μL, followed by measuring the fluorescence intensity over time. A rapid drop in FAM-fluorescence signal indicated successful release of ps3-ODN1826. The slope increased with increasing enzyme concentration demonstrating higher reaction rate (Fig. 4.20,b). Simultaneously, an increase in the Cy5-fluorescence signal

was observed over the reaction time, indicating the accumulation of cDNA by-product, as T7 exonuclease cannot fully degrade it (Fig. 4.20,c). The higher the enzyme concentration, the more rapid the increase in the Cy5 signal and the higher the plateau level was. The two fluorescent signals had opposite trend and mirror-imaged each other's.

We defined two parameters to quantify the system's behavior. First, we calculated the maximum reaction rate, which increased from 0.28 to 0.36 a.u./min with an increase in T7 exonuclease concentration. Second, we defined the reaction efficiency as the percentage change in the fluorescent signal by the end of the reaction time $[(Fl_{t=35}-Fl_{t=5})/Fl_{t=5} \times 100\%]$, indicating the extent to which T7 exonuclease hydrolyzed the cDNA and liberated free CpG ODN. The reaction efficiency increased from 72.8% to 93.1% and showed a linear correlation with the enzyme concentration (Fig. 4.21a,b).

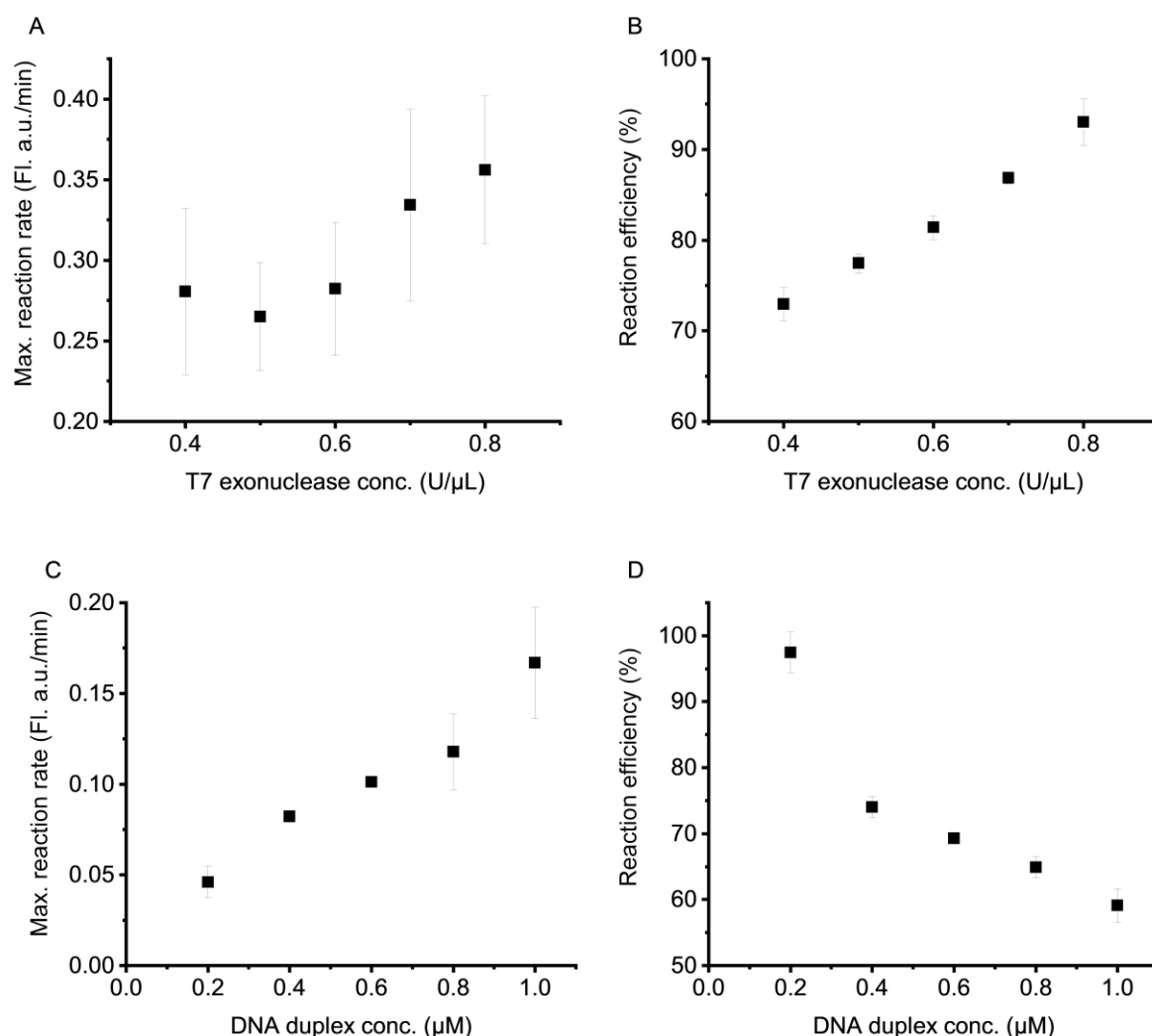


Fig. 4.21. Calculated kinetic parameters of the DNA circuit. **A.** Maximum reaction rate and **B.** reaction efficiency in correlation with T7 exonuclease concentration. **C.** Maximum reaction rate and **D.** reaction efficiency in correlation with DNA duplex starting concentration (Reprinted (adapted) with permission from⁹⁰³ Copyright 2023 American Chemical Society).

Next, we investigated the effect of the starting concentration of the DNA duplex on the kinetics of the DNA circuit (Fig. 4.20,d,e). The DNA duplex concentration was increased from 0.2 to 1 μM while keeping the enzyme concentration fixed at 0.6 U/μL. The FAM-fluorescence signal decreased while the Cy5- fluorescence signal increased, indicating the release of CpG ODN

and the accumulation of residual cDNA, respectively. When the DNA duplex starting concentration increased, a linear increase in the reaction rate was observed (Fig. 4.21c,d). The overall hydrolysis reaction followed second-order kinetics. On the other hand, the reaction efficiency decreased with increasing DNA duplex concentration, which was expected as the enzyme concentration is fixed while the reactant concentration increases.

In summary, these real-time tracking experiments demonstrated the temporal programmability of the DNA circuit kinetics, by adjusting the reaction rate and efficiency through tuning the concentrations of T7 exonuclease and the starting DNA duplex, leading to the controlled release of CpG ODN.

Inactivation-activation of CpG ODN *in vitro*

Before starting with cell culture experiments, we confirmed the activity of T7 exonuclease activity in the presence of cell culture medium. The drop in the FAM- fluorescence signal was demonstrated showing that T7 exonuclease could function in cell culture medium and the liberation of CpG ODN was successful (Fig. 4.22). Furthermore, we tested the hybrids' stability in the presence of DNase I, the main DNase found in mammalian blood that is responsible for at least 90% of deoxy nuclease activity in human plasma⁹⁰⁴, at a biologically relevant concentration of 0.4 U/mL⁹⁰⁵. No significant change in the FAM-fluorescence signal was demonstrated, indicating the stability of our DNA duplex against the enzymatic hydrolysis by DNase I (Fig. 4.22).

To ensure that the utilized CpG ODN concentration fell within the appropriate range to achieve the desired biological activity *in vitro*, we established its dose-response curve for mTLR9 stimulation (Fig. 4.23a). Half-maximal effective concentration (EC₅₀), the concentration of a drug that is required to produce 50% of its maximum effect, was calculated for different CpG ODNs used (Table 4.2). As expected, the potency of CpG ODN increased by increasing the number of PS bonds. This modification enhanced the CpG ODN's stability by rendering it resistant to cellular nuclease activity. On the other hand, the 6A overhang resulted in higher EC₅₀ (*i.e.*, lower potency), potentially due to the hindering of CpG ODN binding with its receptor. We also investigated different concentrations of T7 exonuclease to be used in cell culture, and the optimum concentration was found to be 0.1 U/μL. Further increase of T7 exonuclease did not result in significant increase in mTLR9 stimulation (Fig. 4.23b).

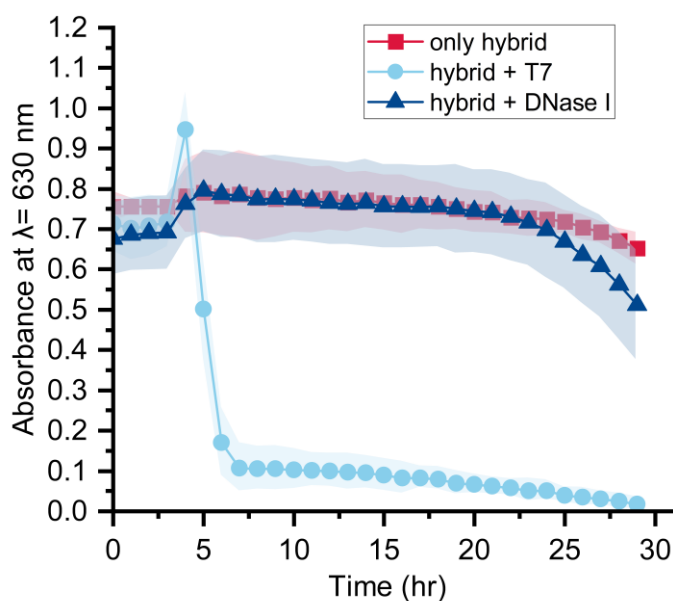


Fig. 4.22. Kinetic tracking of the DNA circuit under different conditions using cell culture medium, by measuring fluorescence intensity of FAM over time. Starting with 0.4 μM ps2-ODN1826 and either T7 exonuclease (0.4 U/μL) or DNase I (0.4 U/μL), followed by the addition of 0.4 μM HP-cODN₄. The duplex alone without any enzyme is used as control.

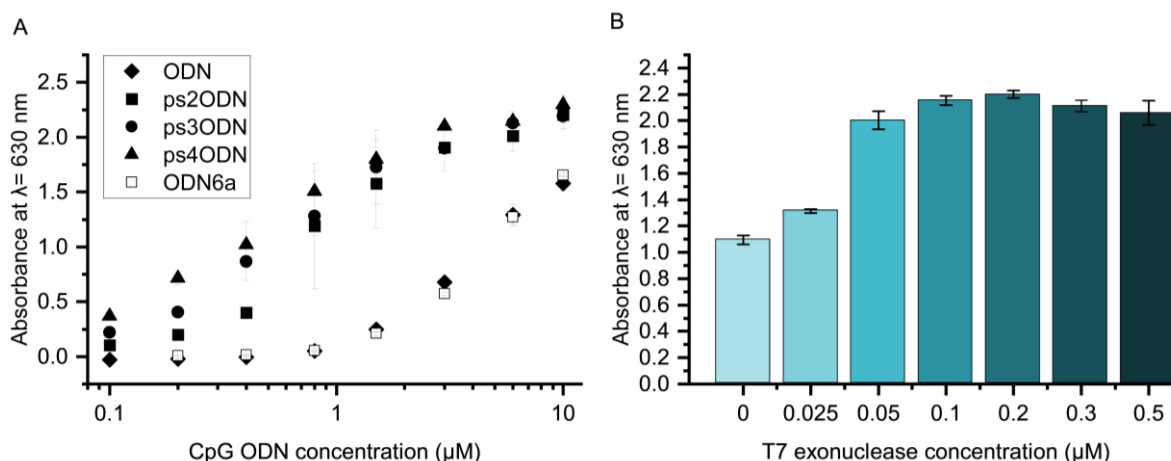


Fig. 4.23. A. Dose-dependent mTLR9 stimulation by different types of CpG ODNs after 18 h of incubation with HEK-Blue mTLR9 cells. Results are expressed as mean $\pm$ SD of triplicates. **B.** mTLR9 stimulation by DNA circuit based on assembly of HP-cODN₁₄ and 0.8 μ M ps2-ODN under different T7 exonuclease concentration ranging from 0.025 to 0.5 U/ μ L. No significant increase in mTLR9 stimulation is observed beyond the concentration of 0.1 U/ μ L. Thus, this concentration was considered to be the optimum one to be used in cell culture *in vitro* experiment.

When we used 6A-ODN1826 in the DNA circuit formation, we did not obtain a significant stimulation of mTLR9 in cell culture. This was consistent with GE results that showed that 6A-ODN1826 was completely hydrolyzed by T7 exonuclease. Thus, we switched to using the second strategy of PS modification of the ODN. Furthermore, the length of the linker in both HP- and GQ-cDNA strands did not lead to a significant difference neither in the loading-release of CpG ODNs nor in the mTLR9 activation at the cellular level. Therefore, only HP-cODN₁₄ and GQ-cODN₁₄ were used as cDNA strands in the following experiments.

To form the duplex, we used either ps2- or ps3-ODN1826 at two different concentrations for each (0.4 μ M and 0.8 μ M). The mTLR9 stimulation was quantified in HEK mTLR9 cells by measuring SEAP enzyme activity using absorption spectroscopy as explained in earlier sections. All DNA duplexes showed a significant decline in mTLR9 stimulation compared to the pristine CpG ODN (Fig. 4.24), indicating successful deactivation of ODNs *via* hybridization of the circuit.

Table 4.2. Calculated EC₅₀ for mTLR9 stimulation by different CpG ODNs

CpG ODN	EC ₅₀ (μ M)
ODN1826	3.799
6A-ODN1826	4.633
ps2-ODN1826	1.078
ps3-ODN1826	0.64

Upon the addition of T7 exonuclease, mTLR9 stimulation was significantly increased, and it was more significant than that of the corresponding pristine CpG ODN at the same concentration. We calculated the activation percent as the ratio of mTLR9 stimulation achieved by the DNA circuit activated by T7 exonuclease to that of the corresponding pristine CpG ODN. Activation percent of different DNA circuits ranged between 40 to more than 200% (Fig. 4.25).

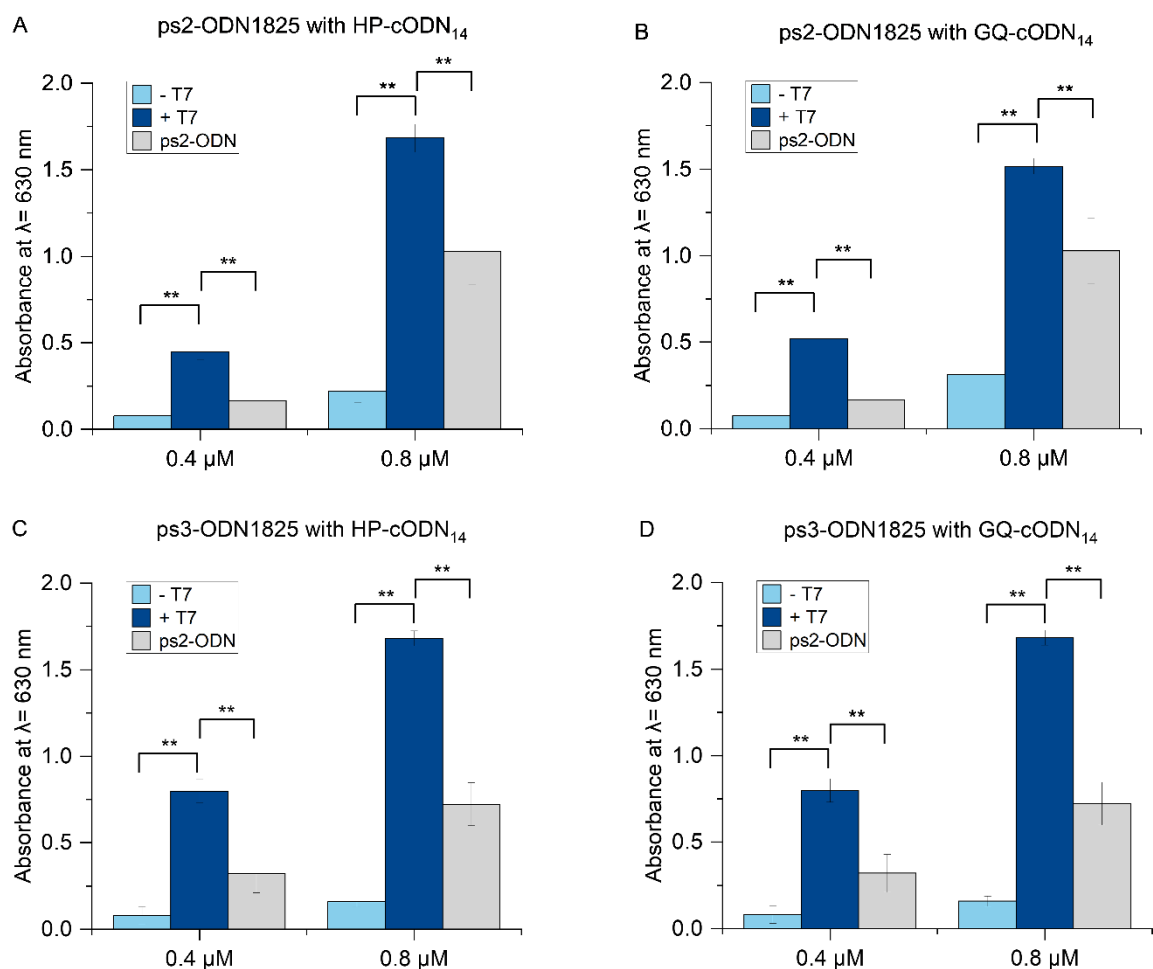


Fig. 4.24. mTLR9 activation by either ps2-ODN1826 released from the DNA circuit with **A.** HP-cODN₁₄ and **B.** GQ-cODN₁₄ strands, or ps3-ODN1826 released from the DNA circuit with **C.** HP-cODN₁₄ and **D.** GQ-cODN₁₄ strands in the presence and absence of T7 exonuclease. Samples were incubated with HEK mTLR9 cells for 18 h. Corresponding pristine CpG ODN and PBS were used as positive and negative controls, respectively. SEAP expression levels by PBS were subtracted and the results are expressed as mean $\pm$ SD of triplicates. Data were analyzed with two-way ANOVA with a Bonferroni post-test. * = $p < 0.01$, ** = $p < 0.001$.

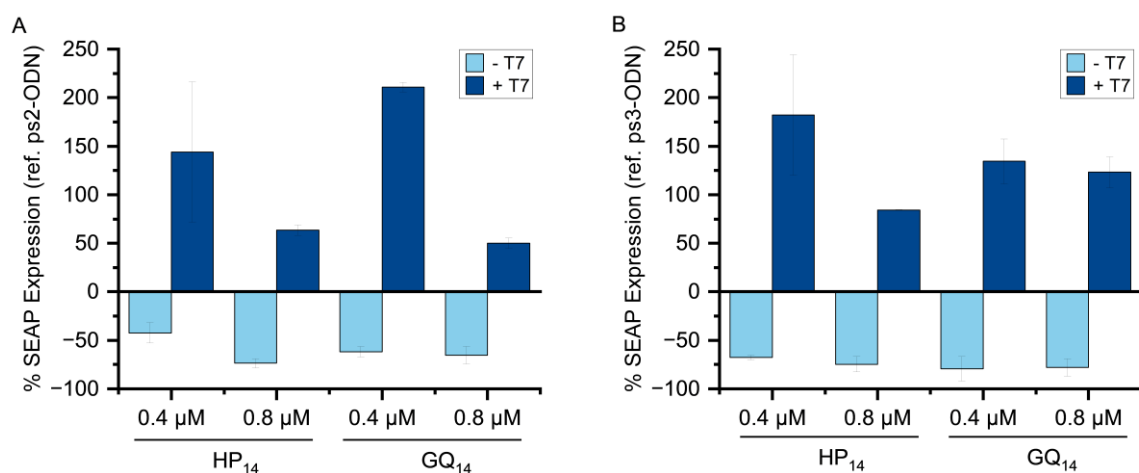


Fig. 4.25. mTLR9 stimulation by DNA circuits loaded with **A.** ps2-ODN1826 or **B.** ps3-ODN1826, at two concentrations (0.4 μM or 0.8 μM), using either HP-cODN₁₄ or GQ-cODN₁₄ as cDNA strands, in the presence or absence of T7 exonuclease. Normalized levels of SEAP expression were calculated relative to the corresponding pristine CpG ODN. Deactivation of the CpG ODN is observed in all DNA duplexes, and activation is observed in all DNA circuits in the presence of T7 exonuclease. Results are expressed as mean $\pm$ SD.

This profound increase in mTLR9 activation when comparing pristine CpG ODN with that released by T7 exonuclease from the DNA circuit could be attributed to the programmed gradual release of the active CpG ODN from its protected hybridized form. This would result in a prolonged half-life leading to higher effective concentration at the target site and therefore an enhanced response at earlier time points. On the other hand, pristine CpG ODN is added directly at once to the cells exposing it immediately to the potential hydrolysis and digestion *via* different nucleases, leading to lower effective concentration of intact CpG ODNs that actually reached the target receptor and stimulated it.

To validate this hypothesis, the same quantity of pristine CpG ODN control was pipetted gradually over incubation time rather than being added at once (Fig. 4.26).

This resulted in an elevated mTLR9 stimulation supporting our hypothesis. Based on these results, we could conclude that the designed DNA circuit could provide an extra layer of temporal control over the release and bioactivity of the CpG ODN *in vitro*.

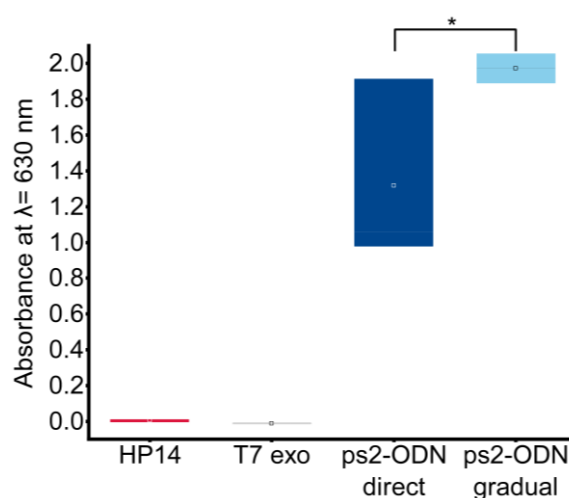


Fig. 4.26. mTLR9 stimulation under different conditions. No stimulation is observed for HP-cODN₁₄ strand or the enzyme T7 exonuclease alone. The gradual addition of pristine ps2-ODN1826 (0.8 μM) resulted in significant enhancement in mTLR9 stimulation compared to direct addition of the same quantity at once.

Kinetic tracking of immunostimulation induced by the DNA circuit *in vitro*

To investigate the time-dependent immunostimulation profile of our DNA circuit, we tracked mTLR9 stimulation over 24 h. Different profiles could be demonstrated when comparing pristine CpG ODN with the DNA circuit activated by T7 exonuclease, especially during the first 12 h of incubation (Fig. 4.27). T7 exonuclease addition promoted an early release of CpG ODN leading to higher level of mTLR9 stimulation. Interestingly, the DNA circuits (*i.e.*, without T7 exonuclease) were not significantly different from the corresponding pristine CpG ODN in the first 9 h of incubation but started to exhibit higher mTLR9 stimulation afterward. Possible explanation would be the slow and gradual hydrolysis of the cDNA strand by endogenous nucleases. In the second half after 12 h of incubation, pristine CpG ODN reached a plateau at a relatively low level of mTLR9 stimulation. However, the DNA circuit, both with and without T7 exonuclease, could reach higher level of mTLR9 stimulation with at least two-fold increase compared to the pristine CpG ODN, consistent with previous results from end-point stimulation experiments.

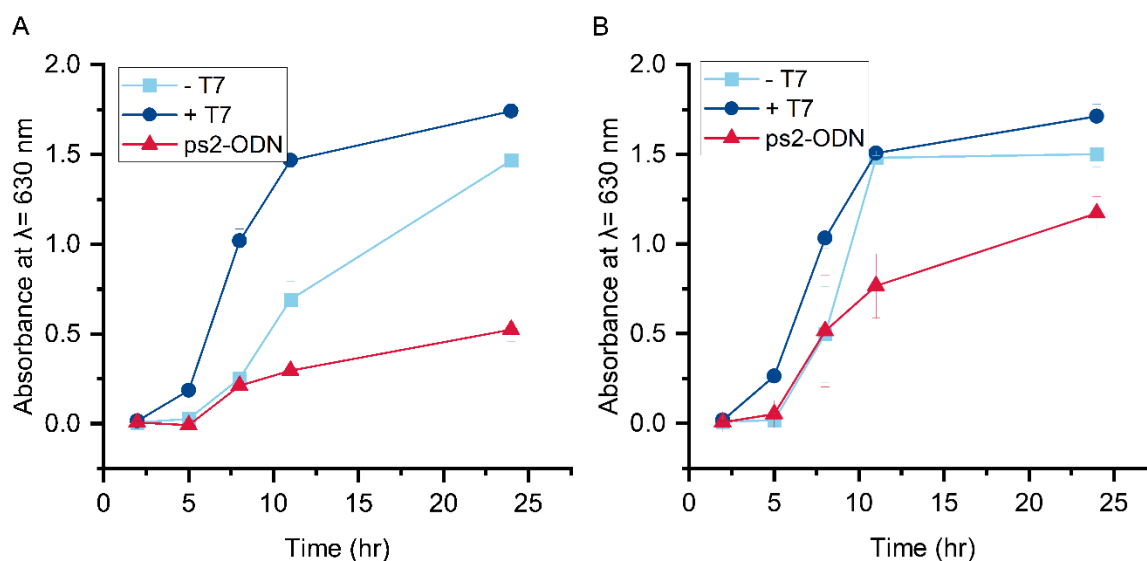


Fig. 4.27. Time-dependent mTLR9 stimulation triggered using the programmed DNA circuit consisting of HP-cODN₁₄ with ps2-ODN1826 at either **A.** 0.4 μ M or **B.** 0.8 μ M in the presence or absence of T7 exonuclease. Pristine CpG ODN and PBS were used as positive and negative controls respectively. SEAP expression levels by PBS were subtracted and the results are expressed as mean $\pm$ SD of triplicates.

To further investigate the immunostimulation triggered by the DNA circuit, we tested our system in J774A.1 mouse macrophages, which are immune cells that express mTLR9 naturally. In response to mTLR9 activation macrophages produce different cytokines and interleukins, ultimately resulting in the activation of the innate immune system. Similar to previous experiments discussed in chapter 2, we could quantify TNF production in the supernatants of macrophages by using HEK TNF reporter cell line.

We used the DNA circuit based on ps3-ODN and GQ-cODN₁₄, which did not result in a significant release of TNF during an incubation period of up to 6.5 h, at concentrations of 0.8 μ M or less (Fig. 4.28a). When the incubation time was prolonged to 24 h, higher TNF production was observed, suggesting a delayed stimulation of mTLR9 by the DNA duplexes, even in the absence of T7 exonuclease. Nevertheless, in time-tracking experiments, the presence of T7 exonuclease led to an early activation of the DNA circuit and a significant peak of TNF release was observed after only 3 h of incubation (Fig. 4.28b).

In the absence of the enzyme, the duplex showed similar behavior to that of pristine ps3-ODN1826 by exhibiting a slow increase in TNF release over the entire incubation time. Additionally, in a control experiment with the step-wise addition of ps3ODN at 0.8 μ M concentration, a distinct activity profile can be observed compared to the direct addition of CpG ODN, with a significant higher stimulation of TNF production (Fig. 4.28c).

To summarize, time-tracking experiments in immune cells demonstrated that the DNA-duplex formation did not entirely deactivated CpG ODNs, but rather delayed its immunostimulatory activity significantly when compared to the functional DNA circuit with T7 exonuclease addition. In the absence of T7 exonuclease, it can be speculated that the observed delayed response is due to the endogenous nucleases activity on the DNA-duplex structure, which could result in its hydrolysis at a slower rate in comparison with the DNA circuit incorporating T7 exonuclease. The addition of T7 exonuclease resulted in an early and potent

immunostimulatory response that could not be achieved even with the pristine CpG ODN. This could be related to the protection of CpG ODN *via* the hybrid system as explained earlier. Gradually releasing CpG ODNs *via* T7 exonuclease resulted in their availability at the receptors binding site at higher effective concentration for a prolonged period compared to that of pristine CpG ODNs, thus leading to higher activity at early stages.

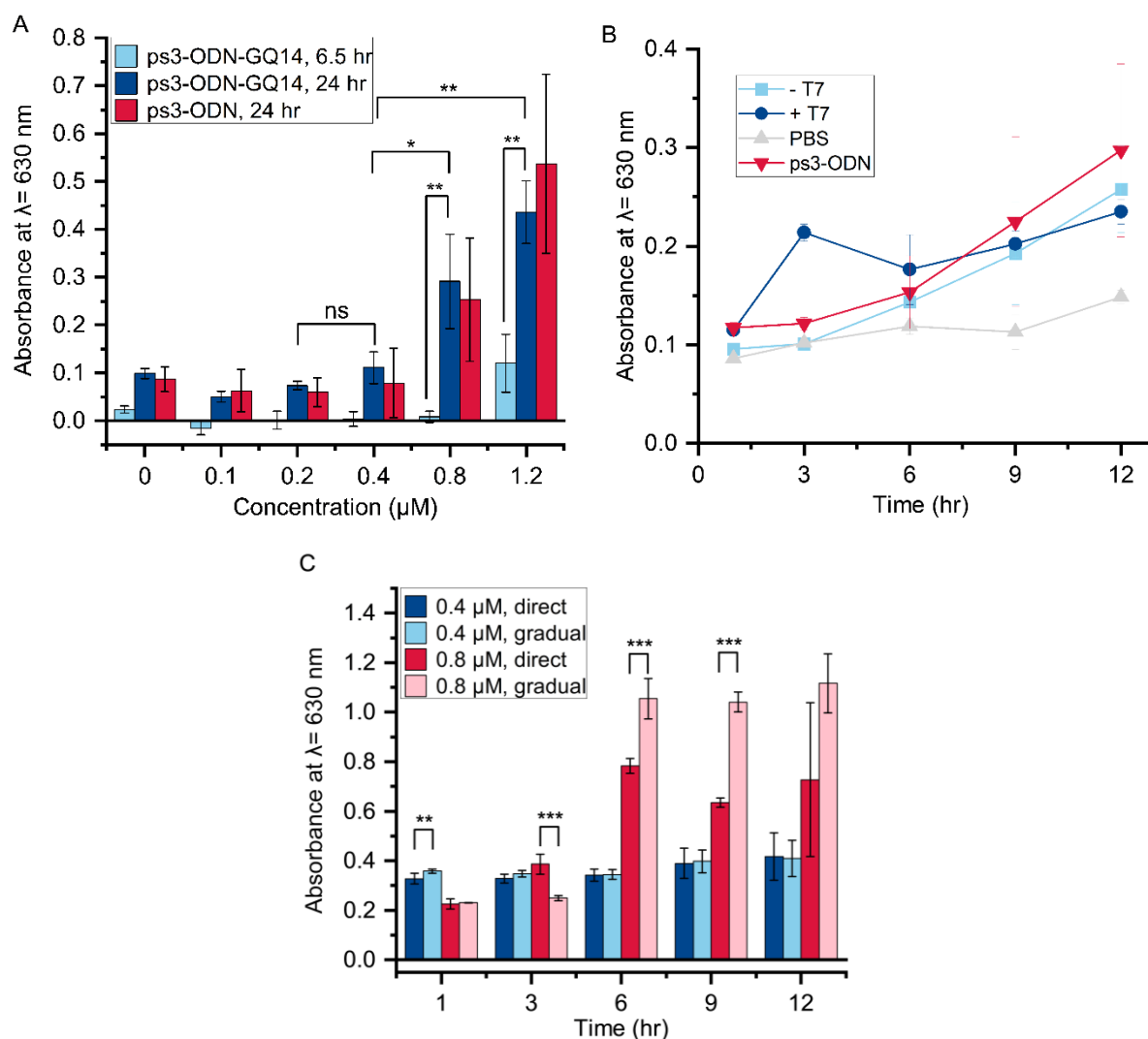


Fig. 4.28. **A.** Dose-dependent TNF- α release triggered by pristine ps3-ODN1826 after 24 h incubation and by the DNA circuit consisting of ps3-ODN1826 and GQ-cODN₁₄ after 6.5 h or 24 h incubation. **B.** Time-dependent TNF release triggered by the same DNA circuit with or without T7 exonuclease. Pristine CpG ODN and PBS were used as positive and negative controls respectively. SEAP expression levels by PBS were subtracted and the results are expressed as mean $\pm$ SD of triplicates. TNF levels produced by the hybrid system in presence of T7 exonuclease were statistically different than the system in absence of T7 exonuclease and ps3ODN with p -value < 0.0001 . **C.** Time-dependent TNF release triggered by either the direct or the gradual addition of pristine ps3ODN at concentrations of 0.4 μ M and 0.8 μ M. Data were analyzed with two-way ANOVA with a Bonferroni post-test. * = $p < 0.01$, ** = $p < 0.001$, *** = $p < 0.0001$.

Dissipative system based on T7 exonuclease activation

To convert the DNA circuit into a DNA-based dissipative system that operates out-of-equilibrium, the system must be reverted to its original state by the addition of cDNA strand that can hybridize again with the CpG ODN to deactivate it. A second cycle of activation *via* T7 exonuclease can then be initiated leading to a second release of CpG ODN. In such dissipative systems, the cDNA strand is termed the ‘fuel strand’.

We investigated the dissipative system performance using the previously described kinetic tracking experiments that utilized FRET pairs. We similarly studied the effects of different concentrations of T7 exonuclease and the fuel strand on the reaction rate and efficiency. However, in these series of experiments, the starting material is a mixture of the CpG ODN and the enzyme, followed by the addition of the fuel strand at certain time points.

First, a series of mixtures containing ps3-ODN1826 at a fixed concentration of 0.4 μM and different concentrations of T7 exonuclease were prepared. To these solutions, equimolar amount of HP-cODN₄-Cy5 was added and the fluorescence of each sample was measured over time. A rapid increase in FAM-fluorescence intensity with subsequent gradual decrease back to the initial value was observed (Fig. 4.29a). This corresponded to the rapid hybridization of DNA strands, followed by gradual hydrolysis of the fuel by T7 exonuclease that released the CpG ODN.

Increasing T7 exonuclease concentration shifted the backward reaction (*i.e.*, hydrolysis) to be faster than the forward reaction (*i.e.*, hybridization), leading to the decrease of the transient lifetime from 28 to 3 min and more complete release of CpG ODN (Fig. 4.29c). On the other hand, the Cy5-fluorescence signal showed an almost inversely symmetrical trend. An initial rapid increase was attributed to the addition of the fuel strand. This was followed by gradual decline indicating the occurrence of the hybridization process. A subsequent increase leading to a plateau phase was then observed, corresponding to the enzymatic hydrolysis that resulted in the accumulation of fuel strand by-products (Fig. 4.29b). These three distinct regions were observed at low concentrations of T7 exonuclease such as 0.1 U/ μL and 0.2 U/ μL . However, when higher enzyme concentrations were used, the hybridization region became less visible as the rapid hydrolysis resulted in a net increase in the fluorescence intensity followed by the plateau phase.

Next, the effect of HP-cODN₄ fuel strand concentration on the behavior of the dissipative system was investigated. For this purpose, the enzyme concentration was fixed at 0.4 U/ μL . The hybridization process took place reaching a peak when all fuel strands were added (Fig. 4.29d). The time needed to reach the peak was almost identical and independent of HP-cODN₄ concentration, showing that the hybridization rate was constant (*i.e.*, zero order kinetics). However, the gradual decline of the fluorescence showed variable rates that slowed down with higher hybrids concentrations. Thus, the lifetime exhibited a linear increase from 0.5 to 15 min with increasing HP-cODN₄ fuel strand concentrations over the range of 0.2 -1.2 U/ μL (Fig. 4.29f). The Cy5-fluorescence intensity in the plateau region was proportional to the fuel strand concentration as expected (Fig. 4.29e). We concluded that the kinetics of the dissipative system could be temporally programmed by tuning the rates of the forward hybridization or the backward hydrolysis, leading to controlled release of CpG ODN.

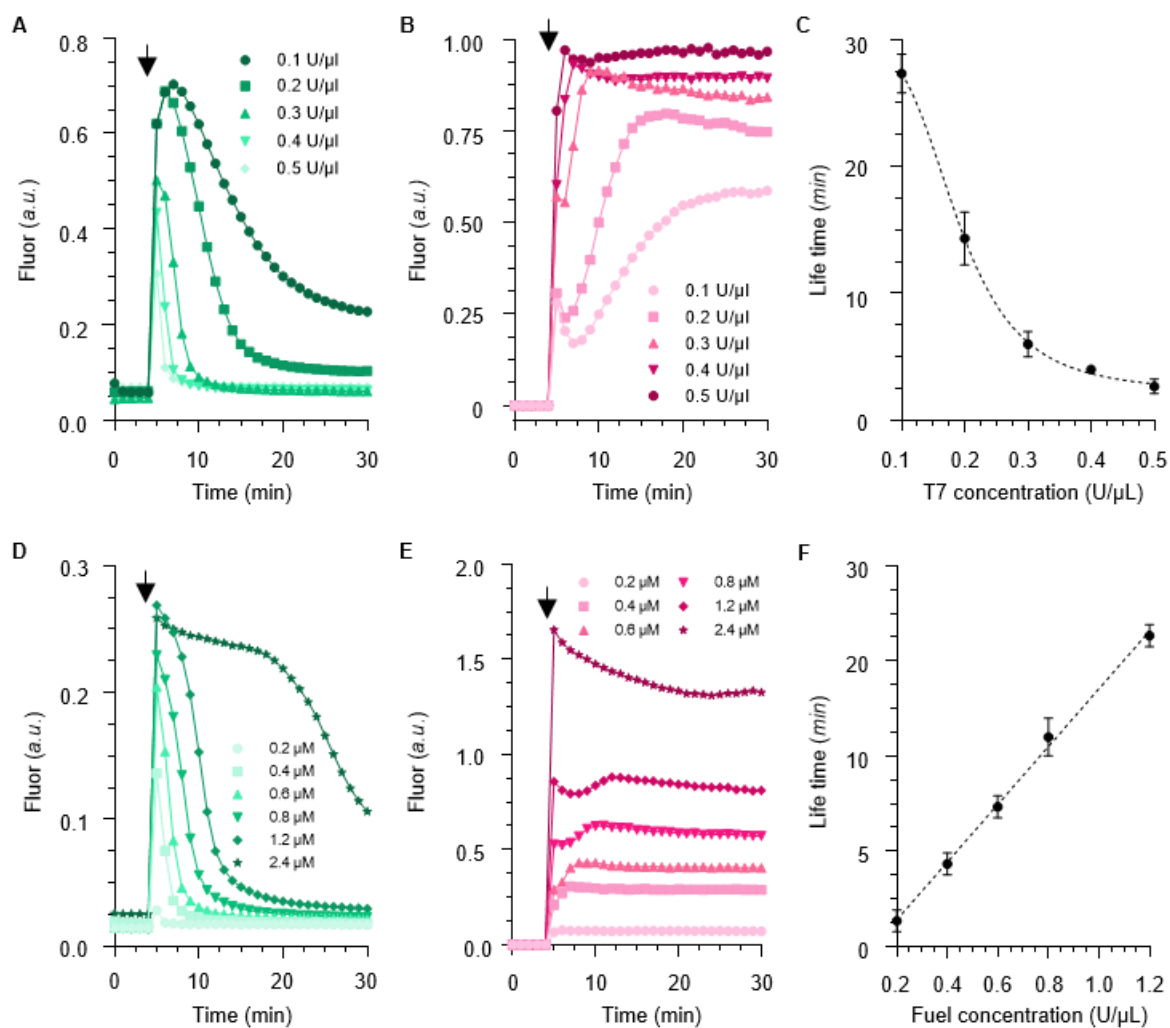


Fig. 4.29. Fluorescence intensity measurement of **A.** FAM and **B.** Cy5 over time. Arrows indicate the addition of the fuel strand. **C.** Calculated transient lifetime in correlation with T7 exonuclease concentration. Fuel strand concentration-dependent kinetic tracking of the dissipative system. Starting with 0.4 μM ps3-ODN1826 and T7 exonuclease (0.4 U/μL), followed by the addition of HP-cODN₄ fuel strand (0.2-1.2 μM). Fluorescence intensity measurement of **D.** FAM and **E.** Cy5 over time. Arrows indicate the addition of the fuel strand. **F.** Calculated transient lifetime in correlation with fuel strand concentration. Reaction conditions: 50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate and 1 mM DTT, 37°C.

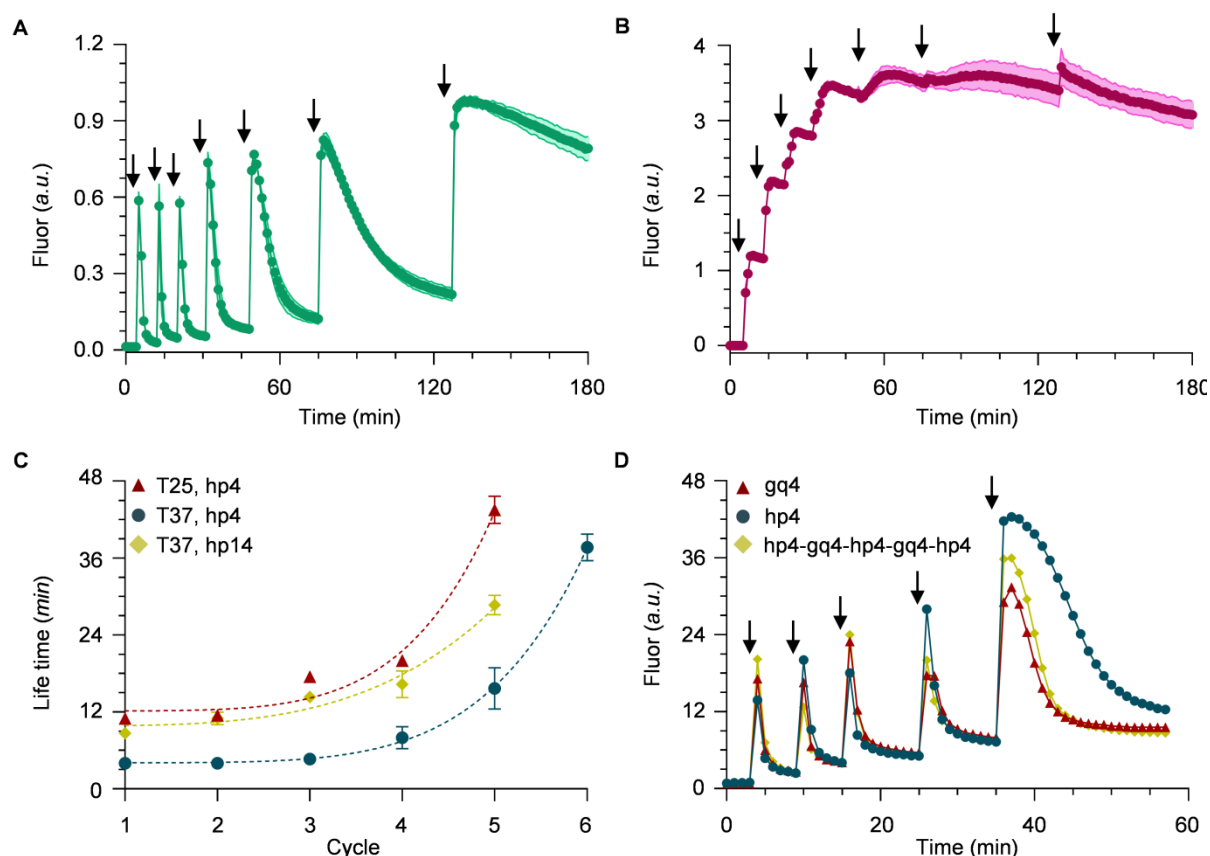


Fig. 4.30. Kinetic tracking of the dissipative system multiple cycles of ps3-ODN1826 (0.4 μM) loading and release, through repetitive addition of HP-cODN₄-Cy5 fuel strand (0.4 μM) in the presence of T7 exonuclease (0.4 U/ μL). Time-dependent measurement of fluorescence intensity of **A.** FAM and **B.** Cy5 at 37°C. **C.** Calculated lifetime for each cycle at 37°C (T37) or 25°C (T25). Reaction conditions: 50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate and 1 mM DTT. **D.** Comparison of dissipative system multiple cycles of ps3-ODN1826 loading and release by time-dependent FAM fluorescence intensity measurement in the presence of T7 exonuclease (0.4 U/ μL). Different fuel strands (0.4 μM) were used; either HP-cODN₄ or GQ-cODN₄, or their alternating addition. Arrows indicate the addition of fuels strand.

The initiation of multiple cycles of the dissipative process was tested by repetitively adding a fixed amount of the fuel strand to the system. We were able to achieve a minimum of six complete loading-release cycles that showed rapid hybridization followed by gradual hydrolysis in all cycles with the hydrolysis reaction becoming slower as the number of cycles progressed (Fig. 4.30a). The plateau region of Cy5-fluorescence intensity increased in length and intensity because of the buildup of by-products through multiple cycles (Fig. 4.30b). An increase in the lifetime became obvious especially after the third cycle. These findings indicated the enzyme's fatigue over time, which could be attributed to the waste accumulation. As expected, using 0.2 μM of HP-cODN₄ fuel strand resulted in similar behavior but with almost 50% less fluorescence intensity of both FAM and Cy5.

Since temperature is anticipated to affect the kinetics of the dissipative system, we compared the system's performance at 25 °C and 37 °C, as the former is the indicated temperature in the enzyme protocol by the producer, and the latter is the physiologically relevant temperature to the intended application of CpG ODN. It was found that the life time of each cycle at 25°C was considerably longer than that at 37°C (Fig. 4.26c). Changing the fuel strand to HP-cODN₁₄ showed similar behavior but with prolonged life-times when compared with HP-cODN₄-based system (Fig. 4.30c). A possible explanation is that increasing the linker's length slowed down

the hybridization process and the by-product had more pronounced effect on the enzymatic fatigue.

To demonstrate the high versatility of the system's design, GQ-cODN was used as a fuel strand instead of HP-cODN. The kinetics of multiple cycles were compared through the addition of either HP-cODN₄ or GQ-cODN₄ fuel strands to the system, or alternating between them in the same system (Fig. 4.30d). Similar behaviors under these conditions were observed indicating that loading-release cycles of CpG ODN from the dissipative system could be achieved with different types of fuel strands.

4.4 CONCLUSION AND OUTLOOK

Although DNA circuits have been extensively investigated for different applications, circuits demonstrating successful delivery of DNA output strands as pharmacologically active moieties in living cells remained elusive. In this context, our designed DNA circuit that involves a biologically active immune adjuvant presents an exciting starting point to create interactive DNA circuits at the interface of synthetic nanodevices and cells for a successful temporal control of TNAs' delivery. The reasons are two-fold: first, the kinetics of the release of the functional motif can be programmed; second, structural evolution of the DNA circuit can be exploited to control biological functions.

Hybridization of CpG ODN with cDNA strand resulted in its inability to bind to its target receptor, thus deactivating its biological function. Subsequently, the selective hydrolysis of the cDNA strand by the enzyme ExoIII or T7 exonuclease resulted in the liberation of the biologically active CpG ODN, with the latter enzyme showing higher selectivity and better biological performance. Effective CpG ODN loading and release could be investigated using FRET-pair real-time tracking experiments. We demonstrated that we could control the kinetics of the DNA circuit by tuning the concentrations of T7 exonuclease and the DNA duplex.

Moreover, the performance of the DNA circuit was tested in cell culture *in vitro*, demonstrating the successful deactivation/activation of CpG ODN for mTLR9 stimulation in HEK-engineered cell line, as well as triggering TNF release in J774A.1 macrophages. Importantly, we could alter the CpG ODN immunostimulation profile *in vitro* by triggering an acute and potent response at an early point of incubation, compared with otherwise a delayed response. This presents the first example of a DDS exploiting a DNA circuit programmed to trigger distinct biological responses of live cells in comparison to a system without such programmed nanodevice. This work lays the cornerstone for the design of future interactive DNA circuits systems that utilize cell-owned signals (*i.e.*, endogenous enzymes) to trigger the DNA circuit and release the bioactive oligomer. These signals would replace the T7 exonuclease in the current conceptual design. Finding proper signals that differ between healthy and diseased tissue would be highly valuable in targeting various disorders to manipulate the biological response in a time-controlled manner. Moreover, we believe that our approach could be readily applicable for controlled activation of other functional nucleic acids such as RNA decoys, ribozymes, antisense oligonucleotides, aptamers and DNazymes.

In addition, we were able to convert the DNA circuit into a dissipative system by the addition of cDNA (*i.e.*, fuel strands) to achieve multiple cycles of activation and deactivation. Kinetics of the forward and backward reactions could be controlled by tuning the concentrations of T7 exonuclease and DNA fuel strands as documented by FRET-pair tracking. Effective CpG ODN loading and release was successfully demonstrated in multiple cycles in a test tube. Further experiments in the future should aim to achieve multiple cycles of assembly/disassembly in biological systems in cell culture.

The vision of such systems is to achieve an oscillating autonomous control without external intervention. A reservoir of the fuel strand needs to be pre-programmed and administered together with the dissipative system (Fig. 4.31). Ideally, a feedback loop from the target cells would signal the release of fuel strand from its reservoir to inhibit additional biological response. A new cycle of the biological response could be then triggered again based on the needs of the target cells. Systems operating in this manner could be valuable in settings where both stimulation and inhibition are needed in balance. For example, in immunostimulation therapeutics, overstimulation of the immune system could lead to undesired side effects such as the cytokine storm syndrome. We believe that further research would be invaluable to improve smart DNA-based DDSs that can interact and respond to their surrounding environment.

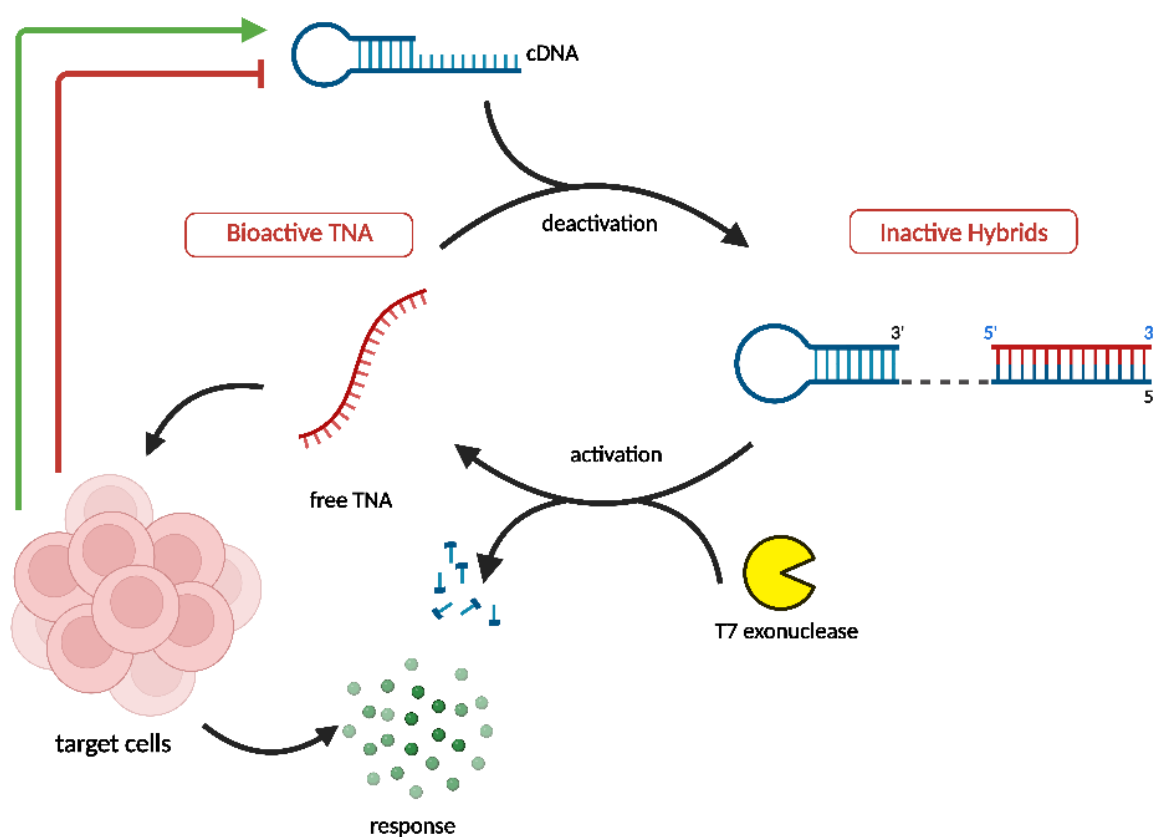


Fig. 4.31. Scheme presenting an oscillating autonomous dissipative system that responds to feedback from the target cells to either initiate or inhibit further cycles and thereby further release of the TNA moiety. Created with BioRender.com

5. Conclusion and Outlook

To summarize, the aim of this thesis was to advance the design and development of smart DDSs that enable controlled drug delivery with the overall aim of improving therapeutic outcome and minimizing side effects. Since TNAs play a critical role in shaping modern medicine, we aimed to harness DNA nanotechnology for their delivery as cargo, including both DNA-based (*i.e.*, CpG ODN) and RNA-based (*i.e.*, siRNA) oligonucleotides.

The first strategy was to design a DDS that can respond to stimulation *via* US, for application in sonopharmacology and sonogenetics. US has been employed for enhancing drug delivery either by thermal or mechanical mechanisms. However, sono-adjuvants such as microbubbles or MS ion channels were needed for the successful use of low-energy US for biomedical applications, and no consensus strategy was exploited to induce specific molecular changes in the DDS that lead to drug release and activation. On the other hand, US was used as a source of mechanical forces to induce specific molecular transformations in polymeric materials in the field of polymer mechanochemistry. Nevertheless, cytotoxic high-energy US was often used in this context.

Therefore, we aimed to bridge this gap and adapt the principles developed in polymer mechanochemistry in the design of a mechanoresponsive DDS that can inherently respond to a low-energy medically compatible imaging US device, without the need of administering sono-adjuvants. We realized this goal by developing an US-responsive DDS that consists of long ssDNA polymers, produced by RCA, that condense into DNA-inorganic-hybrid nanoparticles by co-crystallization with $\text{Mg}_2\text{P}_2\text{O}_7$. The backbone of the RCA polymer was engineered to hybridize with the TNA cargo to protect it in its inactive form, until the exposure to imaging US, which induced covalent and non-covalent bond scission leading to the liberation of the TNA in its active form.

The DDS was extensively characterized and the RCA-ODN combination was thoroughly optimized. Our results demonstrated the importance of the formation of a hierarchically structured hydrogel (composed of a DNA matrix and DNA NFs) for the DDS's responsiveness towards imaging US. Additionally, we analysed the potential mechanisms underlying the system's behaviour, and concluded that on-demand cargo release triggered by US can be caused by cavitation or electrokinetic effects or a combination of both.

In vitro and *in vivo* studies demonstrated successful control over cellular function in different biological systems. For CpG ODNs, controlled immunostimulation was achieved in an engineered HEK cell line that expresses the target mTLR9 exclusively and a macrophage cell line that expresses mTLR9 naturally. Furthermore, we demonstrated successful immunostimulation in an experimental setting involving primary immune cells isolated from human blood donors, namely DCs and T cells, which were co-cultured with a cancer cell line. Finally, we showed profound US-controlled immunostimulation in a mouse animal model *in vivo*. This reports an unprecedented application of polymer mechanochemistry for a therapeutic application *in vivo*. For siRNA, we demonstrated the DDS's function by successful knockdown of *EGFP* as a model in cell culture *in vitro*.

As a future outlook, we foresee that the universal use of this mechanochemically responsive DDS would be feasible by loading other types of cargo, such as small molecules and proteins. This would require modifying the design of the polymer carrier to involve aptamer sequences with high binding affinity to the designated cargo. The use of US as a mechanochemical trigger provides several advantages over other stimuli, such as light in photopharmacology⁹⁰⁶ and optogenetics⁹⁰⁷, due to its high penetration depth and established clinical safety. Thereby, we have developed a technology with broad applicability for on-demand regulation of cellular activities at multiple levels, making it suitable for both fundamental research, aiming to better

understand biological processes, as well as therapeutic applications-oriented research. We envision a seamless coupling of our system with other biotechnological platforms to regulate cellular function at different mRNA and protein levels in the future.

Regarding the second strategy developed in this thesis aiming to achieve controlled drug delivery, we utilized DNA circuits and DNA dissipative systems. These systems can be assembled using different DNA building blocks. Previous use of such systems was either unrelated to drug delivery (*i.e.*, imaging and biosensing applications) or as carriers to deliver small molecule drugs as cargo. In this work, we aimed to utilize DNA circuits and DNA dissipative systems that involve therapeutic DNA building blocks in their design.

Towards this goal, we designed and optimized DNA circuits to achieve controlled delivery of CpG ODNs. Two key elements were used to construct the circuits; the CpG ODN strand and a complementary strand that hybridizes to the CpG ODN and blocks its interaction with its target receptor. Different designs in which steric hinderance due to secondary structures formation were tested. The system was triggered by enzymatic activity of either ExoIII or T7 exonuclease. We demonstrated controlled kinetics by tuning different system parameters. We concluded that the use of T7 exonuclease was the best among the developed systems. We further tested our DDSs in cell culture experiments *in vitro*. The DNA circuit's *in vitro* activation resulted in TLR9 stimulation in HEK-engineered cell line, as well as TNF release by J774A.1 macrophages. By programming the DNA circuit to control the release of CpG ODN, we achieved an altered pharmacological profile with acute and potent immunostimulation, in comparison to a system without controlled CpG ODN release, which exhibited a slow and delayed response.

Moreover, we demonstrated successful conversion of the DNA circuit into a dissipative system that exhibited multiple cycles of deactivation/activation and functions in thermodynamic out-of-equilibrium. The vision for such systems in the future is to achieve oscillating systems with autonomous control of the pre-programmed DDS. This enables the DDS to interact with and respond to the surrounding environment, without external interventions. We expect this envisioned design to be important in therapeutic applications where a balanced activation/inhibition response would be needed.

6. Appendix

6.1 SUPPLEMENTARY TABLES

SUPPLEMENTARY TABLE 1

Sequences of DNA and RNA oligonucleotides used for mechanoresponsive DDS development

Oligomer Name	Sequence (5' to 3')
Primer for RCA-oligomer reaction	TATAATTATAAT
Template for RCA-ODN1826 100% complementarity	ATTATAAAAAAAAAAATCCATGACG TTCCTGACGT TAA AAAAAACGC GCGC GCGC GCGC GCGC GCGC GCGC GCGC AAAAAA AAATTATA
Template for RCA-ODN1826 75% complementarity	ATTATAAAAAAAAAAATCCATGACG TTCCTGA AAAAAAAA ACGCGCGC GCGC GCGC GCGC GCGC GCGC GCGC GCGC AAAAAAAA ATT TA
Template for RCA-ODN1826 50% complementarity	ATTATAAAAAAAAAAATCCATGACGT AAAAAAAAAACGC GCGCGCGC GCGC GCGC GCGC GCGC GCGC GCGC GCGC AAAAAAAA ATTATA
Template for RCA-ODN1826 25% complementarity	ATTATAAAAAAAAAAATCCATA AAAAAAAAAACGC GCGCGC GCGCGCGC GCGC GCGC GCGC GCGC GCGC GCGC GCGC AAAAAAAA ATTATA
Template for RCA-ODN D-SL01 100% complementarity	Phosphate-ATTATAAAAAAAAAAATCGCGACG TTTCG CCCCAGC TTTCGGT AAAAAAAAAACGC GCGCGC GCGC CGCGCGCG CCGCGC GCGC GCGC AAAAAAAA ATTATA
Template for RCA-ODN D-SL01 75% complementarity	Phosphate-ATTATAAAAAAAAAAATCGCGACG TTTCGCCGA CGT AAAAAAAAAACGC GCGCGC GCGC GCGC GCGC GCGC GCGC GCGC A AAAAAAA ATTATA
Template for RCA-senseRNA	Phosphate-ATTATATTTTTTTTTCCTTGAGA AGA TGGTGCTTTTTTTTTTATTATA
Template for RCA-antisenseRNA	Phosphate-ATTATATTTTTTTTGCACCATCTTCTTC AAGGATTTTTTTTTTATTATA
CpG ODN1826	TCCATGACG TTCCTGACG TT
CpG ODN D-SL01	TCGCGACG TTCGCCCCGACG TTTCGGTA
FAM CpG ODN D-SL01	FAM-TCGCGACG TTCGCCCCGACG TTTCGGTA
EGFP siRNA -sense strand	Phosphate-UCCUUGAAGA AGAU GGUGCdTdT
EGFP siRNA -antisense strand	Phosphate-GCACCAUCUUC UUCAAGG AdTdT
EGFP siRNA -sense strand -Cy5	Phosphate-UCCUUGAAGA AGAU GGUGCdTdT-Cy5
EGFP siRNA -antisense strand -Cy5	Phosphate-GCACCAUCUUC UUCAAGG AdTdT-Cy5

SUPPLEMENTARY TABLE 2

Sequences of DNA primers for RT-qPCR

Primer Name	Sequence (5' to 3')
Gapdh	FWD: TGTTGAAGTCACAGGAGACAACCT REV: AACCTGCCAAGTATGATGACATCA
Tnf	FWD: ACCACGCTCTTCTGTCTACTGA REV: TCCACTTGGTGGTTTGCTACG
IL1B	FWD: GAGCTGAAAGCTCTCCACCTC REV: CTTTCCTTTGAGGCCCAAGGC
IL10	FWD: GGCTGAGGCGCTGTCATCG REV: TCATTCATGGCCTTGTAGACACC
Ccl22	FWD: CCGCAAGCCTGGCGTTGTTTTG REV: GGGCTTGCTGAGCCTCCCTG

SUPPLEMENTARY TABLE 3

Sequences of DNA oligonucleotides used in DNA circuits and DNA dissipative systems

Oligomer Name	Sequence (5' to 3')
ODN1826	TCCATGACGTTTCCTGACGTT
ODN1826-6A	TCCATGACGTTTCCTGACGTTAAAAA
ODN1826-ps2	TCCATGACGTTTCCTGACG*T*T
ODN1826-ps3	TCCATGACGTTTCCTGAC*G*T*T
ODN1826-ps4	TCCATGACGTTTCCTGA*C*G*T*T
Ps-ODN1826	T*C*C*A*T*G*A*C*G*T*T*C*C*T*G*A*C*G*T*T
cODN₂₀	AACGTCAGGAACGTCATGGA
cODN₄₀	AACGTCAGGAACGTCATGGAAACGTCAGGAACGTCATGGA
cODN₄₁	AACGTCAGGAACGTCATGGACAACGTCAGGAACGTCATGGA
cODN₄₂	AACGTCAGGAACGTCATGGACCAACGTCAGGAACGTCATGGA
cODN₄₃	AACGTCAGGAACGTCATGGACCCAACGTCAGGAACGTCATGGA
cODN₄₄	AACGTCAGGAACGTCATGGACCCCAACGTCAGGAACGTCATGGA

cODN₁₂₀	AACGTCAGGAACGTCATGGAAACGTCAGGAACGTCATGGAAACGTCAGG AACGTCATGGAAACGTCAGGAACGTCATGGAAACGTCAGGAACGTCATG GAAACGTCAGGAACGTCATGGA
cODN₂₀₀	AACGTCAGGAACGTCATGGAAACGTCAGGAACGTCATGGAAACGTCAGG AACGTCATGGAAACGTCAGGAACGTCATGGAAACGTCAGGAACGTCATG GAAACGTCAGGAACGTCATGGAAACGTCAGGAACGTCATGGAAACGTCA GGAACGTCATGGAAACGTCAGGAACGTCATGGAAACGTCAGGAACGTCA TGGA
HP-cODN₄	AACGTCAGGAACGTCATGGACCCCCCGAAAAAAGCCCTTTTGGGCTTTT TTCGGG
HP-cODN₁₄	AACGTCAGGAACGTCATGGACCCCCCCCCCCCCCCCCCGAAAAAAGCCCTT TTGGGCTTTTTTCGGG
HP-cODN₂₄	AACGTCAGGAACGTCATGGACCCCCCCCCCCCCCCCCCCCCCCCCCGAA AAAAGCCCTTTTGGGCTTTTTTCGGG
HP-cODN₄-Cy5	AACGTCAGGAACGTCATGGACCCCCCGAAAAAAGCCCTTTTGGGCTTTT TTCGGG-Cy5
HP-cODN₁₄-Cy5	AACGTCAGGAACGTCATGGACCCCCCCCCCCCCCCCCCGAAAAAAGCCCTT TTGGGCTTTTTTCGGG-Cy5
GQ-cODN₄	AACGTCAGGAACGTCATGGAAAAAGGTTGGTGTGGTTGG
GQ-cODN₁₄	AACGTCAGGAACGTCATGGAAAAAAAAAAAAAAAAAGGTTGGTGTGGTTGG
GQ-cODN₂₄	AACGTCAGGAACGTCATGGAAAAAAAAAAAAAAAAAAAAAAAAAAGGTT GGTGTGGTTGG
TH1	AGACGTGCGTTAGATATGCTGTACAAGCGCGATCGTGACGACTGCAGAA GTGCTTCACGATTTTCATGATACGAGCTACGCACGTCTACTCTAGGGCGT GGGTGC
TH2	Phosphate-GGAGCGCTGGCCGAATTCGCGCTTGTACAGCATATCTTGCTCGT ATCATGAAA
TH3	Phosphate-TGCGTGTGCGACTCTCGTGCCGGCTTGCGTCCGCGTCGCTAGCA CTTCTGCAGTCGTCACGTTTCGGCCAGCGCTCCGCACCCTGCGGCCCCGGC ACGAGAGCGGACGCAAGGCCGCTCGCCCTAGAGT
Extended TH3	Phosphate-TGCGTGTGCGACTCTCGTGCCGGCTTGCGTCCGCGTCGCTAGGA CTTCTGCAGTCGTCACGTTTCGGCCAGCGCTCCGCACCCTGCGGCCCCGGC ACGAGAGCGGACGCAAGGCCGCTCGCCCTAGAGTAAAAAACGTCAGGAA CGTCATGGA
6A-ODN1826	AAAAAATCCATGACGTTCTGACGTT
ps2-ODN1826	T*C*CATGACGTTCTGACGTT
ps3-ODN1826	T*C*C*ATGACGTTCTGACGTT

BMN-ps2- ODN1826-FAM	BMN-Q620-T*C*CATGACG TTCCTGACGTT-FAM
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BMN-ps3- ODN1826-FAM	BMN-Q620-T*C*C*ATGACG TTCCTGACGTT-FAM
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6.2 LIST OF ABBREVIATIONS

2'-Ara-F	2'-arabino-fluoro
2'-F	2'-deoxy-2'-fluoro
2'-MOE	2'-O-methoxyethyl
2'-OMe	2'-O-methyl
5'-(E)-VP	5'-(E)-vinyl phosphonate
AAT	Alpha-1 antitrypsin
ABs	Antibodies
ADA-SCID	Adenosine deaminase deficiency
ADCs	Antibody-drug conjugates
AGO-2	Argonaute2
AHP	Acute hepatic porphyria
AIDS	Acquired immunodeficiency syndrome
AMA1	Apical membrane antigen 1
APCs	Antigen presenting cells
APIs	Active pharmaceutical ingredients
ARF	Acoustic radiation forces
ARG	Acoustic reporter genes
ASGPR	Asialoglycoprotein receptor
ASIC1a	Acid sensing ion channel 1a
ASO	Antisense oligonucleotide
ATCC	American Type Culture Collection
BBB	Blood-brain barrier
BMDCs	Bone marrow-derived primary dendritic cells
bsAbs	Bispecific antibodies
CAFs	Cancer associated fibroblasts
CALD	Cerebral adrenoleukodystrophy
CAR	Chimeric antigen receptor
CCR7	C-C chemokine receptor type 7
cDNA	Complementary DNA sequence
CHA	Catalytic hairpin assembly
CMV	Cytomegalovirus
CNS	Central nervous system
ConA	Concanavalin A
COVID-19	Coronavirus disease 2019
COX2	Cyclooxygenase-2
CpG ODNs	CpG oligodeoxynucleotides

CRISPR	Clustered regularly interspaced short palindromic repeats
CST	Critical solution temperature
CT	Computed tomography
CTLA-4	Cytotoxic T lymphocyte antigen 4
CTLs	Cytotoxic T lymphocytes
DCs	Dendritic cells
DDSs	Drug delivery systems
DEPC	Diethyl pyrocarbonate
DMD	Duchenne muscular dystrophy
DNA	Deoxyribonucleic acids
DOPE	Dioleoylphosphatidylethanolamine
DOSPA	3-dioleoyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propaniminium trifluoroacetate 2
dsDNA	Double stranded DNA
DSPC	1,2-distearoyl-sn-glycero-3-phosphocholine
dsRNA	Double stranded RNA
DVs	Design variants
EC	European Commission
EC₅₀	Half-maximal effective concentration
ECM	extracellular matrix
EMA	European Medicines Agency
EPR	Enhanced permeability and retention
ER	Endoplasmic reticulum
ESC	Enhanced stabilization chemistry
ExoIII	Exonuclease III
FBS	Fetal bovine serum
Fc	Fragment of crystallizable
FCS	Familial chylomicronemia syndrome
FLT	Fluorescence tomography
FRET	Förster resonance energy transfer
FUS	Focused US
GalNAc	N-acetylgalactosamine
GFP	Green fluorescent protein
GHz	Gigahertz
GNAs	Glycol nucleic acids
GQ	G-quadruplex
GVs	Gas vesicles
HAART	Highly active antiretroviral therapy

hATTR	Hereditary amyloidogenic transthyretin
HBV	Hepatitis B virus
HCR	Hybridization chain reaction
HCV	Hepatitis C virus
HIFU	High-intensity focused ultrasound
HoFH	Homozygous familial hypercholesteremia
HP	Hairpin
HSP70	Heat shock protein 70
hTLR9	Human TLR9
iDCs	Immature DCs
IFN-α	Interferon- alpha
IFN-γ	Interferon-gamma
IgM	Immunoglobulin M
IL-12	Interleukin 12
IL-6	Interleukin 6
ISPPA	Spatial-peak pulse-average intensity
ISPTA	Spatial-peak temporal-average intensity
IV	Intravenous
K2P	Two-pore potassium channel
kHz	Kilohertz
LAMP	Loop-mediated isothermal amplification
LCST	Lower CST
LIFU	Low-intensity focused ultrasound
LNAs	Locked nucleic acids
LNPs	Lipid nanoparticles
LPLD	Lipoprotein lipase deficiency
m1Ψ	Methylpseudouridine
m6A	N6-methyladenosine
mABs	Monoclonal antibodies
MART1	Melanoma-associated antigen recognized by T cells 1
mb	Million bases
mDCs	Mature DCs
MFE	Minimum free energy
Mg₂P₂O₇	Magnesium pyrophosphate
MHC	Major histocompatibility complex
MHz	Megahertz
MI	Mechanical index
miRNA	Micro RNA

M_{lim}	Lower molecular weight threshold
MOE	O-Methoxyethyl
MOP	Methoxypropylphosphonate
MP	Methylphosphonate
MPa	Megapascal
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MS ion channels	Mechanosensitive ion channels
MSC	Mesenchymal stem cell
MscL	Large conductance mechanosensitive ion channel
MSNPs	Mesoporous silica particles
MSP 1	Merozoite surface protein 1
mTLR9	Mouse TLR9
NASBA	Nucleic acid sequence-based amplification
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIR	Near infrared
NK	Natural killer
NSCLC	Non-small cell lung carcinoma
nt	Nucleotides
OFPs	Optical force probes
OVA	Ovalbumin
PAAs	Pathogen-associated antigens
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PD	Pharmacodynamics
pDCs	Plasmacytoid DCs
PD-L1	Programmed death-ligand 1
PDT	Photodynamic therapy
PEG	Polyethylene glycol
PEG-DMG	R-2,3-bis(octadecyloxy)propyl-1-(methoxy PEG 2000) carbamate
PEI	Polyethyleneimine
PFCs	Perfluorocarbons
PFGE	Pulsed-field gel electrophoresis
PhpC	6'- phenylpyrrolocytosine
PI3K	Phosphatidylinositol 3-kinase
PK	Pharmacokinetics
PLA	Polylactic acid

PLGA	Poly(lactic-co-glycolic) acid
PLK1	Polo-like kinase 1
PMO	Phosphorodiamidate morpholino oligomers
PNA	Peptide nucleic acid
PNPs	Polypeptide nanoparticles
pre-miRNA	Precursor miRNA
pri-miRNA	Primary miRNA
PRRs	Pattern recognition receptors
PS	Phosphorothioate
PS2	Phosphorodithioate
PSA	Prostate specific antigen
PTMs	Post-translational modification
PVDF	Polyvinylidene fluoride
qPCR	Quantitative real-time PCR
RBC	Red blood cells
RCA	Rolling circle amplification
RCT	Rolling circle transcription
RGD	Arginylglycylaspartic
RISC	RNA-induced silencing complex
RNA	Ribonucleic acids
RNAi	RNA interference
ROS	Reactive oxygen species
SARs	Structure-function relationships
SAW	Surface acoustic waves
SC	Subcutaneous
SDA	Strand displacement amplification
SEAP	Secreted embryonic alkaline phosphatase
SELEX	Systematic evolution of ligands by exponential enrichment
siRNA	Small interfering RNA
SMA	Spinal muscular atrophy
SMCC	N-succinimidyl 4-(maleimidomethyl)cyclohexanecarboxylate
SPG	Soluble β -glucan schizophyllan
SPIONs	Superparamagnetic iron oxide (Fe ₃ O ₄) nanoparticles
ss	Single-stranded
ssDNA	Single stranded DNA
STC	Standard template chemistry
SUPs	Supercharged elastin-like polypeptide chains
SWCNT	Single-walled carbon nanotube

TAAs	Tumor-associated antigens
TH	Tetrahedral
TI	Thermal index
TLR3	Toll-like receptor 3
TLR8	Toll-like receptor 8
TLRs	Toll-like receptors
TME	Tumor microenvironment
TMSD	Toehold-mediated strand displacement
TNAs	Therapeutic nucleic acids
TNF	Tumor necrosis factor
TPA	Two-photon absorption
TRAAK	TWIK-related arachidonic acid activated K ⁺ channel
TREK-1	TWIK-related K ⁺ channel-1
TREK2	TWIK-related K ⁺ channel-2
TRiM™	Targeted RNAi molecule
TRP	Transient receptor potential
TWIK	tandem of pore domains in a weak inward-rectifying K ⁺ channel
type I IFNs	Type I interferons
UCNP	Upconversion nanoparticles
UCST	Upper CST
UNAs	Unlocked nucleic acids
US	Ultrasound
US FDA	United States food and drug administration
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
WT	Wild type

6.3 LIST OF FIGURES

Figure	Caption
Fig. 1.1	Five classes of therapeutics (small molecules, proteins and peptides, antibodies, nucleic acids and live cells) and their associated delivery challenges. Some challenges are shared among different classes, and others are unique to a given therapeutic class.
Fig. 1.2	Regardless of the class of therapeutic, three delivery paradigms were developed to improve therapeutic function: the modification of the drug or of its environment, and the design of drug delivery systems. Drug delivery systems have adopted one or more strategies for drug modification or environmental modification. Created with BioRender.com
Fig. 1.3	Schematic presentation of different DNA self-assembly methods. Each method is explained in the corresponding text in section 1.1.1.3.1 (Reprinted from ¹⁶⁵ , Copyright (2023), with permission from Elsevier.
Fig. 1.4	Rolling circle amplification process in which the enzyme phi29 DNA polymerase uses a circular template to synthesize a ssDNA polymer. The polymer co-crystallize with magnesium pyrophosphate to form hybrid nanoflowers. Created with BioRender.com
Fig. 1.5	An example of dissipative systems that exist in nature. Microtubules are the backbone of the cytoskeleton and vital to numerous cellular processes. All functions of microtubules are driven by dynamic instability, which is driven by non-equilibrium transitions between the bent (B), straight (S), and curved (C) forms of tubulin monomers and longitudinal interfaces in the two-dimensional lattice of microtubule. The resulting kinetic pathways for these transitions and their corresponding energy landscapes control the different phenomena of microtubules (Reprinted from ²³⁰ , Copyright (2020), with permission from Elsevier).
Fig. 1.6	Comparison between passive and active targeting strategies. A. Passive targeting of cancer cells depends on the enhanced permeability and retention effect due to compromised vascular structure and lymphatic drainage. B. Active targeting of cancer cells utilizing nanoparticles that have surface modifications with targeting ligands that can interact with specific target receptors on the surface of the cancer cell.
Fig. 1.7	Smart drug delivery systems can be designed to respond to different internal and external triggers.
Fig. 1.8	Principles and examples from polymer mechanochemistry. A. The application of force to a polymer backbone results in its stretching and its ultimate scission, preferentially in the middle. B. Schematic diagram of ‘dog bone’ synthetic polymer specimens with spiropyran as a mechanophore. Upon application of tensile force, the colourless spiropyran converts to coloured merocyanine, while the conversion is reversed upon the exposure to visible light, figure recreated from reference ³⁷² . C. Ultrasound-induced mechanochemical scission of a metal complex with polymeric ligands, leading to the activation of a catalyst in which

the metal center catalyzes polymerization, figure recreated from reference³⁷³. Created with BioRender.com

Fig. 1.9 Schematic presentation of the mechanism underlying US-induced polymer chain scission. **A.** Pressure variations induced by the acoustic field lead to the gradual formation of microbubbles. **B.** The rapid collapse of microbubbles generates solvodynamic shear forces that unfold and stretch the polymer (Reprinted (adapted) with permission from³⁸¹, Copyright (2009) American Chemical Society).

Fig. 1.10 Schematic overview of the main biophysical effects occurring during sonoporation generated by microbubbles. **A.** Low acoustic pressure triggers stable cavitation of microbubbles that lead to symmetrical linear oscillations. **B.** Microbubbles undergoing stable cavitation generate volumetric oscillations to exert radiation force and microstreaming-induced shear forces. **C.** Microbubbles undergoing inertial cavitation produce microjets. These different physical forces can result in the permeabilization of the cell membrane, rearrangement of the cytoskeleton and transmembrane ion transport such as K⁺ efflux and Ca²⁺ influx (adapted from Ref.⁴⁴⁰, CC BY 4.0).

Fig. 1.11 US- and microbubble-mediated drug delivery. Microbubbles are either co injected or loaded with the drug. Targeted microbubbles can be achieved by surface modifications. The cavitation of the microbubbles upon US exposure leads to drug release. Furthermore, liquid jetting enhances membranes permeability and therefore increasing drug crossing the membrane (Reprinted from⁵⁰⁷, Copyright (2020), with permission from Elsevier).

Fig. 1.12 US-mediated activation of different drugs based on polymer mechanochemistry principles. Different cargo were loaded onto polynucleic acids carriers leading to their deactivation, and subsequently they become activated by US. **A.** Neomycin B activation by 20 kHz US leads to restoring its antibacterial properties. **B.** Thrombin was activated by LIFU and could catalyze the formation of fibrin from fibrinogen. **C.** DNazymes were activated by HIFU to exert their catalytic activity on labelled probes that become fluorescent after being specifically cleaved by DNazymes in presence of magnesium ions. Figures recreated from references^{527,532} and ⁵³³, respectively. Created with BioRender.com

Fig. 1.13 A general schematic summary of the state-of-the-art sonogenetic tools used for controlling or imaging biomolecular functions. Thermal and mechanical sonogenetics can be used to manipulate cellular functions. In thermal sonogenetics, heat-responsive promoters and repressors are exploited, while mechanical sonogenetics utilize mechanosensitive (MS) ion channels and mechanochemically reactive macromolecular systems. Such strategies eventually result in the control of protein activity, signalling pathways or gene expression. On the other hand, gas vesicles (GVs) have been developed as acoustic contrast agents to image biomolecular functions. Towards that end, GV are either administered exogenously or produced endogenously after genetically engineering the target cells (adapted from Ref.⁵⁴³, CC BY 4.0).

Fig. 1.14 The cancer-immunity cycle, in which the process of generation of immunity to cancer can self-propagate and lead to the accumulation of immune-stimulatory

factors through seven major steps that start with cancer cells releasing antigens and end with killing of cancer cells (Reprinted from⁶¹⁶, Copyright (2013), with permission from Elsevier).

Fig. 1.15 **A.** The monomer structure of unliganded EcTLR9 showing the lateral face (left) and the convex face from the N-terminal side (right). The N-glycan residues are shown in stick representations with their O, N and C atoms coloured red, blue and grey, respectively. The N- and C-terminal halves of EcTLR9 are coloured light green and orange, respectively. **B.** Structure of EcTLR9 in complex with an inhibitory DNA showing the lateral face (left) and the concave face of the N-terminal half (right). Bound inhibitory DNA is depicted in stick representation and semi-transparent surface representation, respectively. **C.** The dimer structure of the complex of EcTLR9 and 12mer CpG ODN1668. EcTLR9 and its dimerization partner EcTLR9* are shown in green and cyan, respectively. The second TLR9 within the dimer and its residues are indicated with asterisks. The CpG ODN 12mer is shown in stick representation and semi-transparent surface representation. **D.** The binding groove of CpG-DNA (Left). An overview of CpG ODN recognition by TLR9 (Middle). A magnified view of the CpG ODN recognition (Right). Reproduced with permission from Springer Nature⁶²⁷.

Fig. 1.16 Schematic presentation summarizing three different approaches used in the development of drug delivery systems for CpG ODNs. These include self. Assembled CpG ODN DDSs, conjugation/complexation of CpG ODN with proteins or peptides and CpG ODN complexation with nanomaterials.

Fig. 1.17 **A.** Schematic representation of a drug delivery system based on self-assembly of lipid-modified DNA that consists of eight regular deoxyribonucleotides and four dodec-1-ynyluracil (U) nucleobases at the 5'-end. To allow its hybridization, CpG ODN was extended with 12 nucleotides complementary to the lipid-DNA. The antigen is ovalbumin peptide epitope (pOVA) that is covalently bound to peptide nucleic acid that is also complementary to the lipid-DNA. **B.** The components self-assemble in aqueous media to form loaded immunotherapeutic nucleic acid (INA). **C.** After subcutaneous injection into tumor mouse models, an antigen-specific immune response is triggered *via* DCs and CD8 α + cells. **D.** This results in successful therapeutic activity (Reprinted from⁷⁰⁰, Copyright (2019), with permission from Elsevier).

Fig. 1.18 Schematic illustrations showing CpG ODN drug delivery system based on the electrostatic complexation of CpG ODN with cationic molecular bottlebrush (CMB). **A.** Presentation of the CMB in which brush chains are spread from the backbone. **B.** Presentation of the CMB-CpG ODN complex in which the latter are trapped on the outer layer because of less crowding chains (Reprinted (adapted) with permission from⁷⁰⁵, Copyright 2023 American Chemical Society).

Fig. 1.19 Schematic summary of five different levels of gene regulation mechanisms. Created with BioRender.com

Fig. 1.20 Schematic presentation of the RNAi mechanism for the regulation of gene expression. **A.** miRNA processing pathway starts with endogenously transcribed primary miRNA (pri-miRNA) which is then processed into precursor miRNA (pre-miRNA) through microprocessor complex and is further processed into

mature miRNA *via* Dicer. Mature miRNA binds to RISC complex and target mRNA with partial complementarity. **B.** siRNA source can be either endogenous or exogenous. Endogenous siRNA results from processing of dsRNA by Dicer. siRNA is recruited by the RISC complex, which then bind to the complementary mRNA sequence and result in its cleavage. Created with BioRender.com

Fig. 2.1 Ultrasound devices used in this work. **A.** One-tip horn Q500 Sonicator® (Qsonica, USA) that operates at US frequency of 20 kHz. **B.** Lumify L12-4 (Philips, USA) broadband linear transducer used for clinical imaging at point-of-care. Ultrasound application (Philips Lumify Ultrasound App) is needed for the operation of the portable L12-4 broadband linear transducer.

Fig. 2.2 **A.** Schematic presentation of the SEAP reporter assay using HEK-Blue mTLR9 cells, which are transfected to express TLR9 exclusively among the TLR family. They are co-transfected with the SEAP reporter gene that operates under the control of the NF- κ B-dependent TLR9 signalling pathway. mTLR9 stimulation by CpG ODNs initiates the signalling pathway resulting in NF- κ B production and thereby inducing the expression of the SEAP reporter gene. Using HEK-Blue detection medium, SEAP levels in the supernatant can be detected by measuring absorbance at $\lambda = 620$ nm, as SEAP converts its substrate to blue/purple product. **B.** Schematic presentation of the experimental setup used to quantify TNF release stimulated by CpG ODN activation of TLR9 in J774A.1 macrophage. TNF is detected in the macrophages' supernatant by co-incubation with an engineered HEK cell line transfected to express the TNF receptor, which upon stimulation triggers a cellular pathway that ends up with NF- κ B production. The latter induces the expression of the SEAP reporter gene which can be quantified by spectrophotometry using suitable detection medium. Created with BioRender.com

Fig. 2.3 Schematic presentation of the DDS production. First, padlock ligation in which a linear ssDNA template is mixed with a primer that has complementary sequences to the template on both 5'- and 3'- ends. T4 ligase is used to seal the bond and convert the linear template into a circular ssDNA, which serves as the starting material for the RCA. In the second step, the RCA polymer is produced from the circular template that is designed to have a hybridization site for the desired TNA cargo. The resulting RCA NFs accommodate and deactivate CpG ODN cargo. Finally, mechanochemical activation of the DDS by imaging US that leads to the release of the CpG ODNs in their active form, to exert their controlled biological activity in cells, enabling on-demand sonopharmacology achieved *via* polynucleotide mechanochemistry. Created with BioRender.com

Fig. 2.4 **A.** Agarose GE demonstrating the successful formation of RCA polymers. Lane 0: kb plus DNA ladder; lanes 1 to 4: RCA polymers using templates that have 100%, 75%, 50% and 25% complementarity to CpG ODN1826, respectively. **B.** Agarose GE showing samples taken from RCA reaction at different time points. Lane 0: kb plus DNA ladder; lanes 1 to 11: RCA samples taken at 5 min, 10 min, 20 min, 40 min, 1 h, 2 h, 4 h, 6 h, 8 h, 24h and 48 h, respectively.

Fig. 2.5 PFGE technique that uses an electric field with changing direction, followed by SYBR Gold staining to visualize the bands. Created with BioRender.com

Fig. 2.6 **A.** Agarose PFGE demonstrating RCA products at the top of the gel after 24 h gel running time. Lane 0: Lambda ladder, Lane 1: *Saccharomyces cerevisiae* ladder; lanes 2,3: Kb plus ladder; lanes 4 to 7: RCA polymers using templates that have 100%, 75%, 50% and 25% complementarity to CpG ODN1826, respectively. **B.** Agarose PFGE demonstrating samples taken from RCA reaction at different time points at the top of the gel after 72 h of gel running time. Lanes 0 and 1: marker with 5.7 mb band; lanes 2 to 9: RCA samples taken at 5 min, 10 min, 20 min, 40 min, 1 h, 2 h, 4 h and 6 h, respectively.

Fig. 2.7 Secondary structures obtained by NUPACK simulations for one unit of the RCA polymer produced using templates that have 100% (**A**) and 75% (**B**) complementarity to CpG ODN1826. Secondary structure formation with high probability at the ODN hybridization site in A is highlighted with the yellow box.

Fig. 2.8 **A.** Agarose GE demonstrating the effect of 20 kHz US on RCA-ODN complexes. Lane 0: Kb plus ladder; lanes 1 to 5: RCA₁₀₀-ODN1826 after exposure to US for 2 min, 4 min, 6 min, 8 min and 10 min, respectively; lane 6: pristine CpG ODN1826 control. **B.** Lane 0: Kb plus ladder; lanes 1 to 5: RCA₇₅-ODN1826 after exposure to US for 2 min, 4 min, 6 min, 8 min and 10 min, respectively; lane 6: pristine CpG ODN1826 control.

Fig. 2.9 mTLR9 stimulation after incubation of HEK mTLR9 cells with different samples. RCA polymers were synthesized using backbones with 100% (Ra100) and 75% (Ra75) complementarity to CpG ODN1826. **A.** The loaded cargo was 5'-Atto488-ODN1826 and *ex situ* sonication resulted in significant activation of the biological response. **B.** The loaded cargo was PS-ODN1826 and no significant difference in biological response was observed after *ex situ* sonication for either systems.

Fig. 2.10 Drug dose-response curve for different CpG ODNs, demonstrating different potency upon using different modifications. **A.** CpG ODN1826 was tested either in PO or PS backbone, either with or without fluorescent tag on the 5'-end. Mixed ODN is PS-modified ODN that has natural PO bonds only at the CG pairs. ODN antagonist is inverted GC sequence. **B.** For PS-ODN and mixed ODN, drug-response curve was further investigated over wider concentration range, as low as 0.001 μ M. **C.** Drug-response curve focusing on PS-ODN performance in both mTLR9 and null cells.

Fig. 2.11 **A.** Agarose GE demonstrating successful production and loading of RCA-PS-ODN1826. Lane 0: kb plus ladder, lanes 1 and 2: reference PS-ODN1826 and double ODN, respectively; lane 3: RCA100 ligated template; lanes 4 and 5: RCA100 polymer; lane 6: RCA100-PS-ODN1826 complex; lane 7: RCA75 ligated template; lane 8: RCA75 polymer; lane 9: RCA75-PS-ODN1826 complex. **B.** Agarose GE demonstrating the effects of 20 kHz US on RCA75-PS-ODN1826. Lanes 1 to 5: RCA75-PS-ODN1826 after US treatment; lane 0: kb plus ladder.

Fig. 2.12 mTLR9 stimulation in HEK-mTLR9 cells after incubation with different samples. *Ex situ* sonication using 20 kHz US was performed.

Fig. 2.13 Quantification of different cytokines and interleukins in the supernatants of primary macrophages that were stimulated by different samples.

Fig. 2.14 mTLR9 stimulation in HEK-mTLR9 cells after incubation with different RCA ODN-DSL01 complexes, either before or after sonication with 20 kHz US. RCA polymers that exhibit 100% or 75% complementarity are used to fabricate **A.** RCA-100 series and **B.** RCA-75 series, respectively. A detailed key to type of RCA-ODN complex is given in Table 2.1.

Fig. 2.15 Agarose GE demonstrating the effect of imaging US on RCA-ODN complex. Lane 0: Kb plus ladder; lane 2; reference ODN-DSL-01; lanes 3,5 and 7: RCA-ODN-DSL-01; lanes 4 and 6: RCA-ODN-DSL-01 after sonication with imaging US; lane 8: RCA-ODN-DSL-01 after sonication with 20 kHz US.

Fig. 2.16 **A.** Calibration curve of FAM-ODN D-SL01, which was used to calculate the concentration of bound and free ODN using the best fit linear regression model. **B.** RCA-CpG ODN loading efficiency increases with longer reaction time. **C.** Quantification of CpG ODN release efficiency after sonication, by calculating the percentage of released CpG ODN that can be detected in the supernatant. Different RCA reactions were performed for different durations followed by sample sonication and analysis.

Fig. 2.17 **A.** Visualization of the phase separation of a gel containing RCA-CpG ODN. Fluorescently labelled CpG ODN is concentrated in the gel phase, as observed under UV light. **B.** Viscosity of RCA-CpG ODN increases with increasing reaction time.

Fig. 2.18 **A.** Oscillation sweep (at frequency of 1 Hz) of RCA-CpG ODN before and after sonication with imaging US. **B.** Rheology of RCA-CpG ODN showing the frequency dependence of G' and G'' in the linear regime (strain amplitude $\gamma_0 = 0.1\%$) before and after sonication with imaging US.

Fig. 2.19 **A.** US-response of RCA-ODN-DSL-01 is related to its viscosity, as evidenced by increased release efficiency. **B.** Rheology of RCA-ODN showing the frequency dependence of G' and G'' in the linear regime (strain amplitude $\gamma_0 = 0.1\%$) for RCA polymers using templates that have 100%, 75%, 50% and 25% complementarity to CpG ODN1826.

Fig. 2.20 **A.** SEM images showing RCA-ODN forming NFs at different magnification powers. **B.** SEM images showing RCA-CpG ODN before and after sonication with either imaging US or 20 kHz US. Characteristic petal-shaped NFs were formed, then fragmented and partially destroyed after exposure to imaging US while being completely destructed after exposure to 20 kHz US.

Fig. 2.21 Optical microscopy images of RCA-CpG ODN demonstrating liquid crystals formation that vanishes after US.

Fig. 2.22 MFE structures and their corresponding Gibbs energies obtained from the NUPACK software, for sequences containing 4 to 8 repeats of the template's complementary sequence (www.nupack.org). The schematic illustration shows the elongated conformation, which induces the lamellar ordering.

Fig. 2.23 Particle size distribution of RCA-CpG ODN before and after exposure to imaging US, showing particles' partial destruction and shifting towards a smaller diameter range after US.

Fig. 2.24 Optical imaging microscopy images for time tracking of the formation of liquid crystals during the RCA-ODN complex formation by examining samples taken at different timepoints during the reaction, demonstrating birefringence starting at 14 h reaction time.

Fig. 2.25 Optical imaging using polarized light microscope for observing the effects of heating on the liquid crystals formed by the RCA-ODN complex. The large particles maintained their birefringence while small particles that did not exhibit birefringence at room temperature showed liquid crystals when heated to 92 °C and then disappeared after cooling down to 84 °C, as indicated by the yellow arrows.

Fig. 2.26 Time tracking of particles' size distribution of RCA-ODN reaction by taking *in situ* measurements at different time points, starting from 30 min and ending with 41 h.

Fig. 2.27 Setup used for temperature measurement *via* thermal sensor during sonication with imaging US. Curve A is the reference well and curve B is the sonication well.

Fig. 2.28 Acoustic measurements. **A.** Experimental setup using Lumify transducer that was fixed ~1 mm above sonographic 96-well plate, while the hydrophone sensor was positioned ~5 mm below the well plate. The plate was mounted on a movable frame of motorized 3D positioning system. **B.** Acoustic pressure values were calculated using hydrophone sensitivity at 7 MHz the burst duration of approximately 200 ns was estimated. The MI of 0.6 was calculated as $p_-/\sqrt{f}$, where p_- is the peak negative pressure (MPa) and f is the frequency (MHz). **C.** The acoustic pressure pulses from the acquisition of neighbouring spatial points. Excess data points between pulses were removed for clarity. **D.** Acoustic pressure pulses at a frame rate of 25 frames per sec (fps) are illustrated. Excess data points between pulses were also removed. The peak sound intensity of ~300 W/cm² can be roughly estimated using formula for harmonic waves $I = p^2/2\rho c$, where p - maximum pressure, ρ - water density, c - speed of sound. The spatial-peak temporal-average intensity was calculated by considering pulses duty cycle (frame rate used, ignoring lower intensity neighbouring pulses) $I_{spta} \approx 1.5$ mW/cm². Assuming a sonication area of ~0.5 cm² and a total processing time of 15 min, the total delivered acoustic energy can be estimated to be ~700 mJ. **F.** Acoustic pressure change over sonication time. The first 4 min were recorded in reference well and the last 15 min were recorded in gel-containing well. The red line indicates the transition between the two wells.

Fig. 2.29 Mechanochemical control of immune response *via* CpG ODN. **A.** Stimulation of mTLR9 in HEK-Blue™ mTLR9 cells. RCA-CpG ODN activated by imaging US exhibited a significant stimulatory effect on mTLR9. Pristine DSL-01 ODN (0.2 µM) and inert RCA, with and without sonication, were used as controls. Imaging US alone did not elicit a significant response in the cells. **B.** SRB assay to demonstrate cell viability after exposure to imaging US.

Fig. 2.30 **A.** Dose-response curve of the HEK-TNF reporter cell line showing the stimulation of the TNF receptor by incubation with increasing concentration of pristine TNF as a positive control. **B.** Production of TNF following stimulation of J774A.1 macrophages. RCA-CpG ODN in combination with imaging US resulted in a significant increase in TNF levels, comparable to that of the pure TNF positive control ($1 \text{ ng}\cdot\text{mL}^{-1}$). Imaging US alone, pristine ODN ($0.2 \text{ }\mu\text{M}$), and RCA-CpG ODN prior to US did not elicit a significant production of TNF.

Fig. 2.31 TNF quantification in the supernatants of primary macrophages in response to stimulation by increasing concentrations of ODN DSL-01, either in the natural PO or modified PS backbone.

Fig. 2.32 **A.** US-mediated activation of CpG ODN and its role in cancer immunotherapy. **B.** Experimental design and timeline of *in vitro* triculture cell experiment with three different stages. In stage 1, primary monocytes are isolated from primary blood mononuclear cells (PBMCs) and differentiated into immature dendritic cells (iDCs), which are then activated to become mature dendritic cells (mDCs). In stage 2, primary CD8^+ T cells are isolated from PBMCs and co-cultured to interact with DCs resulting in T cell proliferation. In stage 3, the DC:T cell mixture is added to pre-seeded B16f10 melanoma cells, and their interaction is analysed after 24 h. Created with BioRender.com

Fig. 2.33 Primary cells isolation from human whole blood. Isolation of monocytes from whole blood using CD14 as surface biomarker. The positive fraction was enriched with CD14^+ cells, while a small percentage of the negative fraction contained CD14^+ cells. The data are representative of three independent experiments.

Fig. 2.34 Analysis of expression of surface biomarkers. CD80, CD83 and CD86 surface biomarkers expressed by monocytes and DCs that were exposed to different conditions.

Fig. 2.35 Primary cells isolation from human whole blood. Isolation of CD8^+ T cells from whole blood using CD8 and CD4 as surface biomarkers. The positive fraction was enriched with $\text{CD8}^+/\text{CD4}^-$ cells, while a small percentage of the negative fraction were $\text{CD8}^+/\text{CD4}^+$. The majority of cells in the negative fraction were found to be $\text{CD8}^-/\text{CD4}^+$. The data are representative of three independent experiments.

Fig. 2.36 **A.** Proliferation tracking of T cells labelled with CellTrace Violet utilizing the dye dilution method. Created with BioRender.com **B.** Flow cytometry histograms for T cells tracking. Unstimulated cells exhibit a single peak with high fluorescence intensity, indicative of a lack of proliferation. Multiple peaks with decreasing fluorescence intensity are observed when the cells are stimulated and undergoing proliferation under different experimental conditions.

Fig. 2.37 TNF production by T cells after co-culture with DCs treated under different conditions. Increased TNF production is observed when T cells were co-cultured with mDCs control, DCs incubated with pristine DSL01 ODN ($2 \text{ }\mu\text{M}$) and DCs incubated with US-activated RCA-CpG ODN DDS.

Fig. 2.38 Viability of CFSE-labelled B16F10 melanoma cells after 2 h incubation with DC:T cells co-cultures that were exposed to different types of stimulation. The samples which have DDS activated with imaging US show significant killing of melanoma cells, as demonstrated by a marked decrease in cell viability

Fig. 2.39 US-controlled immunostimulation *in vivo*. Animal groups used in animal experiments *in vivo*. Group 1 served as a positive control and received pristine CpG ODN. Group 2 and 3 received RCA-CpG ODN with and without imaging US treatment, respectively. Group 4 was left untreated, while group 5 was only exposed to US. Created with BioRender.com

Fig. 2.40 **A.** *In vivo* imaging of RCA-CpG ODN by incorporating Cy5-labeled nucleotides into RCA polymer backbone. Pre-scan before RCA-CpG ODN administration (left) and 60 min after RCA-CpG ODN administration (right). The region of interest for signal quantification is highlighted by the blue dashed circle. **B.** Quantification RCA-CpG ODN accumulation in the liver region of interest using FLT.

Fig. 2.41 Quantitative real-time PCR analysis of inflammatory mediators' expression in whole liver tissue demonstrates successful immunostimulation following the activation of ODN D-SL01 *in vivo* by imaging US.

Fig. 2.42 Immunohistochemistry staining of liver specimens demonstrates no change or distortions, indicating the safety of using both RCA-CpG ODN and imaging US *in vivo*.

Fig. 2.43 Scheme showing the versatility of the developed mechanoresponsive DDS in which it can be adapted to accommodate different types of cargo. **A.** Delivery of CpG ODN and TAA loaded *via* hybridization site and aptamer, respectively, to increase targeting of cancer immunotherapy. **B.** Delivery of CpG ODN and checkpoint inhibitor, loaded *via* hybridization site and aptamer, respectively, to increase efficiency of cancer immunotherapy. **C.** Alternative loading strategy for checkpoint inhibitors that don't have selective aptamers, by conjugation of the protein with a short oligonucleotide sequence that has a hybridization site on the RCA polymer. **D.** Delivery of CpG ODN and small molecule drug loaded *via* hybridization site and aptamer, respectively, to improve cancer therapy. Created with BioRender.com

Fig. 3.1 Schematic presentation of gene knockdown using RCA-siRNA. Two RCA polymers are needed, where each accommodates one ssRNA; either the sense or the antisense strand. The RCA-RNA complexes were purified as described earlier and mixed in 1:1 ratio. After US application, both RNA strands were activated and hybridized to form the dsRNA molecule, which effectively interferes with the mRNA of interest (Egfp in this case) leading to gene knockdown. Created with BioRender.com

Fig. 3.2 Characterization of RCA-RNA. **A.** Agarose GE demonstrating the formation of RCA-RNA complexes. Lanes 1 and 2: reference sense ssRNA and dsRNA, respectively; lanes 3 and 4: RCA loaded with either sense ssRNA or antisense ssRNA, respectively; lanes 5 and 6: products in lanes 3 and 4 after exposure to 20 kHz US; lane 7: mixed RCA-RNA complexes after exposure to 20 kHz US;

lane 8: mixed RCA-RNA complexes. **B.** The calibration curve of Cy5-RNA, which was used to calculate the concentration of both bound and free ssRNA using the best-fit linear regression model. **C.** Absorbance spectra of unbound Cy5-labelled ssRNA strands, with maximum absorption at $\lambda = 650$ nm.

Fig. 3.3	A. Rheological analysis of RCA-RNA loaded with antisense ssRNA strand. B. Rheological analysis of RCA-RNA loaded with sense ssRNA strand. Both hydrogels exhibit elastic behaviour when subjected to increasing frequency at a fixed strain percentage.
Fig. 3.4	SEM images demonstrating the formation of nanoparticles of RCA-ssRNA complex after loading the sense ssRNA strand as cargo.
Fig. 3.5	HeLa cells were successfully transfected to stably express <i>EGFP</i> , as evidenced by A. flow cytometry and B. confocal microscopy images.
Fig. 3.6	Percentage of HeLa-EGFP cells expressing EGFP after incubation with different RNA molecules either in the absence or the presence of lipofectamine. Readout is normalized to untreated samples.
Fig. 3.7	A. Percentage of EGFP expressing HeLa-EGFP cells after treatment with various samples. Treatment with pristine siRNA and RCA-siRNA mixture did not significantly alter EGFP expression. The positive control, siRNA-lipoplex, showed successful gene knockdown as expected. Notably, US-activated RCA-siRNA mixture demonstrated a significantly higher level of gene knockdown compared to the siRNA-lipoplex control. B. MTS cell viability assay demonstrating the safety of RCA-siRNA mixture and imaging US for cells.
Fig. 3.8	Ultrasound-induced RNA delivery can be used to study biological questions in diabetes and aging. The RNA cargo is loaded onto an RCA carrier and injected into the appropriate animal model. Application of US releases RNA strands in their bioactive form to reduce or enhance gene expression, either <i>via</i> the RNAi mechanism or CRISPR/Cas9 system, by the delivery of siRNA and sgRNA, respectively. Created with BioRender.com
Fig. 4.1	DNA circuit design with bioactive CpG ODN1826 as the output strand. Modification at 3'-end with either 6A overhang or PS (indicated by *) bonds aims to protect CpG ODN (red) against ExoIII (yellow). Linear cDNA strand (blue) is designed with two binding sites complementary to the sequence of CpG ODN1826. A linker is indicated by the dashed segment and has a variable length depending on the design. Assembly of cDNA and CpG ODN deactivates the latter. Activation and release of free CpG ODN are achieved <i>via</i> the selective enzymatic activity of ExoIII that starts at the 3'-end of the cDNA strand as indicated by the green arrows. The aim is to achieve control over immunostimulation by utilizing the programmed DNA circuit, where activated CpG ODN stimulates the intracellular TLR9 receptor (orange). Created with BioRender.com
Fig. 4.2	Dose-response curve of ODN1826-6A in mTLR9 and null cells.
Fig. 4.3	Agarose GE to demonstrate DNA circuit assembly using ODN1826-6A and cDNA ₄₀ in different ratios in different media. Lane 0: Orange ruler DNA ladder;

lanes 1 and 2: reference ODN1826-6A and cODN₄₀, respectively; lanes 3 to 5: DNA circuits in different ratios in PBS; lanes 6 to 8: DNA circuits in different ratios in TBE; lanes 9 to 11: DNA circuit in different ratios in water. Target DNA duplex, half-hybrid DNA duplex, excess ss ODN1826-6A and cODN₄₀ are all visualized in the gel and indicated on the right side.

Fig. 4.4 Agarose GE demonstrating DNA circuit assembly in different media at 2:1 ratio of ODN1826-6A:cODN₄₀. Lane 0: Orange ruler DNA ladder; lanes 1 to 4: reference strands of cODN₂₀, ODN1826-6A, cODN₄₀ and cODN₁₂₀, respectively; lanes 5 to 8: DNA circuit in PBS, NEB3 buffer, PBS supplemented with MgCl₂ and cell culture medium, respectively. The excess ODN1826-6A was observed to be minimal when cell culture medium was used.

Fig. 4.5 Agarose GE demonstrating the enzymatic activity of ExoIII when incubated with ODN1826-6A:cODN₄₀ DNA duplex assembled in cell culture medium at 2:1 ratio. Lane 0: Orange ruler DNA ladder; lanes 1 to 3: reference cODN₂₀, ODN1826-6A and cODN₄₀, respectively; lane 4: DNA duplex; lane 5 and 6: DNA duplex after incubation with ExoIII. The target DNA duplex was fully consumed leading to the liberation of free ODN1826-6A, which is observed in the relevant band that exhibited higher intensity after the enzymatic reaction. Half-hybrid DNA duplex band remained resistant to ExoIII.

Fig. 4.6 Agarose GE showing DNA circuit assembly and activation by ExoIII, using ODN1826-6A and cODN₄₀₋₄₄. Lane 0: ULR DNA ladder; lanes 1,2: reference ODN1826-6A and cODN₄₀, respectively; lanes 3 to 7: ODN1826-6A hybridized with cODN₄₀, cODN₄₁, cODN₄₂, cODN₄₃ and cODN₄₄, respectively; lanes 8 to 12: same products from lanes 3 to 7 but after the reaction with ExoIII.

Fig. 4.7 Agarose GE showing DNA circuit assembly and activation by ExoIII, using PS-modified ODN1826 (ps2, ps3, ps4 and ps) and cODN₄₀₋₄₄. Lane 0: ULR DNA ladder; lanes 1,2: reference ODN1826 and cODN₄₀, respectively; lanes 3 to 7: cODN₄₀ hybridized with ODN1826, ODN1826-ps2, ODN1826-ps3, ODN1826-ps4 and ODN1826-ps, respectively; lanes 8 to 12: same products from lanes 3 to 7 but after the reaction with ExoIII.

Fig. 4.8 Activation of mTLR9 HEK and Null HEK cells upon incubation with different materials showing high level of false positive read out when the ExoIII buffer is incubated with both cell types; mTLR9 and null cells, but not with the detection medium.

Fig. 4.9 Agarose GE demonstrating the enzymatic activity of ExoIII in cell culture medium, either in the presence or absence of its buffer. Lane 0: Orange ruler DNA ladder; lanes 1 to 3: reference cODN₂₀, ODN1826-6A and cODN₄₀, respectively; lanes 4 and 6: DNA duplexes hybridized in PBS supplemented with MgCl₂ and in cell culture medium, respectively; lane 5: DNA duplex from lane 4 after incubation with ExoIII; lanes 7 and 8: DNA duplex from lane 6 after incubation with ExoIII.

Fig. 4.10 Stimulation of mTLR9 in cell culture in response to different conditions.

Fig. 4.11 Different designs of the DNA circuit using cODN that has only one binding site to hybridize with CpG ODN1826. The cODN has secondary structures utilizing either HP loop, GQ or TH. Created with BioRender.com

Fig. 4.12 Agarose GE showing DNA circuit assembly and activation by ExoIII, using ODN1826-6A and HP-cODN and GQ-cODN in **A** and **B**, respectively. In gel **A**, lane 0: ULR DNA ladder; lanes 1 to 3: reference ODN1826-6A, HP₄ and HP₂₄, respectively; lanes 4 to 6: ODN1826-6A hybridized with HP-cODN₄, HP-cODN₁₄ and HP-cODN₂₄, respectively; lanes 7 to 9: same products from lanes 4 to 6 but after the reaction with ExoIII. In gel **B**, lane 0: ULR DNA ladder; lanes 1 to 3: reference ODN1826-6A, GQ₄ and GQ₂₄, respectively; lanes 4 to 6: ODN1826-6A hybridized with GQ-cODN₄, GQ-cODN₁₄ and GQ-cODN₂₄, respectively; lanes 7 to 9: same products from lanes 4 to 6 but after the reaction with ExoIII.

Fig. 4.13 Agarose GE showing DNA circuit assembly and activation by ExoIII, using PS-modified ODN1826-6A and either HP-cODN or GQ-cODN series. In gel **A**, Different number of PS bonds was investigated (2,3,4 or full PS backbone) in combination with HP-cODN₄. Lane 0: ULR DNA ladder; lanes 1 and 2: reference ODN1826 and HP-cODN₄, respectively; lanes 3 to 7: HP-cODN₄ hybridized with ODN1826, ODN1826-ps2, ODN1826-ps3, ODN1826-ps4 and full PS backbone ODN1826, respectively; lanes 8 to 12: same products from lanes 3 to 7 but after the reaction with ExoIII. In gel **B**, Only ODN1826 modified with either 2- or 3-PS bonds was used, in combination with HP-cODN series. Lane 0: ULR DNA ladder; lanes 1 and 2: reference ODN1826 and HP-cODN₄, respectively; lanes 3 to 5: ODN1826-ps2 hybridized with HP-cODN₄, HP-cODN₁₄ and HP-cODN₂₄, respectively; lanes 6 to 8: same products from lanes 3 to 5 but after the reaction with ExoIII; lanes 9 to 11: ODN1826-ps3 hybridized with HP-cODN₄, HP-cODN₁₄ and HP-cODN₂₄, respectively; lanes 12 to 14: same products from lanes 9 to 11 but after the reaction with ExoIII. In gel **C**, only ODN1826 modified with either 2- or 3-PS bonds was used, in combination with GQ-cODN series. Lane 0: ULR DNA ladder; lanes 1 and 2: reference ODN1826 and GQ-cODN₂₄, respectively; lanes 3 to 5: ODN1826-ps2 hybridized with GQ-cODN₄, GQ-cODN₁₄ and GQ-cODN₂₄, respectively; lanes 6 to 8: same products from lanes 3 to 5 but after the reaction with ExoIII; lanes 9 to 11: ODN1826-ps3 hybridized with GQ-cODN₄, GQ-cODN₁₄ and GQ-cODN₂₄, respectively; lanes 12 to 14: same products from lanes 9 to 11 but after the reaction with ExoIII. One asterisk symbol (*) is used to indicated one PS bond modification.

Fig. 4.14 DNA circuit utilizing TH-cODN design **A**. Agarose GE of TH DNA assembly. Lane 0: ULR DNA ladder; lanes 1 to 4: reference strand 1, strand 2, strand 3 and extended strand 3, respectively; lane 5: assembly of strands 1,2 and 3; lane 6: TH after ligation of mixture in lane 5; lane 7: assembly of strands 1,2 and extended strand 3; lane 8: TH after ligation of mixture in lane 7. **B**. Agarose GE of ligated and self-assembled tetrahedral structure and extended tetrahedral structure after exposure to different conditions. Lane 0: ULR DNA ladder; lanes 1 and 2: heating at 95 °C for 2 min; lanes 3 and 4: storing at 4 °C; lanes 5 and 6: freezing at 20 °C followed by thawing at room temperature. **C**. Agarose GE showing the hybridization of tetrahedral DNA with CpG ODNs. Lane 0: ULR DNA ladder; lanes 1 and 2: reference ODN1826-6A and mixed ODN, respectively; lanes 3 and

4: TH and extended TH DNA structures; lanes 5 and 6: TH mixed with ODN1826-6A and mixed ODN, respectively; lanes 7 and 8: extended TH mixed with ODN1826-6A and mixed ODN, respectively.

Fig. 4.15 Agarose GE to investigate the enzymatic activity of ExoIII on ssDNA strands in the absence of its buffer. Lane 0: ULR DNA ladder; lanes 1 to 6: reference ps-ODN, ODN1826, ODN1826-6A, GQ-cODN₁₄, HP-cODN₁₄ and cODN₄₀, respectively; lanes 7 to 12: same strands after incubation with ExoIII from a new batch; lanes 13 to 18: same reference strands after incubation with ExoIII from an old batch. Lower intensity of the bands was observed after incubation with ExoIII.

Fig. 4.16 Agarose GE to investigate the enzymatic activity of ExoIII on ssDNA strands. The enzyme buffer was added. Lane 0: ULR DNA ladder; lanes 1 to 6: difference reference ssDNA strands; lanes 7 to 12: same reference strands after incubation with ExoIII from a new batch; lanes 13 to 18: same reference strands after incubation with ExoIII from an old batch. Complete hydrolysis of PO ODN and PO ODN1826-6A was observed. Other ssDNA strands were present in significantly lower intensity after the incubation with ExoIII.

Fig. 4.17 DNA circuit design with bioactive CpG ODN1826 as the output strand. Modification at 5'-end with either 6A overhang or PS bonds aims to protect CpG ODN against T7 exonuclease. cDNA strand is designed in 3 configurations; linear, HP or GQ and contains one or more binding sites complementary to the sequence of ODN1826. A linker is indicated by the dashed segment and has a variable length depending on the design. Assembly of cDNA and CpG ODN deactivates the latter. Activation and release of free CpG ODN are achieved *via* the selective enzymatic activity of T7 exonuclease that starts at the 5'-end of the cDNA strand as indicated by the green arrows. Control over immunostimulation is realized by utilizing the programmed DNA circuit, where activated CpG ODN stimulates the intracellular TLR9 receptor. Created with BioRender.com

Fig. 4.18 Agarose GE showing **A.** 6A-ODN1826 hybridization with cODN₄₀₋₄₄. Lane 0: ULR DNA ladder; lanes 1 to 3 reference 6A-ODN1826, cODN₄₀ and cODN₄₄, respectively; lanes 4-8: 6A-ODN1826 hybridized with cODN₄₀, cODN₄₁, cODN₄₂, cODN₄₃ and cODN₄₄ respectively; lanes 9-13: same duplexes after the reaction with T7 exonuclease. **B.** 6A-ODN1826 hybridization with HP series. Lane 0: ULR DNA ladder; lanes 1 to 3: references: 6A-ODN1826, HP-cODN₄ and HP-cODN₂₄; lanes 4 to 6: 6A-ODN1826 hybridized with HP-cODN₄, HP-cODN₁₄ and HP-cODN₂₄, respectively; lanes 6-8: same duplexes after the reaction with T7 exonuclease. **C.** 6A-ODN1826 hybridization with GQ series. Lane 0: ULR DNA ladder; lanes 1 to 3: references: 6A-ODN1826, GQ-cODN₄ and GQ-cODN₂₄; lanes 4 to 6: 6A-ODN1826 hybridized with GQ-cODN₄, GQ-cODN₁₄ and GQ-cODN₂₄, respectively; lanes 6-8: same duplexes after the reaction with T7 exonuclease.

Fig. 4.19 Agarose GE showing DNA circuit assembly and T7 exonuclease activity for HP series with **A.** ps2-ODN1826 and **B.** ps3-ODN1826, and GQ series with **C.** ps2-ODN1826 and **D.** ps3-ODN1826. In gels **A** to **D**, lane 0: ULR DNA ladder; lane 1: reference CpG ODN1826; lane 2: reference cDNA strand (HP-cODN₂₄, HP-cODN₄, GQ-cODN₂₄, GQ-cODN₄, respectively); lanes 3 to 5: the hybridization

products; lanes 6 to 8: the DNA duplexes after the reaction with T7 exonuclease. One asterisk symbol (*) is used to indicated one PS bond modification.

Fig. 4.20 **A.** Schematic demonstration of FRET-pair-based kinetic tracking of the DNA circuit. CpG ODN is conjugated to a BMN/FAM pair (black/green), and HP-cODN₄ is labeled with Cy5 (red). The waste generated by T7 exonuclease digestion contains mononucleotides and the remaining structural fraction of HP-cODN₄. Fluorescence intensity measurement of **B.** FAM and **C.** Cy5 over time for enzyme concentration-dependent kinetic tracking of the DNA circuit. Starting with 0.4 μM DNA duplex (ps3-ODN1826 and hp4) followed by the addition of T7 exonuclease (0.4-0.8 U/ μL) indicated by the arrow. Fluorescence intensity measurement of **D.** FAM and **E.** Cy5 over time for DNA duplex concentration-dependent kinetic tracking of the DNA circuit. Starting with (0.2-1 μM) DNA duplex, followed by the addition of 0.6 U/ μL T7 exonuclease indicated by the arrow. Reaction conditions: 50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate and 1 mM DTT, 37°C (Reprinted (adapted) with permission from⁹⁰⁰, Copyright 2023 American Chemical Society).

Fig. 4.21 Calculated kinetic parameters of the DNA circuit. **A.** Maximum reaction rate and **B.** reaction efficiency in correlation with T7 exonuclease concentration. **C.** Maximum reaction rate and **D.** reaction efficiency in correlation with DNA duplex starting concentration (Reprinted (adapted) with permission from⁹⁰⁰, Copyright 2023 American Chemical Society).

Fig. 4.22 Kinetic tracking of the DNA circuit under different conditions using cell culture medium, by measuring fluorescence intensity of FAM over time. Starting with 0.4 μM ps2-ODN1826 and either T7 exonuclease (0.4 U/ μL) or DNase I (0.4 U/ μL), followed by the addition of 0.4 μM HP-cODN₄. The duplex alone without any enzyme is used as control.

Fig. 4.23 **A.** Dose-dependent mTLR9 stimulation by different types of CpG ODNs after 18 h of incubation with HEK-Blue mTLR9 cells. Results are expressed as mean $\pm$ SD of triplicates. **B.** mTLR9 stimulation by DNA circuit based on assembly of HP-cODN₁₄ and 0.8 μM ps2-ODN under different T7 exonuclease concentration ranging from 0.025 to 0.5 U/ μL . No significant increase in mTLR9 stimulation is observed beyond the concentration of 0.1 U/ μL . Thus, this concentration was considered to be the optimum one to be used in cell culture *in vitro* experiments.

Fig. 4.24 mTLR9 activation by either ps2-ODN1826 released from the DNA circuit with **A.** HP-cODN₁₄ and **B.** GQ-cODN₁₄ strands, or ps3-ODN1826 released from the DNA circuit with **C.** HP-cODN₁₄ and **D.** GQ-cODN₁₄ strands in the presence and absence of T7 exonuclease. Samples were incubated with HEK mTLR9 cells for 18 h. Corresponding pristine CpG ODN and PBS were used as positive and negative controls, respectively. SEAP expression levels by PBS were subtracted and the results are expressed as mean $\pm$ SD of triplicates. Data were analyzed with two-way ANOVA with a Bonferroni post-test. * = $p < 0.01$, ** = $p < 0.001$.

Fig. 4.25 mTLR9 stimulation by DNA circuits loaded with **A.** ps2-ODN1826 or **B.** ps3-ODN1826, at two concentrations (0.4 μM or 0.8 μM), using either HP-cODN₁₄ or GQ-cODN₁₄ as cDNA strands, in the presence or absence of T7 exonuclease. Normalized levels of SEAP expression were calculated relative to

the corresponding pristine CpG ODN. Deactivation of the CpG ODN is observed in all DNA duplexes, and activation is observed in all DNA circuits in the presence of T7 exonuclease. Results are expressed as mean $\pm$ SD.

Fig. 4.26 mTLR9 stimulation under different conditions. No stimulation is observed for HP-cODN₁₄ strand or the enzyme T7 exonuclease alone. The gradual addition of pristine ps2-ODN1826 (0.8 μ M) resulted in significant enhancement in mTLR9 stimulation compared to direct addition of the same quantity at once.

Fig. 4.27 Time-dependent mTLR9 stimulation triggered using the programmed DNA circuit consisting of HP-cODN₁₄ with ps2-ODN1826 at either **A.** 0.4 μ M or **B.** 0.8 μ M in the presence or absence of T7 exonuclease. Pristine CpG ODN and PBS were used as positive and negative controls respectively. SEAP expression levels by PBS were subtracted and the results are expressed as mean $\pm$ SD of triplicates.

Fig. 4.28 **A.** Dose-dependent TNF- α release triggered by pristine ps3-ODN1826 after 24 h incubation and by the DNA circuit consisting of ps3-ODN1826 and GQ-cODN₁₄ after 6.5 h or 24 h incubation. **B.** Time-dependent TNF release triggered by the same DNA circuit with or without T7 exonuclease. Pristine CpG ODN and PBS were used as positive and negative controls respectively. SEAP expression levels by PBS were subtracted and the results are expressed as mean $\pm$ SD of triplicates. TNF levels produced by the hybrid system in presence of T7 exonuclease were statistically different than the system in absence of T7 exonuclease and ps3ODN with p-value < 0.0001. **C.** Time-dependent TNF release triggered by either the direct or the gradual addition of pristine ps3ODN at concentrations of 0.4 μ M and 0.8 μ M. Data were analyzed with two-way ANOVA with a Bonferroni post-test. * = p < 0.01 ** = p < 0.001, *** = p < 0.0001.

Fig. 4.29 Fluorescence intensity measurement of **A.** FAM and **B.** Cy5 over time. Arrows indicate the addition of the fuel strand. **C.** Calculated transient lifetime in correlation with T7 exonuclease concentration. Fuel strand concentration-dependent kinetic tracking of the dissipative system. Starting with 0.4 μ M ps3-ODN1826 and T7 exonuclease (0.4 U/ μ L), followed by the addition of HP-cODN₄ fuel strand (0.2-1.2 μ M). Fluorescence intensity measurement of **D.** FAM and **E.** Cy5 over time. Arrows indicate the addition of the fuel strand. **F.** Calculated transient lifetime in correlation with fuel strand concentration. Reaction conditions: 50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate and 1 mM DTT, 37°C.

Fig. 4.30 Kinetic tracking of the dissipative system multiple cycles of ps3-ODN1826 (0.4 μ M) loading and release, through repetitive addition of HP-cODN₄-Cy5 fuel strand (0.4 μ M) in the presence of T7 exonuclease (0.4 U/ μ L). Time-dependent measurement of fluorescence intensity of **A.** FAM and **B.** Cy5 at 37°C. **C.** Calculated lifetime for each cycle at 37°C (T37) or 25°C (T25). Reaction conditions: 50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate and 1 mM DTT. **D.** Comparison of dissipative system multiple cycles of ps3-ODN1826 loading and release by time-dependent FAM fluorescence intensity measurement in the presence of T7 exonuclease (0.4 U/ μ L). Different fuel strands (0.4 μ M) were used; either HP-cODN₄ or GQ-cODN₄, or their alternating addition. Arrows indicate the addition of fuels strand.

Fig. 4.31 Scheme presenting an oscillating autonomous dissipative system that responds to feedback from the target cells to either initiate or inhibit further cycles and thereby further release of the TNA moiety. Created with BioRender.com

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Table	Caption
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8. List of Publications

Ishaqat, A., Hahmann, J., Lin, C., Zhang, X., He, C., Rath, W.H., Habib, P., Sahnoun, S. E. M., Rahimi, K., Vinokur, R., Mottaghy, F. M., Göstl, R., Bartneck, M. and Herrmann, A., **2024**. *In vivo* polymer mechanochemistry with polynucleotides. *Advanced Materials*, 2403752.

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Ishaqat, A., Zhang, X., and Herrmann, A., **2024**. DNA Dissipative System for Controlled Release of Immunostimulatory CpG Oligodeoxynucleotides. *In review*.

Hahmann, J., Schüpp, B. N., **Ishaqat, A.**, Selvakumar, A., Göstl, R., Gräter, F., and Herrmann, A., **2024**. Sequence-specific, mechanophore-free mechanochemistry using polynucleic acids. *In review*.