Synthesis of polymeric particles with biological recognition motifs for medical applications

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Von

M.Sc

Anwar Naveed

aus Gujranwala (Pakistan)

Berichter: Universitätsprofessor Dr. rer. nat. Martin Möller

Universitätsprofessor Dr. rer. nat. Andrij Pich

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<tbody>
<tr>
<td>PVA</td>
<td>Poly(vinylalcohol)</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>XRD</td>
<td>X-ray diffraction</td>
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<td>Two-dimensional</td>
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</tr>
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<td>Copper sulfate</td>
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<td>DLS</td>
<td>Dynamic light scattering</td>
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<td>Hydroxyl</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier transform infrared</td>
</tr>
<tr>
<td>ASTM</td>
<td>American society for testing and materials</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>NaBH₄</td>
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<td>CPNs</td>
<td>Conjugated polymer nanoparticles</td>
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<tr>
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<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly(ethyleneglycol)</td>
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<td>Poly(vinylalcohol)-co-(vinylacetate)</td>
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<td>GlcNAc</td>
<td>2-acetamido-2-deoxy-β-D-glucosides</td>
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<td>Clostridium associated disease</td>
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</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>Chemical Symbol</td>
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<td>-----------------</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td>NaHCO₃</td>
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Abstract

The motivation for studying conjugated polymer particles (CPPs) comes from their reasonable photo- and physical and tunable fluorescence brightness. CPPs find applications usually in sensing, cell labelling, and long term imaging. However, use of CPPs in such applications is restricted due to high polydispersity of particles and inertness of CPPs surface. In this thesis, several facile methods of CPPs fabrication with narrow size distribution, characterization and functionalization of their surface with biological recognition motifs are described.

We established a highly versatile method to fabricate extremely bright and light switchable CPPs. This approach can be easily applied to other switchable monomers provided they are dihalogenated to take part in the palladium-catalyzed coupling reactions. They can easily find applications in self-assembled switchable photonics due to their fast switching characteristics, amorphous morphology and high monodispersity. The particles are attractive candidates for use in coatings, where they produce switchable physical color by diffraction or in photonic crystals for wavelength conversion with a switchable band gap or as colloidal laser resonators. We demonstrated a rise in fluorescence intensity together with the change in shape for switching from the trans-to-cis-conformation. All the particles could be used as switchable probes and markers for super resolution imaging in the biomedical field.

In subsequent chapter, we demonstrated an easy approach to prepare monodisperse CPPs using Sonogashira dispersion polymerization bearing acetylene moieties on the surface. These acetylene groups are functionalized with biological recognition motif using thiol-yne click chemistry. The particle size is tunable between 140 nm and 300 nm. We applied the resulting functional particles as fluorescent probes for imaging of endothelial cells, which take up the particles via receptor mediated endocytosis. To employ these particles in-vivo in future, we will develop near-IR emitters to allow excitation and fluorescence detection in the tissue transparency systems. We proved that the particles can bind to αVβ3 integrins on activated endothelial cells under flow and static cell culture conditions showing potential of particles to be applied as non-bleaching near-infrared tumor probes and theranostic agents.
In another study, we synthesized poly(ethylene glycol) (PEG) based microgels for the demonstration of glycans in a multivalent matter. Microgels with variable size, different functional moieties i.e. GlcNc, LacNAc, Galili and numerous concentrations of these glycans were prepared. These microgels can attach both lectin and lectin domain of TcdA due to specific interaction between them and glycans. We proved specific interactions between glycans and protein using FACS analysis. The microgels allow a facile diffusion of 124 kDa protein showing microgels mesh size is large enough. The system may be an ideal substitute for currently available antimicrobial therapy, as it deals directly with the inflammation causing toxin avoiding interruption of the intestinal flora. The versatility of the system can be seen by replacing glycans with different linkers and biomolecules. Altogether, this provides synthesis of highly selective microgels for various biological scavenging applications.
Zusammenfassung


Introduction

Polymer colloids have recently gained increasing attention in the biomedical field due to their easy fabrication, unique optical properties and outstanding performance as imaging and therapeutic agents. Of the particular importance are polyethylene glycol (PEG) based microgels and conjugated polymer nanoparticles.

PEG-based microgels are considered as one of the most widely studied materials in material science and biological applications, such as tissue engineering and drug delivery due to their non-cytotoxicity and non-immunogenicity. Their surface can be tailored through silane, acrylate and thiol linkages depending the applications. PEG-based microgels can be produced from covalent cross-linking of PEG pre-polymers. A very well-known method is the photo-polymerization of PEG-acrylate monomers.

Conjugated polymer nanoparticles (CPNs) are synthesized from benign materials and possess significant advantages in terms of tunable brightness, reasonable photo- and physical stability, good biocompatibility, non-cytotoxicity and facile surface functionalization. These characteristics allow using of CPNs in fluorescence based and biological applications including cellular labeling, imaging, biosensing and single particle tracking. To explore a wide range of applications of CPNs, it is important that CPNs have surface functionality to conjugate a recognition moiety and maintain colloidal stability in biological media.

Way back to early civilizations, humans have made numerous efforts to control ailments, discomfort and pain. They used and tested many naturally occurring substances against the diseases. Tremendous progress in science has made possible to identify the active ingredients of the long used natural products and their structures and mode of action were determined. More and more powerful tools against diseases were tried to discover with the advancement of humanity. The use of polymeric colloids may be an answer to many intractable medical challenges. It already plays a major role in detection, imaging, treatment and prevention of diseases, such as cancer. Polymeric colloids possess unique physiochemical character due to increased surface-to-volume ratio. They are tunable in size so that can pass fine capillaries as well as internalize into the cells and can pass through the intestines without taken up by intestinal mucosa. Hence, polymeric colloids have the potential to resolve many limitations attached to conven-
tional diagnostic and therapeutic device, such as lack of water suitability, non-specific biodistribution and low therapeutic indices. The medical effectiveness is more enhanced with the development of “theragnostic” colloids which combine diagnostic and therapy via integration of drug and dye molecules. Polymer colloids can be made out of various organic and inorganic precursors or combination of both; however conjugated polymer and PEG-based colloids have gained significant attention due to their good biocompatibility, structural diversity and excellent adjustability of their properties. By employing top-down or bottom-up approaches, colloids with well-defined size and shape can be prepared. Despite all these characteristics, the currently available colloids possess some limitations. They are most often not biocompatible and show lower performance due to their restricted loading capacity and their functionality is mostly limited to some extent. Generally, they are bigger than inorganic quantum dots with relatively broad size distribution when applied as bioimaging agents. Therefore, new synthetic approaches need to be explored for the synthesis of colloids with narrow size distribution and higher loading capacity.

The focus of this thesis is to synthesize and characterize the polymeric colloids composed of hydrophobic conjugated polymers and hydrophilic poly(ethylene glycol) (PEG) and their functionalizations with different biorecognition motifs (peptide and glycans). The colloidal entities are subsequently applied as fluorescent probes for imaging of activated endothelial cells and removal of toxins from the body.

Chapter 2 gives a literature overview of different polymeric particles and their biological applications and synthetic approaches involved in this section.

Chapter 3 describes the synthesis of monodisperse conjugated polymer particles with incorporated light-switchable azobenzene moieties in a Suzuki-Miyaura dispersion polymerization and their characterization using SEM, DLS, Confocal microscopy, and XRD. The particles can be applied to responsive organic photonics and as high resolution marker systems for biomedical applications.

Chapter 4 focuses on the synthesis of monodisperse conjugated polymer particles having acetylene moieties on the surface via Sonogashira dispersion polymerization and functionalization of the particles with RGD peptide using thiol-yne click chemistry. The resulting particles are used as fluorescent probes for imaging of endothelial cells.

Chapter 5 deals with the synthesis of microgels form non-immunogenic PEG-acrylate and incorporation of different glycans with different concentration relative to PEG-
acrylate. Furthermore, mesh size of microgels and successful incorporation of glycans is investigated using different proteins with comparable size to toxin A. The microgels can present marked improvement for curing of CDAD disease.

Chapter 6 is concerned with the attachment of different linkers to 2-acetamido-2-deoxy-β-D-glucosides (GlcNAc). The attachment is proved using nuclear magnetic resonance spectroscopy. The attachment of GlcNAc to fluorene and synthesis of BOC-protected amine form benzyl alcohol is also described here.

The colloidal entities become more and more complex by addition of functionality: fluorescent and switchable → fluorescent and biorecognition → biorecognition, porous and uptake
Chapter 1

Literature overview
1.1 Biological applications of polymeric particles

Polymeric particles are interesting materials for biological applications because they are often biocompatible in nature. In the recent decades, they have become very interesting for the scientists and serious effort has been made in designing[1], manufacturing, and their extensive use in many biological [2] and biomedical applications [3]. Polymeric particles of natural, synthetic and semi-synthetic polymeric nature have been greatly used in biological and biomedical fields. However, there are certain conditions that polymers must fulfill to be used in such applications. Polymers must be biodegradable or should be cleared from the body without causing any side effects. They must not have any cytotoxicity and their degradation products must not cause any harm to the body. Synthesis of particles should be easy and their properties should be tunable to regulate the desired properties. Poly (ethylene glycol) (PEG) [4]–[7] and conjugated polymeric particles based microgels fulfill these requirements. This chapter illustrates literature overview of the design, synthesis, functionalization routes and applications of soft microgels made from poly (ethylene glycol) and hard conjugated polymeric particles made from fluorene based polymers. Super resolution microscopy has been extensively used for the investigation of such biological molecules.

1.2 Super resolution microscopy

Fluorescence microscopy has gained a significant importance in the investigation of biological molecules, pathways and events in \textit{vitro} and in \textit{vivo}. Fluorescence microscopy holds the superiority compared to other imaging methods (electron microscopy) because it is quite compatible with living cells, which allows dynamic and minimally invasive imaging experiments. However, it also has a weakness from many years that its spatial resolution is limited to \(~200\) nm only [8].

On the one hand, magnetic resonance imaging, optical coherence tomography and positron emission tomography presented real-time read out from animal or human subjects, but details smaller than \(~1\) mm, \(~100\) \(\mu\)m and \(~19\) \(\mu\)m respectively are still unclear. On the other hand, near molecular-level spatial resolution is achievable with electron microscopy, but cells must be fixed, hence inhibiting dynamic imaging. Fluorescence microscopy gives a range spatial and temporal resolution between these two extremes. Wide-field microscopy and confocal microscopy can resolve certain cellular organelles (nucleus, the Golgi apparatus and endoplasmic reticulum) and can track proteins and other biological entities in live cells. However, they cannot discern the pairs of interact-
ing proteins or the single synaptic vesicles. For example, Bacteria have a size of 1-5 μm
only and traditional fluorescence microscopy is unable to resolve the subcellular fea-
tures [8].

In 1990s new microscopies revolutionized the imaging field by breaking the lateral reso-
lution diffraction limit for the first time. They are collectively termed as super resolu-
tion imaging techniques. For example, far-field super-resolution imaging and near-field
super-resolution imaging. Far-field super-resolution imaging can be further classified
into stimulated emission depletion (STED) [9], photoactivated localization microscopy
(PALM) [10], fluorescence photoactivation localization microscopy (FPALM) [11], and
stochastic optical reconstruction microscopy (STORM) [12].

STED was the first one to be used for the cell imaging among far-field super resolution
imaging techniques [13]. It has been applied for imaging of an ensemble of molecules.
STED microscopy provides 20 nm resolution in the confocal plane [14], and 45 nm
resolution in all three dimensions [16]. Although various applications of STED micro-
copy have been reported [17]–[19]. However, a very complicated instrumentation is still
limiting its widespread use.

For imaging of single molecule, PALM, FPALM and STORM have been applied. These
techniques work on a basic principle that the position of a single molecule can be local-
ized to 1 nm accuracy or better if sufficient photons are collected and there are no oth-
er similarly emitting molecules within ~200 nm. Three-dimensional (3D) imaging is also
possible using STORM and PALM using astigmatism. Huang et al. reported STORM
imaging with 20-30 nm resolution in the xy plane and 50 nm in the axial dimension
[20].

High resolution images using sequential activation and time-resolved localization of
photoswitchable fluorophores have been achieved with the help of STORM, PALM and
FPALM. At a particular moment during imaging, only an optically resolvable subset of
fluorophores is activated to a fluorescent state, such that location of each fluorophore
can be determined with highest precision by finding the central position of the single-
molecule images of particular fluorophore. The fluorophore is then deactivated. The
process is repeated with another subset and imaged. Repetition of this process allows
localization of various fluorophores; hence a super-resolution image can be created from
the image data. Although STORM, PALM and FPALM has been published separately
during a short period of time, but they work on the same principle. Originally, Cy5 and
Cy3 dyes linked to nucleic acid or proteins were used for description of STORM [12].
While photoswitchable fluorescent proteins were applied for the description of PALM and FPALM [10], [11]. Principally, they are applicable to all kinds of photoswitchable fluorophores. STORM imaging can be performed with a single red laser excitation source using stochastic photoswitching of single fluorophores, such as Cy5. The red laser switches the Cy5 fluorophore between the dark state and fluorescent state [21], [22]. A wide variety of dyes have been employed for STORM [23]–[28]. Additionally, this technique is also applied to a dye-pair comprising of a photoswitchable reporter dye (Cy5, Alexa 647) and activator fluorophore (such as Cy3, Cy2) [12], [29], [30]. Here, the activator fluorophore acts as a reactivator for the photoswitchable dye (switches back the fluorescent dye to fluorescent state). Multicolor imaging has been reported by applying different activation wavelengths to distinguish dye-pairs [29]–[31] or using spectrally distinguishing photoswitchable fluorophores either with or without activator fluorophores [23], [32], [33]. Literature survey reveals the use of photoswitchable fluorescent proteins [10], [11], [20], [33].

Another method which has been extensively used in super-resolution microscopy is reversible photoactive fluorescent proteins (PA-FPs). They are also known as photoswitchers. Their emission can be reversibly switched on and off with light. Their main advantage is that imaging of the same fluorophore can be performed multiple times. It is compulsory in RESOLFT imaging, where each molecule is switched on and off multiple times to create a subdiffraction image. For example, PA-FP Dronpa [34] shows a green fluorescence in its bright state. A non-fluorescent form is developed upon intense irradiation with green light with an absorption maximum at 390 nm, which reverts back to its original green-emitting form upon irradiation with 405 nm light. Dronpa can perform 100 such cycles (activation-quenching) with only 25% loss of its original fluorescence [35].
1.3 Biomolecular recognition

Biomolecular recognition occurs at the interface between specific moieties, such as cells or individual molecules. The advancement in chemical technology enables the production of nanoparticles with desired properties. Synthesis of nanoparticles acting as highly specific partners in molecular recognition reactions revolutionized field of nanotechnology. They are composed of solid matter and have a large surface area making them ideal bearer of biological recognitions sites. Now the question is how to change nanoparticle surface to a molecular recognition element? To do this, there are two main strategies. (1) Supramolecular immobilization of biological recognition elements (proteins like antibodies or enzymes). (2) Supramolecular synthesis of surface of the nanoparticles by a specific molecular interaction during the polymerization process (molecular imprinting of polymers).

The surface of nanoparticles must provide molecularly defined binding sites to enable molecular recognition. The nanoparticle surface must be organic in nature. This can be achieved by constructing an organic shell around a core (organic or inorganic). Inorganic nanoparticles have become very popular recently. Cadmium-chalcogenide nanocrystals were applied for the fluorescence imaging of both extracellular and intracellular matrices. Magnetic nanoparticles consisting of metals or metal oxides were applied in magnetic resonance imaging and for thermo therapy of cancer. Although, these colloids are not biomimetic, but can be applied as functional core materials. Silica and organically modified silica are most extensively used core materials for the synthesis of biofunctional nanoparticles. This is due to their relatively simple chemistry, which allows
tuning of composition and finest control over surface properties. Moreover, chemical modification can be easily performed using organofunctional silanes. Additionally, functional components like quantum dots (QDs) magnetic nanoparticles, fluorescent dyes, drugs or combinations thereof can be easily incorporated into the silica cores. This encapsulating property makes them useful for a wide variety of life sciences applications like imaging, diagnosis or therapeutics.

Organic core materials have gained increasing importance for the synthesis of nanoparticles. They are especially advantageous for drug delivery applications. The most important organic systems are polymeric nanoparticles, lipids and fullerenes. They are often selected for envisaged applications like biomarkers, gene delivery system or even biosensors.

The organic shell around the particle core has the biomimetic feature of molecular recognition. These organic shells can also protect the particle core against oxidation and degradation. Additionally, it may control the surface properties of particle core or can prevent aggregations of particles by stabilizing the particle suspension. It can have fluorescent dyes or be loaded with drugs. Briefly, the shell provide the molecular recognition moiety (I) by stabilizing and directing a biomolecular receptor such as an antibody or (II) by being molecular imprinted and thus having the molecularly binding site in an artificial polymer.

During the recent decades, the technique of molecular imprinting is gaining interest of researchers. Typically, molecular imprinted polymers (MIPs) are synthesized by radical polymerization of a functional polymer and a cross-linker using a template molecule. A polymer monolith is achieved, which can be converted to particles by mechanical processing like grinding. Polydisperse particles of micrometer size have been prepared by this method for many years. Recently, alternative methods have been developed and MIPs have been generated. Successful imprinting of small peptides in organic solvents and synthesis of MIPs in water for the recognition of large proteins has been reported. Nanoparticulate MIPs of a definite size and shape are produced via miniemulsion polymerization show a high specific surface area. It can be proved that they can recognize low molecular weight compounds upto biomacromolecules such as proteins. Nanoparticles can approach an organ, a tissue, or specific cell types, such as macrophages, dendritic cells and tumor cells upon conjugation their surface to specific ligands. There are different microscopic techniques which enables researchers to investigate the cells and tissue in larger details.
1.3.1 Live cell imaging

Study of living cells and tissues using time-lapse microscopy is known as live cell imaging. It is quite common in different fields of the life and physical sciences. It is instrumental in providing a better understanding of cellular dynamics and functions [37]. Living cell microscopy has been accessible for decades, as is evident from first time-lapse microcinematographic films of cells made by Julius Ries, exhibiting the fertilization and development of the sea urchin egg [38]. Cell viability is quite important to ensure that the physiological and biological processes under study are not changed during performing such experiments. Exposure of many cells and tissues during their life cycle to light is avoided normally, so it is crucial for microscopy applications to minimize the light exposure, which can cause phototoxicity. Development of many microscopic techniques allowed scientists to investigate living cells in greater detail with minimum effort. For a successful investigation of cellular processes in living cells, the environment of the cells must be chosen in such a way that it does not induce any stress response. Moreover, it allows the alteration of cellular processes of interest. The key factors including type of culture medium and its contents, temperature (must be 37°C for mammalian cells) and pH of the sample must be considered.

Observation of living cells was difficult before the invention of transmitted-light microscopy such as phase contrast or differential interference contrast (DIC). Living cells must be stained to be visible in a conventional light microscope as they are translucent. This can kill the cells unfortunately [39]. Phase contrast microscopy made the detailed investigation of unstained cells possible. The inventor of phase contrast, Fritz Zernike, was awarded the Nobel Prize in 1953. Light throughput and signal-to-noise ratio S/N is not an issue in these techniques due to availability of plenty of light. However, to obtain a high image quality regular cleaning of the optical components is must. Low intensity of white light does not cause any harmful effects. However, high levels of white light should be carefully used due to presence near-UV and near-IR (NIR), which are injurious to cells. Imaging of specific proteins or other organic chemical compounds forming complex machinery of a cell is not possible, which limits its widespread use.

Confocal microscopy provides an excellent alternative to observe such compounds. These compounds are labelled with synthetic and organic fluorescent stains making them observable using confocal microscopy. Confocal microscopy actually increases the contrast and optical resolution of microscopic images using an additional spatial pinhole placed at the confocal plane of the lens to block the extra light (out-of-focus) (see
Figure 1.2). It allows the rebuilding of 3D structures from the obtained images. It has become very popular in many fields especially in life sciences, material science and semiconductor inspection [40].

![Diagram](image)

**Figure 1.2:** Principle of confocal microscopy.

Confocal microscope has the capacity to collect high quality multispectral images across the visible range. One example of high resolution, high-power and multicolor capabilities of the confocal microscope is illustrated in image shown in Figure 1.3. There are four types of commercially available confocal microscopes such as confocal laser scanning microscopes, spinning-disk confocal microscopes, microlens enhanced or dual spinning disk confocal microscopes, and programmable array microscopes.
Optical imaging is an excellent technique, which possesses superior resolution, higher sensitivity and low cost as compared with the other currently available techniques (MRI, CT-Scan). There are two major issues with the current techniques, i.e. auto fluorescence of cells in the visible region (competing with the fluorescence from particles) and prolonged imaging times. So, there is a need of new probes that excite and emit light in the near-infrared (NIR) region ($\lambda>$700 nm) and show more photostability than the currently available fluorophores. Quantum dots (QDs) or semiconductor fluorescent nanocrystals were used as an alternative [41]. Their unique properties such as high brightness and less susceptibility to photo-degradation than organic fluorophores provide an excellent platform for use in cellular imaging and biosensing [42]. However, difficult synthesis process due to high temperature, cytotoxic metal compounds, aggregation in aqueous systems and long term toxicities hinder their use in medical applications [43], [44]. Moreover, they must be coated or incorporated into polymer shells to enhance their chemical and luminescent stability [45], [46]. Conjugated polymer particles are promising materials to overcome these problems due to their excellent optical and electronic properties. They show little photobleaching and hence present ideal materials for long term cellular labeling.

Wu et al. applied multicolor CPNs for cellular imaging in macrophage. They used bare hydrophobic conjugated polymer nanoparticles for labeling of cells, which were taken up by macrophages via endocytosis [47]. Fernando et al. used 18 nm diameter CPNs to
investigate mechanism of cellular uptake of non-capsulated bare poly(fluorene-alt-benzothia-diazole) (PFBT) nanoparticles also in macrophage cells. The detection of nanoparticles can be done at very low loading concentrations of 155 pM (270 ppb). Intracellular co-localization studies of CPNs and Texas red dextran showed that cellular uptake occur through a macropinocytic mechanism [48]. Macropinocytosis is a non-specific mechanism for picking up extracellular fluid, which delivers fluid content to lysosome irrespective of the nanoparticle surface and charge. This mechanism is quite suitable in fluid phase labeling of endosomal and lysosomal organelles.

Poly(lactic-co-glycolic acid) (PLGA) nanoparticles tailored with conjugated polymer have also been applied for cellular labeling. MCF-7 breast cancer cells were used in this study. Particles with diameter 50 nm were delivered into the cytoplasm [49]. The same group used folic acid conjugated particles to categorically target the folate receptor in cultured cancer cells. It was concluded that the uptake of folic acid functionalized particles occurs predominantly as compared with the non-functionalized particles. In the same fashion, conjugated polyelectrolytes and amphiphilic conjugated polymer nanoparticles have also been reported as promising cellular labels [50]–[54]. Cellular uptake of nanoparticles mainly depends on functional surface groups, the specific cell line, and generally take place through both micropinocytosis and receptor mediated endocytosis.

1.3.2 In vivo imaging

Fluorescence imaging can be used for numerous molecular diagnostics and therapeutic applications. Fluorescence-based imaging techniques have been extensively used in small animal research due to their tremendous sensitivity. Photon limiting interferences such as scattering, absorption, and auto fluorescence lessen their capacity to produce effective deep-tissue fluorescence imaging. Near infrared fluorescence (NIR) probes with fluorescence is needed to address these challenges. Conjugated polymer nanoparticles have the ability to overcome these limitations due to their high fluorescence brightness and tunability in fluorescence wavelength. Kim et al. applied a cyano-substituted derivative of poly (phenylenevinylene) to synthesize nanoparticles, which emit in the red to NIR region. They reported in vivo fluorescence mapping of sentinel lymph node (SNL) in a mouse model using nanoparticle of 60±14 nm in size [55]. Chiu et al. produced conjugated polymer nanoparticles with deep red emission (λ = 655 nm) of about 15 nm in size. The probe was decorated with chlorotoxin, a specific peptide ligand for in vivo tumor targeting. Biophotonic imaging and quantitative analysis of bio-
distribution shows that these probes have the capacity to especially target malignant brain tumors. However, to date there are no pure conjugated polymer materials with surface bound ligands, which directly target the cancer cells.

1.3.3 Fluorescence guided surgery

Fluorescence guided surgery (FGS) is an imaging technique used during a surgery for the detection of fluorescently labelled structures. It is also named as fluorescence image-guided surgery, or fluorescence guided resection in specific case of tumor resection. The main purpose of FGS is to provide the surgeon of real time visualization of the operating field. It is cheaper and superior than other medical imaging modalities in terms of number of detectable molecules and resolution [56]. FGS has revolutionized the real-time intraoperative visualization because the information is directly visualized through a microscope using a specialized illuminant.
Figure 1.4: (a) The number of publications in “fluorescence-guided surgery” or “fluorescence guided intersection” in the past 25 years, showing the exponential growth in the field. (b) The Novadaq SPY Elite fluorescence imaging system, which has been at the forefront of the effort to expand fluorescence guided surgery capabilities, leading the commercial market. (c) Laprosopic images acquired under white-light and (d) by exciting indocyanine green which has been pseudocolored blue and overlaid onto C. Reprinted from reference [36].

FGS provides real time information from the color reflectance images and fluorescence emission simultaneously. The sample can be excited and illuminated using one or multiple sources. Optical filters, matching the emission spectrum of the fluorophore are used to collect the light. The final image is created using imaging lenses and digital cameras. Live video processing allows increasing contrast during fluorescence detection and improving signal-to-background ratio. In specific applications, ALA-induced PpIX is highly specific for cancer cells [57]. It has produced good impact on tumor resection. Additionally, it emits red light which means easily visible with naked eye and switched on and off with the push of a button. FGS is a relatively new compared to other image-guided techniques, but clearly showing an exponential development (see Figure 1.4).

Various smart-targeted fluorophores are under pre-clinical investigations including activatable caspass [58], labeled monoclonal antibodies [59]–[61], affibody molecules [62], [63], aptamers [64] and other proteins. A weakness is the penetration depth (only 100 μm in the visible region). However, when excitation wavelengths in the NIR are used, it can penetrate to 1-2 cm.

1.4 Cellular uptake

A carrier must pass through many biological barriers at the cellular level, e.g. channel proteins in the cell membrane. It does not permit the complexes larger than 1 kDa to diffuse. Once a carrier approaches to the targeted tissue or cell population by means of either active or passive targeting or a combination of both, its uptake and internalization can take place through receptor-mediated uptake mechanisms. Uptake occurs especially through receptor-mediated endocytosis but phagocytosis and pinocytosis can also help in the internalization of drug depending on the cell-type (Figure 1.5). It is very crucial to know the exact type of endocytosis because it defines the road of trafficking through different subcellular compartments. As an example, internalization of functionalized polymeric particles through clathrin-mediated endocytosis can occur through lysosomal compartment, whereas it is impossible for those internalized through a caveolin-mediated process [65]. Endosomal escape must take place in clathrin-mediated endocytosis before fusion with a lysosome to inhibit degradation of the
cargo under severe liposomal conditions. In either case, endosomal escape is mandatory so that sufficient carrier can reach the subcellular compartment, whether it is supposed to reach the cytosol, the mitochondria or the nucleus.

The type of cellular internalization heavily depends upon the ligands attached to the surface of functionalized particles. In case of albumin, folic acid and cholesterol, uptake seems to take place via caveolin-mediated endocytosis; however ligands for glycoreceptors facilitate clathrin-mediated endocytosis.

**Figure 1.5:** Different techniques used for cellular internalization of nanoparticles and corresponding size restrictions. (a) Big particles are internalized through phagocytosis. (b) Smaller particles (≥ 1 μm) are internalized nonspecifically by macropinocytosis. Internalization of smaller na-
Nanoparticles can occur through different approaches such as (c) caveolar-mediated endocytosis, (d) clathrin-mediated endocytosis, (e) and clathrin-independent and caveolin-independent endocytosis. All of them are marginally different from each other. Nanoparticles are illustrated by blue circles (> 1 μm), yellow rods (~ 60 nm), yellow rods (~ 60 nm), red stars (~ 90 nm) and yellow rods (~ 60 nm). Reproduced from [66].

1.5 Types of polymeric particles for biological and biomedical applications and synthetic procedures

The following selections of polymeric particles can be used in biological and biomedical applications.

1.5.1 Nano drug carriers

Nano-sized colloids are one main class of tumor imaging [67], [68]. Administration (intravascular) generally occurs through the blood stream where they face the defense mechanism of the body. Long circulation times are required to deliver a drug to its target and release its payload [69]. The nano drug carriers come out of the blood circulation by slow processes. A significant advantage of the long circulation times is that deliverance of the drug at the target due to repeated passage through the target site become easy. The dimension and structure of the vessels greatly affects the design of the colloids, since larger colloids (bigger than few micrometers) find it difficult to pass through lungs whereas small sized colloids could escape from circulation through intercellular junction of health endothelium (e.g. lymph nodes endothelium [70]. Or may be withdraw by the sinus endothelium of the bone narrow [71].

A significant number of in vivo experiments revealed that colloid size of 20-200 nm to be an optimal range for prolonged blood residence. Generally, the nanocarriers may

1. Protect the degradation of drug.
2. Raise the drug absorption by allowing it to diffuse through epithelium.
3. Reshape pharmacokinetic and drug tissue distribution profile
4. Enhance penetration and distribution within the cells.
Most of the “nano” drug carriers, which are being used these days, are remnants of the conventional drug carriers such as liposomes, dendrimers, and nanocrystals (Figure 1.6).

Figure 1.6: Nanoparticle fabrication for different intracellular utilizations. Nanocarriers can be fabricated from different materials composition having distinct physical and chemical properties and decorated with a wide variety of functional groups for biological applications. Such easiness in fabrication permits scientists to decorate nanoparticle for distinct intracellular utilisations as therapeutics and drug delivery agents [72].

1.5.2 Nanoparticles

Generally particles between 1 and 100 nm are called nanoparticles. In the past two decades, polymeric nanoparticles have been extensively employed in biological and medical fields because of their excellent properties e.g. good biocompatibility, smooth and effortless synthesis, diversity of structures and fascinating bio-mimetic character. They play an excellent role especially in smart drug delivery because they can effectively deliver the therapeutic agents to the intended site of action. The optimal requirements which must be fulfilled while synthesizing the nanoparticles are lowest particle size distribution, dispersibility in water, surface chemistry, improved permeation, flexi-
bility and delivering the drug at the site of action in the shortest possible time. Nanoparticles can be made of natural, synthetic and semi-synthetic polymers [73]–[76].

Nanoparticle synthesis can be performed by various polymerization techniques, and a large number of surfactants and polymeric stabilizers are available for their stabilization.

Figure 1.7: Schematic representation of common methods of synthesis of polymeric nanoparticles and their principle involved in the mechanisms. Reprinted from [77].

Polymeric nanoparticles can prove useful in delivering both the hydrophobic and hydrophilic drugs. They can circulate in the body with lowest side effects and maximum half-life. A wide variety of modern techniques such as electrodripping systems [62], interfacial emulsion polymerization, microelectromechanical systems [78], microfluidic systems [79], microneedle based systems [80] and combined systems are available for nanoparticle synthesis (Figure 1.8).
1.6 Development of fluorescent conjugated polymer nanoparticles

Fluorescence spectroscopic techniques along with improved fluorophores are an excellent tool for lowering the limit of detection in biological experiments. Particularly, their use for investigation of bimolecular interaction at single molecule level provides excellent details of biochemical process [81]. Traditional fluorophores being used in biological imaging and bioassays are small molecule fluorescent dyes such as fluorescein, rhodamine and cyanine [82]. These conventional dyes are proven to be so successful in the development of high sensitivity cellular imaging. However, they possess a drawback in
vitro assay especially at the singe molecule level due to their low absorptivity and poor photostability. Therefore, there is a demand to produce fluorophores which could lower the limit of detection and enhance sensitivity. This need is fulfilled by inorganic quantum dots (QDs) \cite{83}, \cite{84} and dye doped silica particles \cite{85}. These nanoparticles are more photostable and show bright fluorescence as compared to conventional organic dyes. However, cytotoxicity (leached metal from the nanocrystal core) \cite{86}, \cite{87} and dark dots presence \cite{88} are main issues associated with quantum dots reducing their use as compared to fluorophores dyes despite their good optical properties. Although this problem has been reduced dramatically by coating the quantum dots with various materials. However, such coatings have their own associated cytotoxic effects \cite{86}, \cite{89}. Fluorescent-dye-loaded polymer latex or silica nanoparticles also show improved brightness and photostability than molecular dyes due to large number of fluorophores per particle \cite{90}--\cite{92}. However, self-quenching of dyes especially at high concentration reduces the overall brightness of such particles \cite{85}.

Consequently, these drawbacks of the current fluorescent nanoparticles result in the development of other classes of nanoparticles, which show lower cytotoxicity but more photostable and bright in fluorescence. A lot of work has been done to architect and prepare nanoparticles, lowering the limitations of current fluorophores for biomedical applications. To that end, one promising strategy is the development of conjugated polymer nanoparticles. CPNs offer high brightness due to enhanced radiation rate and improved absorption cross-section \cite{47}. As these nanoparticles are prepared from relatively benign polymer materials, so there is very low or no observed cytotoxicity. These advantages established CPNs as bright fluorescence probes for improving sensitivity and the limit of detection in biological applications.

For biological applications, the nanoparticles prepared from conjugated polymers can be divided into many different forms. The most common type is the nanoparticles synthesized from hydrophobic conjugated polymer by precipitation method \cite{47}, \cite{48}, \cite{93}--\cite{100}. Water is most commonly used as a dispersion medium for most biological applications. The particles prepared by this method ranges from 10-30 nm, which can be adjusted by changing the concentration of polymer \cite{47}. Competition between the inter chain aggregation and intra chain collapse during the nanoparticle formation process determines the size of the nanoparticles. Synthesis of particles from dilute stock solution limits the possibility of inter chain aggregation, which produce small size particles. The CPNs prepared from hydrophobic polymers are considered to be spherical struc-
ture. This is because of impenetrable packed chromophores and strong hydrophobic interaction [99], [101].

Not only of hydrophobic polymers., nanoparticles of hydrophilic polymers and conjugated polyelectrolytes are also synthesized [50], [102]–[105]. Mixing of poly(p-phenylene ethynylene) (PPE) polymer containing a hydrophilic amine and a PEG linker in dimethyl sulfoxide into saline solution produce particles of much bigger size (500 nm). The nanoparticles can also be prepared from polyelectrolytes by self-assembly and can have particle size of about 80 nm [49], [106]. These CPNs composed of loosely aggregated polymer chains as compared to hydrophobic CPNs, which have a densely packed structure. Similarly, fluorescent amphiphilic polymers composed of hydrophobic polyfluorene backbone having hydrophilic PEGs on side chains produce nanoparticles in water. The micellar nanoparticles form by slowly adding the water into a solution of polymer in THF [107]. The amphiphilic polymers produce particles with a size range 10-100 nm. [51], [108], [109]. These nanoparticles are ideal candidate for cellular labelling and imaging applications.

1.7 Conjugated polymer nanoparticles (CPNs)

Conjugated polymer nanoparticles (CPNs) are an important class of nanoparticles. They have gained increased attention as promising fluorescent nanoparticles due to their high fluorescence brightness. They are mainly prepared by precipitation of hydrophobic conjugated polymers that show bright fluorescence as nanoparticles. They do not possess observable cytotoxicity as they are made of relatively benign polymers [48], [110], [111]. Their high brightness, reasonable photo stability, and no observable cytotoxicity made them ideal candidate for studying as fluorescent labels. These CPNs have extensively been used in techniques like imaging, cellular labeling, biosensing, single particle tracking based on fluorescence [47], [110]–[112]. They have found applications as active materials in light emitting diodes [113], [114], photovoltaic devices [113], [115] and thin film transistors [116], [117].

The synthesis, properties as well as applications of conjugated polymer particles in thin films and solutions have been extensively studied [115], [118]. They have been investigated as fluorescence labels in biological applications.

Colloids of conducting polymer nanoparticles e.g. acetylene [119] and polypyrrole [120] have been demonstrated by Vincent et al. in the early 1980s. They synthesized the par-
ticles by aqueous oxidative polymerization. Later, numerous approaches have been applied to access colloids of conjugated polymers in water.

Two main methods have been applied to prepare conjugated polymer nanoparticles. The method is selected on the basis of molecular weight of the monomer. In case of monomers, direct polymerization is employed in a heterophase system. They can be converted into nanoparticles. Vincent et al. used steric stabilizers to synthesize polya-cetylene particles [119]. Recently, this method has been used to synthesize nanoparticles from a fluorescent semiconducting polymer [121]–[123]. The second method is based on a post polymerization approach. In this method, nanoparticles are synthesized from already prepared high molecular weight polymers. This type of nanoparticle synthesis is based on commercially available polymers and does not demand any polymer synthesis expertise at all. This is a comparatively simple method as compared to direct polymerization.

The post-polymerization method can be further categorized into miniemulsion and re-precipitation.

1.7.1 Miniemulsion

Miniemulsion is a unique case of emulsion. In general, miniemulsion is achieved by shearing a mixture consisting of two immiscible liquid phases e.g. oil and water, surfactant and co-surfactant. Typically, miniemulsion comprise of small and stable droplets in a continuous phase. The emulsion is stabilized by high shearing force provided by ultra-sonication or high-pressure homogenizers. The stabilization of droplets is confirmed by using an amphiphilic component, the surfactant, and the co-stabilizer. The co-stabilizer is normally soluble and homogeneously distributed in the droplet phase. It is less soluble in continuous phase than the rest of the droplet phase, hence developed an osmotic pressure in the droplets (also known as osmotic pressure agents) neutralizing the Laplace pressure. The polymerization occurs inside the droplets or at the surface of the droplets acting as nano- containers, resulting in the production of nanoparticles [124], [125]. Miniemulsion can be applied to produce particles of size between 50 and 500 nm.

Conjugated polymer nanoparticles can be synthesized from the emulsified droplet solution and a water-immiscible solvent is needed generally. Landfester et al. first used the miniemulsion method to synthesize fluorescent conjugated polymer nanospheres which could be utilized to prepare nanoscale multiphase films in photovoltaic and light-
emitting devices [126]–[128]. Generally, a solution of conjugated polymer in an organic solvent usually chloroform was emulsified in water to produce stable droplets. A surfactant usually sodium dodecyl sulphate was mixed in the water already.

1.7.2 Reprecipitaition

The reprecipitation method consists of adding a diluted solution of a conjugated polymer in an organic solvent i.e. tetrahydrofuran (THF) to an excess of water. This process can be supported by sonication for nanoparticle formation. Adding of conjugated polymer dissolved in a good solvent directly into poor solvent causes a sudden change in the solvent environment that results in polymer precipitation. This subsequent removal of the organic solvent at elevated temperature produces nanoparticle dispersion in water. This is a useful method and applicable to all hydrophobic conjugated polymers that show solubility in organic solvent miscible with water.

Mashura et al. reported the reprecipitation method for the synthesis of substituted-polythiophene by mixing a polymer solution in THF into water. The formed particles were between 40–400 nm in diameter measured by dynamic light scattering [129]. McNeill et al. further modified this method to produce nanoparticles of smaller size from hydrophobic conjugated polymers [93]–[98]. Generally, the particles formed by reprecipitation are smaller in size as compared to those prepared by miniemulsion.

1.7.3 Dispersion polymerization

It is a heterogeneous polymerization process which occurs in the reaction medium in the presence of a polymeric stabilizer. It is a kind of precipitation polymerization, so both the monomer and the initiator are soluble in reaction medium (solvent), but the resulting polymer is not soluble (precipitates out). As the polymerization continues, polymer forms the particles, forming a non-homogeneous solution. These particles are locus of polymerization in dispersion polymerization. The monomer is continuously provided to the particles during the whole process. It is identical to emulsion polymerization in this regard (the polymer formation and growth has same features). It is different to precipitation polymerization, where the polymerization is carried out in the continuous phase (solvent solution) rather than particles.

Upon reaching a critical molecular weight, the polymer precipitates in the form of particles. The initially formed particles are not so stable; hence forms the aggregates until the formation of stabilized particles. Further monomer is supplied to the stabilized par-
ticles. Stabilizer attached to surface of the particles as they get bigger in size. Commonly, one end of the stabilizer copolymer has an affinity for the solvent while the second has the affinity for the polymer particle being formed. Mostly graft or block copolymers have been used as stabilizers. These copolymers are quite important in dispersion polymerization because they inhibit the aggregation by forming a hairy layer around the particles [130]. The particles remain separated from each other due to steric hindrance between the outward-facing tails of the stabilizer layers [130].

Dispersion polymerization has been applied to prepare stable colloids in different media[131]. Anionic dispersion polymerization of styrene has been reported [132]–[134]. Although it produces monodisperse particles but the procedure is rather difficult to handle. Free-radical dispersion polymerization of methyl methacrylate [135] using different kinds of polymeric stabilizers including comb [136], [137], diblock [138]–[140] and triblock [141] has been studied. However, these methods lead to high polydispersity making the particles unsuitable for use in photonic crystals and biological applications where monodispersity is very crucial.

We present new one-step condensation dispersion polymerization affording monodisperse conjugated polymer particles using Suzuki-Miyaura[142], [143] and Sonogashira coupling reactions [144]. The method can be employed to produce particles of various conjugated polymers, such as polyfluorene, poly(fluorene-alt-diacetylene) and poly(fluorene-alt-azobenzene). Moreover, tunability of particle size and shape, emission spectrum and surface functionality is easily accessible. Our method opens the new routes for the synthesis of highly monodisperse particles of various polymers which have not been achieved so far. The reaction mixture is homogenous at the start i.e. monomers, initiator, stabilizer are soluble in solvent. The growth of polymer chains occurs, once the polymerization is initiated by the initiator. Upon reaching a critical chain length, particles nucleate through phase separation of the polymer from the solvent[130].

1.8 Recent functionalizations strategies

Polymeric particles prepared from different synthetic routes normally lack functionality on their surface. Functionality on particle surface allows attachment of suitable biomolecules for specific labelling, targeted delivery, and sensitive detection. In addition, it also increases the solubility of the particles in water and improves colloidal stability. The development of numerous functionalization techniques has made it possible to functionalize polymeric particles with different therapeutic agents. The surface modifi-
cations have so far been conducted through adsorption, functional surfactants, emulsification, encapsulation, polymerization, covalently bound functional molecules and various forms of bio-conjugation. For example, encapsulation is the most widely used strategy for functionalization of nanoparticles. Functionality in the conjugated polymer nanoparticles can be inserted by encapsulating the CPNs in functional material such as silica [93], phospholipids [145]-[147] and PLGA polymer [49], [148].

Silica encapsulation has been most greatly used for functionalization of nanoparticle surface [2], [91], [149]. Wu et al. produced nanoparticles of size 10-20 nm with 2-3 nm thick silica shell to incorporate functionality [93]. Green et al. encapsulated quantum dots in phospholipids by using another strategy (miniemulsion) [145]. In another approach, Li and colleagues used PLGA to prepare nanoparticles with the intention of using –COOH functional group of matrix to attach biomolecules. The particles fabricated by using miniemulsion method and loaded with conjugated polymers were extensively used for cellular labelling [49], [148]. Dynamic light scattering revealed bigger size of the PLGA particles loaded with conjugated polymers and the phospholipid encapsulated particles, which restricts their use in many cellular and subcellular targeting applications. A major drawback of encapsulation method is that it reduces the per particle brightness of the particles. Therefore, an alternate method to fabricate nanoparticles with densely packed chromophore is highly needed.

The addition of a functional surface is quite critical for bioconjugation and specific labeling of cellular targets. To that end, Chiu et al. produced numerous methods to incorporate functional moieties in CPNs for bio conjugation [101], [112], [150]. One approach is the association and trapping of amphiphilic polymer chains with functional groups. For example, nanoparticles prepared from the PFBT and with different fractions of amphiphilic comb like polystyrene polymer (PS-PEG-COOH) with carboxyl functional group resulted in functional particles [151]. The carboxyl functional groups are easily available for further attachment of biomolecules by using standard chemistry like carbodimide crosslinking.

However, both encapsulation and association of amphiphilic polymer relies on non-covalent bonding. The functional groups can be readily dissociated due to swelling in high ionic strength solution. So, there is another method based on covalent linkage of functional groups. Chiu et al. used this approach successfully and produced nanoparticles where the covalently bonded functional groups were introduced in polymer chain
before preparing particles from them [101]. This method is known as direct functionalization as no additional step is required to introduce functional moieties.

The polymeric particles are successfully decorated with a wide variety of functional moieties e.g. amine, nitriles, disulfides, thiols etc., [152]–[157] depending upon their applications. The functionalization of particles means reshaping their surface with other special moieties depending upon their application. Functionalization changes the physiochemical character of the particles. Figure 1.9 exhibits numerous functionalizations routes of the nanoparticles for smart drug delivery systems (left side), and a pre-engineered nanoparticle finding tumor cells instead of the healthy ones (right side).

![Diagram](image)

**Figure 1.9:** Schematic diagrams representing the various functionalization pathways of the nano-engines for smart drug delivery systems. Here, the pre-regulated nanoparticle recognized the tumor cells not the healthy ones. Reproduced from [77].

1.9 Uptake of particles by cells

Cell membranes does not allow larger particles to pass through them [158], [159]. They are permeable to particles only if particles have a size from 10 nm to 30 nm [160]. The cell membrane acts as a barrier between external environment and inside of the cells. This barrier can be overcome with the help of two mechanisms i.e. endocytosis and diffusion. However, uptake of particles by cells is influenced by different physical and chemical properties of specific nanoparticles (NPs) (Figure 1.10). These properties include nanoparticle size [75], [97], [161]–[164], surface charge [165], shape [166]–[168],
and nanoparticles hydrophilicity [169], [170]. Here the more important surface properties discussed as they determine the protein corona and thereby possibly the biological impacts [171]. Cellular uptake is also affected by experimental conditions (cell type, aggregation, and opsonization).

![Figure 1.10: Determinants of nanoparticles interactions with cells as determined by experimental conditions (presence of protein and opsonins): shape (spheres, short-long rods, cubes and triangles with different aspect ratios), cell type, size, surface chemistry, and addition, addition of ligands. Reprinted from [160].](image)

Nanoparticles between 20 nm and 50 nm in size are taken up by nonphagocytic cells at fastest rates (109-112). On contrary, phagocytic cells allow internalization of particles between 2 μm and 3 μm [160]. Cells ingest preferentially charged particles as compared to their uncharged counterparts. In case of charges groups on the surface, cationic particles are taken up at faster rate than respective anionic particles. Presence of negative carboxyl groups (-COOH), neutral hydroxyl group (-OH), and positive amine group (-NH₂) on particle surface accelerate the cellular uptake. Similarly, ligands like mannosyl, galactosyl, immunoglobin, or fluoronectin lipoprotein increase particle ingestion by interaction with phagocyte surface receptors [172].
Several non-antibiotic therapeutic approaches have been reported as alternatives to antibiotic treatment for *Clostridium difficile* (*C. difficile*) associated disease (CDAD). These approaches include intraluminal toxin neutralization, immunotherapies and biotherapeutic approaches [173]. The rationale for toxin neutralization is to provide materials with toxin binding sites to inhibit conjugation of toxin to its natural receptor in gastrointestinal (GI) tract, and facilitate their removal from the body. This approach is different from the antibiotic approach since it targets the toxin instead of *C. difficile* as with the current antibiotics treatments. Antibodies, cow’s milk whey protein from *C. difficile* immunized cows, and synthetic polymers were used as neutralizing agents *in vitro* and *in vivo* [174]–[177]. Most studies performed with antibodies and proteins were inconclusive. The main problem using proteins as neutralizing agents is their susceptibility to acid denaturation and enzymatic digestion before they reach their target site. Additionally, they should afford high alkaline stability, once reaching human gut. Tolevamer (Genzyme), an anionic polymer (sodium salt of poly(styrene sulfonate), reached clinical phase-I,II,III trials [178], [179]. Although it was inferior to standard metronidazole or vancomycin treatment. However, patients treated with tolevamer showed significantly lower relapse rates. This proved that it is promising to target the toxin while restoring the normal flora [173], [179]. However, tolevamer could not pass in clinical phase III which may be due to the insufficient stable binding of the toxins by tolevamer in the human gut [180]. Cholestyramine and cholesterol, cationic polymers have also been tested in humans. Although they bind the toxin, they were not recommended for treatment due to their low therapeutic efficiency.

Although, inferiority of previous attempts using polymers is unclear, microgels can overcome the hurdle, since they offer following advantages for specific uptake of bacterial toxins over non-functionalized polymers used previously:

They have high water content and elicit no immunogenic reaction. They are tunable in size so can efficiently remove toxin without uptake by the intestinal mucosa. They have a three dimensional network with a large surface area, which facilitate the conjugation of toxin binding groups. They can be fabricated to enable multivalent interactions between the toxins and toxin binding groups allowing more efficient scavenging of toxins.
1.10 Microgels

Another category of polymeric colloids with remarkable properties are microgels. They have been used as therapeutic drug carriers and diagnostic agents in biology and medicine. They are cross-linked polymeric networks. They are referred as hydrogels if they are made in bulk of hydrophilic polymers. They are biocompatible, contain a huge amount of water and their mechanical properties can be controlled according to the requirement. Their size can be tuned, biomolecules can be incorporated providing a large surface area for multivalent bioconjugation, which are the key factors in biotechnological applications. They swell when immersed in water.

1.10.1 Microgel Synthesis

A gel is a type of soft matter, which consists of cross linked polymers that are swollen with a medium. If the gel size lies in micron- or submicron then it is called as microgel. Microgels are found to be environmentally responsive systems and have been used as carriers for therapeutic drugs and diagnostic agents. Microgels are referred to as hydrogels if they composed of hydrophilic polymers. They are water soluble with different properties from linear molecules having same molecular weight.

![Structural formulae of some polymers employed in microgels.](image)

**Figure 1.11:** Structural formulae of some polymers employed in microgels.

Microgels can be imagined as “hard spheres” if the gel is immersed in a non-solvent. For example, polystyrene microgels have been applied for the investigation of the melting properties of a crystal [181]. They are termed soft materials if the sphere ball contains huge amount of solvent (90 %). Hydrophilic microgels are effective as drug deliv-
ery systems and release carriers. The microgels endowed with thermal or other sensiti-
vity are depicted as smart materials. For example, pH-responsive microgels are respon-
sive to tumor cells and will shrink and release the drug only at the position of the tu-
mor due to decreased pH. Colloidal stability is an additional characteristic of micro-
gels. In general, microparticles have high surface energies due to their large surface ar-
 ea. So they tend to aggregate to lessen their energy making applications in biomedical
systems difficult. The surface energy is mainly due to three forces i.e. electrostatic rep-
pulsive forces, Van der Waals attractive forces and steric repulsive forces. In case of
 microparticles, a high surface charge or tethered polymer chains is required to favor
 repulsion between particles to achieve colloidal stability. So microparticles have to be
tailored with acidic or basic groups or long soluble surface bound polymers for this
purpose. However, these additional functionalities are not essential in microgels where
stability comes from the steric repulsive forces between the polymeric chains pointing
out of the particles. Aggregation of microgels is highly unlikely due to interactions be-
tween polymeric chains of two adjacent particles producing a locally increased osmotic
 pressure, which disassociate the particles. Moreover, their conformation freedom is
greatly reduced due to overlapping of the polymer chains, which provoke an adverse
loss of entropy. Microgels also possess many other distinct features. They exhibit a
larger specific surface area and higher surface reactivity as microparticles. They are
viscous and elastic upon deformation. Eventually microgels are highly versatile and can
be prepared with immense variety of chemical composition, surface epitopes, size and
shape. Different kinds of microgels such as interpenetrated polymer networks (IPN),
hollow and core-shell microgels or Janus particles with specific properties have been
prepared (Figure 1.12) [182], [183].

![Diagram of microgels](image)

**Figure 1.12:** Several commonly available networks of microgels: a. Typical three dimensional structure from one polymer. (b) Interpenetrated network. (c) Hollow (d) core-shell microgels and (e) Janus particles. Reproduced [184].

The target of microgel synthesis includes control over the particle size distribution,
stability of colloids, and the position of distinct functional groups such as cross linker,
charged groups, or reactive sites for further functionalization. Microgels can be synthesized from three possible starting points

- **Monomer.** It is the most frequent approach. Monofunctional monomers can be non-ionic, cationic and anionic. (bottom up)
- **Pre-Polymer.** Aqueous solution of polymer can be emulsified in oil and chemically cross-linked. In another route, colloidal polyelectrolyte complexes can be formed by mixing oppositely charged polymers in dilute solutions. (bottom up)
- **Macrogels.** It has as well being reported that macrogels can be mechanically disintegrated to produce microgels [185]. (top down)

Bottom up microgels synthesis can be divided into two classifications depending on the particle formation mechanisms i.e. physical or chemical cross-linking [186]. Physical methods consist of processing of existing polymers into particles by cross-linking via hydrophobic interactions, e.g. by phase separation and spray drying, or via ionic interactions. Whereas cross-linking emerge from the heterogeneous polymerization of monomers in chemical method, [187], [188]. Different types of polymerizations are conducted such as precipitation, suspension or emulsion polymerization (Figure 1.13).

![Figure 1.13: Principle of different types of polymerizations. Reproduced from [184].](image)

In precipitation polymerization, both the monomer and initiator are soluble in the continuous phase but the resultant polymer is highly insoluble and precipitates out. In suspension polymerization, stabilization of the monomer and initiator occur inside the droplets of an emulsion. This can be achieved through mechanical agitation. Polymerization takes place inside the droplets resulting in the microgels fromation. In case of emulsion polymerization, polymerization occurs within the latex particles forming spontaneously in the fist few minutes in the continuous phase, are fed from the monomer contained in the droplets of an emulsion (reservoir). These latex particles are formed from individual polymer chains and have a size of about 100 nm. The
surfactant prevents the coagulation of the particles by surrounding them. Normally, emulsion polymerization is considered to be better than precipitation or suspension polymerization because it produces particles with narrow size distribution. Microgels are being heavily used in the surface coatings [189]. Microgels show promise in enhanced oil recovery. Their main function is to absorb water [189]. Functionalized microgels found their use in sensing and separation due to their high surface areas and easy surface functionalization strategies. They have been applied in various chemical reactions e.g. catalysis, polymer synthesis and synthesis of nanoparticles. Microgels are considered as ideal candidates for biotechnology biosensing, bioseparation and drug delivery.

Conventional synthesis techniques such as emulsion polymerization [190]–[193] and surfactant free emulsion polymerization [190], [194], [195] help to produce microgels with low polydispersity, but the size of the particles does not usually exceed one micron. Fabrication of microgels having size in several microns is quite challenging and demanding. However, recent developments in droplet microfluidics permit the production of microgels with very low polydispersity and bigger size (several microns)[196]–[198].

1.10.2 Microfluidics

Microfluidic devices can be simply defined as networks of micron-scale channels that are joined together to execute functions. The typical functions can be divided into two main classes. In one class, devices perform chemical and biological assays. Cells, beads, and other reagents are delivered to the device, and the channels merge, mix, and split them, as required for the reaction. This way, various chemical and biological reactions can be performed with great precision, for combinatorial chemical screens, proteomics studies, genetic sequencing, and directed evolution [199]–[201]. In the second class, devices are used to produce monodisperse droplets [202], [203]. Solutions of monomers or crosslinkable polymers are injected and emulsified in the devices, by an immiscible carrier phase. The micro-droplets can later on be solidified by polymerization, crosslinking, or crystallization resulting in solid mono-disperse microparticles.

The main advantage of microfluidic synthesis is that the final particle design is dictated by the fluidics irrespective of the solutions injected to the device. For example, a device which produces anisotropic particles can do so with numerous fluids. This is a significant edge over traditional bulk synthesis approaches. It allows the use of not only
different kinds of materials, but also a variety of structures can be achieved successfully; such as monodisperse spheres, anisotropic magnetic hydrogels, non-spherical Janus particles, and core-shell particles.

Currently, two major types of microfluidic devices are being used for particle synthesis i.e. glass capillary devices and lithographically fabricated poly(dimethylsiloxane) (PDMS) devices. Glass capillary devices offer many distinct advantages for fabricating particles, including higher resistance against chemicals and an excellent coaxial flow focusing geometry, allowing the production of particles with a wide variety of composition and structures. By contrast, PDMS devices offer some other unique advantages for certain application. These devices can be replicated in large numbers, which make them ideal candidate for large scale synthesis applications.

1.10.3 Glass capillary devices

Glass capillary devices can be used to produce single emulsion, double and triple emulsion.

1.10.3.1 Single emulsion particle templating

The first step to produce particles with microfluidic devices is to make equally sized droplets. It can be understood using the example of a water faucet. The water comes out drop by drop, if we switch on the faucet at a low flow rate. Droplet size is the outcome of a balance between weight and surface forces. It is controlled mainly by faucet size and surface tension of fluid. The drops show an unparalleled narrow size distribution because both the surface tension of the fluid and the faucet remains constant throughout the process. A thin water stream or a jet is the result of higher flow rate at the faucet. Although the jet also rips off into drops in the end, they show a larger size distribution [202], [203]. Microfluidic drop maker devices with cross-flow or flow focusing geometries work on the same principle.

Glass capillary devices can be used to produce drops of one liquid in another immiscible liquid with extraordinary narrow size distribution. They are coaxial assemblies of glass capillaries. Their wettability is simply and efficiently governed by a chemical reaction with a silane based suitable surface modifier as they are constructed from glass. For example, octadecyltrimethoxysilane can make the surface hydrophobic, whereas 2-[methoxy(poly-ethylenoxy)propyl]trimethoxysilane can be used to obtain hydrophilic surface. Since glass is both chemically resistant and rigid, these devices allow a truly three-dimension flow, which is quite important for some applications.
One cylindrical capillary is tapered using a micropipette puller in a conventional microfluidic single-emulsion device. The desired diameter is achieved by polishing the tip of the capillary on a sand paper. Afterwards, the round capillary is inserted into a square capillary. Particles with a narrow size distribution can be effortlessly generated from the device. This is called as coflow geometry [204], [205] (Figure 1.14).

Figure 1.14: Schematic representation of a microfluidic device for producing droplets based on coflow geometry. The flow direction of fluids and drops is shown by arrows. Reprinted from [204]

Individual monodisperse drops results from slow flow rates of fluids at the tip of the capillary orifice as depicted in Figure 1.15. a process called dripping.

Figure 1.15: A bright field image of making drops at slower flow rates (dripping regime). Reprinted from [204]

A jet is the result of increasing the flow rate of any fluid after a certain limit as depicted in Figure 1.16. The resulting drops will definitely have a larger size distribution because the drop formation point changes with each drop.
Figure 1.16: A bright field image of a thin jet triggered by pushing the flow rate of the outer phase beyond a certain limit by keeping the flow rate of the inner fluid constant. (b) A bright field image of a thin jet triggered by pushing the flow rate of the inner phase beyond a certain limit by keeping the flow rate of the outer fluid constant. (c) A bright field image monodisperse droplets produced from a microcapillary device. Reprinted from [204].

An alternative to the coflow geometry is the flow-focusing geometry [205], [206]. The two liquids are injected from the two the opposite sides of the same square capillary in flow-focusing device. The inner fluid is hydrodynamically focused by the outer fluid through the narrow orifice of the polished round capillary as depicted in Figure 1.17. The drops are formed when the inner fluid enters the circular orifice. However, high flow rates will also result in downstream causes jetting. A main advantage of the flow-focusing geometry is that it allows small size particles (1-5 μm) with very low polydispersity. It is superior to coflow-geometry especially in case of particulate suspension, where the particles may
Clog the orifice [207]. A large size orifice lessens the possibility of such clogging by the suspended particles or any entrapped debris. Although, the limit for the droplet diameter in the co-flow geometry is roughly the diameter of the orifice. In the flow-focusing geometry the droplet size may be smaller than orifice of the round capillary.

1.10.4 PDMS Microfluidics

1.10.4.1 Introduction

The microfluidic devices expressed so far are made by manually aligning and gluing the microcapillary tubes together. These devices present excellent flow properties especially for templating the double emulsions. However, they have a drawback also. It is not easy to produce many identical devices at the same time since they are constructed manually. Additionally, the nature of fabrication and shaping of the capillary tips by heating and stretching is difficult to reproduce with the same precision. Therefore, it is tremendous work to fabricate identical copies of these devices.

There is an alternative process to construct microfluidic devices, which is based on photolithography and replication in poly(dimethylsiloxane) (PDMS) [198]. It is very precise and allows construction of devices down to a micron in width. Small channels can be constructed, which are ideal for the synthesis of small sized particles and microgels. They also possess greater flexibility during their design, which allows the production of new kinds of structures.

1.10.4.2 Fabricating PDMS devices using photolithography

The basic concept in photolithography is to make microfluidic channel devices from 2D- drawings. First, a drawing of the required device is printed on a transparency slide called a photomask. The photomask are all black except those regions, which need to become channels. A thin layer of photoresist is then expressed through this mask. The height of this photoresist layer governs the height in the PDMS channel. This process is described in the following:
A photoresist generally SU-8, is used to coat the silicon wafer (a few inches in diameter). This can be obtained by simply pouring a glob of the resist on the wafer and spinning it at very high speed. The thickness of the coating can be adjusted to a micron precision depending upon the viscosity of the photoresist, and the spin rate and duration. It is very vital because height of the final microfluidic device is dependent on this thickness. The solvent is removed from the coated wafer by heating and then the coating is solidified by subsequent cooling. The coating can be removed with a proper solvent until this point. The photomask is put on the coated wafer and both are subjected to ultra-violet (UV) light. The transparent regions of the photomask allow the light to pass through, whereas black parts do not. The photons trigger radicals in the photoresist, which cross-link these regions making them hard. The remaining photoresist can be removed by heating and then washing the wafer with a proper solvent. So only the cross-linked parts are left, which are inverse of photoresist. This makes “positive” channels.

The actual PDMS microfluidic device is prepared by pouring Poly (dimehtylsiloxane) (PDMS) onto this “master”. It is baked in an oven, which makes PDMS hard, clear and rubbery material. The solid PDMS is then removed with a suitable scalpel. The resulting block is imprinted with negative features of the SU-8 channels, forming the PDMS micro-channels. Holes are made in the block and the imprinted side is fixed onto a flat surface (either a glass slide or another block of PDMS) thereby enclosing and sealing the channels. The fixing is done by activating the surfaces with an oxygen plasma. When meeting the PDMS block with a glass slide the channels are closed and irreversible Si-O-Si bonds are formed between the PDMS and glass slide. The device is ready to use at this point (the fluids can be injected). Often post-treatment is required to control the surface properties of the channels. Wettability of the device is very important for making emulsions. PDMS allows water-in-oil emulsion by default due to its hydrophobic nature with move solvents. To make it more hydrophilic, it can be treated with Aquapel. Sol-gel and polymer coatings have also been successfully used to make the device chemically resilient.

1.10.4.3 Single emulsion drop formation

“T-junction” drop maker is the first geometry used for PDMS microfluidics [208]. The name “T-junction” because the two channels intersect to form a T-shape. The dispersed phase is introduced from the side channel and the continuous phase from a vertical channel. The drops result at a point where the two channels meet as depicted in Figure 1.18 A.
Figure 1.18: Schematic representation of two typical PDMS microfluidic devices used for formation of drops. (a) The internal phase is inserted from left channel and the outer phase from top channel in case T-junction geometry. Both fluids are injected into nozzle channel, where they meet to form drops. (b) The inner phase is inserted downward from a central inlet, the outer phase from two side inlets in flow-focus geometry. Monodisperse droplets can be produced from both the geometries. The droplet size can be controlled by varying the flow rates only. Reprinted from [204].

Here the physics of drops varies as a function of flow rate. Surface tension is stronger than viscous forces at slow flow rates (<0.01), so that drops formation occurs due to plugging and squeezing [208]: The enlarged tip of the dispersed phase blocks the downstream nozzle, pressing the path of the continuous phase, which is continuously pumped in; this causes a pressure rise in the continuous phase that, in turn, squeezes the on the disperse phase, finally making a drop. In case of high flow rates, when viscous forces become comparable to surface tension (Ca > 0.01), shearing forces starts acting As the dispersed phase goes into the nozzle, it is sheared by the drag of the continuous phase. A drop results when this shear force is equivalent to the tensile force. Monodisperse drops with controllable size can be produced by using this geometry irrespective of the drop formation mechanism used.

Another geometry which is being heavily used to produce drops is the so-called “flow-focus” geometry. This geometry contains many variants but the basic structure is the same for all, in which two channels intersect to result a four-way cross as depicted in Figure 1.18 B. The dispersed phase is introduced from the central inlet and the continuous phase from the two side inlets. The drop is produced at a point in the nozzle where two phases meet each other. The drop size can be adjusted by controlling the flow rates like in the T-junction.
In practice both T-junction and flow focusing show similar behavior, though there are solid differences between them, especially with respect to size and production rate of drops for a given set of flow rates. Additionally, T-junction is better suited for producing monodisperse particles at lower flow rates due to the single continuous phase, whereas flow focus-junction produces monodisperse particles at higher flow rates.

1.10.4.4 The dominance of wetting in PDMS devices

The wettability of the channels plays a very vital role in deciding the types of drop formed irrespective of the geometry of the device used. For example, whether oil-in-water or water-in-oil drops formed. When the disperse phase is injected into the device, it firstly comes in contact with the upper and lower surfaces of the channels. To result into a drop, it must be taken off the surfaces and surrounded by the continuous phase. It depends on the wettability properties of the channels whether this happens. For example, in the case of hydrophobic channels, water will be lifted off the walls by the oil, resulting in the water droplets. By contrast, if the hydrophilic channels are used, then water sticks to the walls, lifting off the oil, forming oil droplets. Wetting is so vital in these devices that even if the inlets for the oil and water are interchanged, drops the same type are produced.

However, there are some ways to relax these restrictions. Either type of drops can be formed in a device with fixed wettability with the help of a combination of geometric control and shear.

The following steps are taken in this thesis to address the resulting problems

1. Development of bright conjugated polymer particles with switchable units (fluorescence ON/OFF) for potential use in super resolution microscopy.


3. Porous, high capacity colloids with toxin recognition for non-antibiotic treatment of toxin infection.

The colloidal entities become more and more complex by addition of functionality: fluorescent and switchable → fluorescent and biorecognition → biorecognition, porous and uptake
1.11 References


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Chapter 2

Light-Switchable and Monodisperse Conjugated Polymer Particles

This chapter has been published as:

Abstract

In this chapter, a novel class of light-responsive particles for fluorescence modulation at low switching doses is described. The monodisperse conjugated polymer particles with incorporated light-switchable azobenzene moieties are prepared by Suzuki-Miyaura dispersion polymerization and exhibit phase-changing and shape-shifting behavior. The influence of the molecular structure and the *trans-to-cis* switching behavior on the photonic performance are investigated. Such polymer particles present ideal candidates for responsive organic photonics and as super resolution for biomedical applications.
2.1 Introduction

Light-responsive moieties are an excellent tool to switch the physical properties of a material system. Azobenzenes are a well-known class of light responsive units. The switching behavior of azobenzenes is well understood [1]. They undergo a conformational transition i.e. *trans*-to-*cis* upon excitation with ultraviolet (UV) light. As a result, alteration in the molecular as well as electronic structure occurs. The azobenzene moiety can be brought back to its original trans-conformation either thermally or by illuminating the system to radiation of the green to yellow spectrum. Azobenzenes have been extensively used in light-responsive films [2], encapsulants [3], and surfactants [4]. In the latter, switching from *trans*-to-*cis* produces a change in the whole molecular structure. As such, switchable surface active agent in the *cis*-state cannot reduce the interfacial energy as well as in *trans*-state so emulsions and encapsulants disintegrate. *Trans*-to-*cis* switch prompts changes in refractive index and absorption, so this makes the switchable materials a powerful candidate for use in biological markers [5] and photonic systems [6]. The change in absorption and refractive index causes the fluorescent enhancement or quenching by Förster Resonant Energy Transfer (FRET) [5], [7]. Switching mechanism of the azobenzene moieties within the π-conjugated polymers has been investigated exclusively in solution [5], [7]–[10]. The absorption and fluorescence spectra can be designated depending upon the interaction of the switchable moieties with the conjugated polymer backbone. The *trans*-to-*cis* switch can either enhance or diminish fluorescence depending on the electronic donor/acceptor characteristics of the comonomer and its bandgaps. Recently, conjugated polymer particles with switchable moieties have been prepared, which can be employed for highly sensitive bio imaging techniques. Particles with switchable moieties have been used explicitly for staining of membrane proteins in cells [5]. The fluorescence signal of the polymer particles can be modulated by UV illumination which will allow elaborated performance in the super-resolution microscopy. However, these conjugated polymer particles were highly polydisperse because they have been synthesized by emulsion processes. Consequently, photonic effects cannot be exploited [11]. Monodispersity is quite crucial for photonic effects because they arise when monodisperse particle self-assemble into periodic structures. Additionally, polydisperse particles are less specific when applied as markers and probes in biological systems. Monodispersity plays a vital role in processes like endocytosis and diffusion, which are size-dependent. It will be quite difficult for large fraction of polydisperse set of particles to enter and stain cells effectively [12]. Recently, the synthesis of highly monodisperse conjugated polymer particles through dispersion
polymerization has been published [13]. Monodisperse conjugated polymer particles functionalized with switchable moieties would facilitate the synthesis of responsive and bistable photonic devices. Self-assemblies of these particles would act as switchable photonic crystals. Fluorescence modulation will allow high sensitivity when these particles are applied as marker in biological system. Furthermore, these conjugated polymer particles will exhibit less bleaching as compared to single molecule emitters. They are not cytotoxic and do not show blinking in contrast inorganic quantum dot probes [14]–[16]. As such, conjugated polymer particles with switchable moieties can be considered as ideal candidate for a novel class of fluorescent biological markers.

2.2 Results and Discussion

Three different kinds of monodisperse conjugated polymer particles were produced with successful incorporation of the azobenzene moieties inside. Particles of alternating fluorene-alt-azobenzene polymers were produced by applying Suzuki-Miyaura dispersion polymerization. The particle size could be easily controlled and trans-to-cis switching resulted in fluorescence enhancement.

Three different kinds of switchable conjugated polymer particles were obtained by applying fluorene diboronic acid ester together with dibromo azobenzene derivatives. Tetrakis(triphenylyphosphine) palladium (0) was used as catalyst, potassium tert-butoxide as a base and 1-propanol a solvent, as depicted in scheme 1. The conjugated polymer precipitates into nuclei upon reaching a critical chain length. These nuclei are then stabilized by Triton X-45 and poly(vinylypyrrolidone-co-vinyl acetate) (PVPVA) [13]. Subsequently, more polymers precipitate and condense onto the existing nuclei. All nuclei grow at the same rate to form monodisperse particles [17]. 1-propanol was used as a solvent because low molecular alcohols were found to be good solvents for conjugated monomers and at the same time non-solvent for the respective conjugated polymers. Additionally, Tetrakis(triphenylphosphine) palladium (0) and potassium tert-butoxide are soluble in 1-propanol. They are not readily soluble in methanol or ethanol. So 1-propanol was used, which provides an excellent reaction medium. A solution of potassium tert-butoxide in 1-propanol was prepared and degassed properly before injection into the reaction flask. To obtain monodisperse particles a suitable stabilizer had to be selected to prevent particle from aggregating (polydispersity).
Scheme 2.1: Suzuki-Miyaura dispersion polymerization of three types of switchable colloids: Incorporating of the Azobenzene unit along the polymer backbone (P1), Perpendicular to the polymer chain (P2), Electronically decoupled from the polymer backbone by means of an alkyl-spacer (P3).

As a stabilizer PVPVA was found to be optimal. The generated monodispersity could be enhanced by using a non-ionic stabilizer Triton X-45. Furthermore, mixing conditions always play a vital role for making monodisperse particles. In this approach combination of mechanical stirring and low-intensity sonication worked very well. Only mechanical stirring or overhead tumbling was not sufficient to achieve sufficient mixing to obtain monodisperse samples. The reaction flask was heated to 70 °C, which was just above the Tg of the resulting conjugated polymer, consequently the surface tension between the polymer and solvent ensured sphericity of resulting colloids.

2.2.1 Synthesis of monomers

Three different monomers were used namely 4,4′-dibromoazobenzene, 3,5-dibromobenzene and 1-(4-((6-(3,5-dibromophenoxy)hexyl)oxy)phenyl)-2-phenyldiazene. 4,4′-dibromoazo-benzene was commercially available. However, both 3,5-dibromobenzene and 1-(4-((6-(3,5-dibromophenoxy)hexyl)oxy)phenyl)-2-phenyldiazene were synthesized in the lab according to literature reports due to commercial unavailability [18], [19].
2.2.2 Synthesis of 3,5-dibromobenzene

\[
\text{NH}_2 \quad \begin{array}{c}
\text{Br} \\
\text{Br}\end{array} \quad + \quad \text{NO} \quad \xrightarrow{\text{CH}_3\text{COOH}} \quad \begin{array}{c}
\text{Br} \\
\text{Br}\end{array} 
\]

**Scheme 2.2:** Schematic of synthesis of 3,5-dibromobenzene.

3,5-dibromobenzene was synthesized according to Mills reaction. Nitrosobenzene was added to a solution of 3,5-dibromoaniline in acetic acid. The resulting mixture was stirred for one day. After washing several times with water and acetic acid, the orange precipitate was dried in vacuo (10 mbar). 3,5-dibromo was obtained in the form of an orange solid. The yield was reasonable (62%). After completely drying the orange solid, the compound was subjected to analysis including NMR spectroscopy.

2.2.3 $^1$H-NMR of 3,5-dibromobenzene

**Figure 2.1:** $^1$H-NMR of 3,5-dibromobenzene.

Additional peaks due to aromatic protons ArH (4-7, 8) were expected in $^1$H-NMR of 3,5-dibromobenzene. So, the presence of peaks at 7.53 ppm and 7.91 ppm confirmed our product.
2.2.4 Synthesis of 1-((6-(3,5-dibromophenoxy)hexyl)oxy)phenyl)-2-phenyldiazene

1-((6-(3,5-dibromophenoxy)hexyl)oxy)phenyl)-2-phenyldiazene was synthesized in a two-step process. Firstly, 1,3-dibromo-5-((6-bromohexyl)oxy)benzene was synthesized, which was further reacted with 4-phenylazophenol to produce the desired product. In the first step, 1,3-dibromo-5-((6-bromohexyl)oxy)benzene was synthesized by following the procedure from literature [10].

![Scheme 2.3: Schematic of synthesis of 1-((6-(3,5-dibromophenoxy)hexyl)oxy)phenyl)-2-phenyldiazene.](image)

3,5-dibromophenol (10.22 mmol) and 1,6-dibromohexane (21.08 mmol) were dissolved in 30 mL toluene. The reaction was done by using 7 mL of a strong base NaOH solution (aq. 50 wt%). Tertiary butyl ammonium bromide (1.04 mmol) was used as a phase transfer catalyst. The resulting mixture was heated to 80 °C under inert atmosphere for one day. The product was purified by using column chromatography using Hexane/CH$_2$Cl$_2$ as a mobile phase and silica gel as the stationary phase. After completely drying the white solid, the compound was subjected to NMR analysis.
2.2.5 $^1$H-NMR of 1,3-dibromo-5-((6-bromohexyl)oxy)benzene

![NMR Spectrum]

Figure 2.2: Comparison of $^1$HNMR of 1,3-dibromo-5-((6-bromohexyl)oxy)benzene with 1,6-dibromohexane.

Additional peaks due to aromatic protons ArH at 7.1 ppm and 7.2 ppm are quite clear in $^1$H-NMR spectra. So, the $^1$H-NMR confirmed the product.

In the second step, 1,3-dibromo-5-((6-bromohexyl)oxy)benzene (3.20 mmol) was reacted with 4-phenylazophenol (3.27 mmol) and tetrabutylammonium bromide (0.29 mmol) were dissolved in 30 mL toluene and degassed for 20 min by bubbling with argon. 6 mL of NaOH solution (aq. 20 wt%) were added. The resulting mixture was heated to 80 °C under inert atmosphere for one day. The product was purified by using column chromatography using Hexane/CH$_2$Cl$_2$ as a mobile phase and silica gel as the stationary phase. After completely drying the white solid, the compound was subjected to NMR analysis.
Scheme 2.4: Schematic synthesis of 1-((6-(3,5-dibromophenoxy)hexyl)oxy)phenyl)-2-phenyldiazene.

2.2.6 $^1$H-NMR of 1-((6-(3,5-dibromophenoxy)hexyl)oxy)phenyl)-2-phenyldiazene

Figure 2.3: $^1$H-NMR of 1-((6-(3,5-dibromophenoxy)hexyl)oxy)phenyl)-2-phenyldiazene.

Molecular weights of all three types of particles were determined via gel permeation chromatography. The molecular weights determine the chain length, upon which the polymer precipitates to form particles. Gel permeation chromatography was performed in chloroform. Reasonably high molecular weights of up to 50 kDa were achieved[20]. The molecular weight of P1 and P2 was about 13 kDa. This is due to the fact that they have rigid side groups. However, critical molecular weight of P3 was about 50 kDa (Table 2.1). This was higher than P1 and P2 because P3 has
<table>
<thead>
<tr>
<th>Entry</th>
<th>$M_w$(Da)</th>
<th>PDI</th>
<th>$\lambda_{\text{max}}^\text{trans}$ (nm)</th>
<th>$\lambda_{\text{max}}^\text{cis}$ (nm)</th>
<th>$D_{\text{PSS}}$ nJ/cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>13423</td>
<td>2.26</td>
<td>444</td>
<td>612</td>
<td>41</td>
</tr>
<tr>
<td>P2</td>
<td>13088</td>
<td>1.14</td>
<td>421</td>
<td>545</td>
<td>22</td>
</tr>
<tr>
<td>P3</td>
<td>49622</td>
<td>2.65</td>
<td>397</td>
<td>528</td>
<td>32</td>
</tr>
</tbody>
</table>

**Table 2.1:** Properties of the switchable conjugated polymer particles.

more flexible side groups, probably increasing solubility and facilitating higher. It can produce much higher molecular weights before polymer chains precipitate into nuclei during dispersion polymerization. The polymerization follows a polycondensation mechanism; however, a PDI of below 2 was not surprising, considering that the molecular weight distribution will have a sharp cutoff toward higher values due to precipitation of the polymer chains at the critical molecular weight for solubility. **P1 and P2** showed higher PDI values as compared to **P3**. This may be due to variations in solubility, the azobenzene monomer purity, or the residual PVPVA stabilizer polymer during GPC analysis. The PDI values were not much different from those which were reported previously during Suzuki-Miyaura dispersion polymerizations [13].
Figure 2.4: SEM images of (a) narrowly dispersed platelets composed of P1, (b) monodisperse P2, (c) P3 particles. The scale bar represents 2 μm. (d) the particles size can be tuned by varying the monomer concentration: P1 (black squares), P2 (red circles), and P3 (gray triangles). The black line is a guide to the eye.

4,4'-dibromoazobenzene was used to get conjugated polymer particles with the azobenzene unit oriented along and in conjugation with the polymer backbone (P1). In this case, switching of azobenzene unit from trans-to-cis causes the extensive and space-demanding motion of the whole backbone. As a result of this, the resulting particles (P1) appeared as thin platelets with square faces. This was not the case with P2 and P3 where monodisperse and perfectly round shape particles were obtained (Figure 2.4a-c). The anisometric shape of P1 particles was surprising but can be explained by a preferential direction of polymer condensation during the growth phase in the dispersion polymerization. Recently, a report has been published on the synthesis of P1 polymer which explains a helical molecular structure of the polymer in the trans-conformation. In solution this helical structure leads to crystallization into aggregates. The aggregates redissolve upon trans-to-cis switching.

By contrast, 3,5-dibromoazobenzene was applied to prepare particles (P2) where the azobenzene unit was perpendicular to polymer backbone. The azobenzene unit is in conjugation with the conjugated polymer backbone but trans-to-cis switching causes the motion of merely the peripheral phenyl ring and not the whole backbone.

1-(4-((6-(3,5-dibromophenoxy)hexyl)oxy)phenyl)-2-phenyldiazene produces particles (P3) where the azobenzene unit is completely decoupled from the π-conjugated polymer backbone. Here, switching of the azobenzene unit does not affect the entire backbone at all. The azobenzene unit is not in conjugation with the backbone but can interact via resonant energy transfer.

2.2.7 X-ray powder diffraction

The anisometric growth of the P1 particles during polymerization will be a result of the reported helical structure of the polymer in trans-conformation. Helices aggregate faster side-by-side than end-to-end, leading to crystallization into the platelet shape.
More probable    Less probable

Figure 2.5: Probability of joining the helices.

X-ray powder diffraction (XRD) analysis was performed on the dried samples of the polymer particles for the verification of our hypothesis. The P1 particles showed distinct XRD peaks which were in agreement with columnar packing (see Figure 2.7).

![XRD pattern](image)

**Figure 2.6:** XRD pattern of the particles composed of P1 (black squares), P2 (red circles), P3 (gray triangles).

By contrast, P2 and P3 showed only broad XRD feature. This explains amorphous polymer morphology consistent with the spherical shape of the particles.
Tunability in particle size is imperative for addressing a broad range of applications. The size of the particles (P1, P2, P3) can be easily adjusted by varying the monomer concentration during synthesis (Figure 1d). The size of narrowly dispersed platelets and monodisperse spherical particles was studied by electron microscopy image analysis, as well as by dynamic light scattering, as reflected by the error bars in Figure 2.4d. The particle size can be easily increased to a maximum value of 1.5 µm by increasing the monomer concentration. The narrow particle size distribution (standard deviation below 4%) was observed for particles prepared from a concentration of below 8 mM in case of P2 and P3. The shaded area in Figure 2.4d represents the concentration range where standard deviation governs the particle dispersity. Here, secondary nucleation started to appear during synthesis, limiting monodispersity (see Figure 2.4d).

UV/Vis extinction and fluorescence spectroscopic experiments were performed to study the tran-to-cis switching characteristics of the polymer particles. Structurally, the azobenzene unit is moving away from the polymer back bone from P1 to P3. In P1, it is part of the polymer back bone. In P2, it is moved to the periphery and it is completely decoupled from the polymer backbone in case of P3. This displacement reflects in the spectral properties of the polymer particles. The extinction and fluorescence maxima shifted from longer wavelengths to higher energies from P1 to P3. This is effect of changing the electronic push-pull system along the polymer chain for P1 to a backbone-azobenzene FRET pair in case of P3. A broadband UV source with an emission maximum at 380 nm was used to study the switching behavior of all the three kinds of particles and changes in extinction were investigated over time. By using confocal microscopy, the fluorescence of individual particles was recorded at an excitation 405 nm (see Figure 2.7 and Figure 2.8).
Figure 2.7: Switching of azobenzene conjugated polymer particles. Confocal microscopy images of (a) P1, (b) P2, and (c) P3 particles before (above) and after UV irradiation (middle) and absorption and fluorescence spectra of the particles (below). The scale bar represents 5 μm.

The extinction of dispersed particles decreased due to trans-to-cis switching upon UV-illumination. An increase in absorption at lower energies due to n → π* transition of the cis-conformation was not noticeable here. The band is usually very weak and will be concealed by the extinction peaks, which broadened due to scattering of the particles. However, a consequent increase in extinction is observed at higher energies (342 nm) for P3. The characteristics n → π* transition of the cis-conformation is noticeable due to the electronic decoupling of the conjugated polymer backbone and azobenzene moiety [1]. A raise in fluorescence was noticed with trans-to-cis switching for all three kinds of particles. This can also be detected by using the confocal microscope, where the particles show increased fluorescence after illumination at 405 nm (see Figure 2.7a-c). This behavior indicates fluorescence quenching between the fluorene donor and the trans-azobenzene acceptor units. In the trans-conformation fluorescence quenching can occur due to the conical intersection in the S₀/S₁ energy landscape of the azobenzene isomers [21]. Nonradiative decay via the conical intersection is more probably for azo-
benzene in the trans-conformation [22]. However, as a result of switching of the azobenzene unit into the cis-conformation, their electronic bandgap gets slightly widened and overlaps integrals between the fluorescence of the fluorene donor and the absorption of the azobenzene acceptor will be higher. This increased overlap integrals makes the shift of energy into the cis-azobenzene unit easier. So the azobenzene unit fluoresces in the visible range of the spectrum with less chance to decay nonradiatively. This explanation predicts the fluorene and azobenzene units as separate entities; however, in the real polymer particle there will be an extended-conjugation and interchain effects, which influence the quenching and energy transfer processes. Some more work needs to be done to completely interpret the underlying molecular orbital structures and corresponding quenching and intensification mechanism in detail.

Switching of the azobenzene unit is quite slower for P1 particles than for P2 and P3 (see Figure 2 and table 1). This is attributed to the molecular structure of the polymers inside the particles. A very simple explanation is based on the fact that the whole backbone of the polymer molecule and diazobenzene monomer units needs to move in concert in order to switch diazobenzene monomer units in case of P1. The crystalline platelets change their shape during switching as it becomes clear in confocal microscopy image in figure 2a. This is clearly an effect of the molecular rearrangement of the crystallized polymer chains, while the azobenzene units isomerize to the cis-form. Both P2 and P3 attain their photo stationary state at lower irradiation doses (D_{pss}) without any effect on their spherical shape (see Figure 2 and Table 1). For P2 and P3 types of particles, switching of the azobenzene unit from trans-to-cis does not affect the geometry of the polymer backbone at all. The azobenzene cis-form switches back to transform upon irradiation with a green light of 561 nm on the particle samples. This isomeric switching for P2 and P3 is quite obvious from a sharp decrease in fluorescence at low doses. However, this decrease in fluorescence intensity rate is quite slower in case of P1 particles. Furthermore, the P1 do not regain their original platelet shape, but remain deformed, and cis-to-trans switching is not completely reversible (see Figure 2.7 and Figure 2.8).
Figure 2.8: Dose vs. fluorescence intensity: Switching of the trans-to-cis shows the increase and cis-to-trans back switching the decrease in fluorescence, both at $\lambda_{\text{max}}$. P1 (black squares), P2 (red circles), P3 (gray triangles).

The shift from crystalline to an amorphous morphology is usually attributed with the glass transition, which is usually traversed by heating the material. Here the transition is produced by using the light, which provides an intriguing system to study phase-change materials.

A highly versatile method to prepare such switchable conjugated polymer particles was established. This method can easily be applied to other switchable monomers provided they are dihalogenated to take part in the palladium-catalyzed coupling reaction. Both P2 and P3 can be considered as ideal applicants for self-assembled switchable photonics due to their fast switching characteristics, amorphous morphology and high monodispersity. The particles can be used into coatings [23], where they produce switchable physical color by diffraction or in photonic crystals for wavelength conversion [24] with a switchable band gap or as colloidal laser resonators [5], [25]. An increase in fluorescence intensity together with the change in shape for switching from the trans-to-cis conformation is observed for P1 particles. This will make them an ideal candidate to be used in phase-change materials or shape shifting. All three types of particles could be used as switchable probes and markers for super resolution imaging in the biomedical field [16], [26].
2.3 Experimental:

2.3.1 3,5-Dibromobenzene

3,5-dibromobenzene was synthesized following a protocol from literature [1]. Nitrobenzene (257 mg, 2.4 mmol) was added to a solution of 3,5-dibromoaniline (500 mg, 2.0 mmol) in 2.5 mL of acetic acid. The precipitate was filtered and washed with acetic acid and water. The product was dried in vacuo (1 mbar).

Yield: 62% (orange solid)

\[^1\text{H-NMR}\ (400\ \text{MHz, CDCl}_3)\ \delta (\text{ppm}) = 8.01\ \text{(d, 2H, ArH), 7.92}\ \text{(m, 2H, ArH), 7.76}\ \text{(t, 1H, ArH), 7.54}(\text{m, 3H, ArH}).\]

2.3.2 1,3-dibromo-5-\((6\text{-bromohexyl)oxy})\text{benzene}\n
1,3-dibromo-5-\((6\text{-bromohexyl)oxy})\text{benzene}\ was synthesized according to a protocol from literature [2]. 3,5-dibromophenol (2.575 g, 10.22 mmol) and 1,6-dibromohexane (5.143 g, 21.08 mmol) were dissolved in 30 mL toluene. Tetrabutylammonium bromide (0.335 g, 1.04 mmol) and 7 mL of NaOH solution (aq. 50 wt%) were added. The mixture was degassed by bubbling with argon for 20 min. The mixture was heated to 80 °C for 24 h under a nitrogen blanket. The mixture was left to cool. The organic phase was separated and washed three times with water. The organic phase was dried over magnesium sulphate, filtered and concentrated in vacuo. The product was purified by column chromatography (silica gel, Hexane/DCM) and obtained as a white solid after removal of solvent.

Yield: 31% (white solid)

\[^1\text{H-NMR}\ (400\ \text{MHz, CDCl}_3)\ \delta (\text{ppm}) = 7.23\ \text{(t, 1H, ArH), 6.98}\ \text{(t, 2H, ArH), 3.92}\ \text{(t, 2H, Ar-O-CH2-R), 3.43}(t, 2H, R-CH2-Br), 1.89\ \text{(m, 2H, R-CH2-Br}), 1.80\ \text{(m, 2H, Ar-O-C-CH2-R), 1.5}\ \text{(m, 4H, R-CH2-CH2-R}).\]

2.3.3 1-(4-\((6\text{-}(3,5\text{-dibromophenoxy})\text{hexyl)phenyl})\text{phenyl)-2-phenyl-diazene}\n
1,3-dibromo-5-\((6\text{-bromohexyl)oxy})\text{benzene}\ (1.3276 g, 3.20 mmol), 4-phenylazophenol (0.6477 g, 3.27 mmol) and tetrabutylammonium bromide (0.0942 g, 0.29 mmol) were dissolved in XX mL of toluene and degassed for 20 min by bubbling with argon. 6 mL
of NaOH solution (aq. 20 wt%) was added. The mixture was heated to 80 °C for 24 h under a nitrogen blanket. The mixture was left to cool. The organic phase was separated and washed three times with water. The organic phase was dried over magnesium sulphate, filtered and concentrated in vacuo. The product was purified by column chromatography (silica gel, Hexane/DCM 1:1) and obtained as a orange solid after removal of solvent.

Yield: 50.2% (bright orange solid)

$^1$H-NMR (400 MHz, CDCl$_3$) $\delta$ (ppm) = 7.92 (dd, 3H, ArH), 7.55-7.37 (m, 4H, ArH), 7.23 (t, 1H, ArH), 7.02-6.99 (m, 4H, ArH), 4.06-3.94 (m, 4H, Ar-O-CH$_2$-R), 1.82 (m, 4H, Ar-O-C-CH$_2$-R), 1.56 (m, 4H, R-CH$_2$-CH$_2$-R).

2.3.4 General synthetic protocol for fluorene-co-azobenzene polymer particles prepared by Suzuki-Miyaura dispersion polymerization

Equal molar amounts of 9,9-dioctylfluorene-2,7-diboronic acid bis(1,3-propanediol) ester and the perspective dibromo-azobenzene monomer. Triton X-45 (800 mg) and PVPA (720 mg) were dissolved in 6.6 mL of 1-propanol. The mixture was filtered using a syringe filter (0.45 μm, PTFE) into a precleaned 25 mL flask. After degassing for at least 10 min by bubbling with argon, 1 mg of tetrakis(triphenylphosphine)palladium(0) was added. The reaction flask was then charged with a stirrer bar, sealed with a rubber septum and degassed again for 10 min with argon. The nozzle of tip-sonicator was inserted through an opening in the septum so that the sonicator tip was ca. 2 mm beneath the surface of the reaction flask. The flask was then transferred into a 70 °C oil bath and stirred magnetically (1300 rpm). While the tip-sonicator was operated continuously at 12 W. after 5 min, all the catalyst had dissolved and 1.4 mL of a degassed solution of 5 g/L potassium tert-butoxide in 1-propanol were added to initiate polymerization. The reaction mixture turned turbid after 30 min and the reaction was left to completion for 1 h. The particles were washed by repeated centrifugation, redispersion cycles, and changing the supernatant for 1-propanol with 0.5 %wt. Triton X-45. The yield varied between 40 and 65 % depending upon how many purification cycles were performed. GPC data is presented in table 1.

P1: $^1$H-NMR (400 MHz, CDCl$_3$) $\delta$ (ppm) = 8.25-7.20 (m, 14H, ArH), 2.09 (broad, 4H, CH$_2$), 1.11 (broad, 20H, CH$_2$), 0.77 (broad, 10H, CH$_2$, CH$_3$).
**P2:** $^1$H-NMR (400 MHz, CDCl$_3$) $\delta$ (ppm) = 8.25-6.75 (m, 14H, ArH), 2.08 (broad, 4H, CH$_2$), 1.10 (broad, 20H, CH$_2$), 0.80 (broad, 10H, CH$_2$, CH$_3$).

**P3:** $^1$H-NMR (400 MHz, CDCl$_3$) $\delta$ (ppm) = 7.81-6.75 (m, 18H, ArH), 4.20-3.90 (m, 4H, Ar-O-CH$_2$), 2.03-1.80 (m, 8H, CH$_2$), 1.06-0.77 (m, 34H, CH$_2$, CH$_3$).

### 2.4 Analysis:

#### 2.4.1 Gel permeation chromatography

The polymer particle samples were dissolved in chloroform to obtain polymer solutions to determine the critical molecular weights. The molecular weight determination was carried out using a high pressure liquid chromatography pump (JASCO PU-2080 Plus) and a refractive index detector (JASCO RI-2031 Plus) at 25°C. The eluting solvent was chloroform. Five columns with styrene-divinylbenzene (MZ SD-plus) gel were applied. The length of the first column was 50 nm and 300 nm for the other four columns. The diameter of each column was 8 nm. The diameter of the gel particles was 5 µm and nominal pore widths were 50 Å, 100 Å, 103 Å, and 104 Å, respectively. Calibration was achieved using narrowly distributed poly(methyl methacrylate) standards.

#### 2.4.2 X-ray diffraction

Experiments were performed on dried dispersion (powders) of particles at Cu kα radiation in the Bragg Brentano geometry.

#### 2.4.3 Confocal microscopy

Bright field and fluorescence images were recorded on a Leica SP8 confocal microscope. The switching of the particles and dose curves were recorded by setting fixed laser intensities for the respective laser lines: (405 nm for trans-to-cis, 561 nm for cis-to-trans) and defining a region of interest of known area in the field of view incorporating 5-10 particles. The region of interest was then irradiated at 405 nm and the intensity at the fluorescence maximum was recorded over time. The photo-stationary-state was assumed to have been reached when the fluorescence did not increase any further upon switching form trans-to-cis. The dose, at which this intensity was reached, was determined to be $D_{RSS}$. After this dose was reached the laser line was changed to 561 nm as to switch the particles back from cis-to-trans.
By defining a dose per area and normalizing the fluorescence intensity to the intensity of the photo-stationary-state, the dose curves become independent of the number and the size of the particles in the observed area.
2.5 References


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Chapter 4

RGD-decorated conjugated polymer particles as fluorescent bio-medical probes prepared by Sonogashira dispersion polymerization

This chapter has been published as:

Abstract

In this chapter a facile one-step Sonogashira dispersion polymerization is described. The process results into monodisperse conjugated polymer particles bearing accessible acetylene moieties on the surface. These acetylene groups are easily functionalized with biological recognition motif using thiol-yne click chemistry. The resulting functional particles are applied as fluorescent probes for imaging of endothelial cells, which take up the particles via receptor mediated endocytosis.
3.1 Introduction

The potential of conjugated polymer particles is extensively increasing in cell labeling, in-vivo imaging, particle tracking and drug or gene delivery due to their non-cytotoxic nature [10]–[15]. To date, mostly small size conjugated polymer particles (diameter < 100 nm) are being used in biomedical studies. These particles are easily synthesized from solutions of conjugated polymers via hot-injection and precipitation or miniemulsification followed by solvent evaporation of the dispersed phase [10], [16]–[18]. Alternatively the required particles can be prepared from mini-emulsion; where palladium catalyzed cross-coupling polymerization is performed inside of the droplets [17]. However, particles prepared by these systems are usually very polydisperse. Additionally, it is highly difficult to decorate these particles with bimolecular recognition motifs to explicitly target desired cellular epitopes due to their inert character. Click-chemistry is a reasonable coupling procedure, which can be easily applied to attach a manifold of biological recognition motifs [19]. However, one major drawback with click chemistry is the use of copper due to its cytotoxicity. Mostly azide/alkyne click-reaction has been performed by using copper as a catalyst [20]. Furthermore, glycans with azide functionality were coupled to an alkyne functionalized conjugated polymer particles via click chemistry. The glycans are applied as recognition motifs because they target glycoproteins on breast carcinoma cells [15]. However, copper residue from the functionalization creates problems because it is very toxic to biological entities like cells. Polydispersity is a major problem of current preparation methods for conjugated polymer particles. If endocytosis is a mechanism of the staining system, the polydisperse particles are taken up over an extended period of time depending on the cell type smaller particles are taken up much faster than the larger particles [21]. So monodispersity is crucial for these particles when applying for biomedical applications. It is impossible to differentiate between non-specific endocytosis and receptor mediated uptake resulting in disruption of analysis [22]. In the work group there have been reports on the synthesis of highly monodisperse particles using the Suzuki-Miyaura cross coupling reaction via dispersion polymerization [3], [8], [9], [23]. The particle size can be easily tuned from 100 nm up to the micron scale. The particles are monodisperse with standard deviations below 5 %. The only problem with these particles is that we cannot link bio molecular recognition motifs due to unavailability of functional groups on the surface of these particles. This inhibits the use of these conjugated polymer particles in the fields of fluorescence based cell labeling, analysis and imaging.
3.2 Results and discussion

In this chapter the synthesis of conjugated polymer particles via Sonogashira dispersion polymerization having the acetylene moieties on the surface is reported. These acetylene moieties can be used for attachment of biomedical recognition motifs. The biomolecules with thiol functionality can be attached to the surface of these particles via copper-free thiol-yne click chemistry [24]–[27]. This is a highly versatile method as a large variety of biological epitopes with thiol-functionality is commercially available. For our purpose here thiol-containing cysteine was selected which can be easily incorporated at one of the termini of the biomolecule or it is intrinsically carrying this moiety. Acetylene units on the surface of the particles are quite prone to oxidation during the thiol-yne click chemistry. To minimize this problem, the reaction is performed under inert atmosphere. However, the oxidation of acetylene units does not affect conjugation in the polymer backbone and fluorescence is retained by the resulting vinylene bond in the polymer backbone. The facile one step synthesis followed by one step thiol-yne click chemistry makes it a versatile preparation of a material toolbox for fluorescent monodisperse particles. The cysteine containing biological recognition motifs can be used to bind especially to certain epitopes on cell surfaces, for example those with bio-medical importance, which are only present or over-expressed under pathological conditions, allowing for their detection and 3D imaging [28]–[31].

Monodisperse particles were synthesized via Sonogashira dispersion polymerization. The reaction represents a cross coupling reaction between terminal acetylene and aryl or vinyl halides in the presence of a palladium or other transition metal catalysts [32]. It is one of the most esteemed and extensively used sp²-sp carbon-carbon bond formation reactions in organic synthesis. It is being regularly applied in synthesis of natural products, biologically active molecules, heterocycles, molecular electronics, dendrimers and conjugated polymers or nanostructures.

![Chemical Reaction Diagram]

\[ \text{cat. [Pd(0)] amine or inorganic base} \]

\[ \text{Cu(I) co-catalyst} \]

\[ \text{H} = \text{Aryl, Hetaryl, Alkyl, } \text{SiR}_3 \]

\[ \text{R}_2 = \text{Aryl, Hetaryl, Vinyl} \]

\[ \text{X} = \text{I, Br, Cl, OTf} \]
Scheme 3.1: Schematic representation of copper-catalyzed Sonogashira coupling.

The reaction can be easily done at room temperature in the presence of palladium source such as PdCl₂(PPH₃)$_2$ as catalyst and a co-catalyst CuI [33]. The base is an amine or other inorganic base could also be used. The use of CuI is really beneficial in a sense that it raises the reaction rate to a very high degree. However, it has some shortcomings too. On the one hand, inert working conditions are required because presence of oxygen can produce undesirable formation of alkyne homocoupling through a copper mediated Glaser/Hay reaction [34]. On the other hand, presence of Cu residual is highly toxic for cells. So copper has to be completely removed before any further functionalization, which is not an easy task.

3.2.1 Mechanism

Scheme 3.2: Proposed mechanism for copper-cocatalyzed Sonogashira reaction.

The real mechanism of palladium/copper-catalyzed Sonogashira reaction is still not fully understood. It is due to the presence of two metal catalysts. It is really difficult to analyze the combined action of two catalysts, although it is considered to occur through two independent catalytic cycles [35]. The first cycle is called the palladium cycle (cycle A) representing classical C-C cross coupling [36]. It begins by the catalytically active species Pd(0)L₂, which can be of colloidal nature and/or a low ligated Pd(0) species. The presence of corresponding bis(phosphane)palladium and other in-
volved species has been confirmed by negative-ion electrospray ionization mass spectrometry in the gas phase while using phosphane as ligands. There are two possibilities for the creation of this \([\text{Pd}(0)L_2]\) complex. It can either be formed from \text{Pd}(0) complexes such as \text{Pd}(\text{PPh}_3)_4 or from \text{Pd}(II) complexes such as \text{PdCl}_2(\text{PPh}_3)_2. Additionally, amines can also take part in the reduction of \text{Pd}(II) to \text{Pd}(0) through formation of iminium cations which has already been reported [37].

After formation of the \([\text{Pd}(0)L_2]\) complex, the first step in the catalytic cycle is started by oxidative addition of the aryl or vinyl halide. This is the rate limiting step of the Sonogashira reaction. The barriers of oxidative addition of \text{ArX} (X= Cl, Br, I) increase in the order of \text{ArI} < \text{ArBr} < \text{ArCl} [35], [38].

The copper cycle which is denoted as cycle \text{B} is not yet very well understood. The base (organic or inorganic) is considered to support copper acetylide formation with the assistance of a \(\pi\)-alkyne copper complex. Due to this complex formation, the terminal proton of the alkyne becomes more acidic in nature.

3.2.2 Conditions usually applied in Sonogashira-cross-coupling polycondensation

3.2.2.1 Catalysts selection

Although a wide variety of catalysts such as \text{PdCl}_2(dppe) [39], \text{PdCl}_2(dppp) [40], \text{PdCl}_2(dppf) [41], have been applied in Sonogashira coupling. However, \text{PdCl}_2(\text{PPh}_3)_2, \text{Pd}(\text{PPh}_3)_4 are two most extensively used catalysts at the moment. Among these two, \text{PdCl}_2(\text{PPh}_3)_2 is more preferred being more stable and soluble [32]. Additionally, in case of \text{Pd}(0) complexes ligands needed to stabilize \text{Pd}(0) invariably have coordinating properties which would cause hindrance in production of the active \text{Pd}(0) catalyst. Hence, \text{Pd}(II) salts provides an excellent alternative with the capability of producing a more active catalyst [35], [42]. Trifluoroacetylated iodophenethylamine 1 was alkynylated with trimethylsilylacetylene (TMSA) under typical Sonogashira reaction conditions to produce silylated acetylene 2 [43]. (See scheme 3.3)
Scheme 3.3: Synthesis of silylated acetylene under typical Sonogashira conditions.

Another example is the reaction of aryl iodide 3 with propargyl alcohol 4 to produce acetylenic system, which gives estrogren receptor (Z)-tamoxifen 5 in multi steps process [44]. (See scheme 3.4)

Scheme 3.4: Synthesis of estrogren receptor (Z)-tamoxifen under typical Sonogashira conditions.

3.2.2.2 Selection of base

The role of base in Sonogashira reactions is quite crucial. Amines especially triethylamine (usually applied as a solvent or in excess) are proven to be quite efficient [32], [35], [42]. It should be noted that the base should not be strong enough to abstract pro-
ton from the terminal alkyne, hence forming a copper acetylide in the presence of Cu (1) salt. In contrast, they form a π-alkyne complex as depicted in Scheme 3.2 [45], which makes the alkyne proton more acidic for easier abstraction. For example, vanillin-derived iodoarene 6 was cross-coupled with alkyne 7 in the presence of typical Sonogashira conditions to produce diarylalkyne 8.

\[ \text{Scheme 3.5: Synthesis of diarylalkyne under typical Sonogashira conditions.} \]

### 3.2.2.3 Reactivity order of the monomers

The general reactivity of the sp² species lies in the order: aryl iodide > aryl triflate > aryl bromide > aryl chloride. Hence, Sonogashira works nicely in the presence of unstable aryl iodide as compared to corresponding bromides and chlorides. Aryl bromides are not much favorable for coupling reactions [32], [35], [46].

Sonogashira coupling was used to synthesize monodisperse conjugated polymer particles via dispersion polymerization from simple molecules like 1,3-diethynlybenzene and 1,3-dibromobenzene.
Scheme 3.6: Schematic representation of copper-catalyzed Sonogashira coupling using 1,3-diethynylbenzene 1,3-dibromobenzene as monomers and diethyl amine as a base.

The reaction was carried out similar to the previously reported Suzuki-Miyaura dispersion polymerization [3]. CuI was added as a co-catalyst and potassium tert-butoxide was replaced with a softer base diethyl amine due to the requirements of Sonogashira coupling. However, the stabilizers and other conditions remained. In a typical reaction, both reactants and stabilizers were mixed in 1-propanol. The resulting mixture was filtered using a syringe filter (0.45 μm, PTFE) and degassed with argon. After proper degassing, the catalyst, tetrakis(triphenylphosphine)palladium(0) was added. The reaction flask was then charged with a stirrer bar, sealed with a rubber septum and degassed again with argon. The nozzle of a tip-sonicator was inserted through an opening in the septum so that the sonicator tip was ca. 2 mm beneath the surface of the reaction flask. The flask was then transferred into a 70 °C oil bath and stirred magnetically (1300 rpm), while the tip-sonicator was operated continuously at 12 W. When all catalyst had dissolved a degassed solution of diethyl amine in 1-propanol was added to initiate the polymerization. The reaction mixture was heated for 4h under nitrogen atmosphere in which time the solution turned. The presence of inert conditions is very crucial in Sonogashira coupling because oxygen can produce undesirable alkyne homocoupling through a copper mediated Glaser/Hay reaction as mentioned above. By contrast, Suzuki-Miyaura dispersion polymerization is not as susceptible to oxygen. The particles were washed by repeated centrifugation redispersion cycles while exchanging the supernatant for 1-propanol with 0.5 %wt. Triton X-45. However, the result was not as expected. The particles were highly polydisperse as shown by scanning electron microscopy images (see Figure 3.1).
3.2.3 Change in concentrations of monomers

To tune the particle size and reduce the polydispersity, the monomer concentrations were varied. The concentrations of monomers were varied i.e. 5 mM (Figure a), 6.25 mM (Figure b), 8.75mM (Figure c), 12.5 mM (Figure d), 15mM (Figure e), 20 mM (Figure f), while keeping the base, catalyst and other reaction parameters constant.
This was done to see the effect of changing the concentration on the particle size. But change in concentration does not increase the particle size or reduce the polydispersity. However, the conditions did not prove to be fruitful. The particles started to aggregate instead of getting monodisperse and bigger in size as proved by SEM image analysis (see Figure 3.2).

### 3.2.4 Change in concentration of surfactants

After testing the effect of monomer concentrations on particle polydispersity, effects pertaining to the stabilizer concentrations were checked. The concentrations of both the Triton X-45 and PVPVA were varied stepwise (see Table 3.1), but the results obtained were also not much different from the previous ones. The particles were highly polydisperse. (see Figure 3.3)

<table>
<thead>
<tr>
<th>Figure number</th>
<th>PVPVA concentration [mg]</th>
<th>Triton X-45 concentration [mg]</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>864</td>
<td>960</td>
</tr>
<tr>
<td>B</td>
<td>792</td>
<td>880</td>
</tr>
<tr>
<td>C</td>
<td>936</td>
<td>1040</td>
</tr>
<tr>
<td>D</td>
<td>1008</td>
<td>1120</td>
</tr>
</tbody>
</table>

**Table 3.1:** Different concentrations of both PVPA and triton X-45 and respective figure numbers.
3.2.5 Change of monomers

After varying the concentrations of monomers and stabilizers to reduce polydispersity, the other possibility was to change the monomers. 1,3-dibromobenzene was replaced with 9,9'-dioctyl-2,7-dibromo fluorene while keeping the other parameters constant (see Scheme 3.7).

Scheme 3.7: Schematic representation of copper-cocatalyzed Sonogashira coupling using 9,9'-dioctyl-2,7-di-bromo fluorene, 1,3-dibromobenzene as monomers and bis(triphenylphosphine)-palladium dichloride as catalyst.
Figure 3.4: Scanning electron microscopy images of resulting particles.

SEM image analysis showed no reduction in polydispersity (see Figure 3.4).

As we know that general reactivity order is ArI > ArBr > ArCl. We changed one monomer system to iodo-functionalities. The obtained results showed enhancement of monodispersity compared to the bromo monomers (see Figure 3.5). In a typical reaction, 1,3-diethynylbenzene was reacted with 1,3-diiodobenzene while keeping other parameters constant (see Scheme 3.8).

\[
\text{Pd(PPh}_3\text{)}_2\text{Cl}_2 \\
\text{CuI} \\
\text{TEA} \\
\text{1-propanol} \\
\text{surfactants} \\
\rightarrow
\]

**Scheme 3.8:** Schematic representation of copper-cocatalyzed Sonogashira coupling using 1,3-diethynylbenzene, 1,3-diiodobenzene as monomers and bis(triphenylphosphine)palladium dichloride as catalyst.
The resulting particles were highly monodisperse as shown by SEM image analysis (see Figure 3.5). However, their fluorescence was very weak. As we are aiming for further functionalization of the particles with RGD-peptides for biomedical applications, fluorescence intensity of the particles is crucial. We chose a diiodofluorene monomer. Fluorene based conjugated polymers are known for their high fluorescence yields (see Scheme 3.9).

To gain a wide range of applications tunability of particle size is imperative. To tune the particle size, the concentrations of monomers were varied from 5mM to 20 mM. Monodisperse particles of different sizes were obtained as depicted by SEM image analysis (see Figure 3.6a-d).
3.3 Mechanism of Sonogashira Dispersion Polymerization

Scheme 3.9: Mechanism for the Sonogashira dispersion polymerization. Bifunctional monomers are polymerized in a Pd-catalyzed cross-coupling polycondensation in 1-propanol. As the molecular weight of the resulting polymer approaches the critical point of the solvation the polymers nucleate. Subsequently formed polymers condensate onto existing nuclei during the growth period leading to a set of monodisperse particles.

The reaction is done in 1-propanol because it is a good solvent for both monomers and a non-solvent for the resulting polymer. When the resulting polymer approaches a critical molecular weight of solvation, it precipitates into seeds, which are stabilized by the PVPVA and further improved by non-ionic surfactant Triton X-45. The stabilizers in-
hibit aggregation of the seeds and promote growth through condensation of more polymers approaching the critical molecular weight, as is typical in dispersion polymerization (see Scheme 3.9).

The tunability of particle size is very vital to access a wide range of applications. The particle size was adjusted by varying the monomer concentration while keeping all other parameters constant. The size of particles can be easily adjusted between 140 nm and 300 nm with polydispersity value lower than 5 %.

![Figure 3.7](image.png)

**Figure 3.7:** By varying the monomer concentrations in Sonogashira dispersion polymerization, the particle size can be tuned. Above 14 mM of the monomer, the dispersion polymerization affords polydisperse particles. The dashed line is a guide to eye.

The size of narrowly dispersed spherical particles was studied by electron microscopy image analysis, as well as by dynamic light scattering, as shown by error bars in Figure 3.7. The narrow particle size distribution (standard deviation below 20%) was observed for particles prepared from a concentration of below 14 mM. The dispersion was quite stable for long time. There were no aggregates observed even after three months. A particle-size histogram was constructed from measurements of ~100 individual particles from electron micrographs (see Figure 3.8). The particle size can be easily increased to a maximum value of 300 nm by increasing the monomer concentration. The particles remained monodisperse until a concentration of 14 mM. After reaching this value, the particles started to become polydisperse due to secondary nucleation as indicated by gray area in Figure 3.7.
**Figure 3.8:** Size distribution histograms constructed after from measurements of ~ 100 individual particles from electron micrographs. The SEM images of respective particles are depicted in insets. Scale bars depict 4 μm everywhere except 10 mM, here it describes 5 μm.

The particles are stable in relevant media such as deionized water, Phosphate-buffered saline (PBS) and cell culture medium and keep their fluorescence profile after storing for three months (see Table 3.2 and Figure 3.9).
**Figure 3.9:** Fluorescence spectra (ex = 405 nm) of particles in a) deionized water, b) PBS buffer and c) cell culture medium. The green data represents particles which are freshly prepared, the gray data after exposure to 10 minutes of exposure to 370 mW laser radiation at 405 nm and black squares represents data taken after 10 days of storage in the different media.

<table>
<thead>
<tr>
<th>Media</th>
<th>Diameter (nm)</th>
<th>Standard deviation (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PH= 1</td>
<td>262.5</td>
<td>2.3</td>
</tr>
<tr>
<td>PH= 3</td>
<td>269.6</td>
<td>2.9</td>
</tr>
<tr>
<td>PH= 5</td>
<td>271.2</td>
<td>7.5</td>
</tr>
<tr>
<td>Deionized water (pH= 7)</td>
<td>245.1</td>
<td>3.7</td>
</tr>
<tr>
<td>PBS buffer (pH = 7.4)</td>
<td>246.0</td>
<td>5.8</td>
</tr>
<tr>
<td>PH= 9</td>
<td>251.9</td>
<td>5.7</td>
</tr>
<tr>
<td>PH= 11</td>
<td>261.3</td>
<td>4.5</td>
</tr>
</tbody>
</table>

**Table 3.2:** DLS measurement of the particle size in different media.

Photo-bleaching of the particles is tested by exposing them to 405 nm laser radiation (730 mW) and recording the fluorescence over 10 minutes. The medium does not influence the photo-bleaching effect. The fluorescence is firstly reduced to about 60% within a few minutes and remains constant afterwards. This is due to photo-oxidation on the
surface, while the particle core remains unaffected and fluorescence remains the same. The particles exhibit more stability and reduced photo-bleaching as compared to Pyrromethene 564 doped polystyrene particles (Figure 3.10).

**Figure 3.10:** Bleaching study. 10 minutes of exposure to a 730 mW, 405 nm laser. The left side confocal images depict the sample before and the confocal images on the right after the bleaching experiment. The green data represents an average over the entire frame. The three gray
lines represent single particles bleaching traces as indicated by the white circles in the confocal microscopy images. a) Particles in deionized water, b) in PBS buffer, c) cell culture medium. Data in d) represent PS particles dyed with PM 546 in deionized water for comparison. (Some particles attach and detach from the substrate over the course of 10 minutes. As a result the averaged data (green) can also rise over time as non-exposed particles enter the field of view. The three marked particles in each experiment are stationary at the substrate over the entire experiment.

Particles smaller than 200 nm were used for further functionalization as HUVECs internalize smaller particles much better than larger particles. The particles were purified by repeated centrifugation and solvent exchange from 1-propanol to water.

![Chemical structure](image)

**Figure 3.11:** Acetylene units on the surface of the particles can be addressed using thiol-yne click chemistry. Here the particles are functionalized with thiolated PEG (6000 g/mole) and cRGD.

Thiol-terminated cyclic RGD (cRGD) peptides were attached to the surface of particles by thiol-yne click chemistry. To achieve this, particles were heated with cRGD in the presence of 2,2,6,6-tetramethylpiperidine-1-yl)oxidanyl (TEMPO) and cesium carbonate in dimethyl sulfoxide as solvent [35]. But X-ray photoelectron spectroscopy (XPS) analysis showed that there were no cRGD covalently attached to the surface. As an alternative, photo click chemistry was used. Different photoinitiators (Azobisisobutyronitrile, potassium persulfate, 1-hydroxy cyclohexyl ketone) were investigated but did not lead to the desired RGD functionalized particles. However, when we applied 2,2-dimethoxy-2-phenylacetophenone attachment of cRGD was successful. The attachment was proven by XPS.

Thiol-terminated cyclic RGD (cRGD) peptide was applied in 10⁶ molar excess to a dispersion of the fluorene acetylene phenylene co-polymer particles in DMSO [26]. The resulting mixture was stirred for 12 h while being irradiated with UV light (7.5-Watt) to initiate the photoclick reaction. The particles were also functionalized with thiolated oligo ethylene glycol (PEG) to obtain a control sample. After performing the thiol-yne
click reaction with either cRGD or PEG, the particles were washed several times with Milli Q water to remove additional initiator. For the verification of successful surface functionalization of the particles with cRGD or PEG, XPS analysis was performed on particles prepared without the PVPVA stabilizer. In case of cRGD functionalized particles, XPS clearly showed the emergence of nitrogen (N), oxygen (O) and sulphur (S) signals. (see Figure 3.12).

![XPS spectrum showing RGD functionalized particles](image)

**Figure 3.12**: XPS measurement on cRGD functionalized particles showed surface modification.

The XPS spectra of PEG-coated particles showed peaks only for oxygen and sulphur, which was in agreement with the expected result as there are no N signal in the particles on the surface coating.
XPS analysis was also performed on particles without any functionalization. The peaks for nitrogen and sulphur were clearly missing. The oxygen peak arose from residual oxygen from the applied Triton X-45 stabilizer on the particle surface. So XPS confirmed the successful attachment of both PEG and cRGD on particle surface.

The resulting surface functionalized particles experience similar stabilization and surface oxidation, rendering their fluorescence almost similar. (see Figure 3.15)
Figure 3.15: Fluorescent spectra of PEG (black line) and cRGD (green line) functionalized particles excited at 350 nm, showing almost identical fluorescence profiles with a maximum at 520 nm.

To check whether the cRGD surface functionalization is accessible for bio-recognition cell culture tests were performed on human umbilical vein endothelial cells (HUVECs). HUVEC cells overexpresses αVβ3-integrins on their cell membrane, which binds to the RGD motif. We performed this study in collaboration with Wiltrud Lederle from UK Aachen. HUVEC cells were incubated with either cRGD functionalized or PEG-functionalized control particles. The difference in cell-labeling of the cRGD functionalized versus the control particles was significant after 4-6 h. More cRGD functionalized particles were localized near nuclei of HUVECs (stained by DAPI) as compared to the control particles. This clearly showed the surface activity and recognition of membrane bound integrins by the RGD functionalized particles as compared to the particle without recognition motif.
Figure 3.16: HUVECs incubated with control particles. No attachment or endocytosis is visible after 6 hours. Cells are stained with DAPI.

Figure 3.17: cRGD functionalized particles incubated with HUVECs for 6 hours. Most particles are either adhering or have been taken up by the cells. Cells are stained with DAPI.
Figure 3.18: Cytotoxicity test: HUVECs were incubated for 24 hours with either no particles, cRGD functionalized conjugated particles and non-functionalized particles (particle concentration 250 μg/mL). PEG and cRGD functionalized particles were flown over adherent HUVECs. The average number of particles bound per cell was plotted on the y-axis. As a competition experiment, an excess of the free cRGD was added to the cRGD functionalized particle dispersion. Replacement of the particles with free RGD proved binding of cRGD to αVβ3 integrins on the HUVEC cell membrane.

Binding studies in a flow chamber setup were also performed to further test the binding of cRGD functionalized particles to αVβ3 integrins at the cell membrane. The particles were flown over adherent HUVECs and binding of the particles to the cells was studied. The binding of the PEGylated control particles versus cRGD functionalized particles was recorded. Very low binding was observed in case of the PEG functionalized control particles. However, cRGD functionalized particles show a threefold increase in particle binding to HUVECs (see Figure 3.18 ). A competition experiment was performed by adding an excess of non-particle bound cRGD molecules, which showed reduced binding similar to the non-binding PEG-functionalized particles. This illustrated the binding specifically originated from the cRGD motif to αVβ3 integrins on the cell surface of the HUVECs. The relatively large standard deviations originate from batch to batch heterogeneity of the HUVEC cells as > 2000 cells (see Figure 3.18) were analyzed. Bonferroni corrected unpaired t-tests were used to check the reliability of the binding study and high significance was achieved in all cases (p < 0.0001). A 3-dimensional scan through HUVEC was performed (already incubated with cRGD func-
tionalized particles for 6 hours). The scan clearly showed an uptake of particles via endocytosis (see Figure 3.19).

![Confocal microscopy z-scan of two HUVECs from the bottom (z = 0) to the top (z = 7) of the cell. Cell nuclei are stained with DAPI. The scale bar represents 5 μm.](image)

**Figure 3.19:** Confocal microscopy z-scan of two HUVECs from the bottom (z = 0) to the top (z = 7) of the cell. Cell nuclei are stained with DAPI. The scale bar represents 5 μm.

Endocytosis mediated by αVβ3 integrin binding was accelerated; and much less uptake of the control particles at the same point of time (see Figure 3.19) was observed. It is already reported that HUVECs internalize particles with 200 nm in size much better than larger particles. It was also observed that the uptake of conjugated polymer particles of 200 nm in diameter was much quicker than the particles with 300 nm in diameter. When 300 nm particles with cRGD-functionalization to non-functionalization of the same size were compared, the difference was only evident after 24 hours. This clearly is a huge difference as compared to the 200 nm particles where difference can be observed after 4-6 h only. This clearly shows the importance of monodispersity to obtain representative results for *in-vitro* integrin binding and cellular uptake and the necessity to work with particles of 200 nm to enable early discrimination between cells with integrin overexpression and normal cells.

We moved on to test the cytotoxicity of our RGD functionalized particles. The particles were incubated with HUVECs for 24 hours to check their cytotoxicity. The cell viability was tested by using Trypan Blue Staining. Both the surface functionalized (cRGD) and particles without any surface functionality all proved to be non-cytotoxic.
This clearly illustrated the biocompatibility and non-cytotoxicity of this novel particle system (see Figure 3.18).

3.4 Conclusion

In the future, to drive this field of conjugated polymer diagnostics further towards in-vivo application, near-IR emitters will have to be developed to allow excitation and fluorescence detection in the tissue transparency window. The results presented here showcase an easy approach to produce highly selective particle systems, which bind selectively to αVβ3 integrins on activated endothelial cells under flow and static cell culture conditions, demonstrating the potential in this type of particles, which will eventually be applied in non-bleaching near-infrared tumor probes and theranostic agents.
3.5 Experimental work

3.5.1 Materials:

All chemicals, solvents and materials were obtained from Sigma Aldrich apart from cRGD, which was obtained from Bachem Holding AG (Switzerland). All chemicals were used without further purification.

3.5.2 General synthetic protocol for fluorene diacetylene phenylene co-polymer particles prepared by Sonogashira dispersion polymerization

Equal moles of 9,9-dioctyl-2,7-diiodofluorene and 1,3-diethynlybezene, Triton X-45 (800 mg) and PVPVA (720 mg) were dissolved in 6.6 mL of 1-propanol. The resulting mixture was filtered into a 25 mL flask with a syringe filter (0.45 mm, PTFE). After degassing for 15 min by bubbling with argon, bis(triphenylphosphine) palladium(II)dichloride (1.5 mg) and CuI (3 mg) were added. The reaction flask was then charged with a stirrer bar, sealed with a rubber septum and degassed by using argon for another 10 min. The nozzle of a tip sonicator was inserted in the flask through a hole in the septum so that the tip was ca. 2 mm below the surface of the reaction mixture. The reaction mixture was heated to 70 °C with an oil bath and stirred (at 1300 rpm), while the tip sonicator was running consistently at 12 W. After all the catalyst had dissolved, 1.6 mL of degassed solution of triethylamine (15% in 1-propanol) was injected to start the polymerization. The reaction mixture turned turbid after ca. 30 min. The reaction mixture was left to completion for 2 h. The particles were purified by washing three times with each (1 %wt. Triton X-45 in propanol and 1- propanol). The yield was varied between 30 to 50% depending on the purification procedure.

3.5.3 Functionalization of fluorene diacetylene phenylene co-polymer particles with cRGD

A 10 mL glass vial with rubber septum was charged with a mixture of 12 mg of fluorene diacetylene phenylene co-polymer particles, 2, 2-dimethoxy-2-phenylacetophenone (30 mg) and 3 mL of dimethyl sulfoxide. The reaction mixture was degassed for at least 15 min by bubbling with argon. Cyclo(-Arg-Gly-Asp-D-Phe-Cys) solution (1.5 mg in 1mL DMSO) was injected to the reaction mixture and degassed again for 10 min. The reaction mixture was irradiated with a UV lamp with an intensity maximum at 365 nm
at room temperature under magnetic stirring overnight. The particles were purified by washing with distilled water several times.

3.5.4 Functionalization of fluorene diacetylene phenylene co-polymer with PEG-thiol

A 10 mL glass vial with rubber septum was charged with a mixture of 12 mg of fluorene diacetylene phenylene co-polymer particles, 2,2-dimethoxy-2-phenylacetophenone (30 mg) and 3 mL of dimethyl sulfoxide. The reaction mixture was degassed for at least 15 min by bubbling with argon. PEG-thiol (2.5 mg) was injected to reaction mixture and degassed again for 10 min. The reaction mixture was irradiated using a UV lamp with an intensity maximum at 365 nm at room temperature under magnetic stirring. The particles were purified by washing with distilled water several times.

3.5.5 Cell culture

Human umbilical vein endothelial cells (HUVEC, Promocell, Germany) were cultured using Vasculife basal medium (Lifeline, Germany) supplemented with 2% growth supplements, 3% fetal calf serum and 1% Pen/Strep (10,000 U/ml penicillin; 10,000 µg/ml streptomycin, Invitrogen, Germany). Cells were cultured in T75 cell culture flasks (Cell star, Greiner, Germany) and incubated at 37°C, 5% CO2 and 95% relative humidity.

3.5.6 Cell viability assay

The toxicity of the particles on HUVEC was investigated by trypan blue staining. 3 x 105 cells were seeded in 6-well plates (Greiner, Germany) and cultured for 24h. Medium was removed and each well was washed with 2ml of phosphate buffered saline (PBS, Invitrogen, Germany). Cells incubated with cell culture medium served as negative controls. Non-functionalized and functionalized particles were diluted in cell culture medium to a concentration of 250 µg/ml. 3 ml of cell culture medium or medium containing particles was added to the cells and the cells were incubated for 24h. Three samples per condition were analyzed. After incubation, medium was removed and transferred to 15 ml reaction tubes (Greiner, Germany). Cells were washed once with PBS and subsequently trypsinized with 0.25 % Trypsin/ 0.05 % EDTA (Invitrogen, Germany). The cell suspension was centrifuged at 1000 rpm (Multifuge, Thermo scientific, Germany) for 5 min and the supernatant was removed. The cell pellet was dissolved in 0.5 ml of cell culture medium. 50µl of the cell suspension were mixed with 50 µl of the Trypan blue staining solution (Sigma-Aldrich, Germany). Trypan blue positive cells (dead cells) were counted using the Cedex XS cell counter (Innovates AG,
Germany). The percentage of positive cells as a function of the total cell number was calculated.

3.5.7 Cellular uptake of non-functionalized and functionalized particles in-vitro

Cellular uptake of nanoparticles by HUVEC was tested using fluorescence microscopy (Axio Imager Z2, Carl Zeiss, Germany). 3 x 105 cells were seeded in 6-well plates (Greiner, Germany) and cultured for 24h. Four hours prior incubation with nanoparticles, cells were stimulated to express a higher amount of αvβ3-integrin using 4 ng/ml of human recombinant tumor necrosis factor-alpha (TNF-α) (ProSpec-Tany TechnoGene Ltd, USA). Medium was removed and each well was washed with 2 ml of phosphate buffered saline (PBS, Invitrogen, Germany). HUVEC incubated with cell culture medium served as negative controls. Non-functionalized and functionalized particles were diluted with cell culture medium to a concentration of 250 μg/ml. 3 ml of cell culture medium or medium containing particles was added to the cells and incubated for 6 h. 3 samples per condition were analyzed. After incubation, medium was removed and cell nuclei were stained with DAPI (4’,6-diamidino-2-phenylindole, Vector Laboratories, Germany). Cells were washed with PBS and subsequently trypsinized as described above. Cells were transferred to glass slides (Langenbrinck, Germany) and mounted with Mowiol mounting medium (Sigma-Aldrich, Germany). Fluorescent micrographs were captured using an epifluorescence microscope (Axio Imager.M2, Carl Zeiss, Germany) equipped with a high resolution camera (AxioCam MRm Rev.3; Zeiss, Germany). Quantification was performed by counting the amount of intracellular nanoparticles within five randomly selected micrographs for each condition.

3.5.8 In vitro binding study (flow chamber experiments)

Binding of nanoparticles on the surface of HUVEC was evaluated using flow chamber experiments. 3 x 105 cells were seeded in 35 mm Petri dishes (Greiner, Germany) and cultured for 24 h. Four hours prior incubation with nanoparticles, cells were stimulated to express a higher amount of αvβ3-integrin using 4 ng/ml of TNF-α. One hour prior incubation, cell membranes were stained with 5 μg/ml of wheat germ agglutinin Alexa Fluar 594 conjugate (Life technologies, Germany). Four hours after the addition of TNF-α, the 35 mm Petri dishes were mounted to a customized flow chamber. Subsequently, a suspension of either 250 μg/ml of non-functionalized or functionalized nanoparticles in cell culture medium, or a combination of 250 μg/ml functionalized particles
together with free cRGD in excess (10,000 fold, for competition) was injected into the tube connecting both ends of the flow chamber. A peristaltic pump (Gilson Inc., USA) allowed circulation of the nanoparticles for 10 minutes at a flow rate of 0.25 ml/min. After incubation, the loop was opened and unbound nanoparticles were washed out with cell culture medium for 5 minutes using the same flow rate. Nuclei were stained with DAPI. Fluorescence microscope images were acquired using the Axio Imager.M2 (Carl Zeiss, Germany). For each condition (non-functionalized and functionalized nanoparticles, and competition with free cRGD), n=6 flow chambers were used. The mean number of bound nanoparticles per cell was determined by manually counting all nanoparticles and cells in the images.
3.6 References


Chapter 4

Glycofunctionalized microgels for scavenging *Clostridium difficile* toxin A

This work has been performed in collaboration with Ruben Rosencrantz (Helmholtz Institute, RWTH Aachen) and Ana Mandic (Uniklinik, RWTH Aachen).
Abstract

A novel biocompatible microgel platform for biomedical application is presented in this chapter. Monodisperse PEG based microgels are synthesized via microfluidic emulsification and incorporation of glycans takes place during microgel synthesis. This results in bioactive, ligand functionalized microgels for binding to the cell surface receptor domain of the bacterial enterotoxin A of *Clostridium difficile*. The amount of incorporated glycans in the microgels is determined by magic angle spinning solid state NMR. Incorporation degrees of up to 1.5 in molar ratio as compared to macromonomer have been achieved. Various glycans including the trisaccharide galili have been incorporated. Galili represents a ligand for the enterotoxin A of Clostridium difficile. The microgels obtained are 20 μm in diameter. They are in the same range as mammalian cells and therefore flow cytometric analysis of single microgels was possible. This way, highly specific and selective binding of bacterial enterotoxins to the microgel could be proven. This multivalent polymeric platform can be used as a novel non-antibiotic approach for overcoming the rising numbers of infections caused by pathogenic bacteria.
4.1 Introduction

Pathogenic bacteria like *Clostridium difficile* (*C. diff*) are causing an enormous burden on the healthcare system worldwide. *C. diff* has been identified as the main cause of antibiotic associated diarrhea[1]–[4]. In mild cases, it can also cause loss of appetite and fever [5], [6]. When *C. diff* overgrows in the colon (large intestine), *C. diff* associated diseases (CDAD) can occur. CDAD is very common among the elderly in hospitals and nursing home patients, however numbers of young patients are increasing [7]–[9]. The problem is that after broad-spectrum antibiotic therapy, the natural intestinal flora is disturbed and *C. diff* can overgrow. *C. diff* produces two toxins, enterotoxin (TcdA) and cytotoxin (TcdB), which cause diarrhea and inflammation. This manifests in necrosis and formation of pseudo-membranes, or in the ultimate state in life-threatening megacolonitis [11], [12]. CDAD is treated again with antibiotics and relapse of CDAD occurs in ~20% a high percentage of patients [2], [15]. Considering the rise of various bacterial strains, an alternate therapeutic strategy is badly needed [16].

Both TcdA and TcdB are large proteins of approximately 308 kDa and 270 kDa respectively [19], [20]. They are multi-domain proteins having a similar domain assembly. Both exhibit a N-terminal glycosyltransferase domain considered the toxic domain, joined by a autoprotease domain which is cleaved to deliver the toxins ability to disrupt the intestinal cell membrane produce transmembrane domain. A C-terminal domain comprise of multiple repetitive sequences and is cell surface receptor binding domain. In case of TcdA, it has been reported to be lectin like. The mode of toxin action is thought to begin with binding of the receptor binding domain to the cell surface, followed by endocytosis and pH-acidification in early endosomes. At this point the β-sheet rich domain builds-up a transmembrane part and the toxic glycosyltransferase domain is cleaved off in the cytosol by autoproteolysis, where it leads *in ultimo ratio* to a disruption of the cytoskeleton[22], [23]. Taking all steps together a potential weak point may be the binding to the cell surface. Screening of TcdA revealed various putative glycan ligands for the receptor binding domain. Among them the so called Galili-structure, a trisaccharide consisting of Galα1,3Galβ1,4GlcNAcβ [24]–[26]. Binding of multiple glycan galili moieties to the single toxin would lead to multivalent interactions which increase the binding strength exponentially with every additional binding even. Upto seven glycan binding domains exist in TcdA. The aim of this work is to scavenge the toxin TcdA in vivo by galili functionalized microgels.
Microgels are widely studied candidate for biomedical applications, drug delivery systems and for imaging [38]–[43]. Their periphery can be functionalized with a wide variety of functional groups, which may be used for subsequent bioconjugation. They can be made biocompatible through the selection of water soluble compounds [44]. They contain large amounts of water and swell when immersed. Microgels made out of PEG are non-cytotoxic and non-immunogenic [48]. Moreover, properties like tunability in size, charge alteration, variable mesh size, selection and distribution of functional groups make them ideal candidate to be used for scavenging materials. There are numerous approaches available for designing microgels. Free radical polymerization is the main route of preparation used in majority of these approaches, where relatively low molecular weight monomers are crosslinked to make gels with different functional moieties [49], [50]. In general, there are three known synthetic strategies towards microgels i.e. homogeneous nucleation, emulsification and complexation. In homogeneous nucleation, the gel forms from homogenous solutions of monomers. Emulsifications, where pre-gel droplets are formed and later crosslinked to be stabilized; complexation, which describes the mixing of two polymers in water that form complexes. Recently, microfluidic reactions become of high interest and where also used for the synthesis of microgels [51], [52]. This mainly emulsification-based method yields a high number of gels in a short time, with precise control of shape, functionality and size-distribution. Because of the tight control of the tunable characteristics of microgels, microfluidic synthesis are often considered as superior to conventional batch syntheses, when it comes to high performance gels in biomedical applications.

In this chapter microgel synthesis and modification is described which can be used as a potential candidate for scavenging TcdA. Acrylate functionalized six armed polyethyleneoxide-stat-polypropyleneoxide (EO-stat-PO) star-shaped [53] polymer (mol wt. 18 kDa) was used for designing microgels from macromonomers. It was done in pressure controlled microfluidic devices using inverse emulsion as templates for the microgel colloids (water in oil). Glycan-functionalized microgels were prepared by successfully incorporating the N-acetyllactosamine (LacNAc) and Galili epitope with allyl linker, which were fabricated via a chemo-enzymatic cascade reaction of recombinant glycosyl-transferases. Magic angle spinning solid state NMR (MAS-NMR) was used to validate the presence of glycans in the microgels. To check whether the mesh size of microgels is large enough to allow TcdA to diffuse inside, experiments were conducted with fluorescently labelled model proteins of comparable sizes to TcdA. Highly sensitive flow cytometry (FACS) and wellplate format was used to check the binding of the LacNAc
specific lectin from *erythrina cristagalli* (ECL) [54] and binding of recombinantly expressed TcdA receptor binding domain (TcdA-R). The influence of different glycans contents (in the gels) with respect to protein specificity and uptake was studied to find the optimum parameters for future *in vivo* experiments.
4.2 Results and Discussion

To incorporate the glycans into the microgels, a polymerizable linker had to be incorporated into the microgel network. To achieve this, first allyl-functionalized GlcNAc glycoconjugates were prepared in a three step synthesis from 2-acetamido-2-deoxy-d-glucose. It was further used as a substrate for the enzymatic reactions with recombinant glycosyltransferases. The enzymatic synthesis offers advantage over the chemical synthesis of oligosaccharide in such a way that no protection and deprotection steps are required. The enzymatic synthesis was performed in collaboration with Ruben Rosenkrantz from Helmholtz Institute Aachen. The synthesis of allyl-functionalized GlcNAc glycoconjugates was performed according to the literature [55]. In the first step, protection of the −OH groups and replacement with chloride of 2-acetamido-2-deoxy-d-glucose 1 was done using acetyl chloride. A huge advantage of this step is that both the protection and replacement takes place in a single step. $^1$H NMR and $^{13}$C NMR confirmed the product. Then compound 2 was heated with allyl alcohol in the presence of silver perchlorate and silver carbonate to attach the allyl linker. Finally, the deprotection was performed using sodium methoxide and methanol as a solvent (see Figure 4.1).

![Figure 4.1: Schematic representation of chemical synthesis of GlcNAc-allyl (4) (yellow) and production of LacNAc (5) and the galili-structure (6) using glycosyltransferases via enzymatic cascade reaction.](image-url)
Both allyl functionalized LacNAc (5) and galili (6) were obtained in very good yields after enzymatic conversion. NMR and ESI-MS (5[M-H]$^{-}$ = 422 m/z, 6 [M-H]$^{-}$ = 584 m/z) were used to confirm the obtained products. The glycosyltransferase system allows a wide variety of substitutions at the anomeric C-atom of GlcNAc and in most cases, the reaction was completed within 24 h [56], [57]. The products were purified by high performance liquid chromatography (HPLC) with product loss of 20 %. The generation of microgels was achieved by applying two different devices i.e. glass capillary devices and pressure driven devices with polydimethylsiloxane (PDMS) flow cell having a channel diameters of 20 µm. Glass capillary devices were used to produce large size microgels with diameter ranging from 300-500 µm. Here the liquids were supplied to the device by means of syringe pumps.

![Microgels](image)

**Figure 4.2:** (a) Bright field microscopic image of a glass capillary device for producing droplets based on flow-focusing geometry. (b) A close-up of the same image showing the production of droplets. (c) Bright field micrograph of microgels produced by the same device.

Microgels were prepared from acrylate terminated six-arm star shaped PEG prepolymer using ammonium persulfate as initiator and tetramethylethylenediamine (TEMED) as a catalyst. PEG-acrylate was selected due to its biocompatibility, non-cytotoxicity and anti-fouling characteristics [58], [59]. The aqueous solution of star PEG was emulsified in the microfluidic devices to obtain monodisperse droplets in the
micro- and millimeter ranges. The emulsion was stabilized with Span 80. Continuous phase consisted of paraffin oil with the surfactant. The microgels were water in oil functionalized with different glycans e.g. GlcNAc, LacNAc, and Galili. In the inverse emulsion pre-polymer and initiator were dissolved in the aqueous phase. The catalyst and surfactant were dissolved in the continuous phase. The size of the microgels was controlled by adjusting the flow rates and diameter of the inner capillary of the glass capillary device or the channel diameter in the PDMS devices.

PDMS device was applied to synthesize smaller sized microgels (diameter ranging from 10-50 μm) while the same dispersed phase formulations was applied. However to gain better control over the polymerization, the thermally active radical initiator was replaced with a photoinitiator i.e. 2-hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone (HEMP). 50% of n-Hexane was added to paraffin oil to reduce the viscosity of continuous phase. This is necessary due to viscous and capillary forces which arise due to smaller channel diameters in the PDMS devices. Before use the dispersed phase was purged with nitrogen for 15 min to ensure oxygen-free reagents. The surfactant was added to the continuous phase consisting of paraffin oil and n-Hexane. The throughput in the PDMS device was much lower than in the glass capillary devices. Pressure driven flow was applied to be able to control the flow rates precisely over several days and collect monodisperse droplets in the acceptable volumes.

Figure 4.3: (a) Bright field microscopic image of a typical PDMS microfluidic device based on flow focusing geometry. (b) A close-up of the same image showing the production of droplets.
(c) Bright field micrograph of microgels produced by the same device. (d) A close up of image C. Scale bars denote 100 μm.

To initiate the photoinitiator and start crosslinking of the star-PEG precursor a UV-lamp was placed on the collection flask to initiate the polymerization and to cross-link the droplets. Various concentrations of allyl-glycans relative to PEG-acrylate were incorporated into the microgels. This *in situ* bioconjugation has the advantage of omitting the necessity of free functional groups on the microgels and circumventing subsequent coupling reactions.

To check successful incorporation and evaluate the amount of attached glycans, MAS-NMR was applied. The assigned glycan signals could be integrated to obtain molar ratios of incorporation. It was concluded that adding a 4-fold molar excess of glycan per macromonomer produces a 1:1 final ratio in the microgel as the allyl functionality will react much more slowly than the acrylate moieties in star-PEG (see Figure 4.4).
Figure 4.4: MAS-NMR spectrums of A) microgels tailored with GlcNAc, with annotation of the signals used for the ratio determination, B) spectrum of PEG-Acrylate, C) spectrum of GlcNAc with allyl linker.

By doubling the initially added molar excess produce microgels with a 2:1 ratio of glycan per PEG-acrylate. The impact of sugar attachment on protein binding with different glycan incorporation ratios was studied. There should be a boost in protein binding for increasing molar ratio of glycan. This is due to two main reasons: First, there should be higher affinity because of multivalent binding effects. Secondly, more binding sites are available for the attachment of lectins and the CD-toxins. The mesh size of the microgels is large enough to allow access and diffusion of lectins and the toxin, diffusion experiments with differently size lectins were performed. The lectins and proteins were fluorescently labelled to enable observation in confocal microscopy. However, microgels with only fluorescent labels on the surface as well as those with also a fluorescent core were observable in confocal microscopy. It was concluded that if all the microgels are exposed to UV for different periods of time, it produces microgels with different crosslinking densities resulting in different diffusion profiles especially in case of larger proteins (see Figure 4.5). To guarantee the same UV exposure for each microgel and thus generate a uniformly crosslinked ensemble of particles, the microfluidic set up was modified, so that the droplets were irradiated over a fixed distance while flowing out of the microfluidic device. Microgels of very low polydispersity with uniform crosslink density could be achieved. They allowed up to 124 kDa protein to diffuse in without any hindrance (see Figure 4.5).
The microgels fluoresce over their entire volume proving good access for 500 kDa Thyroglobulin protein. Binding experiments were conducted with ECL and TcdA-R using microgels with incorporated ligands 4, 5 and 6. Two different strategies were applied. Recording of fluorescence signals in a wellplate format showed glycan-based specific recognition. In a typical experiment, the microgels were incubated in the dark with 20 μg mL⁻¹ of proteins (TcdA, ECL) in PBS. After 1 h the microgels were centrifuged and the supernatant was removed. For qualitative determination, the microgels were washed with PBS containing 0.05% Tween-20. The microgels were analyzed by fluorescence spectroscopy and the intensity of fluorescence provided by the bound and tagged proteins was recorded. ECL was bound to 5 and TcdA-R to the Galili-structure exclusively. However, this approach was found to be of insufficient sensitivity. In the second approach, FACS analysis was performed, which proved to be an excellent alternative. FACS even allowed the quantification of the exact number of tested microgels. The samples are analyzed using FACS to be able to record fluorescence as well as scattering of the analyzed microgels. This allows discrimination between non-fluorescent and fluorescent microgels and determination of the fluorescence intensity. Microgels where the glycan binding motif and the specific lectin match should exhibit a strong fluorescence signal. Combinations with glycan to lectin mismatch or where unspecific binding occurs should exhibit weaker fluorescence. Using FACS, which is usually applied to investigate and sort populations of cells, we can investigate thousands of microgels in a short period of time. FACS analysis was performed in collaboration with Ana Mandic from UKA Aachen. Quantification and measurement of the fluorescence intensity per microgel is
possible. It is also a highly sensitive method compared to wellplate format due to its single particle tracking character.

Figure 4.6: FACS-plots for lectin binding to glycofunctionalized microgels. A: neutral microgels, B: GlcNAc (4) microgels, C: LacNAc (5) microgels, D: galili (6) microgels. Black: PBS, light blue: ECL, green: TcdA-R, violet: eGFP

Figure 4.6 depicts FACS-plots of different fluorescently labeled proteins binding to the microgels functionalized with no sugar (neutral), functionalized with GlcNAc or LacNAc and functionalized with Galili-structure. PBS and eGFP are selected as nonspecific references. The selection of eGFP is based on the highly fluorescent nature and lack of interaction with the microgels. A remarkable difference in fluorescence intensity is noted for two cases. The ECL binds specifically to LacNAc and TcdA-R binds specifically to Galili. However, no fluorescence shift is noticed for binding to microgels without any sugar moiety (neutral). A histogram was constructed from the FACS-plots for the quantification of specificity (see Figure 4.7).
Figure 4.7: Mean fluorescence intensity (MFI) for A) varying glycans and B) variation of the lectin concentration and amount of incorporated sugars. For read-out the same numbers of gel particles were investigated.

A substantial rise in binding signal is observed in cases of ECL and TcdA-R for the relevant ligands (see Figure 4.7). Subsequently the loading capacity for different glycan
functionalization was investigated. Galili was incorporated into the microgels at different molar rations and the binding interaction with fluorescently labeled TcdA-R and non-binding eGFP was investigated. The most prominent signal is detected with a 1.5 mole% Galili to macromonomer ratio. The signal due to non-specific binding between the microgels and the galili is negligible. Very low signal from unspecific binding proves not only glycan specific interactions but selectivity also. Specific binding of TcdA-R to glycan incorporated microgels is five-times greater than unspecific binding to neutral microgels at this concentration. No interactions between neutral microgels and eGFP are observed even at μM concentrations showing either a TcdA-R mediated adhesion to the gel or some protein size related effects.

4.3 Conclusion

A biocompatible microgel based platform for the demonstration of glycans in a multivalent matter is described here. Due to the flexibility of the synthetic route, microgels with variable size, different functionality i.e. GlcNAc, LacNAc, galili and various concentrations of these glycans were synthesized. These glycans present specific interactions for lectin interactions and the lectin domain of TcdA. Highly selective and specific interactions between protein and microgels are verified by FACS analysis. The microgel mesh size is large enough to give access for the 124 kDa protein to diffuse in. Approval may provide an excellent substitute for previous antimicrobial therapy, as it avoids the interruption of the intestinal flora and deals directly with the inflammation causing toxin. The versatility of the system can be easily envisioned attaching different linkers or other biomolecules than glycans. This leads to production of extremely specific microgels for numerous biological scavenging applications.
4.4 Experimental Section

4.4.1 Materials

All chemicals for preparation of buffers were from Carl Roth (Karlsruhe, Germany) and used without further clean-up. Chemicals for SI-ATRP were from Sigma-Aldrich (Steinheim, Germany). No additional purification was applied. Nucleotide sugars were from Carbosynth (Berkshire, UK). FITC-labeled ECL was from VectorLabs (Burlingame, CA, US). All other origins of materials are stated within the text.

4.4.2 Production of TcdA-R

For expression of TcdA-R the amino acids 1833-2710 of TcdA bearing the receptor/lectin domain were reverse translated into DNA in silico, compared to reported sequences (Swiss. Prot.: P16154.2 GenBank:X51797.1) and the coding sequence was optimized for expression in E. coli.[1,2] The gene of 2631 bp with appropriate restriction sites (NcoI, NdeI and NotI) was synthesized by Genescript (Piscataway, NJ, USA) and provided in pUC57. After plasmid propagation in E. coli NEB Turbo (New England Biolabs, Frankfurt a. Main, Germany) the TcdA-R gene was cloned using NdeI and NotI into pET28a (Novagen, Schwalmbach, Germany), which was previously prepared to code an N-terminal eGFP-fusion. His₆-eGFP-TcdA-R was expressed in E. coli BL21 (DE3) plysS (Novagen, Schwalmbach, Germany) in a 1.5 L Minifors fermenter from Infors (Bottmingen, Switzerland). TB-Medium was used adding 50 μg mL⁻¹ kanamycin. Growth was carried out at 37°C with 1100 RPM and maximum aeration rate. By reaching the stationary phase the temperature was decreased to 20°C and the expression was induced by adding 0.1 mM IPTG, maintaining aeration and mixing for 24 h. Additionally, the cells were fed with 50% glycerol in water at a flow rate of 0.1 mL min⁻¹. Cells were harvested by centrifugation, lysed via sonication and the protein of interest was purified using HisTrap HP columns as well as Aeka Systems (GE Healthcare, Solingen, Germany). The buffer was exchanged by dialysis to give TcdA-R in PBS with 25 % (v/v) glycerol. The protein was stored at -20°C and used after dilution in PBS.

4.4.3 Production of glycosyltransferases

The utilized glycosyltransferases were expressed and purified as described elsewhere. Briefly, β4GalT was expressed in E. coli Shuffle T7 Express (New England Biolabs, Frankfurt am Main, Germany) to enhance the activity, α3GalT was expressed in E.
coli BL21(DE3) (Novagen, Schwalm-Bach, Germany). Purification was done by using HisTrap HP columns and Aekta Systems (GE Healthcare, Solingen, Germany). β4GalT was used directly after purification, α3GalT needed an exchange of buffer to 25 mM MES-NaOH pH=6.0, 25 mM KCl via diafiltration or dialysis. Both enzymes were stored at 4°C.

4.4.4 Glycosyltransferase reaction

The reaction was generally performed as described elsewhere for β4GalT [55]. Briefly, up to 500 mg of 4 was dissolved at a concentration of 5 mM in a solution containing final concentrations of 7.5 mM UDP-α-D-galactose, 25 mM HEPES-NaOH, pH=7.6, 2 mM MnCl₂, 5 U alkaline phosphatase (life technologies, Darmstadt, Germany) and 5 U β4GalT. The reaction was incubated at 30°C for 24 h – 48 h. As no residual substrate was detected anymore by RP-HPLC-MS the reaction was terminated by heating and the precipitated, denatured enzyme was filtered off. The product 5 was achieved after preparative HPLC and freeze-drying as white powder with a yield of 85 %. Analysis was carried out by ESI-MS and NMR. 5 were utilized as acceptor substrate for the second reaction with α3GalT. Up to 500 mg of 5 was dissolved in 25 mM MES-NaOH pH=6.0, 25 mM KCl, 2 mM MnCl₂, 7.5 mM UDP-α-D-Galactose, 5 U alkaline phosphatase and 5 U of α3GalT at a final concentration of 5 mM. The reactions were carried out at 30°C for 24 h – 48 h. Analysis and purification of the galili-structure was carried out as described before. The product 6 was achieved after freeze-drying as off-white powder with a yield of 60 %.

4.4.5 Synthesis of microgels

The continuous phase A is prepared by the mixing of paraffin oil and n-hexane that is added to reduce the viscosity, in the ratio of 1:1. To this mixture is added as surfactant, 8 wt% of Span 80 and the mixture is stirred vigorously for 10 min to achieve a homogeneous mixture.

The discontinuous phase B is prepared by adding Ac-sPEG (10 wt %) to deionized water and stirred vigorously for 10 minutes or until the solution process is completed. As the next step, 2-Hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone (HEMP) (0.5 wt %) is added to the mixture and is also stirred vigorously for 10 minutes. At the point of addition, the solution must be protected from light to suppress an uncontrolled polymerization, as by wrapping the snap lid glass with aluminum foil.
Both solutions A and B are placed into separated pressure vessels that are connected via tubing to the device to the appropriate inlets. With the aid of a microscope the cross section is observed and the pressure is adjusted so that droplets are formed by the ideal flow rates of A and B corresponding to the channel diameter. Through the outlet, connected with a tubing, the droplets are polymerized by passing an irradiation source with \( \lambda = 380 \text{ nm} \). The microgels are collected in a snap lid glass with B under continuous stirring.

The clean-up procedure is achieved in three steps. The collected emulsion is transferred into a tube. In the first step, the sample is washed by adding hexane and is subsequently centrifuged. The excess solvent is removed by the means of a pipette and the obtained microgel re-dispersed in hexane. This operation was repeated three times with hexane, isopropanol and deionized water. The purified microgels are stored at 8 °C.

For glycofunctionalized microgels the sugar compound must be added to a mixture of B before adding the initiator. The weighed sugar is added under vigorous stirring to the solution until it has completely dissolved, which results in C. Following the initiator is added and the solution is stirred for 10 minutes. The snap lid glass was covered with aluminum foil to prevent an uncontrolled polymerization.

### 4.4.6 Diffusion experiments

For diffusion experiments microgels were incubated in the dark with 20 \( \mu \text{g mL}^{-1} \) of proteins (eGFP, TcdA, ECL) in PBS. After 1 h the gels were centrifuged and the supernatant was removed. After that the gels were analyzed via confocal laser scanning microscopy. For qualitative determination of binding the same experiment was performed, but with washing of the gels after incubation with PBS containing 0.05 % Tween-20.

### 4.4.7 Specific binding of proteins by microgels

Binding studies were carried out in a wellplate format and for more sensitive and quantitative analysis FACS was applied. For the wellplate format a procedure basically as described for diffusion experiments was carried out with additional washing steps. After that, the gels were analyzed by fluorescence spectroscopy and the intensity of fluorescence provided by the bound and tagged proteins was recorded.

### 4.4.8 Synthesis of 1-allyl-N-acetylglucosamine

The synthesis of was carried out according to the procedure previously published [55].
4.4.9 Synthesis of 2-acetamido-2-deoxy-3,4,6-trio-O-acetyl-α-D-glu-copyranosyl chloride

A round bottom flask equipped with a magnetic stirrer bar was charged with acetyl chloride (100 mL). 2-acetamido-2-deoxy-d-glucose (25 g, 0.11 mol) were dissolved to the acetyl chloride. The reflux condenser was installed and the resulting mixture was stirred for 20 h at room temperature. Chloroform (200 mL) was added and the solution was poured under vigorous stirring on 200 g ice in 50 mL water. The organic layer was neutralized with 200 mL ice-cold saturated NaHCO₃ solution. The washing procedure was done in 10 min. The organic phase was dried with anhydrous sodium sulfate, filtered and washed with dichloromethane afterwards. The filtrate was concentrated to 40 mL and 250 mL dry ether was rapidly added. The crystallization took place at room temperature after 12 h. The solid was filtered and washed with dry ether. The product was dried at high vacuum (yield 64%).

4.4.10 Synthesis of 2-propenyl 2-acetamido-2-deoxy-3,4,6-trio-O-acetyl-β-D-glucopyranose

A round bottom flask equipped with a magnetic stirrer bar was charged with molecular sieve (0.3 mm pore size), dichloromethane (in excess), 2-propen-1-ol (3 eq. 8.1 mmol, 0.48 g) and purged with nitrogen. Silver carbonate (7.6 g, 2.77 mmol, 1 eq.) was added and resulting solution was stirred for 2 hours. 2-acetamido-2-deoxy-3,4,6-trio-O-acetyl-D-glucopyranosyl chloride (10 g, 2.77 mmol, 1.0 eq.) and catalytic amount of silver perchlorate monohydrate (30 mg) were added and mixture was stirred overnight. The solids were filtered over celite 545 and washed with dichloromethane. The filtrate was washed with saturated NaHCO₃ solution and distilled water. The organic phase was dried over sodium sulphate, filtered and concentrated under reduced pressure. The residue was purified by flash column chromatography using silica gel as stationary phase and ethyl acetate/n-hexane as mobile phase (yield 71%).

4.4.11 Synthesis of 2-propenyl 2-acetamido-2-deoxy-β-D-glucop-yranose

A round bottom flask equipped with a magnetic stirrer bar was charged with dry methanol (3 mL). 2-propenyl 2-acetamido-2-deoxy-3,4,6-trio-O-acetyl-βD-glucopyranose (200 mg, 0.4 mmol), NaOMe (8.7 mg, 2.0 eq) were added to the methanol. The resulting mixture was stirred for 5 hours at 0 °C. The reaction was controlled with thin layer
chromatography. After all the reactant was consumed, water (5 mL) was added to the mixture. Methanol and water were evaporated under reduced pressure. The NaOMe was removed by column chromatography using ethanol as an eluent. The product dried under high vacuum (82 %).
4.5 NMR Spectra

4.5.1 2-acetamido-2-deoxy-3,4,6-trio-O-acetyl-α-D-glucopyranosyl chloride

4.5.2 $^1$H NMR

Figure 4.8: $^1$H NMR of 2-acetamido-2-deoxy-d-glucose.

Figure 4.9: $^1$H NMR of 2-acetamido-2-deoxy-3,4,6-trio-O-acetyl-α-D-glucopyranosyl chloride.
4.5.3 $^{13}$C NMR

Figure 4.10: $^{13}$C NMR of 2-acetamido-2-deoxy-d-glucose.

Figure 4.11: $^{13}$C NMR of 2-acetamido-2-deoxy-3,4,6-trio-O-acetyl-α-D-glucopyranosyl chloride.
4.5.4 2-propenyl 2-acetamido-2-deoxy-3,4,6-trio-O-acetyl-\(\beta\)-D-gluco-pyranose

4.5.5 \(\textsuperscript{1}H\) NMR

**Figure 4.12**: \(\textsuperscript{1}H\) NMR of 2-acetamido-2-deoxy-3,4,6-trio-O-acetyl-\(\beta\)-D-glucopyranosyl chloride.

**Figure 4.13**: \(\textsuperscript{1}H\) NMR of 2-propenyl 2-acetamido-2-deoxy-3,4,6-trio-O-acetyl-\(\beta\)-D-glucopyranose-se.
4.5.6 $^{13}$C NMR

Figure 4.14: $^{13}$C NMR of 2-acetamido-2-deoxy-3,4,6-trio-acyl-β-D-glucopyranosyl chloride.

Figure 4.15: $^{13}$C NMR of 2-propenyl 2-acetamido-2-deoxy-3,4,6-trio-acyl-β-D-glucopyranose.
4.6 2-propenyl 2-acetamido-2-deoxy-β-D-glucopyranose

4.6.1 $^1$H NMR

**Figure 4.16:** $^1$H NMR of 2-propenyl 2-acetamido-2-deoxy-3,4,6-trio-O-acetyl-β-D-glucopyranose.

**Figure 4.17:** $^1$H NMR of 2-propenyl 2-acetamido-2-desoxy-β-D-glucopyranose.
4.6.2 $^{13}$C NMR

**Figure 4.18:** $^{13}$C NMR of 2-propenyl 2-acetamido-2-deoxy-3,4,6-trio-acetyl-$\beta$-D-glucopyranose.

**Figure 4.19:** $^{13}$C NMR of 2-propenyl 2-acetamido-2-deoxy-$\beta\beta$-D-glucopyranose.
4.7 References


Naveed Anwar

Address: Holtenauer Straße 213, 24106 Kiel, Germany | Mobile: +49 176 63863220 | email: naveed.anwar@rwth-aachen.de

Nationality: German
Date of Birth: Oct. 10, 1983
Marital Status: Single

Education

Jan. 2016-Feb. 2017 Postdoc, Organic Chemistry Christian Albrecht University (Kiel, Germany)

Synthesis of multivalent glycoclusters

Sep. 2011–Jan. 2016 PhD, Polymer Chemistry DWI-Leibniz Institute for Interactive Materials, RWTH Aachen University (Aachen, Germany)

PhD thesis: Polymeric particles with biological recognition motifs for medical applications
Promotor: Prof. Martin Möller

Oct. 2008–Aug. 2011 MSc, Organic Chemistry Technical University Braunschweig, (Braunschweig, Germany)

Master’s thesis: 2-Cyanoperchlorobutadiene as a synthetic building block for highly substituted thiophenes

Sept. 2003–Feb. 2006 MSc, Chemistry University of the Punjab (Lahore, Pakistan)

Sep. 2000–June. 2003 BSc, Chemistry University of the Punjab (Lahore, Pakistan)

Work Experience

Jan. 2016-Feb. 2017 Postdoc, Organic Chemistry Christian Albrecht University (Kiel, Germany)

Synthesis of multivalent glycoclusters
Investigation of carbohydrate binding to the bacterial lectin FimH
Synthesis of glyco-SAMs
**Research associate**  
DWI-Leibniz Institute for Interactive Materials, RWTH Aachen University  
(Aachen, Germany)

- Polymeric particles with biological recognition motifs for medical applications
- Synthesis of microgels with glycan epitopes using microfluidics and their use for efficient uptake of toxin
- Fabrication of conjugated polymer particles via Sonogashira dispersion polymerization and decoration with RGD for medical imaging
- Synthesis of conjugated polymer particles with switchable moieties via Suzuki coupling
- Good knowledge of characterization techniques like NMR, SEM, GPC, XRD, UV/Vis, IR and Raman spectroscopy

**Research assistant**  
Technical University Braunschweig, (Braunschweig, Germany)

- Institute of Technical Chemistry
  - Analysis of polymers
- Institute of Nanochemistry
  - Synthesis and characterization of nanoparticles
- Institute of Biochemistry and biotechnology
  - Enantioselective synthesis of ketones

**Sr. Color Chemist**  
Nishat Chunian Ltd. (Lahore, Pakistan)

- Handling the matters of lab dipping
- Reactive, vat, disperse dyeing on continuous methods
- Researching and developing on new dyes and chemicals
- Developing the highly cost effective recipes
### Skills

**Technical**

- Nuclear Magnetic Resonance Spectroscopy \(^1\)HNMR, \(^1\)CNMR, (Including HMBC, HSQC, H-H Cosy)
- Organic synthesis, Purification techniques
- Mass spectrometry, Solvent purification systems
- High-Performance Liquid Chromatography
- Fluorescence and UV-Vis spectroscopy
- Scanning electron microscopy
- Gas permeation chromatography
- Microfluidics (Glass capillary device and PDMS device)
- Bright field microscopy, Fluorescence microscopy, Zetasizer, Dynamic light scattering

**Computer**

- Office Tools: MS Office

**Languages**

- Miscellaneous: ChemBioDraw, MESTREc, Topspin, GIMP, EndNote, OriginLab, ImageJ

- German (B1), English (fluent), Urdu (fluent), Punjabi (mother tongue)

### Interests

- Playing cricket, snooker, reading books, travelling, fitness
List of Publications

Development of glycan-functionalized microgels for specific binding of bacterial enterotoxins. (Manuscript in preparation).


