

**"Self-Assembled
Bionanoparticle-Polymer-Conjugates for Building
Soft Composite Membranes"**

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The only limitation is our imagination.

J. -C. Nièpce

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A ma famille

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Posters

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List of abbreviations

Chemicals and bionanoparticles

BIBA	2-Bromo isobutyric acid
CPMV	Cowpea mosaic virus
DCC	1,3-Dicyclohexal carbodiimide
DCM	Dichloromethane
DMAc	Dimethylacetamide
DMF	Dimethylformamide
DMIAAm	2-(dimethyl maleimido)- <i>N</i> -ethylacrylamide
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DOMA	Diocetadecyldimethyl ammonium bromide
DTT	Dithreitol
EDC	(1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride)
ELP	Elastin-like polymer
HABA	2,5-dihydroxybenzoic acid
HMTETA	Hexamethyltriethylenetetramine
HPMA	<i>N</i> -(2-hydroxypropyl)methacrylate
HSF	Horse spleen ferritin
Me ₆ TREN	Tris(2-dimethylaminoethyl)amine
MPC	2-Methylacryloyloxyethyl phosphorylcholine
NaTFA	Sodium trifluoroacetate
NHS-BIBA	<i>N</i> -Hydroxysuccinimide-2-bromo-2-methylpropionate
OEGMA	Oligo(ethylen glycol) methacrylate
PCF	Polycationic ferritin
PEG	Poly(ethylen glycol)
PMDETA	Pentamethyldiethylene triamine
PNAS	Poly(<i>N</i> -acryloxysuccinimide)
PNIPAAm	Poly(<i>N</i> -isopropylacrylamide)
PNMS	Poly(methacryloyloxysuccinimide)
PVDF	Polyvinylidene fluoride
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
Sulfo-NHS	<i>N</i> -hydroxysulfosuccinimide
THF	Tetrahydrofuran
TNBS	2, 4, 6,-Trinitrobenzenesulfonate or Picrylsulfonate acid

Technical terms

AFM	Atomic force microscopy
ATRP	Atom transfer radical polymerization
BAM	Brewster angle microscopy
CEVS	Controlled-environment vitrification system
Cryo-TEM	Cryo-transmission electron microscopy
CRP	Controlled/living radical polymerization
DLS	Dynamic Light Scattering
LBL	Layer-by-layer
LCST	Lower critical solution transition
LTM-AFM	Liquid tapping mode-atomic force microscopy
LRP	Living radical polymerization
LS	Light scattering
MALDI-ToF	Matrix-assisted laser desorption ionization time-of-flight
MS	Mass spectrometry
MWD	Molecular Weight Distribution
NMP	Nitroxide-mediated polymerization
OWLS	Optical waveguide lightmode spectroscopy
PDI	Polydispersity index
pH	Negative common logarithm of proton concentration ($-\lg[H^+]$)
pI	Isoelectric point
PRE	Persistent radical effect
QCM	Quartz crystal microbalance
RAFT	Reversible addition-fragmentation transfer
RI	Refractive index
RSA	Random sequential adsorption
SAM	Self-assembled monolayer
SDS-PAGE	Sodium dodecyl sulfate-poly(acrylamide) gel electrophoresis
SEC	Size exclusion chromatography
SEM	Scanning electron microscopy
SPR	Surface plasmon resonance
TEM	Transmission electron microscopy
TIRE	Total internal reflection ellipsometry
TIRF	Total internal reflection fluorescence
UV-Vis	Ultraviolet-visible
wt%	weight percent
XPS	X-ray photoelectron spectroscopy
XRD	X-ray diffraction

Physical symbols

Symbols that are generally used and not explained within the equations:

T	absolute temperature [K]
R	molar gas constant: $8.314510 \text{ J}\cdot\text{mol}^{-1}\cdot\text{K}^{-1}$
N_A	Avogadro constant: $6.0221367 \cdot 10^{23} \text{ mol}^{-1}$
k_B	Boltzmann constant: $1.380658 \cdot 10^{-23} \text{ J}\cdot\text{K}^{-1}$
g	gravitational acceleration: $9.80665 \text{ m}\cdot\text{s}^{-2}$

1 Summary

This project was focused on building a smart nanoporous membrane based on an assembly of bionanoconjugates. Horse spleen ferritin was chosen as a model protein scaffold to graft thermoresponsive polymers as Poly(*N*-isopropylacrylamide) (PNIPAAm) to it using the *grafting from* approach. PNIPAAm is a well known thermo-responsive polymer exhibiting a LCST of 32°C. After developing the well controlled atom transfer radical polymerization (ATRP) of NIPAAm in water at low temperature, the in-situ grafting of PNIPAAm from the ferritin was achieved.

The *grafting from* approach consisted of two steps: the modification of ferritin into a macro-initiator by coupling the N-hydroxysuccinimide activated 2-bromo isobutyric acid ATRP initiator to the 72 addressable ϵ -amino end groups around ferritin polypeptide chains. It allows the modification of the majority of the amino sites of ferritin and induces a polymerization resulting in a higher grafting density. The condition of this chemistry has to be mild to prevent the denaturation of the proteins.

The challenge was the preparation of monomeric conjugates with low polydispersity as ferritin aggregates in solution and perturbs the polymerization process. The resulting conjugates are well defined and show a thermo-responsive behavior with a T_c of about 31.5 °C.

The building of the membrane requires the crosslinking of the polymer matrix. The crosslinking was realized by a random copolymerization of NIPAAm with the monomer DMIAAm, (2-(dimethyl maleimido)-*N*-ethylacrylamide), a photocrosslinker. This photocrosslinker hosts a maleimide group able to form cyclobutane rings activated of UV irradiation and a sensitizer thioxanthone. The photocrosslinker is fully assimilated in the copolymer and decreases the lower critical solution temperature depending on its ratio.

The assembly of the ferritin-P(NIPAAm-DMIAAm) conjugates at the solid-liquid

1 Summary

interfaces on a mica sheet or silicon wafer creates a homogeneous thin polymer-protein film. After crosslinking under UV irradiation while cooling the sample, the polymer matrix undergoes protein denaturation by the use of chaotropic agents and temperature to create nanopores in the membrane. The final membrane is a thin polymer film with monodisperse pores of about 16 nm of diameter and 2 nm depth carrying different functional groups.

Such conjugates can also be assembled at the liquid-liquid interfaces forming porous micro-capsules as they reduce the interfacial tension and are able to stabilize oil droplets in solution. The ferritin-P(NIPAAm-DMIAAm) conjugates are interesting building blocks with adjustable properties able to produce functional and switchable nanoporous membranes and capsules.

2 Zusammenfassung

Dieses Projekt konzentrierte sich auf den Aufbau intelligenter, nanoporöser Membranen durch die Anordnung von Bionanokonjugaten. Horse Spleen Ferritin wurde als Modellprotein gewählt, um an dessen Gerüst thermo-responsive Polymere wie Poly-(N-isopropylacrylamid) (PNIPAAm) mittels *grafting from*-Technik aufzupolymerisieren. PNIPAAm ist als thermo-sensitives Polymer hinreichend bekannt und zeigt eine LCST von 32°C. Nach der Entwicklung einer kontrollierten Atom Transfer Radical Polymerization (ATRP)-Methode von NIPAAm in Wasser bei niedriger Temperatur, konnte die Polymerisation von NIPAAm ausgehend von der Ferritinoberfläche (*grafting from*-Technik) verwirklicht werden.

Die *grafting from*-Methode setzt sich aus zwei Schritten zusammen: Ferritin wird durch die Kupplung des ATRP Initiators (N-hydroxysuccinimid-aktivierte 2-Bromisobuttersäure) an die 72 zugänglichen ϵ -Amino-Endgruppen entlang der Ferritin-Polypeptidketten in einen Makroinitiator umgewandelt. Anschließend erfolgt die Polymerisation von NIPAAm ausgehend von der Ferritin-Oberfläche. Das *grafting from* erlaubt die Modifikation einer Mehrzahl der Amino-Funktionalitäten des Ferritins, so dass die anschließende Polymerisation zu einer höheren Dichte der Ketten an der Oberfläche führt. Die Bedingungen für diese Reaktionen müssen aufgrund der Anwesenheit des Proteins sehr mild sein.

Da Ferritin Aggregate bildet und diese den Polymerisationsprozess negativ beeinflussen, bestand die Herausforderung darin, monomere Konjugate mit niedriger Polydispersität herzustellen. Die resultierenden Konjugate sind genau definiert und zeigen thermo-responsives Verhalten mit einer T_c von 31.5°C.

Der Aufbau von Membranen erfordert eine Vernetzung der Polymermatrix. Diese Vernetzung konnte durch eine statistische Copolymerisation von NIPAAm mit DMIAAm (2-(Dimethylmaleimido)-N-ethylacrylamid), einem Photovernetzer, ermöglicht werden. Der erwähnte Vernetzer besitzt eine Maleimid-Funktionalität, die, aktiviert durch UV-Bestrahlung in Gegenwart eines Thioxanthones als Sen-

sibilisator, in der Lage ist, Cyclobutanringe zu bilden. Der Photovernetzer wird vollständig in das Copolymer eingebunden und verringert die Phasenübergangstemperatur in Abhängigkeit von seinem Einbauverhältnis.

Die Anordnung der Ferritin-P(NIPAAm-DMIAAm)-Konjugate an einer Fest-Flüssig-Grenzfläche auf einem Glimmerplättchen oder Silizium-Wafer führt zur Ausbildung eines dünnen, homogenen Polymer-Protein-Films. Nach Vernetzungen mittels UV-Strahlung können die Proteine im Polymerfilm chemisch denaturiert werden und ergeben monodisperse Poren von 2nm Tiefe. Solche Konjugate können auch an flüssigen Grenzflächen angeordnet werden. Oberflächenspannungsmessungen haben hierbei eine dramatische Erniedrigung der Grenzflächenspannung ergeben. Anschließende Vernetzung der Aggregate liefert robuste, poröse Mikrokapseln.

Ferritin-P(NIPAAm-DMIAAm)-Konjugaten sind interessante Bausteine, die durch ihre schaltbaren Eigenschaften zum Aufbau von funktionalen und variablen nanoporösen Membranen und Kapseln beitragen.

3 Introduction

At the dawn of the 21st century, it was recognized that reducing the size to the nanometer scale enabled the creation of novel materials. Great technological achievements were possible due to newly discovered mechanical, optical and electronic properties of nanosized and nanostructured materials. In the traditional *top-down* approach, materials are structured by physical or chemical means from initially unpatterned materials. The *bottom-up* approach uses the self-assembly properties of nanosized (macro)molecular and colloidal building blocks to build functional nanodevices with medical applications, as the devices can enter the cells and treat them specifically. Currently, technological limitations of the top-down approach indicate that bottom-up approaches are very promising, supported by great achievements¹ in the synthesis of novel nanoparticle building blocks.

Water solubility, biocompatibility, non toxicity and bioactivity represent the requirements for smart materials and devices for medical applications. Thus nucleic acids and proteins are wonderful tools for materials science. They have many facets, which make them very useful to create any kind of hybrids with specific properties required for future bionanotechnology fields such as novel materials for sensing, diagnostic and therapeutics applications. Proteins can be considered as macromolecules as they consist of repeating amino acids. Modern microbiological and biochemical methods like recombinant DNA make possible the precisely defined synthesis of novel proteins, such as the natural polymer Elastin-Like Polymer (ELP), with predefined specific properties^{2,3}.

Proteins have nanometric size and that is why they can be considered as well defined nanoparticles. It is then normal to conjugate them to inorganic nanoparticles. Proteins have different types of structure, such as globular or tubular. They can be used as template for creation of perfectly defined hybrids, scaffolds or surfaces^{4,5}. Another positive feature is their perfect monodispersity in shape but also in addressable chemical groups. The conjugation of proteins to inorganic nanoparticles combines the best of two worlds: valuable optical, electronic and

magnetic properties of inorganic nanoparticles with biocompatibility and specific biological functions of proteins. The reaction tool set onto proteins is called bio-conjugation that enables conjugation to dye labels, gold nanoparticles, enzymes or the grafting of polymers.

Numerous polymer based nanodevices for drug delivery were developed. Today challenges lie in increasing the efficiency of the drug carrier to reach the target site in order to avoid side effects of cancer treatments for example. In the beginning, low molecular weight drugs were conjugated to water soluble, non toxic, non immunogenic and degradable polymers through exhibiting side groups. Intensive studies were led on *N*-(2-hydroxypropyl)methacrylate (HPMA) copolymers and many different structural and architectural variations such as star-like or dendritic polymers. The control of structure allows a better transport as well as slower and more controlled release of the drug. Polymers were also conjugated to proteins in order to stabilize them and prolong their biological half-life, resulting in less frequent administration to the patient. Conjugation of linear or branched poly(ethylene glycol) (PEG) to side-specific amino acids such as the amino or carboxy end of a protein dramatically changes the properties and biological behavior. This technology called pegylation has become an important tool for synthesis of novel pharmaceuticals e. g. in cancer treatment⁶. A further step is the utilization of polymers whose properties can be non-invasively triggered by external stimuli⁷ such as magnetic field⁸, glucose concentration⁹, pH¹⁰ or temperature¹¹ for pulsatile drug release systems.

The synthesis of protein-polymer conjugates through the reaction of defined groups remains a challenge. Reaction statistics are highly unfavorable for the reaction of the very few functional groups compared to the large polymeric chain. Many polymers cannot be used, as they are not water soluble and adopt conformations that bury the functional groups. Some problems can be solved with the advent of extremely efficient crosslinking reactions like *click-chemistry*¹². Bovin Serum Albumin proteins were coupled by azide-alkyne chemistry reactions to poly(*N*-isopropylacrylamide)¹³. The goal of this thesis is to graft various water soluble polymers on bionanoparticles such as viruses or proteins and to create a membrane with well-defined pores. Horse spleen ferritin was chosen as a model protein because of its shape, size, stability and commercial availability. The techniques of *grafting to* and *grafting from* have been investigated. The polymers chosen have to be thermoresponsive and biocompatible, so *N*-Isopropylacrylamide (NIPAAm) and Oligo(Ethylenglycol) Methacrylate (OEGMA) are polymerized in water by Atom Transfer Radical Polymerization (ATRP). The composite polymer-

protein materials are then employed in the construction of a nanoporous responsive membrane with hydrophilic monodisperse pores suitable for drug delivery applications. In order to do so, the obtained conjugates are then assembled at the solid-liquid interface. Crosslinking is achieved by photocrosslinking of the polymer film which is a copolymer of PNIPAAm or PEGMA and a photocrosslinker. The used photo-crosslinker is 2-(dimethyl maleimido)-N-ethylacrylamide (DMIAAm). It is copolymerized in a small ratio with NIPAAm and OEGMA to form a polymer matrix. The proteins are then denaturated using a chaotropic reagent allowing the formation of the membrane, which may still contain chemical functions that are left over by the protein shell and can furthermore be modified chemically. The construction of nanoporous membranes by self-assembly processes is efficient and scalable not only to large areas but also to various scaffolds or interfaces. An assembly around nanoparticles and mesoporous colloids can serve as biocompatible and porous coating¹⁴, thus novel theranostics (therapeutics and diagnostic at the same time) can be developed. An assembly and crosslinking at oil-water interfaces are efficient means to trap water or oil soluble drugs.

The content of this thesis:

- Characterization and purification of horse spleen ferritin
- ATRP of NIPAAm in water
- ATRP of NIPAAm-DMIAAm in water
- Transformation of horse spleen ferritin into a macro-initiator
- Grafting PNIPAAm/PEGMA from horse spleen ferritin
- Assembly of the conjugates at the solid-liquid interface
- Crosslinking of the self-assembled film by UV irradiation
- Denaturation of the conjugates & formation of the membrane

4 Fundamentals

4.1 Horse Spleen Ferritin

Ferritin is a globular protein with an important metabolic function as an iron storage protein. Its ability to sequester iron provides it with a double function of detoxification and iron supply. Ferritin is present in the monocytes-macrophages (type of leukocyte, part of the immune system) of the liver, the spleen and in the bone marrow. It is also present in the cytosol (internal fluid of the cell) of many cells: hepatocytes, heart, lung, testicle, kidney, placenta, red blood cells and leukocytes. The ferritin, used in this study, is the horse spleen ferritin (HSF), found in the spleen of horses.

4.1.1 From Primary to Quaternary Structure

Eukariotic ferritins are made of a mixture of two different types of assembled subunits: the monomer L (Light chain) and the monomer H (Heavy chain); they have a molecular weight of roughly 20 kDa and 21 kDa, respectively. There are differences between the subunit amino acid sequences. Ratios between H- to L- chain subunits in ferritin molecules are found to vary between organisms but horse spleen ferritin is a mixture of roughly 15% H- and 85% L- chain subunits. The L-chain is composed of 174 amino acids, which are represented with different colors respectively to the different amino acid residues (cf. Fig. 4.1). The same unit is represented with different degrees of hydrophobicity, influencing the folding of the globular packing.

The two subunits H and L are structurally similar. Each subunit consists of a bundle of four α -helices (labeled A, B, C, D) composed of two pairs of anti-parallel

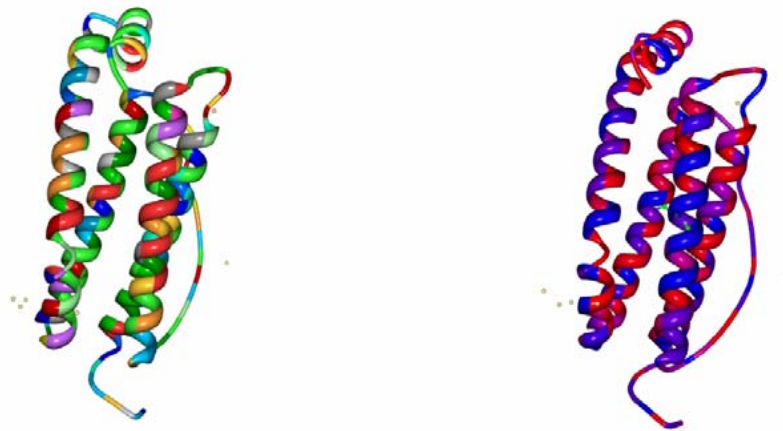


Figure 4.1: Secondary structure of L- chain subunit of cubic symmetry of horse spleen ferritin. Right side: colored part depending on the residues. Orange corresponds to lysine, while glutamic acid is red and cysteine is pink. Left side: the color gradient shows the hydrophobicity of the residues from blue (least hydrophobic) to red (most hydrophobic) (adapted from the Protein data bank (<http://www.rcsb.org>)).

helices (AB, CD). Helices B and C are connected by a loop (L) of 17 residues, while the connections between the other helices are short turns. The bundle of four helices is capped by a fifth helical section (E), which lies at roughly 60° with respect to the bundle axis as illustrated Fig. 4.2. There are 2-8 residues extending beyond helix E to the C-terminal end. At the other N-terminal end of the polypeptide resides a stretch of 8-12 residues. The difference in molecular mass between H- and L- subunits is due to the short extensions¹⁵ at both N- and C- termini of the H-chain.

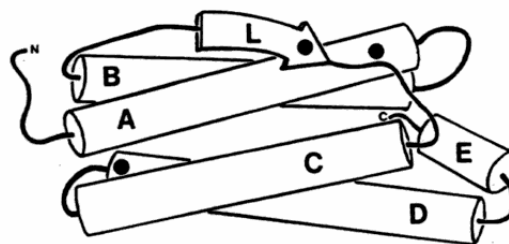


Figure 4.2: Subunit conformation of horse spleen apoferritin, showing the bundle of helices (A, B, C, D) with the short helix (E) and the loop (L), adapted from¹⁵.

On one side, horse spleen ferritin is known to crystallize in three different space groups: cubic (F432), tetragonal (P4₂12) and orthorhombic (P2₁2₁2) as shown in Fig. 4.3. On the other side, the remaining types of ferritin only crystallize in the cubic space group (F432). The protein shell of all different ferritin proteins is made of the assembly of 24 subunits (a mixture of H and L). The protein shell, called apoferritin has a diameter of 125 Å delimiting a cavity with a diameter of 60-80 Å. In the ferritin shell (cubic symmetry), there are eight hydrophilic channels along the threefold axes which allow iron uptake into the ferritin cavity^{16,17}. There are also 6 hydrophobic channels along the fourfold axes whose function has not yet been identified.

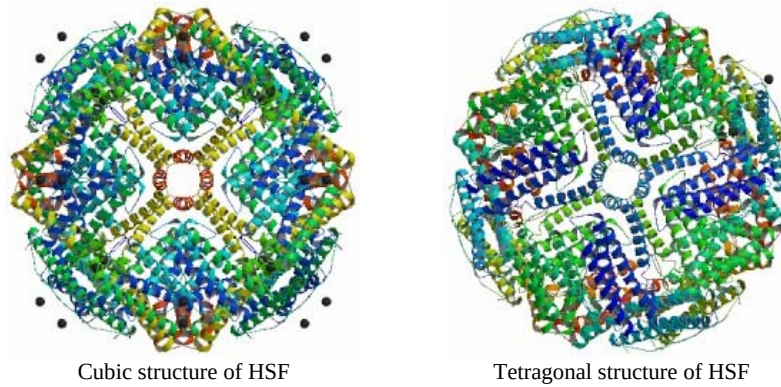


Figure 4.3: Two different crystallographic quaternary structures of horse spleen ferritin, source from protein data bank (<http://www.rcsb.org>).

Granier *et al.*¹⁸ studied the different types of crystallization of horse spleen ferritin by synchrotron X-ray radiation. The orthorhombic symmetry has not been studied yet. Table 4.1 summarizes the collected data.

Table 4.1: X-ray data collection of different ferritin crystallizations.

Symmetry	Cubic	Tetragonal	Orthorhombic
Space group	F 432	(P4 ₂ 12)	(P2 ₁ 2 ₁ 2)
Crystal size (mm)	0.6x0.5x0.6	0.5x0.3x0.25 0.5x0.35x0.25	-
Lattice parameters (Å)	a=182.9	a=b=147.23 c=152.58	-

In this study, horse spleen ferritin has a cubic structure, and is considered as a monodisperse nanoparticle with very well defined addressable chemical func-

tions: ϵ -amino groups from lysine residues, carboxylate groups from glutamic acid residues and sulphhydryl groups from cysteine residues are available all around the apoferritin shell. Many studies tried to determine the number of the addressable groups depending on the type of ferritin^{19,20}. After denaturation from horse spleen apoferritin with guanidine hydrochloride, Wetz and Crichton determined 1.0 ± 0.1 cystein residue per subunit, 4.4 ± 0.4 lysine residues and 11 ± 0.4 carboxyl groups per subunit (7.0 ± 0.7 per subunit on its exterior surface which gives 168 surface COOH groups)²¹. Those numbers do not correspond to the numbers available for the addressable groups because they are estimated while the apoferritin is denaturated into subunits, so the unavailable groups (residing at the interior) are also counted while the apoferritin is reassociated.

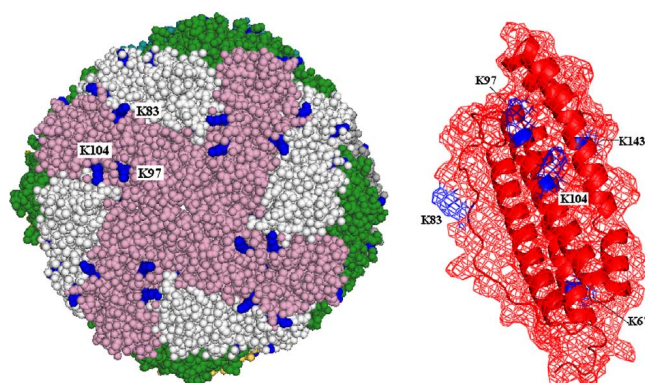


Figure 4.4: Left: Structural model of HSF of cubic symmetry (a dodecahedron), four subunits around the four-fold axis highlighted in violet, exposed lysine residues highlighted in blue. Right: Structural model of one L- subunit of HSF with reactive lysines exposed to surface (K97, K83 and K104) shown in blue color. It consists of a bundle of four long helices lying parallel or anti-parallel to one another, together with a much shorter helix which lies perpendicular to the bundle and a loop of extended chain on the outer surface of the cages²².

Zeng *et al.* determined 24 addressable ϵ -amino end groups using the dye 5-carboxyfluorescein by UV-Vis. Being a rather big molecule, only one per subunit can be attached²³. However, Zeng *et al.* found 9 lysines per L- subunit, of which three are exposed to the surface (K97, K83, and K104) and may be addressed, as seen in Fig. 4.4. Altogether ferritin possesses 72 ϵ -amino which are potentially addressable²².

4.1.2 Function: Iron Storage Protein

Iron is an essential nutrient for the synthesis of iron-porphyrin proteins, such as hemoglobin. The iron molecules are insoluble in their native form under physiological conditions. They are encapsulated as a ferrihydrite core by ferritin. Ferritin plays a significant role in iron detoxification and acts as a large reservoir for iron in a bioavailable form^{24,25}.

Iron is localized inside apoferritin, a spherical macromolecule with a central cavity able to stock iron as a micelle of iron oxide hydroxide-phosphate of an approximate composition $(\text{FeOOH})_8(\text{FeOPO}_3\text{H}_2)$. Ferritin can contain a maximum of 4500 atoms of iron per molecule²⁶. Hydrophilic and hydrophobic pores penetrate the capsid at the eight threefold and the six fourfold axes. Iron enters the capsid via the hydrophilic and hydrophobic pores located at the threefold axes of the capsid and oxygen enters through the hydrophobic pores at the fourfold axes.

The iron uptake depends on the composition of L- chain and H- chain subunits of the ferritin. The ratio can vary between the different types of ferritin from 2 : 22 to 20 : 4 of L- and H- chain respectively^{27,28}. H- rich ferritins catalyze the oxidation of iron (II), while L- rich ferritins promote the nucleation and storage of iron (III). Iso-ferritins rich in H- chain are associated with a rapid iron uptake, and have a protecting role in H rich tissues such as the heart, the brain and the red blood cells. L- rich ferritins have a slower iron uptake and have larger mineralized cores and are found in the liver, spleen or plasma. Generally, L- rich ferritins are more stable against chemical and heat denaturation²⁹.

Horse spleen ferritin has a composition of 85 % of L- chain and 15 % of H- chain subunits³⁰. There are no data about the H- chain of the horse spleen ferritin because this gene has not been cloned yet. Nevertheless, crystallographic studies of Hempstead *et al.*^{31,32} compared the L- chain subunit of the horse spleen ferritin HoLF and the H- chain subunit of human ferritin HuHF (cf. Fig. 4.5). HuHF and HoLF exhibit 53 % identity in primary sequence that influences the 3D structure of the subunit folding and assembly properties and of course the shell stability and the function of the proteins.

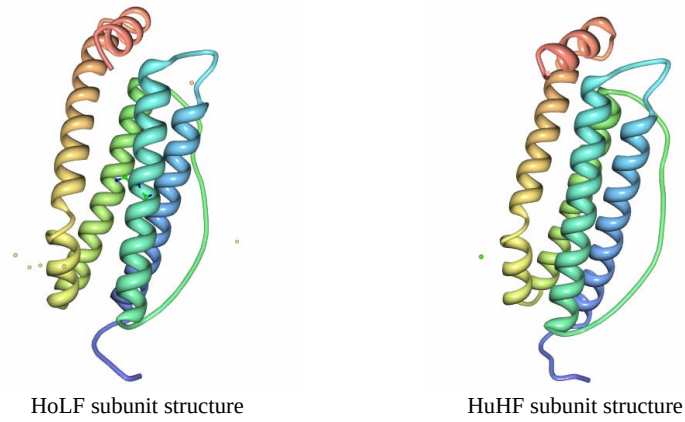


Figure 4.5: Difference in the secondary structure of the subunits HoLF (L- chain) and HuHF (H- chain), adapted from protein data bank.

Finally, Table 4.2 recapitulates the main structural properties of the horse spleen ferritin.

Table 4.2: Structural properties of Horse Spleen Ferritin.

Molecular Mass	440 kDa
Subunits	L- chain 20 kDa (85 %)
Molecular mass	H- chain 21 kDa (15 %)
Radius (Core)	125 Å (60-80 Å)
Symmetries	Cubic (F 432) Tetragonal (P4 ₂ 12) Orthorhombic (P2 ₁ 2 ₁ 2)
Molar absorptivity (per ferritin)²⁹	$\epsilon_{280}=4.8 \cdot 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$ $\epsilon_{280}=1.09 \cdot 10^3 \text{ mL} \cdot \text{mg}^{-1} \cdot \text{cm}^{-1}$
Isoelectric point	pI=4.5 ^{33,34}
dn/dc	in water 0.187 (from Wyatt ^[1]) in SEC buffer 0.02428 ^[2]
For apoferritin	in SEC buffer 0.1805 ^[2]
Addressable groups	Lysine (-NH ₂): 72 Cysteine (-SH): 24 Glutamic acid (-COOH): 168

[1] given by Wyatt size exclusion company for calculation

[2] measured using a RI detector knowing the constant K_{RI} : $(RI) = K_{RI} * c * (dn/dc)$

4.1.3 Denaturation of Horse Spleen Ferritin

The denaturation consists of breaking the quaternary structure so that the protein is separated into tertiary structure subunits. The denaturated state is defined by a breakdown of its native conformation and is accompanied by the loss of biological or biochemical activity. High temperatures break the weak non covalent interactions, that stabilize the folded protein, and convert the folded structure to a largely unfolded one with remarkably different properties e.g. optical rotation, viscosity or UV absorption.

Another way to induce the denaturation of protein is the use of chemical denaturants (also called chaotropic agents) such as urea or guanidinium hydrochloride in high concentrations or detergents like sodium dodecyl sulfate (SDS). These compounds are thought to unfold proteins in large part by competing for hydrogen bonds with the polar groups of the backbone and of the side chains. Denaturing compounds can hydrophobically associate with the non polar residues and expose them to the surface. Thus they lead to a breakdown of the native structure by disrupting the optimized internal hydrophobic interactions. Listowsky *et al.*³⁵ showed that HSF can be denaturated by addition of aqueous guanidine chloride at a concentration of 7 M at pH 7.5 and Zeng²² used urea (6 M) and β -mercapto-ethanol (10 mM). This phenomenon is reversible upon removal of the guanidinium hydrochloride.

4.1.4 Chemistry & Materials Science Involving Horse Spleen Ferritin

Horse spleen ferritin is a powerful tool for materials science. It is perfectly monodisperse in size and in its composition of addressable chemical groups. It is relatively cheap and easily accessible making it a perfect model protein. Moreover, horse spleen ferritin is highly stable regarding temperature (up to 85 °C) and pH range (from 3 to 9). It is water soluble but can undergo a mixture of water and organic solvent in minor quantity such as DMSO and DMF for a short time. HSF has been used as a multivalent nanoplatfrom in a large number of hybridization procedures³⁶.

4.1.4.1 Self-Assembled Monolayers (SAM) & Two-Dimensional Array

Nanoparticles have the ability to self-assemble at different interfaces (solid-liquid-air) creating organized monolayers (c.f. Chapter 4.4). This property is used to create self-assembled monolayers (SAM) on S-layer proteins. S-layers are crystalline, monomolecular (glyco)protein arrays representing one of the most commonly observed surface structures in eubacteria and archaea. They show oblique, trigonal, square or hexagonal lattice symmetry³⁷. Pum *et al.* used polycationic ferritin (PCF) to label the negatively charged hexagonal S-layer lattice, resulting in well ordered PCF arrays following the same orientation of the S-layer³⁸ as shown in Fig. 4.6.

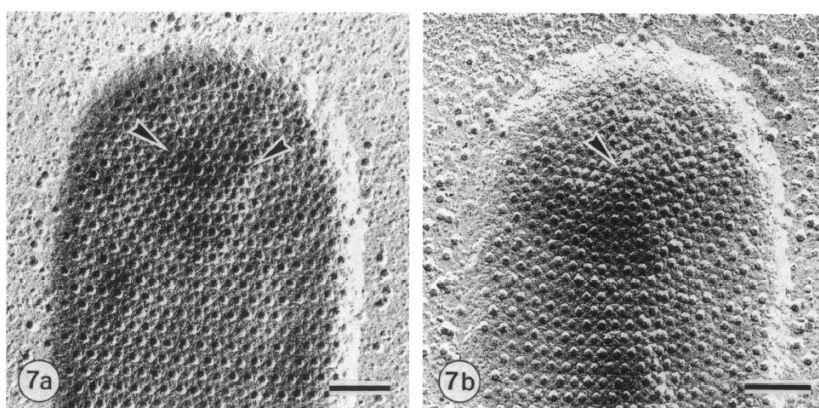


Figure 4.6: Self-assembly of polycationic ferritin on lipid hexagonal S-layer, fixed by glutaraldehyde. (a) and (b) indicate different wedge disclinations in the lattice. Scale bars: 100 nm, adapted from³⁸.

Ferritin can be used for nanopatterned molecular arrays on lipid layers playing with the biotin/streptavidin interaction by utilizing the self-assembly and chemical conjugation of the addressable groups of the shell³⁹ (cf. Fig. 4.7).

Because of its iron storage function, HSF is also used to create two-dimensional arrays for quantum electronics applications^{40,41}. It is indeed easy to realize the self-assembly at the solid-liquid interfaces onto a silicon wafer with previous chemical modification of the surface in order to improve the absorption of the particles. By heat treatment, the organic shell of the ferritin is removed and the Si-wafer is covered with monodisperse iron oxide nanodots that are well organized on a hexagonal lattice.

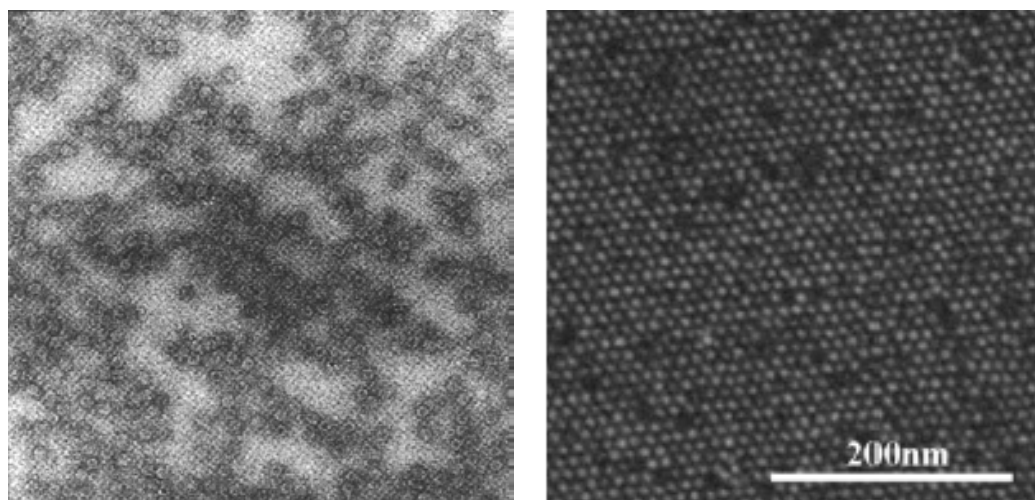


Figure 4.7: Different ferritin arrays. Left: Electron microscopy of a negatively stained, ordered array of streptavidin labeled with biotinylated ferritin. Scale: bottom edge corresponds to $0.53\ \mu\text{m}$; adapted from³⁹. Right: HR-SEM of the two dimensional array of ferritin on Si substrate coated with a hydrophobic layer sintered until $700\ ^\circ\text{C}$, resulting in iron nanodots, adapted from⁴⁰.

4.1.4.2 Self-Assembled Nanoreactor

Reactors allow a confined reaction environment, thus controlling the reaction pathway but also the size and the morphology of the product. Cells were first used for this purpose, then synthetic reactors such as vesicles and micelles formed from self-assembled macromolecules (phospholipids) were developed⁴².

Apo ferritin can be considered as a nanocapsule due to the possibility of removal of the iron atoms from the hydrophobic core of the ferritin through the channels that penetrate the shell by reductive dissolution^{43,44}. Mann *et al.* used ferritin as a nanoreactor to produce monodisperse metal particles from metal different of the natural hydrated iron (III) oxide like manganese oxide, uranyl oxohydroxide and iron sulphide particles (cf. Fig. 4.8). It is a protein cage with the potential to act as constrained reaction environment in the synthesis of inorganic materials of nanometer dimension. It allows different mineralization pathways, such as metathesis mineralization or hydrolysis polymerization (depending on pH) thanks to mineral nucleation sites inside the cavity⁴⁵. Later, ferromagnetic nanocrystals of magnetite (Fe_3O_4) and magnetite/magnemite ($\text{Fe}_3\text{O}_4/\gamma\text{-Fe}_2\text{O}_3$)

were synthesized within the ferritin cavity to construct a magnetic protein called magneto-ferritin^{30,46,47}. By further bioconjugation with biotin and playing with the biotin/streptavidin interaction, an array based on magneto-ferritin particles could be created^{48,49}. Its potential lies within applications in magnetic storage and nanoelectronic devices.

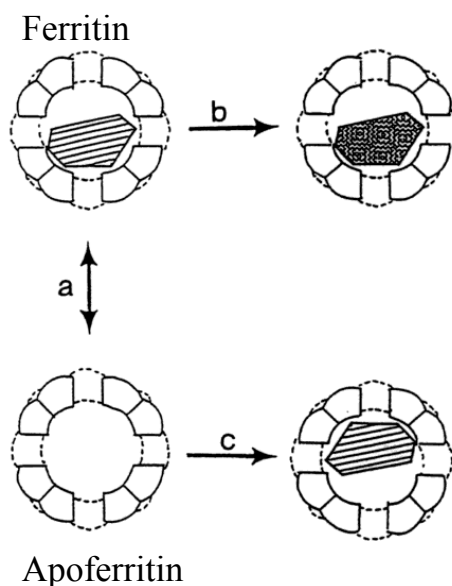


Figure 4.8: Possible reaction pathways for nanoscale synthesis using the protein ferritin: (a) mineralization/demineralization, (b) metathesis mineralization; (c) hydrolysis polymerization, adapted from¹⁵.

4.2 Atom Transfer Radical Polymerization

In this study, Atom Transfer Radical Polymerization (ATRP) was used to build bio-nanoconjugates with horse spleen ferritin. Among all the different techniques of controlled/living radical polymerizations (CRP), ATRP is one of the most accessible polymerization techniques as it includes a large range of available monomers and macro(initiators), a simple reaction setup, and the ability to conduct the process over a large range of temperatures, solvents, and dispersed media. It allows the synthesis of a wide variety of well-defined polymers. Moreover, ATRP is an

adequate technique for the grafting approaches allowing the synthesis of many different structures and applications^{50–58}.

4.2.1 Controlled/Living Radical Polymerization

A polymerization is described as "living" when the formed macromolecular chains are able to grow further if some extra amount of monomer is added to the system, i.e. the active species are stable and the transfer reactions or termination are negligible. The molecular mass increases linearly with the conversion and the distribution of the mass of the chains is narrow.

4.2.1.1 ATRP

Principle

ATRP consists in the exchange of living and dormant species^{59–61}. The living species are growing radical chains. They are activated by a transition metal catalyst such as Cu, complexed by a ligand (M_t^n/Ligand). The change of oxidation state occurs due to the initiation with an alkyl halide ($R-X$) cf. Fig. 4.9. The copper is used as a metal catalyst due to its stability at a higher oxidation state ($\text{Cu(I)}/\text{Cu(II)}$), favoring the dormant species. The monomer is added to the chains, while the radical is propagating. It is deactivated by reacting with the oxidized transition metal halide ($X-M_t^{n+1}/\text{Ligand}$) to reform the initial transition metal catalyst and an oligomeric X-terminated chain P_n-X .

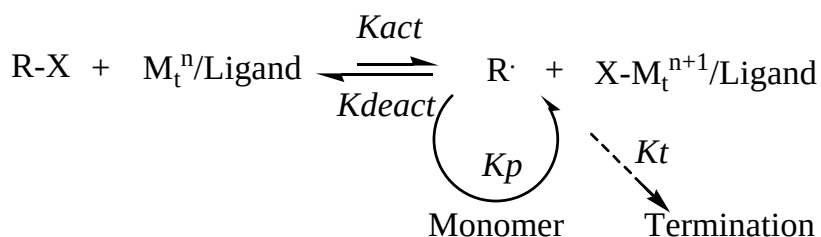


Figure 4.9: Transition metal catalyzed ATRP scheme.

This process occurs with a rate constant of activation K_{act} and deactivation K_{deact} . Polymer chains grow at a rate constant of propagation K_p of the radicals. Termination reactions (K_t) also occur in ATRP through radical coupling and disproportionation but to a smaller degree than other techniques.

In summary the ATRP system is composed of a monomer, an initiator with a transferable halogen, and a catalyst system with a ligand. The solvent and the temperature are also important elements to control the system.

Mechanism

A large variety of monomers has been polymerized by ATRP such as styrenes, (meth)acrylates, (meth)acrylamides and acrylonitrile. Each monomer has its own atom transfer equilibrium constant for its living and dormant species. In the absence of side reactions, the equilibrium constant K_{eq} determines the polymerization rate:

$$K_{eq} = K_{act}/K_{deact} \quad (4.1)$$

ATRP will not occur if K_{eq} is too small, whereas a large K_{eq} will lead to a large amount of termination. For every monomer the concentration of propagating radicals has to be adjusted in order to control the polymerization. The initiator concentration determines the number of growing polymer chains. If the initiation is fast and the side reactions are negligible, the number of chains is constant and equal to the initial concentration of the initiator. The theoretical molecular weight or degree of polymerization (DP) increases reciprocally with the initial concentration of initiator in a living polymerization:

$$DP = [M]_0/[I]_0 \cdot \text{conversion} \quad (4.2)$$

Simultaneously the molecular weight of the polymer chains increases linearly following the conversion while the polydispersity (M_w/M_n) decreases. The alkyl halide ($R-X$) must rapidly and selectively migrate between the growing chain and the transition metal complex. In ATRP, when X is a bromide or a chloride, the molecular weight control is at its optimum. When the initiating moiety R is attached to macromolecular species or proteins, macro-initiators are formed and

can be used to synthesize block/graft copolymers. The catalyst system composed of the transition metal complexed by a ligand is the key of control over polymer chains, as it determines the position of the atom transfer equilibrium and the dynamics of exchange between the dormant and living species. The metal center must possess at least two accessible oxidation states separated by one electron, and it should have a reasonable affinity towards the halide. The ligand should complex the metal strongly and quickly. ATRP can be carried out in solution, in bulk or in heterogeneous systems (emulsion, suspension). Chain transfer to the solvent should be minimal. Interactions between the solvent and the catalytic system should be considered due to the fact that the structure of the catalyst can change in dependence of the solvents. Polymerizations in polar media, such as water are accelerated. It is possible to add an amount of transition metal complex at the higher oxidation state to shift the equilibrium towards the dormant species. The temperature also plays an important role as the rate of polymerization increases with increase in temperature. Moreover at higher temperature the termination is more pronounced and the catalyst can be decomposed. Prolonged reaction time leads to nearly complete monomer conversion. This may not increase the polydispersity but will induce loss of end groups. To obtain polymers with an end group functionality, the conversion should not exceed 95 %.

Kinetics

Assuming negligible contribution of termination and using a fast equilibrium approximation which is necessary to get low polydispersities, the concentration of radicals is constant and a kinetics of first order can be observed:

$$v_p = -d[M]/dt = K_p \cdot [M] \cdot [R\cdot] = K_{app} \cdot [M] \quad (4.3)$$

$$\text{with } [R\cdot] = \text{const and } K_{app} = K_p \cdot R\cdot$$

so

$$\ln([M]_0/[M]) = K_{app} \cdot t \quad (4.4)$$

It is a linear variation of conversion with time in a semilogarithmic plot (cf. Fig. 4.10).

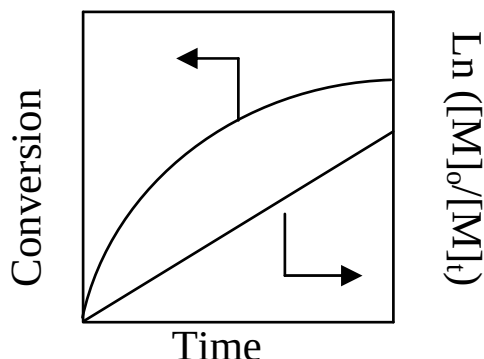


Figure 4.10: Scheme of the time-dependence of the conversion in linear and semi-logarithmic coordinates.

In reality the kinetics laws are more complicated at the beginning of the polymerization due to the spontaneous formation of the complex of the catalyst system via the persistent radical effect (PRE). Indeed, if the initial amount of oxidized transition metal halide ($X - M_t^{n+1}/\text{Ligand}$) is not sufficient to deactivate the living species rapidly, its concentration is increasing due to the irreversible termination of the small amount of chains in the first instant of the polymerization. The persistent radical effect allows the regulation of the transfer equilibrium: if this equilibrium is shifted far towards the living species, the concentration of radicals is too high. This leads to termination reactions, which increase the concentration of the oxidized complex and the deactivation rate and shifts the equilibrium towards the dormant species and allows the control of the polymerization.

4.2.2 Grafting Approaches

ATRP is well known to be one of the most suitable techniques for the grafting of block copolymers, gradient polymers, and brushes. The grafting techniques⁶² have two different approaches: the *grafting to* and the *grafting from* strategies (cf. Fig. 4.11). The *grafting to* strategy includes polymerization of free polymer chains and use of some functional groups to attach them to another polymer chain, to surfaces or to nanoparticles. The functional group can be at the end of the chain as it comes from the initiator, ($R - P_n - X$) in ATRP, but it can also be part of the

chain as the monomer possesses addressable groups. The *grafting from* approach uses a macro-initiator such as a polymer chain ($P_n - R^\cdot$) which has to be reactivated in order to allow further polymerization with the same monomer (block extension) or a different one (block copolymer). ATRP, being a living radical polymerization technique, is particularly suitable for the control of the grafted polymer.

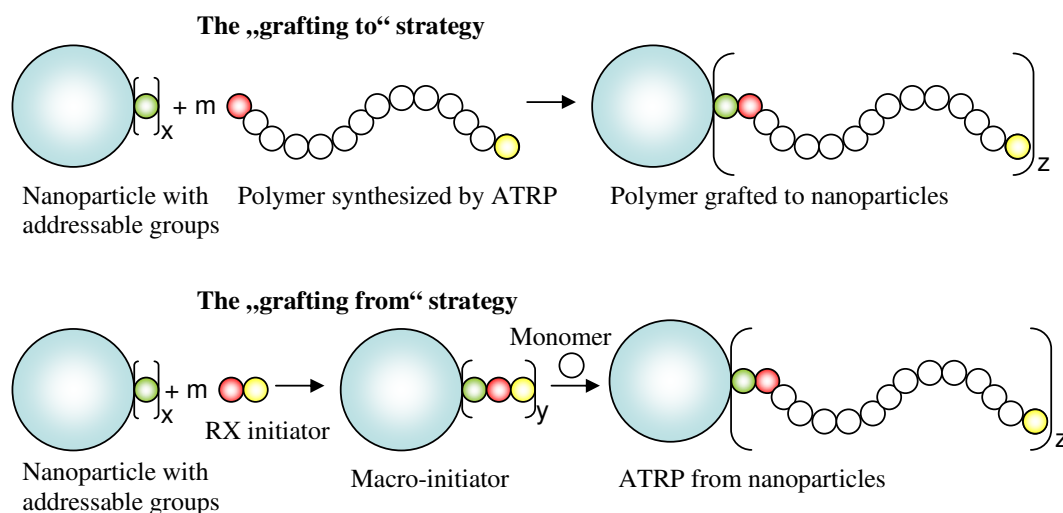


Figure 4.11: The *grafting to* and *grafting from* strategies applied to nanoparticles.

Both approaches have their advantages and drawbacks. The *grafting to* approach is faster as it is achieved in only a single step and preserves the nanoparticles properties. However, it can lead to a poor yield of modification and polydisperse distribution in the number of polymer chains per particles. The *grafting from* is performed in two steps and the conditions of polymerization are limited, but a high yield of modification can be expected with a monodisperse distribution of the polymer around the nanoparticles. Finally the main difference between both approaches is the grafting density that can be achieved^{63,64}.

4.2.3 Thermoresponsive Polymers: PNIPAAm and PEGMA

Smart polymers are macromolecules that display a dramatic physico-chemical change in response to small changes in environment called stimulus. They can be classified according to the external stimuli they respond to. Among them are

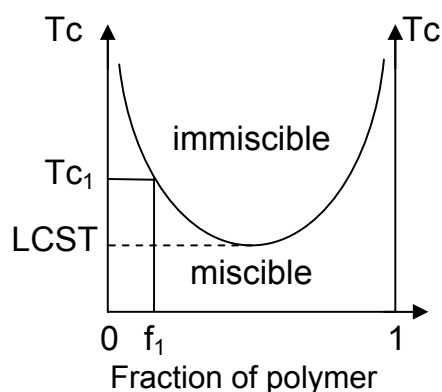


Figure 4.12: Phase diagram to evaluate the LCST and the cloud points T_c .

temperature, pH, solvent, magnetic field, ions and pressure. The ones responding to temperature are called temperature sensitive or thermoresponsive and can be widely used in drug delivery systems⁶⁵.

Thermoresponsive polymers show abrupt changes in their solubility as a function of temperature. At a lower critical solution temperature (LCST), the hydrophilic water soluble polymer becomes hydrophobic in solution and the solution turns turbid. The interaction forces between water molecules, as hydrogen bonding, and polymer become unfavorable compared to polymer-polymer and water-water interactions and a phase separation occurs as the polymer dehydrates. The sharp transition is reversible and influences the viscosity of the solution. The transition occurs in aqueous solution, in the absence of an organic solvent, which makes them attractive for the drug delivery system. Indeed, while the polymer undergoes the phase transition it shrinks and excludes the encapsulated drug. The polymer swells again when the temperature decreases. Two thermoresponsive polymers are particularly promising due to their LCST close to the body temperature of 37 °C. Poly-*N*-isopropylacrylamide (PNIPAAm)^{66–68} shows a sharp LCST of 32 °C and polyethylene glycol methacrylate (PEGMA) exhibits a LCST which can be shifted between 26 °C and 90 °C, depending on the number of ethylene glycol units of the oligomer from two to nine⁶⁹.

In the literature, the term "LCST" is often used for "cloud point". The cloud point is the temperature at which a polymer solution becomes immiscible at a fraction f_1 of polymer in solution; in this study the cloud point was estimated at 50 % of

the normalized absorbance abrupt shift. The cloud point depends on the molecular weight of the polymer but also on its polydispersity. The LCST is the lowest temperature of the limit miscible/immiscible of the phase diagram as shown in Fig. 4.12.

4.3 Bioconjugation and (Thermoresponsive) Polymer-Bioconjugates

4.3.1 Bioconjugation of Ferritin

Bioconjugation is the chemical modification of biological molecules with target crosslinking and derivatizing reagents. Through specific target groups, DNA, amino acids, proteins, viruses, carbohydrates, lipids, synthetic polymers and other macromolecules can be labeled or crosslinked. This provides infinite possibilities to intelligently design a modification or conjugation strategy. Ferritin, as other proteins, possesses addressable carboxylic, amino and sulfhydryl groups because amino acids, such as glutamic acid, lysine, or cysteine respectively, are exposed to the surface and thus can easily be derivatized. Carboxylate groups in proteins can be derivatized through the use of amide bond forming agents or through active ester or reactive carbonyl intermediates. The carboxylate becomes the acylating agent to the modifying group. Amine containing nucleophiles can couple to an activated carboxylate to yield amide derivatives (cf. Fig. 4.13). Hydrazide compounds react similarly to amines. Sulfhydryls, while reactive and resulting in a thioester linkage, form relatively unstable derivatives, which can exchange with other nucleophiles such as amine or hydrolyze in aqueous solutions. Ferritin has been used for different types of derivatization utilizing the exposed amino groups. The ϵ -amino groups are mostly derivatized by alkylation and acylation. In alkylation, an active alkyl group is transferred to the amine nucleophile with loss of one hydrogen. In acylation, an active carbonyl group undergoes an addition to the amine. Alkylating reagents are highly varied and the reaction with an amine nucleophile is difficult to generalize. Acylating reagents usually proceed through a carbonyl addition mechanism.

Horse spleen ferritin was derivatized by many means in order to modify its surface and use its properties. Water-soluble horse spleen ferritin has been coupled

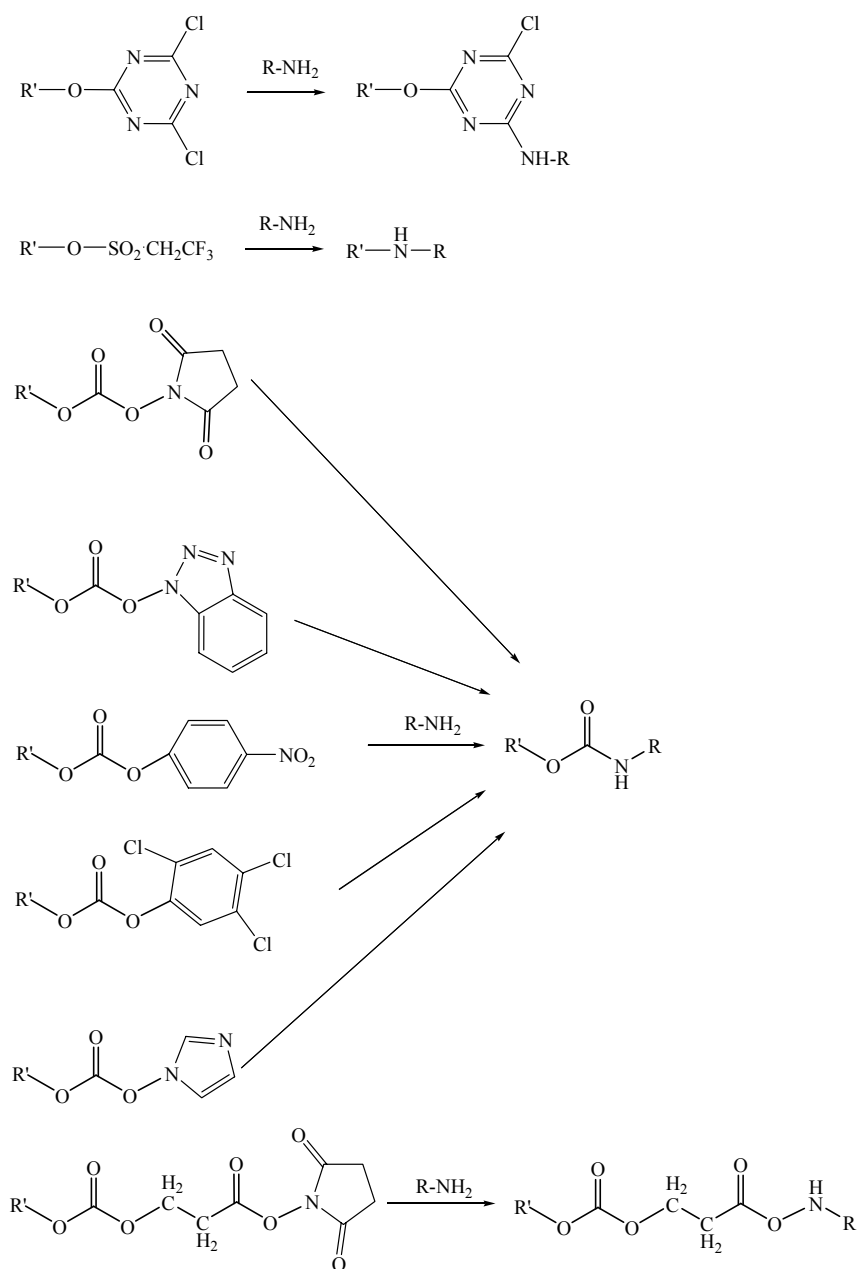


Figure 4.13: Different amine reactive molecules used for bioconjugation reactions towards ϵ -amino groups from lysine, R' can also represent polymers such as PEG⁷⁰.

to alkyl chains (hexyl, nonyl, lauryl chains) through its addressable carboxylic acid group using EDC, a zero length crosslinker, in a mixture of water/THF. The resulting ferritin was hydrophobic and soluble in dichloromethane (DCM) without disruption of its quaternary structure⁷¹.

4.3.2 Design of Polymer-Bioconjugates

Polymer-bioconjugates are a new trend to design drug delivery systems. Nucleic acids, oligopeptides, proteins, enzymes, carbohydrates, viruses or cells are conjugated to polymers using their targeting sites through *grafting to* and newly *grafting from* approaches. With different strategies, living radical polymerization techniques were exploited for the synthesis of a new generation of polymer-bioconjugates. Using different living radical polymerization (LRP) such as RAFT or ATRP, very well defined conjugates were obtained. Tables 4.3 and 4.4 give an overview of the work already done.

Table 4.3: Different syntheses of polymer-bioconjugates by *grafting to* strategy.

Bio-template	Target groups	Polymer	LRP	Refs
Lysozyme	amine/cystein	NHS-PEGMA	ATRP	72
BSA	cystein	P(HEMA)	ATRP	57
Papain	amine	MPC	inifer mediated LRP	73
Peptide (GGR) and BSA	Click chemistry	PS-N ₃	ATRP	74
Peptide (GGRGDG)	Click chemistry	Branched PEG-N ₃	ATRP	75
Glycine methyl ester	amine	PNMS	ATRP	76
Glycine leucine	amine	PVO- <i>block</i> -PNIPAAm	RAFT	77
Galactamine	amine	PNAS	ATRP	78
CPMV	amine	PEG	ATRP	79
Ferritin	amine	PEG	-	80

Different investigations have been already performed on the synthesis of protein-polymer conjugates or hybrids and in case of the temperature sensitive polymers, the thermoresponsive behavior of the hybrids was proven⁸¹. Lele *et al.* were the first to assay the *grafting from* approach to synthesize uniform protein-polymer conjugates using chymotrypsin and poly(MPEG-MA) by ATRP⁶⁴. At first an ATRP initiator was attached to the lysine groups of the protein and the polymer successfully grew from the protein. In order to compare both strategies, the conventional *grafting to* was also assayed; MPEG-SPA was grafted to chymotrypsin by

ester chemistry. As a result, it was shown that *grafting to* leads to broader distribution of the conjugates than *grafting from* approach. Maynard *et al.* were the first to use the *grafting from* approach to synthesize thermoresponsive hybrids. Bovine serum albumine (BSA) and strepavidin were both modified into macro-initiator using the amino end groups of both proteins and then NIPAAm was polymerized by ATRP in DMSO as those proteins are stable in the organic solvent. Turbidity measurements showed thermoresponsive behavior of the hybrids^{82,83}.

Table 4.4: Different syntheses of polymer-bioconjugates by *grafting from* strategy.

Bio-template	Initiator	Monomer	LRP	Refs
α -chymotrypsin	2-bromoisobutyryl bromide	OEGMA	ATRP	64
BSA and lysozyme	pyridyl disulfide initiator	NIPAAm	ATRP	84
BSA	maleimide initiator	OEGMA	ATRP	85
Lysozyme	N-hydroxysuccinimide initiator	DMAEMA	ATRP	85
Peptide	N-hydroxysuccinimide initiator	<i>tert</i> -butyl acrylate	ATRP	86
Ferritin	N-hydroxysuccinimide initiator	OEGMA	ATRP	23
Peptide	CTA	nBA	RAFT	87
BSA	pyridyl disulfide CTA	(EO)A	RAFT	88

Horse spleen ferritin was pegylated in different studies to change its solubility or incorporate the resulting particles into polymer matrices. Polyethylene glycol (PEG) was prepared with a functional NHS-ester end group with a molecular weight of $2000 \text{ g} \cdot \text{mol}^{-1}$ and was grafted to the ferritin using its ϵ -amino groups. PEG is extensively used to modify proteins because of its solubility in water and in organic solvents, its lack of toxicity and biocompatibility. Pegylated-ferritin is more stable and soluble and prevents denaturation in organic solvent⁸⁰. Lin *et al.* demonstrated the effect of incorporated nanoparticles⁵ on the crystallization of poly(ethylene oxide) domains of a block-co-polymer by using pegylated ferritin. There are other ways to bind ferritin to polymer. One is to use biotinylated ferritin and to add it to a non compressed monolayer of strepavidin-polystyrene conjugates⁸⁹. Zeng *et al.* used successfully *click chemistry*⁹⁰ to address selectively the ϵ -amino groups of the horse spleen ferritin. It could be derivatized with alkyl chains but also with a dye reagent fluorescein-NHS. His work was dedicated to show how many amino groups are addressable but also to prove that the MALDI-ToF MS technique is suitable to study the reactivity and regioselectivity of biological nanoparticles towards chemical modification^{22,23}.

4.4 Assembly of Nanoparticles at Solid-Liquid Interfaces

When a protein solution is spread on a charged surface, the particles adsorb on the surface instantaneously. The process of protein adsorption depends on the properties of the surface, the nature of the proteins and the solution properties. The surface of a protein is complex due to the exposed amino acid residues that influence the protein hydrophobicity and charges⁹¹. Most of proteins are amphiphilic and therefore highly surface active. Globular proteins are more hydrophilic as the apolar residues are located in the inside shell. A major factor influencing the adsorption is the surface energy and it has been reported that the hydrophobic surface adsorbs more proteins than hydrophilic ones⁹². Proteins are still able to change their conformation to bind to the surface. The driving force of the binding reaction described by Norde^{93,94} is an increase of the entropy, due to conformational changes of the protein resulting in the loss of the secondary structure. Adsorption is electrostatically controlled at charged surfaces and the maximum of adsorbed amount is often found at the isoelectric point of the protein, it is due to a minimum of the intramolecular and/or lateral repulsion⁹⁵. Capillary force also influences the binding of proteins at solid-liquid interfaces. It is resulting in a closed packed monolayer of protein⁹⁶.

4.4.1 Adsorption Process

The adsorption process of protein molecules on a surface comprises various aspects: kinetics, type of binding, adsorbed amount, and structure of the adsorbed layer and of the individual molecules⁹⁵. The different steps, as shown in Fig. 4.14, of through that an adsorbing and desorbing protein molecule passes are: (1) the transport to the surface, (2) adsorption/deposition on the surface, (3) relaxation of the molecule, (4) detachment from the surface, (5) transport away from the surface and (6) possible restructuring of the molecule.

Step (1): Transport to the surface. In order to adsorb, the protein has to be transported from the bulk phase to the surface by diffusion or convection. Even in a well-stirred system there is a stagnant layer close to the surface through which the protein has to migrate by diffusion.

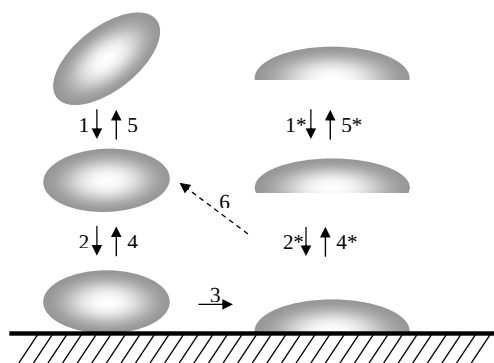


Figure 4.14: Adsorption/desorption process of a protein molecule.

Step (2): Adsorption. The adsorption reaction at the surface can be the rate-determining step. As the surface fills with adsorbed proteins, the adsorption rate becomes a function of surface coverage and decreases below the rate of diffusion and thereby becomes a controlled surface-reaction.

Step(3): Time dependent structural changes. The conformation of the adsorbed protein can be vitally important for its function. Changes in conformation can occur immediately upon adsorption, but time-dependent conformational changes are also evident. Conformational changes have been suggested to be greater at low surface coverage where other molecules impose a negligible influence on the ability of a protein to adopt different conformations. Once the protein molecule has been attached, it relaxed towards its equilibrium structure because of the altered environment that in general is different from the native structure in solution. But the relaxation is slow because of the strong internal coherence of the protein. Structural relaxation implies optimization of protein-surface interactions and involves a certain degree of spreading of the protein molecule over the sorbent surface, developing larger number of protein-surface contacts. As a consequence, after relaxation the detachment of the protein from the sorbent is more difficult. When the spreading occurs more quickly the adsorbed molecules are flattened. If the flux of particles to the surface increases, the conformation of proteins is more globular and the adsorbed mass per unit surface area is higher.

Step(4): Desorption of adsorbed protein or exchange. Protein adsorption is often irreversible or partially irreversible. However, the protein can be desorbed by changing the pH, or increasing the ionic strength. The effect of pH and salt concentration are usually more pronounced with hydrophilic surfaces than hy-

drophobic ones⁹². The absorption reaches a saturation after a certain time, and depends strongly on the flux. Adsorption isotherms (Adsorbed mass per unit sorbent surface γ versus c for globular proteins display well defined plateau values that are reached at $\gamma(c_{eq})$ (concentration at the equilibrium). The plateau is comparable to the absorbed amount in a closely packed monolayer of molecules. There is a hysteresis between the adsorption and desorption isotherms, manifesting an almost irreversible protein adsorption process. This can be explained by the fact that the protein is bound to the surface by several weak bonds. Although the formation and breakage of such bonds is a dynamic process the likelihood of all bonds between the protein and its binding sites on the surface breaking at the same time is low. However, the energy needed to disrupt one of these bonds will be quite small and another protein may then bind to the surface, replacing it. The desorbed molecules can re-adsorb at a slightly different location, leading to surface mobility of a particular molecule.

Step(5): Transport away from the surface. This is the reverse of step (1) although the protein may be conformationally altered compared to the native state. The protein is less prone to absorb a second time.

The 3D structure of globular protein molecules is only marginally stable, so interaction with a sorbent surface may induce rearrangements in the protein structure. The thickness of the monolayer of adsorbed protein is comparable to the dimensions of the native protein molecule, which proves that the structure undergoes small rearrangements. After adsorption, at one side of the protein molecule the aqueous environment is replaced by the sorbent material. As a consequence, intramolecular hydrophobic interactions become less important as a structure stabilizing factor, as apolar parts of the protein that are buried in the interior of the dissolved molecule may become exposed to the sorbent surface without making contact with water. Hydrophobic interactions between amino acid residues support the formation of α -helices and β -sheets. A destabilization of the secondary structure may form hydrogen bonds with the sorbent surface. Then a decrease in ordered secondary structure would result in an increased conformational entropy of the protein and hence an increased adsorption affinity.

4.4.2 Capillary Forces between Colloidal Particles

Capillary interactions between colloidal particles can produce the formation of organized two-dimensional arrays of submicrometer particles such as globular proteins. They appear between particles protruding from a liquid film and its origin is the capillary rise of the liquid along the surface of each particle. This situation will inevitably occur during the drying process of adsorption of a liquid drop of particles on a solid substrate. As the solvents evaporate, the particles on the surface begin to show three-phase contact line.

It is necessary to distinguish two different lateral capillary forces: the floating and the immersion force. The former depicts the interaction between floating particles in an aqueous film due to particle weight and Archimedes force, it has no effect on very small particles. The latter one depicts the interaction between very small particles (even down to 10 nm size) partially immersed in a thin aqueous film. The transition from disordered state forward to ordered state appears suddenly in the moment, when the particle tops protrude from the thinning liquid films. The capillary forces are entirely governed by the surface forces (those, which give rise to the disjoining pressure and the three-phase contact angle), the gravity effect being negligible.

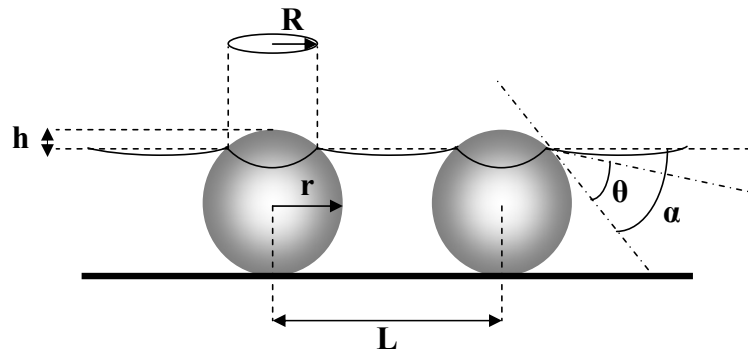


Figure 4.15: Two particles partially immersed in a thin liquid film. Attractive immersion capillary forces evolve between them, defined by the contact angle θ , the separation distance h , and the particle radius r .

Capillary effects are of special importance in systems containing three-phase boundaries, e.g. containing solid surfaces, a liquid and a gas (vapor) phase, as it is the case of one drop of particles adsorbing on a solid surface. This implies three types

of interfacial energies: the solid-vapor interfacial free energy γ_{SV} , the liquid-solid one γ_{LS} and the liquid-vapor one γ_{LV} . If the drop has reached the equilibrium state, the change in surface free energy will be zero and Young's equation can be derived:

$$\cos(\theta) = \frac{\gamma_{SV} - \gamma_{SL}}{\gamma_{LV}} \quad (4.5)$$

where θ is the contact angle that evolves between the surface and the drop. If the surface free energy γ_{SV} is higher than the interfacial free energy between the liquid and vapor phase γ_{LV} , the liquid will spread on the surface resulting in a low contact angle. In the reverse case, the liquid will form a drop with a contact angle larger than 90° . A surface is wetted if the contact angle is 0° . The surface tension at interface evolves with the capillary pressure, which can be defined as:

$$\Delta p g h = \frac{2\gamma \cdot \cos(\theta)}{r} \quad (4.6)$$

where Δp is the difference in density between liquid and fluid (vapor phase), g the acceleration gravitational, h the height of the meniscus and γ is the interfacial tension between liquid and fluid (vapor phase).

Theory of Immersion Capillary Force

The cause of the lateral capillary forces is the deformation of the liquid surface, which is supposed to be flat in absence of the particles. The larger the interfacial deformation created by the particles is, the stronger the capillary interaction between them. In the case of particles partially immersed in a liquid layer on a substrate, the deformation of the liquid is due to the wetting properties of the particle surface, i.e. to the position of the contact line and the magnitude of the contact angle⁹⁷. The contact angle plays an important role in the formation of the water meniscus. The immersion capillary forces even exist if the contact angle θ is zero. Kralchevsky *et al.* propose the following analytical expression for the lateral capillary forces between two particles on a surface^{98,99}:

$$F = 2\pi \cdot \gamma \cdot Q_1 Q_2 \cdot q k_1 \cdot (qL) \quad (4.7)$$

where L is the separation distance of the particles, γ is the surface tension between liquid and fluid (vapor) and k_1 is a modified Bessel function of first order and Q_i is defined as followed:

$$Q_i = R_i \sin(\alpha_i) \quad (4.8)$$

where R is the radius of contact line (of particle 1 and 2) and α is the meniscus slope angle as shown in Fig. 4.15. q^{-1} is the Debye length defined as:

$$q^{-1} = \left(\frac{\gamma}{\Delta p \cdot g} \right)^{1/2} \quad (4.9)$$

Under the assumption that $R \ll L \ll q^{-1}$, Eq. 4.7 simplifies to:

$$F = 2\pi \cdot \gamma \cdot \frac{Q_1 Q_2}{L} \quad (4.10)$$

4.4.3 Self-Assembly of Colloidal Particles

If particles absorb on a surface (e.g. by electrostatic interactions), capillary forces are present during the drying step and tend to influence the particle array produced in suspension. The adsorption parameters can be tuned (e.g. concentration of particles, temperature, hydrophilicity of the surface, pH (addition of salt), rate of evaporation) and affect the interparticle distance such that it suddenly is low enough for capillary forces to start acting on clustering the particles^{100,101}.

Capillary forces occur in all particle self-assembly processes as soon as the evaporating solvent layer is thinner than the particle diameter^{102,103}. The mechanism governing their 2D colloidal assembly is in two steps: first, the immersion of capillary forces attract nearby particles and start to form the nucleus of the monolayer. The second step of that process is the formation of a colloidal monolayer or crystal due to the hydrodynamic force which drags particles to regions of thinner liquid layers¹⁰⁴. The reason for that particle flux is a hydrodynamic flux, which transports matter to regions where evaporation rates are the highest (which is at

the three-phase contact lines and thus where liquid films are the thinnest). If a drop of a suspension dries, the three-phase contact line will be the edge of the drop and particles will be dragged to the edge of the drop where they assemble, as shown in Fig. 4.16. It may lead to a monolayer of particles (such as globular proteins), which may form some tetragonal or hexagonal packing (as it is the case for ferritin molecules) depending on the geometry of the particles^{105,106}.

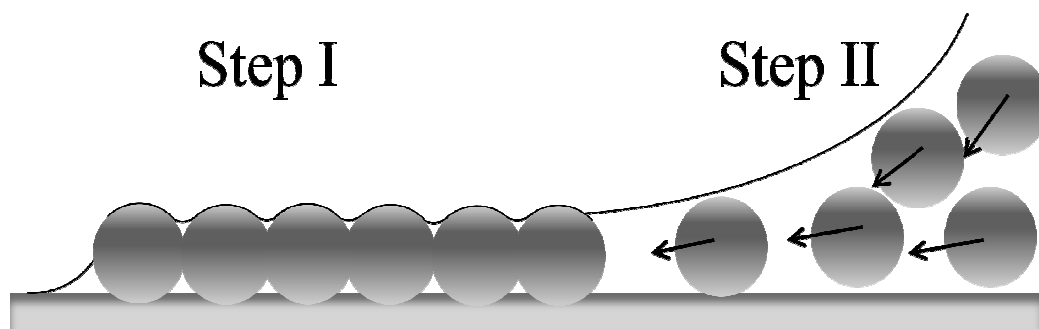


Figure 4.16: Monolayer formation of particles due to capillary forces in 2 steps: step I evaporation of the solvent and nucleus formation; step II hydrodynamic flux of particles.

4.4.4 Adsorption of Ferritin at Solid-Liquid Interfaces

Protein adsorption at liquid-solid interfaces is an important phenomenon as it is a process in biofouling, dental plaque or biointegration of implanted materials. Moreover, ferritin is widely used for immunodiagnoses. The concentration of ferritin in the blood plasma is a very sensitive indication of iron deficiency. Ferritin radioimmunoassays have been identified as most efficient and have become a standard test¹⁰⁷. Many methods have been developed to follow and quantify the deposition of ferritin onto solid substrates. As summarized in Table 4.5, basic characterization methods up to very sophisticated ones have been used to understand if the kinetics process of adsorption is following the Langmuir adsorption theory, (assuming that a reversible monolayer of protein is formed) or the random sequential adsorption model (RSA) (assuming the irreversible character of the adsorption).

Both models have been discussed to interpret the results of different studies. Protein adsorption is a complex process as it involves factors like the hydrophilic/hy-

drophobic character of the sorbent substrate, the concentration of protein, ion-exchange, and the electrostatic forces comprising pH, ionic strength and surface charge density. It seems that depending on the sorbent surface a different kinetics model has to be established for the adsorption of ferritin. Even if the phenomenon is not fully understood, the applications of adsorption of ferritin onto solid substrates are multiplied through molecular imprinting.

Table 4.5: Studies of ferritin adsorption at different liquid-solid interfaces.

Sorbent surface	Properties	Characterization methods	Ref.
SiO ₂ (oxidized silicon wafer)	Hydrophilic/ $\ominus$	Ellipsometer, TEM	108
Methylated quartz	Hydrophobic	QCM, AFM, SPR, XPS	109
Freshly cleaved mica sheet	Hydrophilic/ $\ominus$	AFM	110
Si(Ti)O ₂ chip	Hydrophobic	AFM, OWLS	111
Amphiphilic cyclodextrin	Hydrophilic	TEM, surface pressure, QMC	112
DETA-coated glass	Hydrophobic / $\oplus$	LTM-AFM	113
Charged lipids (DOMA)	Hydrophobic/ $\ominus$	TIRE, BAM	114
Gold-coated glass	Hydrophobic	SPR, TIRE	115

Molecular imprinting of proteins was imagined using the specific molecular recognition that offer proteins (or host) to ligands (guest molecules) through a binding process. This fundamental process controls biological form and function, mediated by proteins^{116,117}. Molecular imprinting is a strategy to prearrange the recognition site around the target molecule and template. 2D and 3D strategies have been developed to offer alternative ways in synthetic molecular recognition field using protein adsorption at liquid/air interfaces but also liquid-solid interfaces¹¹⁸. The amphiphilic components of the Langmuir monolayers serve as "host molecules" whereas the non-surface-active species dissolve in the aqueous sub-phase as "guest molecules". The molecular recognition event occurs due to the interaction between the dissolved guest-component and the host component as Langmuir monolayer. Du *et al.* investigated the reverse system. Two lipid components cationic (dioctadecyldimethylammonium bromide DOMA)/ non-ionic (methyl sterate SME) Langmuir monolayer were used as a "host" to follow ferritin binding and the possible template induction through local demixing. The enhanced binding of ferritin to fluid mixed Langmuir monolayers was accentuated in fluid having non-ionic SME as principal component¹¹⁹.

4.5 Nanoporous Membranes

Membranes are interphases between two adjacent phases acting as a selective barrier, regulating the transport of substances between the two compartments. They are low energy consumption devices for separation processes and can be performed isothermally at low temperature. Depending on the pore size and pore density, membranes offer different separation processes such as dialysis, gas separation, reverse osmosis, nanofiltration, ultrafiltration, microfiltration and electrodialysis. Through all different membrane applications such as water treatment or blood detoxification, many approaches have been developed and the industrial market of membrane is growing. After producing the first membrane made of metal (aluminium oxide), ceramics or liquid membrane, some greater attention is focused onto *polymer membranes* which allow a wide variability of barrier structures and properties. The first generation of membrane made of biopolymers (cellulose) tried to mimic the kidney function (hemodialysis). Since then great efforts have been made to improve selective transport through biological membranes enabled by highly specialized macromolecular and supramolecular assemblies, based on molecular recognition. It was focused on the synthesis of novel polymers with well-defined structure as "tailored" membrane materials, advanced surface functionalization, use of templates, preparation of composite membranes for the synergetic combination of different functions and novel processing of polymers for membranes¹²⁰.

In the case of porous membranes, the transport rate and selectivity are mainly influenced by viscous flow and sieving or size exclusion. Interactions of solutes with the membrane (pores) surface may alter the membrane performance. With meso- and microporous membranes, selective adsorption can be used for an alternative separation mechanism. In theory, porous barriers could be used for very precise continuous permselective separations based on subtle differences in size, shape, and/or functional groups. The permeability and selectivity can integrally be controlled by concentration polarization or membrane fouling. The selectivity can be switched by an external stimulus or can adapt to the environment/process condition which opens new applications such as analytics, screening, membrane reactor or bio-artificial membrane systems. One more essential challenge will be the minimizing of the thickness of the membrane barrier layer and the upscaling production.

The polymer membrane technology involves different approaches resulting in dif-

ferent separation mechanisms. Among them, track-etching, photolithography and phase separation micro-moulding techniques produce membranes with barrier thickness from 1 μm to 35 μm , pore size from 10 nm to several μm , with different rigidity and density. Track-etched polymer membranes are prepared from polycarbonate (PC) or polyethylene terephthalate (PET) films. They have a rather low porosity or pore density in order to reduce the probability of defects. They are limited in the preparation of pores with diameters in the lower nanorange. To reduce the pore size and increase the stability and the selectivity of the membranes, complex polymer structures, architectures and functionalities were investigated. Tailored polymers such as poly(pyrrolone-imide)¹²¹ can overcome the lack of intrinsic microporosity of polymers due to their ultra rigid backbone structure which can alternate "open" and "bottle neck" selective regions. Block-copolymers as building blocks for ordered three dimensional structures are also very attractive.

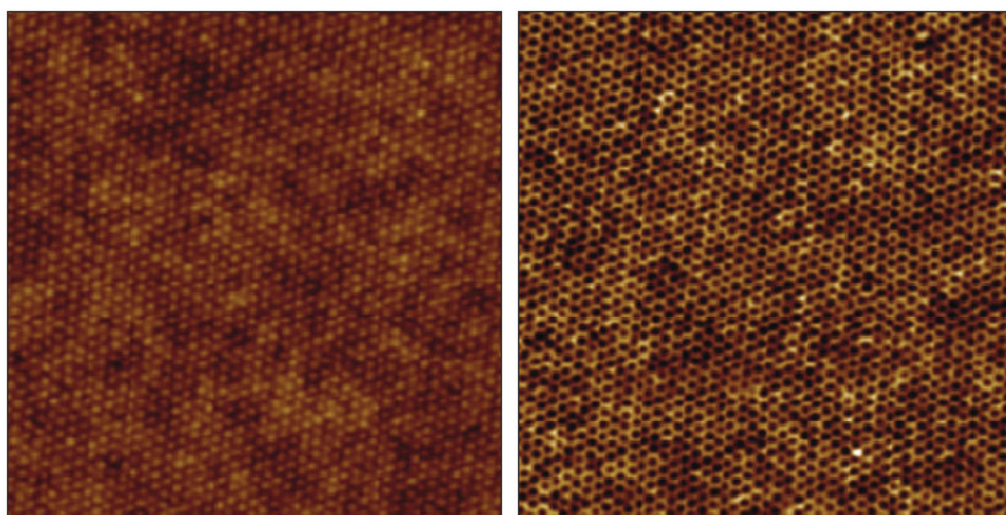


Figure 4.17: Tapping-mode AFM topography images of the highly ordered tri-block copolymer PS-P2VP-PtBMA before (left) and after (right) exposure to ultra-violet radiation¹²².

As an example, Ludwigs *et al.*^{122–124} created a responsive nanoporous membrane based polystyrene-*block*-poly(2-vinylpyridine)-*block*-poly(*tert*-butyl methacrylate). The structure of this triblock copolymer is a P2VP-PS-P2VP sheet perforated by PtBMA channels. UV treatment removes the acrylate matrix phase, and develop a highly ordered hexagonal array of hollows, as the PtBMA is removed from the perforations in the P2VP-PS-P2VP layer. P2VP is responding to pH and its

quaternization can lead to a water-soluble coverage of PS-core. However, the *wettability* remains a challenge as the general problem of the block-copolymer based membrane is that they do not exhibit a *sufficient permeability* and required surface functionalization.

Indeed, surface properties can be triggered by using functional (responsive) polymer as a matrix or a blend in copolymer mixture during the membrane formation. Grafting to and from strategies are used to modify the surfaces but also the pore walls of the membrane. As an example, Hester *et al.* prepared block-copolymers, via ATRP graft copolymerization of PEG methacrylates onto the membrane polymer of PVDF. Such polymers are promising additives for surface modification¹²⁵. However, the functionalization of nanopores is rarely achieved and despite the impressive ordered porous morphologies based on di- or triblock copolymers, a direct demonstration of the membrane function, that is, permeability measurements or even a selective permeation controlled by the nanoporosity of the polymer film, is yet to be accomplished¹²⁶. The use of "supramolecular" templates for the preparation of materials with controlled pore size had been explored in many variations¹²⁷. Beginn *et al.* reported the synthesis of supramolecular channel membranes with pore mimicking biological ion-channels^{128,129}. The approach was based on the gelation of acrylate monomer solutions which form non-porous blocks and do not shrink upon polymerization-by string-like supramolecular assemblies of functional gelator molecules, and the subsequent fixation of these gels by an in-situ polymerization followed by removal of the gelator fibers thus finally yielding pore channels predetermined by the size and the shape of the template.

Mechanical stability is also a weak point of polymer membrane but the crosslinking of polymer and polymer composite membrane can enhance it. Thin-film composite membranes raise attention even if the preparation of defect-free selective membranes with thickness of less than 50 nm seems to be fundamentally difficult. The attempt to use monolayers of functional amphiphilic molecules (Langmuir-Blodgett (LB) technique) as ultra-thin selective barriers were unsuccessful because of their instability and the non reproducibility of defect-free composite membrane^{130,131}. The Layer-by-layer (LBL) technique based on assembly of charged macromolecules in a vertical order with nanometer precision has an advantage on the lateral one (assembly of small molecules in LB films) because possible defects can be healed within few layers¹³². *Responsive and switchable membranes* also provide an interesting tool because they allow a reversible switching of the permeability, by grafting of pH and thermoresponsive polymers such as

polyacrylic acid and PNIPAAm^{133–135}. For the function of such responsive membranes, the defined anchoring of grafted polymer chains or crosslinked polymer systems into the wall's pores is very essential, even if it is still challenging.

Finally, it is important to optimize the *biocompatibility* of the membrane. The main biomedical applications of the membrane technology are hemodialysis, plasmapheresis and oxygenation. For the majority of current relevant processes, the behavior of the membrane in contact with blood is crucial. Minimizing the non specific adsorption of proteins is important in order to preserve the performance of the membrane. Surface modifications have to be performed in order to reduce it and improve biocompatibility to suppress the pathophysiological defense mechanisms.

Our approach brings an alternative to block copolymer membrane to circumvent wettability problems while controlling the pore size and selectivity. Ferritin is used as a sacrificial template to control the size of the nanopores of the future membrane. After grafting P(NIPAAm-DMIAAm) from ferritin molecules, the obtained bionanoconjugate ferritin-P(NIPAAm-DMIAAm) are used as building blocks to generate the matrix by assembly on solid substrates. PNIPAAm is well-known for its biocompatibility and thermoresponsive behavior. The polymer matrix can be crosslinked by UV enhancing the mechanical stability. The subsequent denaturation of the proteins will create the aqueous pores. This approach allows the control of the nanoporous pore size at about 10 nm and its functionality. The subsequent polypeptide chain left in the pore can lead to further modification of the pores for a better selectivity (attachment of responsive polymer) or construction of a filter.

5 Characterization Methods

Horse spleen ferritin (HSF) was first characterized in its native state to learn about its properties, establish a standard protocol for each technique and use the results as a reference. The protein was characterized in solution by UV-Vis and a light scattering technique (Dynamic light scattering (DLS) and size exclusion chromatography (SEC) with online light scattering, ultraviolet (UV) and differential refractive index detection), onto substrates by transmission electron microscopy (TEM), scanning electron microscopy (SEM) and atomic force microscopy (AFM). The denaturated state was investigated by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-ToF MS) and sodium dodecyl sulfate-poly(acrylamide) gel electrophoresis (SDS-PAGE) to learn about the sub-unit molecular weight.

5.1 Characterization in Solution

HSF is bought as a concentrated saline solution from Fluka. It has a high tendency to aggregate, forming dimers, trimers and n-mers. The protein must be purified in order to separate those aggregates and collect the monomeric ferritin by size exclusion chromatography using only the UV detector (sodium phosphate buffer 0.025 M at pH 7, flow of 0.25 mL·min⁻¹). The concentration and the stability of the proteins were determined and followed by UV-Vis and DLS.

5.1.1 Light Scattering Technique

5.1.1.1 Dynamic Light Scattering (DLS)

Dynamic light scattering is also known as photon correlation spectroscopy^{136,137}. It is used to determine the size of particles like proteins in the range of 1-1000 nm. Shining a monochromatic light beam, e.g. a laser, onto a solution with spherical particles in Brownian motion causes a Doppler shift when the light hits the moving particle, which changes the wavelength of the incoming light. The measurement consists on following on the time scale of the molecular fluctuations. This scattering effect is related to the size of the particle and allows the description of the particle's motion in the medium by measuring the diffusion coefficient of the particle and using an autocorrelation function.

The set up is composed of a laser which passes through a collimator lens and hits the cell containing the solution. The light is scattered and detected by a photomultiplier that transform a variation of intensity into a variation of voltage. The photomultiplier is usually positioned at a scattering angle of 90°.

According to light scattering theory, when light impinges the matter, the electric field of the light induces an oscillation polarization of electrons in the molecules. Hence, the molecules provide a secondary source of light and subsequently scattered light. The frequency shifts, the angular distribution, the polarization, and the intensity of the scatter light are determined by the size, the shape and the molecular interactions in the scattering material.

The intensity of the scattered light is dependent of spatial arrangement of the scattering centers at any instant in time. The particles (macromolecules) are undergoing constant motion due to collisions with solvent molecules. The instantaneous value of I_{tills} fluctuates in time about the average intensity, where the rate at which these spontaneous fluctuations decay to the equilibrium value is directly dependent upon the dynamics of the molecules. The autocorrelation function $C(t)$ can be defined as:

$$C(t) = \langle I(t)I(t+\tau) \rangle = \lim_{T \rightarrow \infty} \frac{1}{T} \int_{-\frac{T}{2}}^{\frac{T}{2}} x(t')x(t'+t) \cdot dt' = \langle x(0)x(t) \rangle \quad (5.1)$$

with $x(t)$ a set of values depending on time.

The second order autocorrelation curve is defined as followed:

$$g^2(\vec{q}, \tau) = \frac{\langle I(t)I(t+\tau) \rangle}{\langle I(t) \rangle^2} = \frac{C(\tau)}{\langle I(t) \rangle^2} \quad (5.2)$$

where $g^2(\vec{q}, t)$ is the autocorrelation function at a particular wave vector $\vec{q}$ and delay time t and I is the intensity. The correlation is linked to the delay time: for large delay times the Brownian movement of the particle makes it far away from the initial position, and so the correlation decrease exponentially and vice versa for short delay times. This exponential decay is then related to the diffusion coefficient, depending on the polydispersity of the particle. In order to fit the data, it is necessary to define the first order autocorrelation function:

$$g^2(\vec{q}, \tau) = 1 + \beta[g^1(\vec{q}, \tau)]^2 \quad (5.3)$$

where the parameter β is a correction factor that depends on the background, the geometry and alignment of the laser beam in the light scattering setup. For monodisperse, hard spherical particles, g^1 corresponds to a single exponential decay curve:

$$g^1(\tau) = e^{\Gamma \cdot \tau} \text{ and } \Gamma = D_o \cdot \vec{q}^2 \quad (5.4)$$

where Γ can be related to the translational self-diffusion coefficient D_o with the wave vector $\vec{q} = 4\pi n/\lambda \sin(\theta/2)$, where λ is the incident laser wavelength, n the refractive index of the medium, θ is the scattering angle and D_o is given by the Einstein-Stokes relation:

$$D_o = \frac{k_B T}{6\pi\eta R_h} \quad (5.5)$$

with k_B the Boltzmann constant, η the viscosity of the medium and R_h the hydro-

dynamic radius which is defined as the radius of a sphere with the same hydrodynamic properties as the particle under investigation.

Dynamic light scattering is a method to determine some properties of protein particles and macromolecules. However, it depends drastically on the polydispersity of the sample. After purification and collection of the monomeric ferritin, the collected particles were characterized by dynamic light scattering. The result was a radius of 6.7 ± 2 nm (cf. Fig. 5.1).

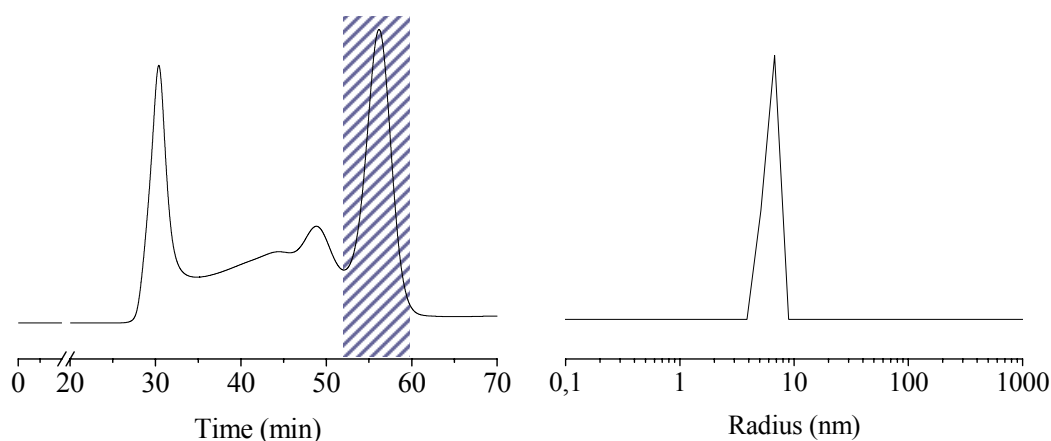


Figure 5.1: Left: SEC measurement of horse spleen ferritin (UV detector); the monomeric peak is colored in blue. Right: DLS measurement of collected monomeric ferritin ($R_h=6.7 \pm 2$ nm).

Stability studies could be followed by DLS. Horse spleen ferritin is highly stable versus pH and temperature. Monomeric ferritin is stable towards aggregation and denaturation from pH 2 to 9, up to at least 60°C , and towards UV light and sonication. The addition of a small amount of organic solvent (volume ratio 1:5 (v:v)) miscible to aqueous solution (DMSO, DMF) does not perturb the monomeric ferritin distribution, while a higher amount of organic solvent precipitates the aggregation of protein particles.

5.1.1.2 Size Exclusion Chromatography with Online Light Scattering, Ultraviolet (UV) and Differential Refractive Index Detection

Size exclusion chromatography (SEC) coupled with online light scattering (LS), refractive index (RI) and ultraviolet (UV) detection provides a non destructive method to determine the molecular weights of proteins and their complexes in solution, as well as their sizes^{138,139}. The sample passes through the porous *stationary phase* in the column which is mostly a gel medium based on polyacrylamide, dextran or aragose. Particles with different sizes elute through the stationary phase at different rates. Large molecules cannot enter the pores of the column and elute faster. Smaller molecules are retained by the pores of the gel and therefore come later. Very large molecules, which cannot enter any pore but remain in the void volume between the gel particles, elute unresolved at the earliest elution volume, the so-called upper exclusion limit. The properties of the stationary phase determine the quality of separation, especially at low molecular weight.

The molecular weight M_w depends only on the downstream LS and RI detectors and is independent of the elution position. The "two detectors" method (LS and RI) can be applied when the dn/dc is known. If this is not the case, the "three detectors" method is used. For globular proteins a dn/dc value of $0.185 \text{ mL} \cdot \text{g}^{-1}$ is normally used, but it can also be measured with a RI detector in batch mode.

The amount of light collected by the LS detector is proportional to the product of the weight average molar mass and the solute concentration $LS \approx M_w c$. This relationship is based on Zimm's formalism of the Rayleigh-Debye-Gans light scattering model for dilute polymer solutions. The relation between the excess scattered light and the molecular weight is given by the Equation:

$$\frac{K^* c}{R(\theta)} = \frac{1}{M_w \cdot P(\theta)} + 2A_2 c \quad (5.6)$$

where

- $R(\theta)$ is the excess intensity of scattered light at an angle θ (i.e. the excess Rayleigh ratio of the solution as a function of θ and concentration of the sample. It is directly proportional to the intensity of the scattered light in

excess of the light scattered by the pure solvent.)

$$R(\theta) = \frac{I(\theta)r^2}{I \cdot f \cdot V} \quad (5.7)$$

with I the intensity of the incident light, $I(\theta)$ total intensity of scattered radiation observed at an angle θ at a distance r from the point of scattering, V the scattering volume and f a factor compensating polarization phenomena

- c is the sample concentration ($\text{g} \cdot \text{mL}^{-1}$)
- M_w is the weight average molecular weight (molar mass $\text{g} \cdot \text{mol}^{-1}$)
- A_2 is the second virial coefficient ($\text{mL} \cdot \text{mol} \cdot \text{g}^{-2}$)
- K^* is an optical parameter equal to $4\pi^2 n^2 (dn/dc)^2 / \lambda_o^4 N_A$; with n being the solvent refractive index and dn/dc being the refractive index increment. N_A is Avogadro's number and λ_o is the wavelength of the scattered light in vacuum (cm)

To solve the Equation 5.6, the second virial coefficient term ($2A_2c$) can be neglected when $2A_2c \ll 1$. This condition is met at the relatively low concentration ($0.1 \text{ mg} \cdot \text{mL}^{-1}$). The function $P(\theta)$ describes the angular dependence of scattered light. The expansion of $1/P(\theta)$ to first order gives:

$$1/P(\theta) = 1 + (16\pi^2/3\lambda^2) \langle r_g^2 \rangle \sin^2(\theta/2) + \dots \quad (5.8)$$

The term $[(16\pi^2/3\lambda^2) \langle r_g^2 \rangle \sin^2(\theta/2)]$ is neglected for protein with $\langle r_g^2 \rangle$ smaller than 15 nm (r_g as the radius of gyration) which includes proteins and their complexes with M_w smaller than 5,000 kDa.

The "two detectors" method is based on the LS and the RI detection. It is necessary to introduce K_{LS} , the calibration constant of the LS detector. The measured intensity of scattered light at a given angle θ :

$$(LS) = K_{LS} \cdot c \cdot M_w \cdot (dn/dc)^2 \quad (5.9)$$

Similarly K_{RI} can be introduced, the calibration constant of the RI detector:

$$(RI) = K_{RI} \cdot c \cdot (dn/dc) \quad (5.10)$$

From the Equations 5.9 and 5.10, it results in the relationship:

$$M_w = \frac{(LS)K_{RI}}{K_{LS}(RI)} \cdot \left(\frac{dn}{dc}\right)^{-1} = K' \frac{(LS)}{(RI)} \quad (5.11)$$

with K' the instrument calibration constant, determined by analyzation of protein standards.

Alternatively, M_w can be determined directly from the absolute light scattering measurements by solving the Rayleigh-Debye-Gans equation (cf. Eq 5.11), by independently calculating the concentration of the eluting protein. The Astra software (Wyatt Technology Corporation, Santa Barbara, CA) package provides different fitting methods (including calculation of polynomial fitting): Zimm, Debye and Berry fitting methods. The Zimm method is applied for mid-sized molecules (20-50 nm) and consists of plotting the curve $K^* c / R(\theta)$ against $\sin^2(\theta)$ and yields the determination of $1/M_w$ as the intersection and $\langle r_g^2 \rangle^{1/2}$ as the slope at zero angle. The Debye method consists of plotting the curve $R(\theta) / K^* c$ against $\sin^2(\theta)$ and yields the determination of M_w as the intersection and $\langle r_g^2 \rangle^{1/2}$ as the slope at zero angle. This method is applied for a wider range of M_w than the Zimm method. The Berry fitting method constructs a plot of the square root of $[K^* c / R(\theta)]$ against $\sin^2(\theta)$. It is useful for large macromolecules.

The "three detectors" method is used when the dn/dc is not known, introducing the UV detection to determine the concentration. The extinction coefficient ϵ is then needed.

$$(UV) = K_{UV} \cdot c \cdot \epsilon \quad (5.12)$$

K_{UV} is the calibration constant of ultraviolet detector and ϵ is the extinction coefficient in $\text{mL} \cdot \text{g}^{-1} \cdot \text{cm}^{-1}$.

By combining the Equations 5.11 and 5.12, it is obtained:

$$M_w = \frac{K_{RI}^2}{K_{LS}K_{UV}} \cdot \frac{(LS)(UV)}{\epsilon(RI)^2} \quad (5.13)$$

The extinction coefficient ϵ can be determined at one λ using solutions of proteins at different known concentrations by UV-Vis measurement.

SEC allows the fragmentation of the n-mer of ferritin, and as said before the collection of monomeric ferritin as seen in Fig. 5.1. Moreover, the molecular weight and the radius of gyration of the protein were determined by the three detector method. In order to estimate the molecular weight of the sample, the light scattering detectors (with MiniDawn TREOS at three different angles: 49° , 90° and 131°) are coupled to the ultraviolet detector as the intensity of the RI signal is often too small to be used. The extinction coefficient is given in the literature ($\epsilon_{280}(\text{HSF}) = 1.09 \cdot 10^3 \text{ mL} \cdot \text{g}^{-1} \cdot \text{cm}^{-1}$).

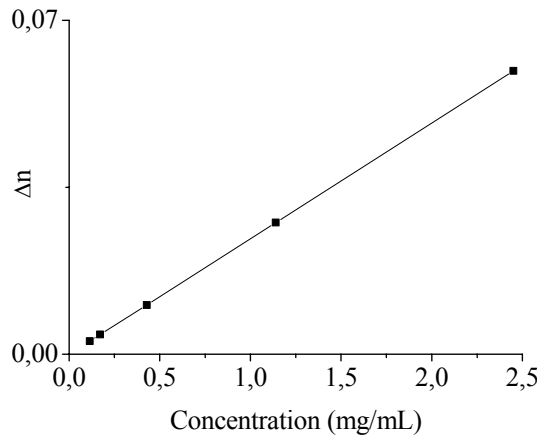


Figure 5.2: Estimation of dn/dc of horse spleen ferritin in SEC buffer (sodium phosphate buffer pH 7.4) using RI detector: $dn/dc = 0.02428 (\pm 7.4 \cdot 10^{-4}) \text{ mL} \cdot \text{g}^{-1}$.

In order to use the RI detector, when the signal is of sufficient intensity, it is necessary to determine the dn/dc of the sample also called specific RI increment. The dn/dc of monomeric horse spleen ferritin in the SEC buffer at 25.2 °C is 0.02428 ($\pm 7.4 \cdot 10^{-4}$) mL·g⁻¹ (cf. Fig. 5.2).

The estimation of the molecular weight of monomeric horse spleen ferritin gives $4.488 \cdot 10^5$ ($\pm 0.041 \cdot 10^5$) g·mol⁻¹. The radius of gyration is estimated at $R_g = 5.9$ (± 4.8) nm, the relatively large error results from detector errors at small angle (49 °). As it can be seen in Fig. 5.3, both measurements are in coherence with data retrieved from literature^{16,17}.

After separation through the column of the aggregates and the monomeric particles, SEC measurements of apoferritin allow the determination of the molecular weight and the hydrodynamic radius of the particles. As a result, a molecular weight of $3.25 \cdot 10^5$ g·mol⁻¹ and $R_g = 8$ (± 0.1) nm (with the $dn/dc = 0.1805$ estimated in SEC buffer) was calculated using the Berry model. The result of the molecular weight is smaller than the one retrieved from literature, while the hydrodynamic radius is larger. This derived from the calibration of the detectors.

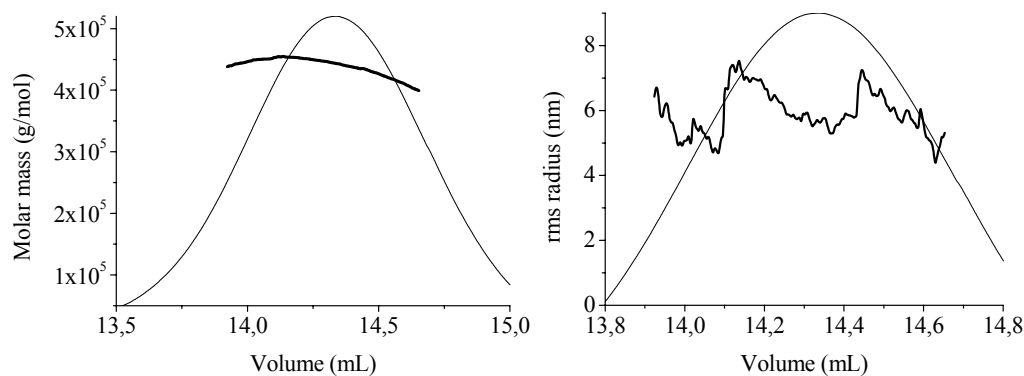


Figure 5.3: Left: Molar mass calculated from the Zimm model of horse spleen ferritin using UV detector to calculate the concentration and, LS detector at 90 °. $M_w = (4.488 \pm 0.041) \cdot 10^5$ g·mol⁻¹. Right: Radius of gyration calculated from the Zimm model of horse spleen ferritin using LS detector at 49 ° and 131 °. $R_g = 5.9 \pm 4.8$ nm.

5.1.2 UV-Vis Spectroscopy

UV-Vis is a molecular spectroscopic method, derived from the Bouguer-Lambert-Beer law, allowing the quantitative evaluation of absorption measurements¹⁴⁰.

The Bouguer-Lambert-Beer law forms the basis of light absorption measurements on gases and solutions in the UV-Vis and IR region:

$$\log \frac{(I_o)}{(I)_v} = lg \left(\frac{100}{T(\%)} \right) = A_{\tilde{\nu}}(\lambda) = \epsilon_{\tilde{\nu}} \cdot c \cdot d \quad (5.14)$$

where $A_{\tilde{\nu}}(\lambda) = \log \frac{(I_o)}{(I)_v}$ is the absorbance and $T_{\tilde{\nu}} = \frac{I_o}{I} \cdot 100$ in % is the transmittance, $\epsilon_{\tilde{\nu}}(\lambda)$ (in $\text{L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$) is the molar extinction coefficient depending on the wavelength. I_o is the intensity of the monochromatic light entering the sample and I is the intensity of light emerging from the sample, c is the concentration of the light-absorbing substance and d is the pathlength of the sample in centimeter.

The Bouguer-Lambert-Beer law is limited by dilution, as $\epsilon_{\tilde{\nu}}$ is no longer constant for concentrated solutions but depends on the refractive index of the sample. According to Eq. 5.14, the application of the Bouguer-Lambeer-Beer law presupposes a measurement of the relationship between the light intensities I_o and I . However, when measuring in quartz cuvettes, part of the light is lost due to reflection at the cuvette surfaces. In order to eliminate this source of error, a reference measurement is performed in an empty cuvette with the same path length. Usually, the pure solvent is used as reference, and it should ideally not absorb in the spectral region under investigation.

As plant viruses consist of both nucleic acids (mostly RNA) and proteins, their absorption spectra consists of a superposition of the absorbance of both components: nucleic acid (minimum at 230 nm; maximum at 260 nm) and protein (minimum at 250 nm; maximum at 280 nm). Nucleic acids absorb much stronger than protein. The peak maximum of a virus preparation is therefore typically at 260 nm and shifts to higher wavelengths with higher protein/nucleic acid ratio. The ratio A_{260nm}/A_{280nm} of the absorbances at the two wavelengths characterizes the protein content (empty capsids or capsids with RNA) and is a measure for the purity of the preparation (presence of plant protein contamination). UV-Vis can be applied to proteins, since some of the aromatic amino acids such as tryptophan,

contribute to its adsorption at 275-280 nm on a mole-per-mole basis. The folding of the protein will influence on the extinction coefficient¹⁴¹.

From collected monomeric ferritin, the concentration is estimated by UV-Vis measurement, using the Beer-Lambert law ($Abs < 1$), and knowing the extinction coefficient at 280 nm ($\epsilon = 4.8 \cdot 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$). The concentration of protein can also be determined by following the Lowry protocol¹⁴² using a Folin-Ciocalteu reagent solution which binds to the phenolic group of tyrosine and tryptophan residues and produces a blue purple color complex with a maximum absorption at 660 nm.

Table 5.1: Labeling of primary amino groups of horse spleen ferritin with 5-carboxyfluorescein succinimidyl ester and picrylsulfonate acid (TNBS) characterized by UV-Vis.

Fluorescent dye	Buffer (25 mM)	pH	Molar excess	Incubation time (min)	T (°C)	Degree of labeling per $\epsilon\text{-NH}_2$ (per particle)
NHS-fluorescein ^[1]	K ₂ PO ₄ /KH ₂ PO ₄	7.8	15x	1	RT	3-4
	K ₂ PO ₄ /KH ₂ PO ₄	8.2	20x	2	RT	19-20
	K ₂ PO ₄ /KH ₂ PO ₄	8.5	100x	12	4	12-13
	K ₂ PO ₄ /KH ₂ PO ₄	8.5	200x	24	4	9-10
	Borat buffer	8.5	200x	2	RT	24
TNBS ^[2]	K ₂ PO ₄ /KH ₂ PO ₄	8.5	200x	2	37	60-61

[1] 5-carboxyfluorescein succinimidyl ester: $M_w = 473.14 \text{ g} \cdot \text{mol}^{-1}$

[2] TNBS also called Picrylsulfonate acid: $M_w = 293.17 \text{ g} \cdot \text{mol}^{-1}$

UV-Vis also allows the determination of the conjugation of dyes with proteins and estimates their different reactive groups. In the case of ferritin, ϵ -amino groups were assayed. The number of addressable ϵ -amino groups was determined by performing two different assays: one using a fluorescent dye (5-carboxyfluorescein succinimidyl ester) in excess, and one following the TNBS (picrylsulfonate acid) assay described by Habeeb^{143,144}, purified by large dialysis against buffer and analyzed by UV-Vis spectrometry. The two different dyes have different molecular weights and their different steric conformations can explain the difference in the results. Different conditions were tested in order to determine the maximum of amino groups as seen in Table 5.1). With the 5-carboxyfluorescein succinimidyl ester, it is possible to label a maximum of 24 amino groups per ferritin particle (1 per subunit), while with picrylsulfonate acid, it is possible to label a maximum of 60-61 ϵ -amino groups per ferritin particle (2.5 per subunits), which is in agreement with the MALDI-ToF MS study of Zeng *et al.*, which predicts 3 addressable

ϵ -amino groups per subunit.

The labeling of primary amino groups of horse spleen ferritin with fluorescent dyes characterized by UV-Vis allows the determination of their number per ferritin. With 5-carboxyfluorescein succinimidyl ester, the maximum is at 494 nm. A correction factor CF of 0.3 at 280 nm is used to correct the absorption of ferritin. With a maximum at 494 nm, 5-carboxyfluorescein succinimidyl ester extinction coefficient is $\epsilon_{494}=7 \cdot 10^4 \text{ M}^{-1}\cdot\text{cm}^{-1}$. Picrylsulfonate acid (or 2, 4, 6,-trinitrobenzenesulfonate TNBS) has a maximum at 340 nm, the extinction coefficient is $\epsilon_{340}= 6.5 \cdot 10^2 \text{ M}^{-1}\cdot\text{cm}^{-1}$. The formula used to calculate the ϵ -amino groups is :

$$\text{Moles fluor per mole protein} = \frac{A_{\text{max of the labeled protein}} \cdot \epsilon_{\text{protein}}}{\epsilon_{\text{fluor}} \cdot (A_{280} - (A_{\text{max}} \cdot CF))} \quad (5.15)$$

5.2 Characterization by Microscopic Techniques

In order to control the ferritin adsorption onto solid substrates, zeta-potential measurements need to determine the isoelectric point of ferritin, as the adsorption is influenced by pH and leads to the adsorption of the particles on the solid surface. It was investigated with horse spleen ferritin to confirm the literature data^{33,34}. Solutions of different concentrations were prepared at different pH-values and the zeta potential was measured. The resulting isoelectric point of horse spleen ferritin is between 3.5 and 4.5, and is in coherence with the literature value of 4.5^{33,34}. The structure of horse spleen ferritin was also investigated by TEM (cf. Fig. 5.5), SEM (cf. Fig. 5.7), and AFM (cf. Fig. 5.9).

5.2.1 Transmission Electron Microscopy (TEM)

The history of electron microscopy is well known and rewarded. In 1986, Ruska *et al.* received the Nobel Prize for Physics for their work on electron optics and the design of the transmission electron microscope. One of the earliest samples investigated were virus suspensions¹⁴⁵, and since then, TEM has become an indispensable method for the investigation of specimen in biology and soft matter science^{146,147}.

5.2.1.1 Principle of TEM

The major advantage of using electrons rather than light is the resolution, which can be enhanced up to 0.2 nm. The electron beam is generated by thermo-ionic emission of electrons from a tungsten V-shape filament. A strong electric field of up to 200 kV, accelerates the electrons to high energies, while electromagnetic lenses focus and direct the beam towards the anode. The so-called Wehnelt assembly, a metal surrounding the filament, has an additional focusing action on the beam.

The image is generated by interaction of the electrons with the atoms of the sample. While transmitting the specimen, some electrons are scattered. The inelastically scattered ones are removed by apertures, while the elastically scattered produce the adsorption contrast in the image on the fluorescent screen below the sample. The usual "bright field imaging" mode uses the directly transmitted electrons for image recording. The darker areas correspond to denser regions of the specimen that scatter larger numbers of electrons. The intensities in the final image are closely connected to the thickness and the distribution of matter.

Biological samples can be spread as particles directly from aqueous solution on carbon filmed copper or gold grids, which were previously made hydrophilic by a short glow discharge. Larger entities such as cells must be embedded and cut into thin sections before deposition on a grid. As typical biological samples do not contain heavy atoms which can provide enough contrast, staining with solutions of heavy metal ions is necessary. Usually, uranyl acetate, tungsten or lead solutions are used. The salt penetrates all accessible volumes and forms an amorphous structure around the viruses. As a consequence, the sample appears bright against the background covered with salt (negative staining)¹⁴⁸.

Ferritin has the unique property of having an iron core which makes it visible for TEM. The surrounding polypeptide shell however can not be imaged that simply, here it is necessary to apply a negative staining.

5.2.1.2 Cryo-TEM

The chemical reactions of the staining process and the drying of the sample can damage the structure of the specimen. To avoid preparative artifacts, the sample can be flash-frozen, physically preserving the structure of the specimen, and measured by Cryo-TEM^{149,150}.

Biological solutions are usually aqueous. Water has, when in the solid state, a crystalline (hexagonal or cubic) structure, called ice. Ice crystals are harmful for the microstructure of specimen; they can damage tissue or capsid of viruses by perforation. To avoid this problem, the specimen must be frozen sufficiently quick to preserve the water in a vitreous glass-like state, the cooling rate for vitrification of water being about $100,000 \text{ K}\cdot\text{s}^{-1}$. Vitrification is a process of converting the surface area-to-volume ratio of the specimen to the maximum by using thin film. A proper cryogen is required for successful vitrification. It has to be at a low temperature far above its boiling point, to avoid the formation of a gas film around the specimen during the immersion. It should also have enough thermal conductivity. Liquid nitrogen is a poor cryogen because of the narrow temperature between its freezing and boiling points. Liquid ethane or propane are good cryogens. The crust of ice that can be formed during the transfer would be sublimated once inside the microscope.

The electron beam cannot be produced in a gas-filled environment. Even if it was possible, the electron beam could not be stable because ionized gas molecules would create a random discharge. It would also burn out the filament if reactive gases, as oxygen, would interact with the heated electron emitter. Moreover, the transmission into the column would be hindered by the presence of too many gas molecules. Vacuum should be strong enough to prevent the collision between electrons and gas molecules. A typical vacuum in an electron microscope is about 10^{-4} Pa .

Thin vitrified films are produced directly onto grids coated with a lacey carbon film. The cryogen, liquid ethane, has to be first cooled enough in its small metal vessel by the surrounding liquid nitrogen reaching -130°C . A pair of tweezers above the vessel of liquid ethane holds the grid. A droplet of the aqueous biological solution, about $2\text{--}5 \mu\text{L}$ is placed onto the grid to form a thin film of about 200 nm . Then, after blotting for $1\text{--}2 \text{ s}$ with a filter paper, it is directly plunged into the cryogen. The blotting time depends on the viscosity of the sample and has

to be adapted every time. The grid is plunged into the liquid ethane. The frozen grid with the vitrified sample is then transferred to liquid nitrogen, draining off the excess liquid ethane and then placed into the cryo-sample holder, also cooled below -140°C . It is important to keep the sample below this temperature to prevent crystallization of the vitrified water. The holder is then transferred into the microscope under inert gas to avoid contamination. For a controlled preparation, it is advised to use the Controlled-Environment Vitrification System (CEVS). This apparatus is a polycarbonate closed chamber (cf. Fig. 5.4), where the temperature, the humidity and the blotting time can be adjusted, ensuring the environmental preservation of the specimen.

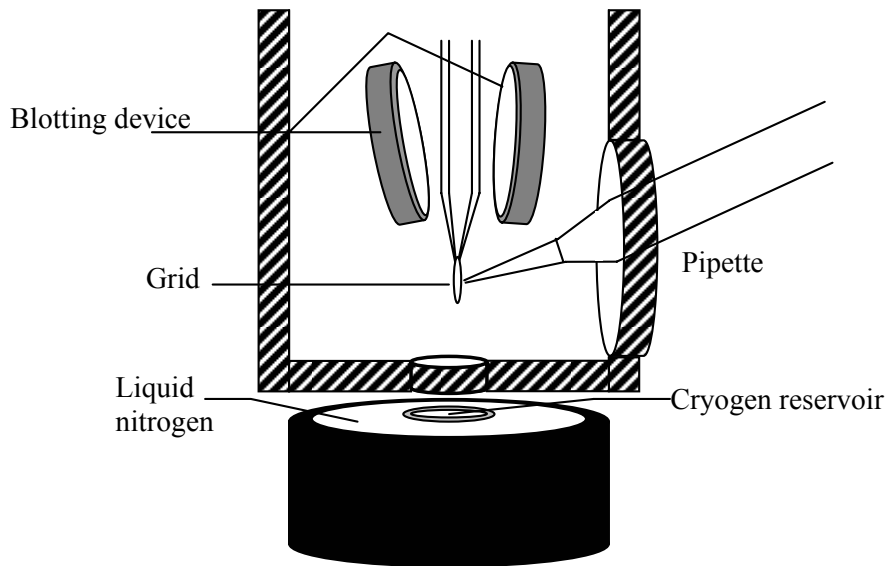


Figure 5.4: Schematic cross-section of a CEVS.

The preparation, from the droplet application to the blotting, is performed inside the chamber and assisted by software. The cryogen reservoir is outside the chamber. The grid held by the pair of tweezers is plunged into it, and the rest of the procedure is then the same as for the preparation without the CEVS.

Electron beam radiation damage, or radiolysis, is an inevitable consequence of the electron beam interaction with the specimen. The process is resulting from free radical chain reactions started by ionization of specimen molecules by the high-energy electron beam. The radiolysis is severe with vitrified specimen, that

contain organic compounds. It can destroy both sample and film. However, it can be used to determine whether certain structural elements are part of the structure or contaminating elements. Recording at the lowest magnification possible and minimizing the electron dose received by the specimen before the image is recorded can reduce it.

Cryo-TEM is used for ferritin-conjugates which are very sensitive and can be easily destroyed by the electron beam as it needs a long exposure time to obtain a good contrast between the iron core and the polymer chain.

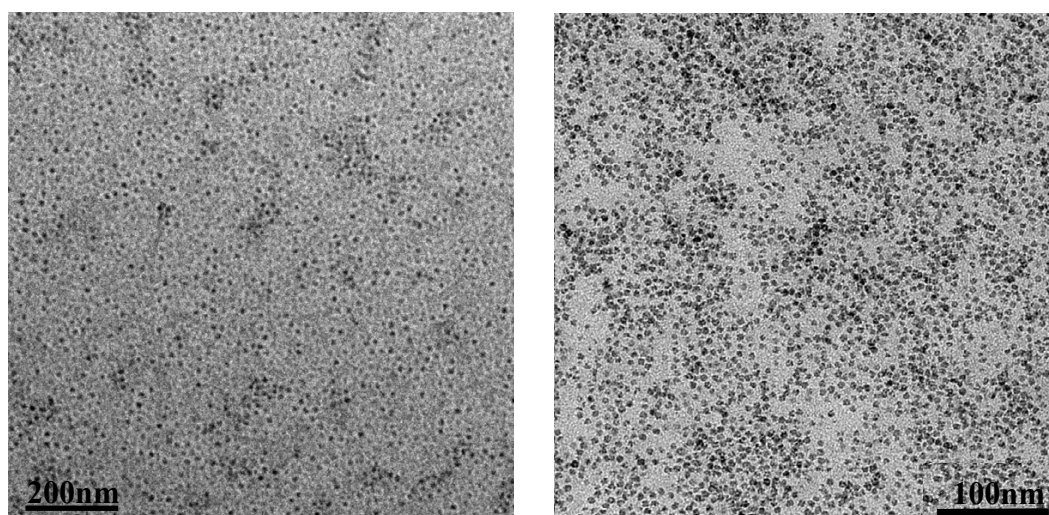


Figure 5.5: TEM micrographs of monomeric horse spleen ferritin on formvar coated copper grids at different concentrations (left: monolayer of ferritin).

TEM sample was prepared with monomeric ferritin solution drops coated onto a pretreated copper grid by glow discharge. The iron core of horse spleen ferritin gives a very high contrast in electron microscopic, so the proteins can be found easily as they appear as dark spots on TEM image (cf. Fig. 5.5). Those spots are about 6 nm in diameter, which corresponds to the core. However, the polypeptide shell is not visible. TEM micrograph of apoferritin has less contrast but shows a spherical particle of about 10-12 nm, which corresponds to the literature data. Freshly prepared diluted monomeric ferritin particles are not aggregating on the surface of the grid, but higher concentrations of ferritin are resulting in a monolayer of self-assembled particles in a hexagonal structure^{151–154}.

5.2.2 Scanning Electron Microscopy (SEM)

In comparison to light microscope, scanning electron microscope (SEM) featured a greater depth of field, a higher resolution and magnification¹⁴⁶. Its advantage, compared to the TEM, is the easier preparation of the samples (measurement in bulk, not only film), and the bigger surface that can be examined. As in TEM, the image is formed by the interaction between electron beam and sample; whereas it has lower resolution, as the spatial resolution of the SEM depends on the size of the electron spot, which depends on both wavelength of the electrons and the magnetic electron-optical system which produces the scanning beam. As a result, resolution of SEM is not high enough to image individual atoms, as is possible in the shorter wavelength (i.e. higher energy) TEM. The resolution is somewhere between less than 1 nm and 20 nm depending on the instrument. SEM is a surface characterization technique. For image generation mostly secondary electrons are used.

A focused electron beam (1-2 nm) is scanning over the sample. When the electrons of the scanning beam interact with the surface of the sample, it undergoes a series of complex interactions with the nuclei and electrons of the atoms of the sample. These interactions produce secondary electrons of different energies, X-rays, heat and light, which can be used to produce an image or gain information on the composition of the sample.

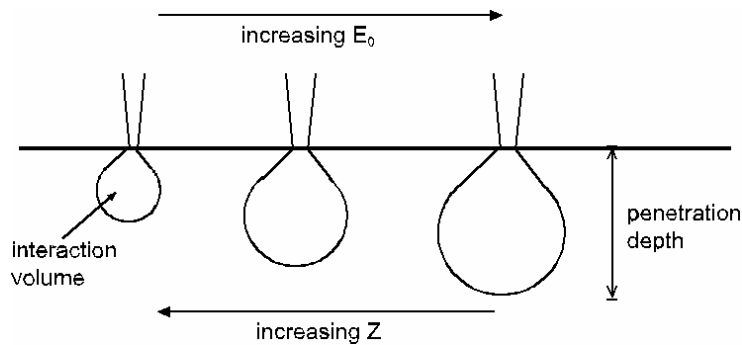


Figure 5.6: Scheme of the interaction volume as a tear drop.

When an electron beam interacts with atoms, each interaction causes the scattering of the incident beam. The interaction volume (depth and width) depends on

the acceleration voltage: the higher the beam energy, the larger the interaction volume which in turn is inversely related to the average atomic number of the sample (cf. Fig. 5.6).

As said before, samples can be measured in bulk, not only as film or foil. For good imaging the atomic number has to be high enough, as otherwise no contrast can be detected. Staining methods are also used for SEM. But it is used to sputter the sample with some electrical conductive metal such as gold or platinum, carbon coating can also be done. However, such manipulation may destroy the sample (polymer for example). Samples should also be dehydrated for the stability of the vacuum. The environmental scanning electron microscope allows working in highly humid conditions, e.g. to visualize biological samples, due to the development of a special detector.

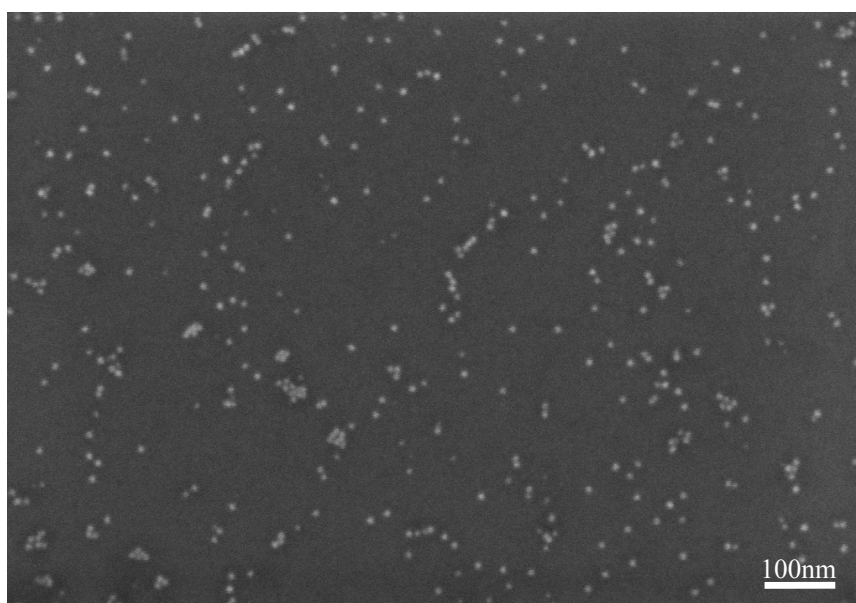


Figure 5.7: SEM image of monomeric horse spleen ferritin on silicon wafer (magnification 100 kX, EHT 1 kV).

Due to its iron core, ferritin produces a high contrast in SEM images. With the resolution being limited from 1 to 20 nm, it is not easy to focus on the 6 nm iron core, but the particles appear clearly as white dots as shown in Fig. 5.7.

5.2.3 Atomic Force Microscopy (AFM)

AFM is scanning force microscopy (SFM) method developed by Binnig, Quate and Gerber in 1986¹⁵⁵. A sharp probe is scanned across a surface and tip-sample interactions are monitored. AFM is running in three primary modes: contact mode, non-contact mode and tapping mode (cf. Fig. 5.8). The principle of AFM is to scan an attached tip at the end of a cantilever across the sample surface while monitoring the change in cantilever deflection with a split photodiode detector. The distance the scanner moves vertically is stored by the computer to form the topographic image of the sample. The piezo crystal expands and retracts proportionally to an applied voltage and allow the scanner to move in x, y, and z directions. The scanning parameters can be controlled: set point (the feedback loop, which amplitude to maintain during scanning), integral gain (controls the amount of the integrated error signal used in the feedback calculations) and scan rate (number of trace and retrace scan lines performed by second (Hz)). The scanning results in three images: height, phase and amplitude images.

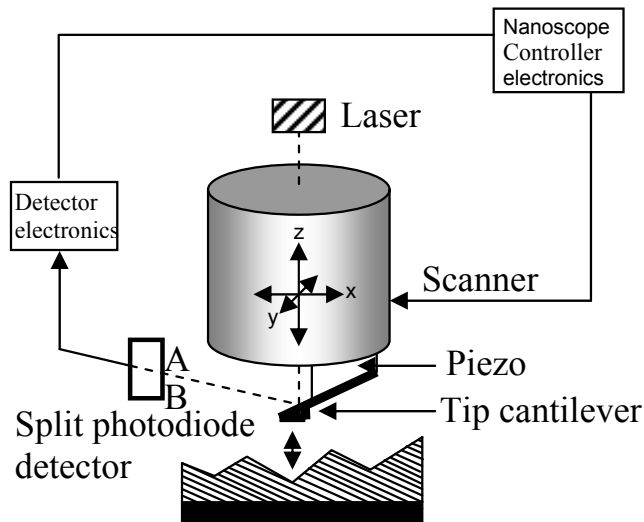


Figure 5.8: Working principle of Atomic Force Microscope.

In the contact mode, the feedback loop maintains a constant deflection between the cantilever and the sample by vertically moving the scanner. So the force between the tip and the sample is constant. $F = -kx$, with k spring constant, and x the cantilever deflection. F is in the range of 0.01 to $1 \text{ N}\cdot\text{m}^{-1}$. In tapping mode, the

tip is attached to an oscillating cantilever scanning across the sample surface. The cantilever is oscillated at its resonance frequency with an amplitude ranging from 20-100 nm. The tip taps on the sample surface, contacting the surface at the bottom of its swing. The feedback loop maintains constant oscillation amplitude by keeping a constant RMS of the oscillation signal acquired by the split photodiode detector. The vertical position of the scanner (with a constant set point) is stored by the computer to form the topographic image of the sample surface. By holding constant oscillation amplitude, a constant tip-sample interaction is maintained during measuring.

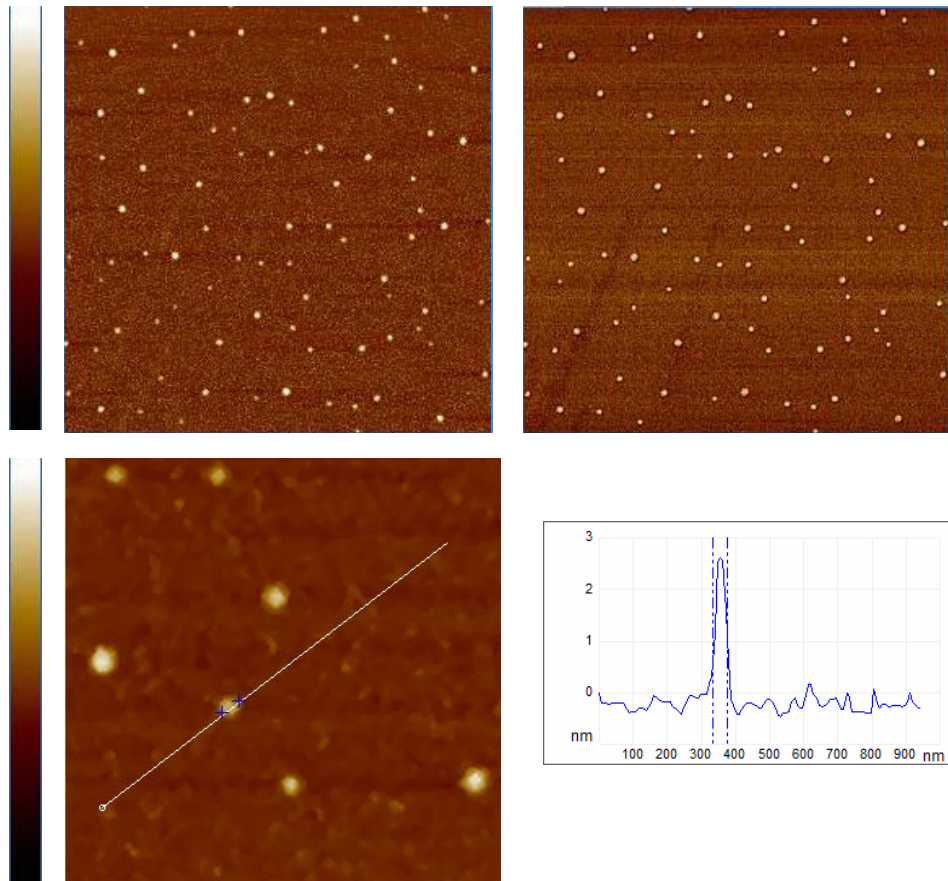


Figure 5.9: Top: AFM image of monomeric horse spleen ferritin on freshly cleaved mica sheet over $100 \mu\text{m}^2$, left image (A) height z-range: 10 nm, right phase image (B) z-range: 20° . Bottom: AFM height zoom image (C) over $1 \mu\text{m}^2$. The last image (D) shows the horse spleen ferritin diameter of about 20 nm.

In the non constant mode, the feedback loop maintains a constant oscillation amplitude or frequency by vertically moving the scanner until "setpoint" amplitude or frequency is reached. The cantilever is oscillated at a frequency which is above the cantilever's resonance with amplitude of a few nanometers to obtain an AC signal from the cantilever. The tip does not contact the sample surface, but oscillates above the absorbed fluid layer on the surface during scanning. The cantilever's resonance frequency is decreased by Van der Waals forces which extend from 1 to 10 nm above the absorbed fluid layer.

The protein solution was spincoated onto a freshly cleaved mica sheet, washed with water to dissolve the top-layer of salt. On the AFM phase picture, HSF particles are homogeneously distributed as seen in Fig. 5.9. The iron core of ferritin allows a high contrast, while on the height phase, particles about 20-25 nm are homogeneously spread. Protein particles are enlarged on the AFM image because the tip is flattening the proteins due to their soft nanoparticle character.

5.3 Characterization in Denaturated State by MALDI-ToF MS & SDS-PAGE

Ferritin containing a high percentage of L chains, as it is the case for horse spleen ferritin, is more stable towards denaturation than other proteins. Indeed every attempt to denaturate horse spleen ferritin, avoiding high temperature, failed so far. Horse spleen ferritin is stable over 24 hours under UV-light, under sonication. When dissolved into different solvents (methanol, acetone) the particles aggregate but are not denaturated. When mixed with 2-mercaptoethanol (a disulfide bonds reducer), and chaotropic agents such as guanidine hydrochloride and urea, the protein does not defold neither. The method leading to the denaturation of the quaternary structure of horse spleen ferritin is to combine urea (7 M) and 2-mercaptoethanol or dithiothreitol (10 mM). A further enzymatic digestion with trypsin, chymotrypsin or V8 protease allows cleaving the subunit into smaller peptides^{22,156,157}. Trypsin is a serine protease found in the digestive system, where it breaks down proteins. It predominantly cleaves peptide chains at the carboxyl site of the amino acids lysine and arginine. Chymotrypsin preferentially cleaves peptide chains at the carboxyl site of the amino acids phenylalanine, tyrosine, tryptophan and methionine, V8 protease cleaves aspartic and glutamic acid. 100 μ L of

concentrated horse spleen ferritin or apoferritin was added to a 300 μ L solution of urea at 7 M and 10 μ L of 2-mercaptoethanol. The digestion was achieved by adding 5 mg of trypsin to the solution, then incubated for 12 hours at 37 °C. The denaturated HSF was characterized by MALDI-ToF MS and SDS-PAGE.

5.3.1 Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-ToF MS)

MALDI-ToF mass spectrometry was especially developed for the analysis of large polymers and proteins. It is a mass spectrometry method which is an analytical technique to measure the mass-to-charge ratio of ions¹⁵⁸. MALDI-ToF is a soft ionization technique which allows for the sensitive detection of large, non volatile and labile molecules such as biomolecules (proteins, peptides and sugars) and macromolecules (polymers and dendrimers) which are more fragile and fragmented than when they are ionized by more conventional methods. Molecules are vaporized, in the MALDI-ToF process, within a volatile light-absorbing matrix, composed mostly of small organic molecules, with the energy-input of a laser. Ideally, the analyte in the gas phase has acquired one or only few charges, and is then accelerated with an electrical field of known strength and thus the flight time is measured. Thus, molecules with the same mass to charge-ratio will be simultaneously detected.

A mass spectrometer consists of three parts: an ion source, a mass analyzer and a detector. In the ion source, ions are produced by evaporation of the analyte, which was applied in a mixture with the matrix onto a steel target with the aid of a laser. Ideally, the sample is surrounded by the matrix, which forms a crystal lattice. Matrices have to be chosen according to the sample. To make ion formation easier, alkaline salts are added to the matrix/sample mixture or acidic solutions are used. In most cases, the analyte molecules pick up a proton and turns into a positively charged gas phase. Multiple peaks arise because an analyte can be multiply charged (mass appears to be lower) or if it forms complexes but picks less than one charge per molecule (masses appear to be larger). Usually the structures of proteins stay intact and the proteins are charged after interaction with the laser.

The mass analyzer is separating ions with different mass-to-charge ratios. Then the numbers of different ions are detected by the detector. In the time of flight-

mass analyzer, it measures the time it takes for the ions to fly from one end of the analyzer to the other and hit the detector. The velocities of ions are proportional to their mass-to-charge ratio. There are two different kinds of mass analyzer modes: linear and reflectron mode. In the former, the MALDI-ToF MS analyzer measures the time of flight for an ion to fly from one end to the other. In the so-called reflectron mode, the ions are reflected at the end of the analyzer tube by an electric field and turn back to the detector located close to the beginning of the flight tube. Thus, not only the flight path is prolonged and resolution increased but the reflectron mode has an additional action: it focuses ions with the same m/z values, and makes them reach the detector at the same time, which results in more accurate detection. The linear mode lacks in resolution but is more suitable for the analysis of very high molecular weights (> 100 kDa). MALDI-ToF MS is a very sensitive method with absolute results, but is strongly dependent on the sample/matrix combinations and the quality of the analyte preparation. Further, broad polydispersity will adversely affect the analysis.

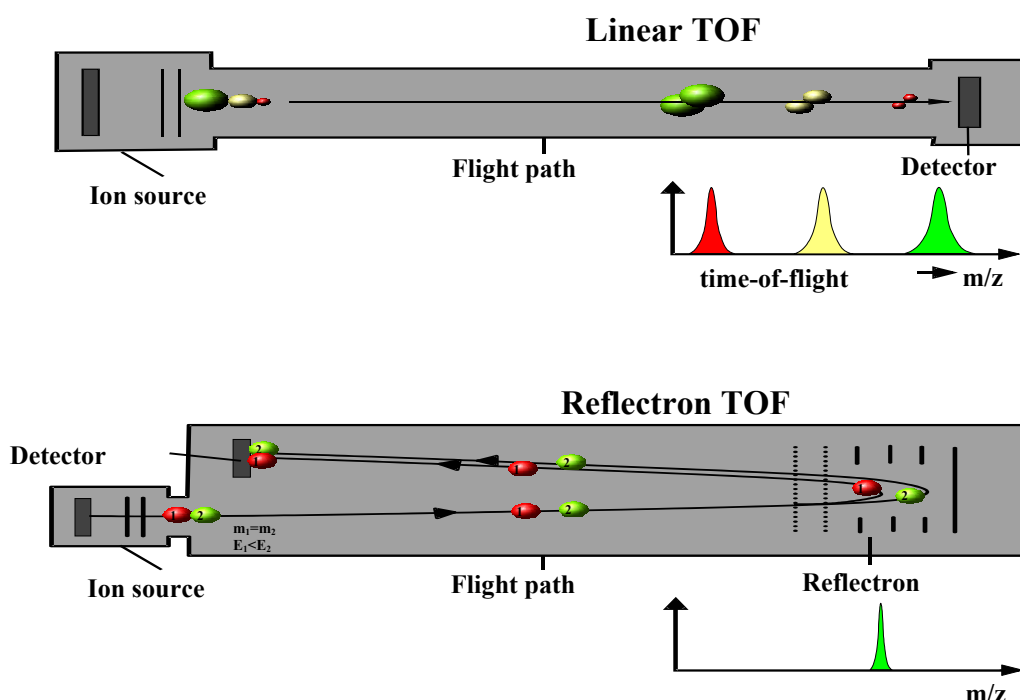


Figure 5.10: Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Analyzer scheme in linear and reflectron modes.

5.3.2 Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE)

In electrophoresis, the migration of charged biomolecules in a matrix within an applied electric field is used as separating principle, based on the size of the analyte (principle of gel filtration) as well as the electrophoretic mobilities. As there is no solvent space in the gel beads, as analogously in between the gel beads of gel chromatography, the separation of proteins occurs reverse i.e. the movement of large molecules is impeded relatively to the small ones. SDS-PAGE is a high resolution technique^{159,160} for the separation of proteins using their electrophoretic mobility. The SDS-PAGE of protein chains having identical charge-to-mass ratios results in fractionation by size. It is able to separate up to 100 components per gel.

The technique is a powerful and convenient method to separate complex and large mixtures of proteins of 1000 up to 200,000 Da. However, the determination of molecular weights is usually performed by comparison with a protein standard mixture. A prerequisite to achieve high resolution is to reduce disulfide bonds by addition of reducing agents (such as dithiothreitol, or 2-mercaptoethanol) and to cleave protein complexes completely into their polypeptide chains by preparation with urea and SDS. The latter is an anionic detergent which denatures secondary and non-disulfide-linked tertiary structures, and applies a negative charge to each protein in proportion to its mass. It allows to obtain approximately uniform mass-to-charge ratio for most proteins, so that the distance of migration through the gel can be assumed to be directly related to only the size of the protein. A tracking dye (bromophenol) may be added to the protein solution to allow tracking of the progress of the protein solution through the gel during the electrophoretic run.

A polyacrylamide gel is used as it is chemically inert and easy to crosslink into gel with different pore sizes and can withstand high voltage. A stacking gel with large pores polyacrylamide gel of 4 % is cast on the top of the resolving gel. The proteins are then stacked at the same height starting zone. The resolving gel can be of uniform pore size or have a gradient crosslinking density and is chosen according to the range of the interesting molecular weights.

The gel is then placed into an appropriate running buffer, and an electric current is applied across the gel, causing the negatively-charged proteins to migrate across the gel towards the anode. Depending on their size, each protein will move

differently through the gel matrix: short proteins will more easily fit through the pores in the gel; while larger ones will have more difficulty (they encounter more resistance), so proteins are separated according to size. After electrophoresis, the proteins are stained by Coomassie Brilliant Blue or a silver staining complex, allowing the visualization of separated proteins, and the determination of the molecular weight, thanks to a run molecular marker, which contains different proteins with different known molecular weights. The calibration of the gel allows an accurate estimation the molecular weight, by comparing the standard proteins in a separate parallel line. The calculation of the mobility has to include the length of the gel before and after staining as well as the mobility of the protein and the marker dye. The mobility can be calculated as followed, assuming the swelling of the gels¹⁶¹:

$$\text{Mobility} = \frac{\text{distance of protein migration}}{\text{length after destaining}} \cdot \frac{\text{length before staining}}{\text{distance of dye migration}} \quad (5.16)$$

The mobilities are logarithmically proportional to the molecular weights so the migration can be expressed as:

$$M_w = k \cdot (10^{-bx}) \quad (5.17)$$

where x is the distance of migration and b is the slope.

SDS-PAGE is a powerful technique to determine the protein subunit molecular weight and derivatized conjugates. It was used to determine the protein subunit and protein chemical modification. One must be aware that non-protein blocks in the conjugates may not behave like peptide sequences and thus give different apparent sizes. Molecular weights must be determined with caution.

MALDI-ToF MS measurements lead to subunits of the protein with two different peaks at m/z -values about about 19.9 and 21.4 kDa (cf. Fig. 5.11) which correspond to the molecular weight of L- and H-subunits of 20 and 21 kDa respectively. The peak at 23.3 kDa corresponds to the excess of trypsin. For SDS-PAGE, horse spleen ferritin was denaturated and digested with different preparations. All the samples proved to be denaturated as a band for each sample was found to be at the same height than the marker at 20 kDa (sample 1: HSF was denaturated as de-

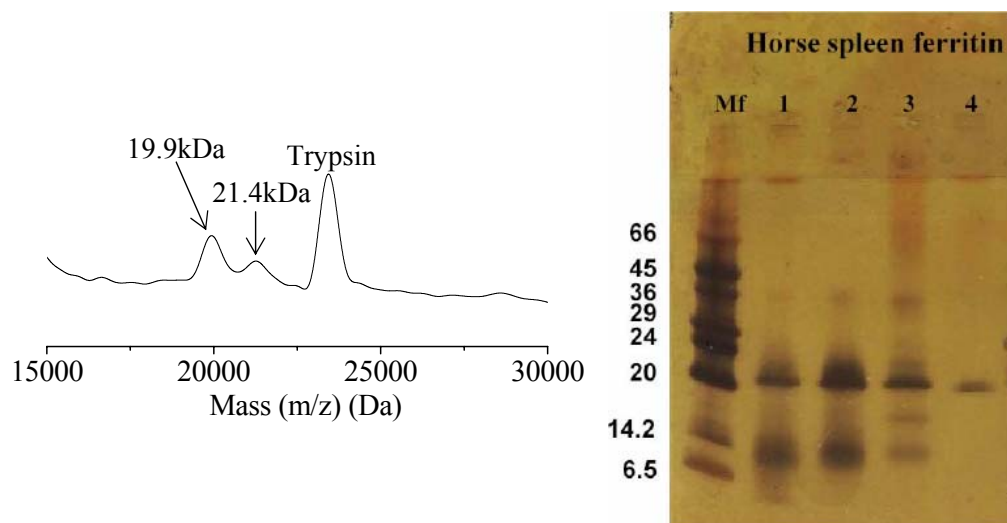


Figure 5.11: MALDI-ToF MS measurement of apoferritin after denaturation and digestion and SDS-PAGE of ferritin after denaturation, dyed with silver solution (1-4 are different preparations of denaturated ferritin solution. Mf is a standard protein marker from 6.5 to 66 kDa).

scribed before; sample 2 was denaturated and boiled; sample 3 was denaturated with urea 8 M and 10 μ L of 10 mM solution of DTT; sample 4 was denaturated and digested as described earlier).

6 Controlling the Fast ATRP of *N*-Isopropylacrylamide in Water

6.1 Introduction

Poly(*N*-isopropylacrylamide) (PNIPAAm) is a well-known thermo-responsive polymer⁶⁸ and exhibits a lower critical solution temperature (LCST) of 32 °C in water. It adopts a random coil structure (hydrophilic state) below the LCST and a collapsed globular structure (hydrophobic state) above. Because of this defined reversible transition, this polymer finds a vast array of applications, e.g. in the delivery of therapeutics, bioseparations and biosensors^{162–164}. NIPAAm is generally polymerized via free radical polymerization. However, conventional free radical polymerization does not allow control of the molecular weight and to reach a narrow molecular weight distribution (MWD). For sophisticated PNIPAAm-containing materials, especially NIPAAm conjugation to biomacromolecules like ferritin, defined molecular weight and end-group but also low polydispersity index are highly desirable.

Controlled free radical polymerization techniques have been intensively investigated during the past ten years. Nitroxide-mediated polymerization (NMP)^{165–167}, reversible addition fragmentation chain transfer (RAFT) polymerization^{168–170} as well as atom transfer radical polymerization (ATRP)^{56,171} are the main radical polymerization techniques that allow the preparation of polymers with defined molecular weights and narrow polydispersities. Several groups have developed strategies to carry out NIPAAm polymerization with a good control. Schulte *et al.* performed this synthesis via NMP with a sterically hindered alkoxyamine and detailed mass spectrometry analysis¹⁶⁶. In our laboratory RAFT polymerization was employed in pure water to obtain high molecular weight PNIPAAm with very good control and without irreversible termination even at high conversion¹⁷².

Working in water is a great challenge and exhibits a high potential; it is an environmentally friendly solvent and also allows the presence of biological compounds like viruses, polypeptides or proteins in the polymerization process^{84,173–175}. The synthesis of well-defined bioconjugates for biomedical applications has been an active area of research for many years. However, before investigating the synthesis of biohybrids based on PNIPAAm, the homopolymerization in pure water has to be optimized to reach the best possible control. During the past few years, several studies have been realized in this field. Among them, Masci and co-workers were the first to report the successful ATRP of NIPAAm using a DMF /water mixture. However the experiment performed in pure water failed, as a gel was formed immediately after the addition of the catalyst¹⁷⁶. Due to the low solubility of usual ATRP initiators like methyl 2-bromopropionate or ethyl 2-bromoisobutyrate in water, a pure organic solvent or an aqueous mixture are commonly used yielding satisfactory results. Thus, Stöver and co-workers studied the influence of different alcohols and demonstrated good polymerization control especially in isopropanol by the use of CuCl/Me₆TREN as catalyst system¹⁷⁷. Nevertheless, to perform the polymerization in pure water another applied strategy was to start from a macro-initiator, which is soluble in water. Kim *et al.* have succeeded to prepare linear PEG-*b*-PNIPAAm diblock copolymers and also hydrogel nanoparticles by ATRP of NIPAAm in water at 25 °C and 50 °C using a PEG macro-initiator¹⁷⁸. Another example was recently described by Kizhakkedathu and co-workers. They synthesized mikto-arm star copolymers of poly(dimethylacrylamide) (PDMAAm) and PNIPAAm by sequential RAFT and ATRP from a multi-initiator-functionalized polyglycerol. The ATRP of NIPAAm was conducted after the RAFT polymerization of DMAAm in the presence of CuCl/Me₆TREN in pure water. Monomodal and narrow MWD were achieved¹⁷⁹. Additionally, several studies were performed to polymerize NIPAAm from surfaces, like gold, carbon black, polystyrene (PS) or dextran particles, directly in water^{180–184}. Brooks *et al.* prepared PNIPAAm brushes by surface-initiated ATRP from polystyrene particles. High molecular weights (up to 800 kg·mol⁻¹) and narrow MWD were obtained^{185,186}. However, there is no known report on the polymerization of NIPAAm via ATRP in pure water with low molecular weight water-soluble initiators. Therefore, in the current study, a novel strategy is described to obtain PNIPAAm via ATRP in pure water by using a fully soluble low molecular weight initiator. The influence of the ratio CuBr/CuBr₂ or CuCl/CuCl₂ and of the choice of the ligand are studied in details, to access this polymer with an excellent control, without irreversible termination even at high conversion and demonstrate the livingness of the process by a successful chain extension.

6.2 Experimental

6.2.1 Materials

All chemicals and solvents were purchased from Sigma-Aldrich, Acros and Fluka at the highest available purity and used as received unless otherwise noted. *N*-isopropylacrylamide (NIPAAm) (99 %, Acros) was purified by two successive recrystallizations in a mixture of *n*-hexane and benzene (4:1 (v:v)). CuBr (98 %, Aldrich) and CuCl (97 %, Aldrich) were purified by stirring with acetic acid overnight. After filtration, they were washed with ethanol and ether and then dried in vacuo. N,N,N',N'',N''-pentamethyldiethylene-triamine (PMDETA; 99 %, Aldrich) and 1,1,4,7,10,10-hexamethyltriethylenetetramine (HMTETA; 97%, Aldrich) were distilled before use. Tris(2-dimethylaminoethyl)amine (Me₆TREN) was prepared as described in the literature¹⁸⁷.

6.2.2 Polymerization Procedure

NIPAAm and 2-bromo-isobutyric acid (BIBA) were dissolved in 19 mL of pure water. Then CuBr/CuBr₂ or CuCl/CuCl₂, respectively were added. Monomer concentrations and monomer/BIBA/Cu(I)/Cu(II)/Ligand ratios are given in Table 6.1 and Table 6.2. The vial was capped with a rubber stopper to allow addition of the ligand and placed in an ice bath. In a second small flask, 2 mL of aqueous ligand solution was prepared. Then both were deoxygenated by purging with nitrogen gas for 15 min. Afterwards 1 mL of ligand solution was withdrawn with a degassed syringe and placed in the polymerization flask to start the reaction, which was stopped after a pre-selected time and quenched with air. PNIPAAm samples were purified by freeze-drying to remove the solvent. Then the solid was dissolved in dichloromethane and passed through a silica gel column to remove the ATRP catalyst. Finally, PNIPAAm was precipitated from this solution into a 20-fold excess of diethyl ether before further analysis. The conversion of each sample was determined directly after freeze-drying by ¹H-NMR (in CDCl₃) from the relative integration of peaks associated with the monomer in relation to those associated with the polymer. For NIPAAm, the monomer peak chosen as reference was its vinyl peak at $\delta = 5.72\text{--}5.8$ ppm (dd, CH(H)=), which was compared to the proton peak of the isopropyl group at 4.1–3.8 ppm (m, CH(CH₃)₂) of the polymer and monomer.

6.2.3 Characterization

Polymers were characterized by size exclusion chromatography (SEC) using a solution at 0.05 M of LiBr in 2-N-methylpyrrolidone (NMP) as eluent. PSS GRAM columns (300 mm·8 mm, 7 μ m): 10³, 10² Å (PSS, Mainz, Germany) were thermostated at 70 °C. A 0.4 wt% (20 μ L) polymer solution was injected at an elution rate of 0.72 mL·min⁻¹. RI and UV (λ =270 nm) were used for detection. Polystyrene standards were used to calibrate the columns, and methyl benzoate was used as an internal standard. ¹H-NMR spectra were recorded on a Bruker AC-25 spectrometer in CDCl₃ (reference peak δ = 7.26 ppm) at room temperature.

6.3 Results and Discussion

6.3.1 Homopolymerization of *N*-isopropylacrylamide

N-isopropylacrylamide (NIPAAm) was polymerized in the presence of 2-bromoisobutyric acid (BIBA). This initiator was mainly chosen due to its high solubility in water. In addition, it has the advantage to introduce a carboxylic group to allow protein modification by active ester chemistry or post-polymerization modification¹⁷⁴. Common ATRP initiators like methyl 2-bromopropionate and ethyl 2-bromoisobutyrate were also used but the resulting polymers always exhibited a high polydispersity index (PDI < 1.7) and a multimodal distribution (results not shown). This absence of control can be explained by the very poor solubility of these initiators in water, which leads to a slow initiation. BIBA, as a weak acid, can partially protonate the tertiary amines used as ligands, which are weak bases¹⁸⁸ and thus destabilize a part of the copper complexes¹⁸⁹. However the quantification of this phenomenon, which may affect the activation equilibrium of ATRP, is not investigated here due to the very efficient activity of the copper species involved in this polymerization. Another important parameter allowing for a successful polymerization is the reaction temperature. When polymerizations were carried out at room temperature with Cu(I) or with a high ratio Cu(I)/Cu(II), kinetics were extremely fast, typically less than a minute for full conversion. This very high rate of polymerization was also observed by Narumi *et al.* in DMF/water at 20 °C. In this less polar solvent mixture, conversions of 98 % were obtained after only 30 min¹⁹⁰. Moreover, due to the exothermic character of the propagation,

the temperature in the medium increased drastically which accelerated the rate of polymerization but more importantly, the temperature raised at least above the LCST of PNIPAAm, leading at the polymer collapse and a total loss of control. This increase in temperature during the polymerization of NIPAAm via ATRP was already described by Kuckling and co-workers for a DMF/water system. However for this solvent mixture, an increase of 5-10 K was observed which was not sufficient to observe the collapse of PNIPAAm¹⁹¹. To avoid this problem, a rather low monomer concentration, typically $[M]_0=0.5$ M and an ice bath were used to control the heat evolution. Under these conditions all polymerizations were successfully achieved even in the absence of Cu(II).

A suitable selection of the ligand is crucial to reach a good control of NIPAAm polymerization¹⁹². Inspection of the data given in Table 6.1 clearly indicates that *N,N,N',N'',N''*-pentamethyldiethylenetriamine (PMDETA) and 1,1,4,7,10,10-hexamethyltriethylenetetramine (HMTETA) are not the right choice to obtain a polymer with a low polydispersity when CuCl₂ is not present in the media. The SEC profiles of the polymers made in the presence of PMDETA and HMTETA with CuCl only is strongly skewed towards low molecular weights, which might indicate a slow initiation step. Moreover, the polymers have a higher molecular weight compared to those made in presence of Me₆TREN. A reasonable explanation is a low efficiency of this initiator in the presence of these two former ligands. This problem was already described in the case of BIBA¹⁹³. By adding Cu(II) to the system, for PMDETA and HMTETA, kinetics slow down and the molecular weight distributions of the resulting polymers become sharper but still not below 1.2 (results not shown). Under these conditions, these two ligands do not seem to be suitable to obtain a well controlled PNIPAAm. Me₆TREN globally yields the best results for the different ratios of $[CuCl]_0/[CuCl_2]_0$ employed and was the ligand of choice for the continuation of the study. This ligand is known to be very active for ATRP compared to HMTETA and PMDETA¹⁹². Unfortunately it is also highly sensitive to oxygen which can drastically slow down the kinetics. Hence, kinetic reproducibility was difficult to achieve. However, the ATRP of NIPAAm in water did not exhibit any or less than few percents of termination even at full conversion. This property allowed us to solve the reproducibility problem. Therefore the reactions were always carried out at longer times than normally required to follow the kinetics, and then well defined polymers with narrow molecular weight distribution at full conversion were obtained. The complex of Cu(I) with Me₆TREN is also known to disproportionate in aqueous media into Cu(0) and Cu(II)¹⁹⁴. This reaction generally leads to a loss of control of the overall process. In all our experiments with this ligand, Cu(0) particles were not observed. Thus this reaction is

possibly limited by two factors: (i) the general use of Cu(II) which displaces the equilibrium of disproportionation and (ii) by the presence of monomer and polymer which can coordinate to the complex and prevent this side-reaction.

Table 6.1: Influence of the ligand on the ATRP of NIPAAm in water at 4 °C^[1].

Ligand	Time (min)	$M_{n,exp}^{[2]}$ (kg·mol ⁻¹)	PDI ^[2]
PMDETA	95	32	1.79
HMTETA	130	29	1.94
Me₆TREN	76	23	1.30

[1] $[M]_0 = 0.5 \text{ mol} \cdot \text{L}^{-1}$ with $[M]_0/[BIBA]_0/[CuCl]_0/[L]_0 = 100/1/1/1$. Monomer conversion, determined by ¹H-NMR in D₂O > 99%. The theoretical number average molecular weight, evaluated according to the formula, $M_n^{th} = M_M \cdot \text{conv} \cdot [M]_0/[BIBA]_0 + M_{BIBA} = 11.5 \text{ kg} \cdot \text{mol}^{-1}$.

[2] measured by size-exclusion chromatography (SEC) using polystyrene standards in 2-N-methylpyrrolidone (NMP) as eluent.

The influence of the catalyst system was also investigated by comparing CuCl- and CuBr-based systems at different ratios of $[Cu(I)]/[Cu(II)]$. The results are summarized in Table 6.2. In all cases CuBr provides a narrower molecular weight distribution of the resulting polymer than CuCl for the ATRP of NIPAAm in water, independently of the ratio $[Cu(I)]/[Cu(II)]$ used. This effect is generally observed for acrylate polymerization. When CuCl is used, the low rate of activation of the dormant species combined with the high reactivity of the secondary propagating radical lead to a lower control as compared to CuBr¹⁹⁵. Moreover, the deactivation rate constants for the CuBr₂ complexes are generally higher than for the chloride-based complexes¹⁹⁶. As a result of the faster deactivation, better defined polymers are obtained with the bromide system. In the case of the bromide system, already without addition of CuBr₂ to the reaction mixture, the polydispersity index is lower than 1.2. Here, already the addition of only a small amount of CuBr₂ leads to a drop in PDI to around 1.1. This proves the excellent ability of this system to polymerize NIPAAm in a controlled fashion. For the chloride-based ATRP, the MWD is broader but narrows by raising the amount of CuCl₂. For both catalytic systems and for all the different ratios of $[Cu(I)]/[Cu(II)]$, the SEC traces are monomodal and symmetrical. However, at full monomer conversion, especially for a low concentration of CuBr₂, some SEC traces of CuBr mediated ATRP show a small amount of coupling which is the predominant termination reaction for acrylamide-based monomers. This termination was never detected even at full conversion in the case of CuCl mediated ATRP.

Table 6.2: Influence of the ratio Cu(I)/Cu(II) on the ATRP of NIPAAm with Me₆TREN as ligand in water at 4 °C^[1].

Catalyst	[BIBA] ₀ /[Cu(I)] ₀ / [Cu(II)] ₀ /[L] ₀	Time (min)	M _{n,exp} ^[2] (kg·mol ⁻¹)	PDI ^[2]
CuCl/CuCl₂	1/1/0/1	76	23	1.30
CuCl/CuCl₂	1/0.6/0.4/1	85	26	1.25
CuCl/CuCl₂	1/0.5/0.5/1	90	16.5	1.20
CuBr/CuBr₂	1/1/0/1	60	17	1.19
CuBr/CuBr₂	1/0.85/0.15/1	100	18.5	1.08
CuBr/CuBr₂	1/0.7/0.3/1	115	18	1.08
CuBr/CuBr₂	1/0.6/0.4/1	130	19	1.09

[1] [M]₀ = 0.5 mol·L⁻¹ and [M]₀/[BIBA]₀=100. Monomer conversion, determined by ¹H-NMR in D₂O > 99%. The theoretical-number-average molecular weight, evaluated according to the formula, $M_n^{th} = M_M \cdot \text{conv} \cdot [M]_0 / [BIBA]_0 + M_{BIBA} = 11.5 \text{ kg} \cdot \text{mol}^{-1}$.

[2] measured by size-exclusion chromatography (SEC) using polystyrene standards in 2-*N*-methylpyrrolidone (NMP) as eluent.

Based on the above results, the CuBr-based catalyst with BIBA as initiator was chosen to study the kinetics due to its excellent ability to control the polymerization of NIPAAm in water at low temperature. The results are summarized in Fig. 6.1 The polymerization was carried out in presence of Me₆TREN and with an equal ratio of [CuBr] and [CuBr₂]. This relatively high amount of CuBr₂ is needed to slow down the kinetics which otherwise proceeds in less than a minute. This very fast ATRP in water was already observed for several types of monomers like poly(ethylene glycol) methacrylate, sodium 4-vinylbenzoate or dimethylacrylamide¹⁹⁷. Tsarevsky *et al.* explained this phenomenon by some side reactions which occur during the ATRP in protic media, such as reversible dissociation and substitution (by a solvent or possibly by a polar monomer molecule) of the halide ligand from the deactivating Cu(II) complex. These reactions lead to an inefficient deactivation and therefore to faster polymerizations with unsatisfactory control¹⁹⁸.

In our case the first-order time-conversion plot (Fig. 6.1) is linear at least up to 90 % and an apparent first-order dependence on monomer concentration can be found during the major part of the polymerization. This tendency indicates the absence of side reactions. Fig. 6.1B depicts the molecular weight and the polydispersity index evolution with the conversion. It is obvious that the molecular

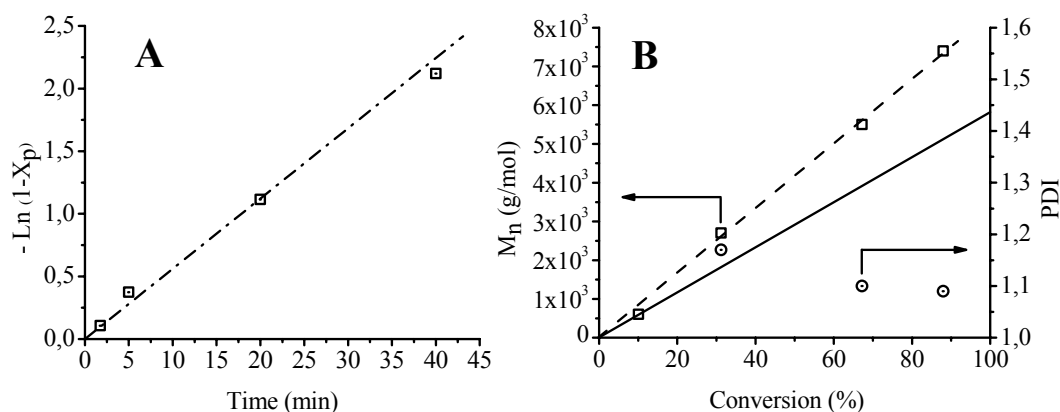


Figure 6.1: Kinetics of ATRP of NIPAAm (0.5 M) in water at 4 °C with $[M]_0/[BIBA]_0/[CuBr]_0/[CuBr_2]_0/[Me_6TREN]_0 = 50/1/0.5/0.5/1$. (A) First-order time-conversion plot. (B) Molecular weight and polydispersity index vs conversion. (—) theoretical number average molecular weight.

weight increases linearly with the conversion demonstrating the controlled fashion of the process. The difference between the theoretical and the experimental molecular weight can be assigned to the calibration of the SEC on the basis of polystyrene standards. The resulting polydispersity indices are low ($PDI < 1.2$) and decrease with the conversion. Even at high conversions (close to 90 %), the PDI is low (< 1.1). Moreover the SEC traces (not shown here) are always unimodal and symmetrical and do not show any trace of termination by recombination of growing radicals.

To prove the versatility of this process, different molecular weights of PNIPAAm were synthesized. Fig. 6.2 indicates that an increase of the ratio of monomer/initiator leads (at a comparable conversion) to a linear increase of the molecular weight. The SEC traces display unimodal and narrow peaks. Moreover a large range of molecular weights from rather low ($DP=30$) to rather high ($DP=400$) were achieved. In all cases the PDI remains below 1.2 at full conversion, without any trace of termination. All these criteria indicate the controlled fashion of the ATRP of NIPAAm in water.

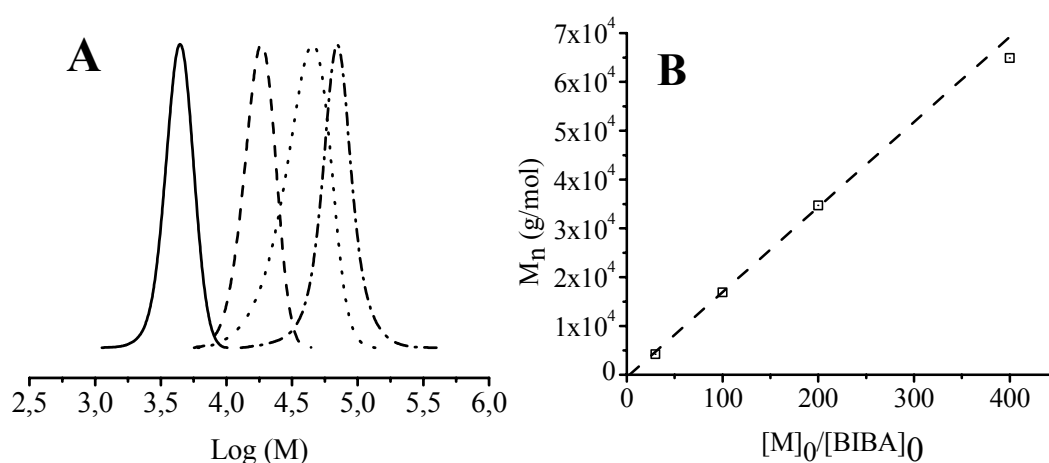


Figure 6.2: Influence of the ratio $[\text{monomer}]/[\text{initiator}]$ of the ATRP of NIPAAm (0.5 M) in water at 4 °C with $[BIBA]_0/[CuBr]_0/[CuBr_2]_0/[Me_6TREN]_0 = 1/0.7/0.3/1$. (A) MWD at a ratio $[M]_0/[BIBA]_0 =$ (—) 30, (---) 100, (···) 200, (- · -) 400. (B) Dependence of M_n on the ratio $[M]_0/[BIBA]_0$.

6.3.2 Chain Extension Experiments

To further demonstrate the livingness of the process a chain extension of PNIPAAm was carried out. The initial block was obtained by using a ratio $[M]_0/[BIBA]_0/[CuCl]_0/[CuCl_2]_0/[Me_6TREN]_0$ of 120/1/1.6/0.4/2 with a NIPAAm concentration of 0.5 M. Then the block copolymer was synthesized by sequential addition after 38 minutes of a degassed aqueous solution of monomer (0.5 M) without purification of the macro-initiator. A CuCl-based catalyst was chosen to perform the reaction to avoid any termination. Indeed, in water, bromide-terminated polymers can be sensitive to halogen abstraction by nucleophilic substitution. Then with CuCl the resulting polymer-halide bound C-Cl is much stronger and there is lower possibility of halogen abstraction. It was also sometimes observed a tiny amount of termination by recombination of growing radical in the ATRP of NIPAAm with CuBr at full conversion. This termination was not really detected on any SEC traces in the case of CuCl catalyst. For the chain extension, a strategy of direct addition of a second monomer solution was used, the first block has to be polymerized up to full conversion. In order to reduce these, two different types of termination CuCl combined with Me_6TREN were selected as catalyst system.

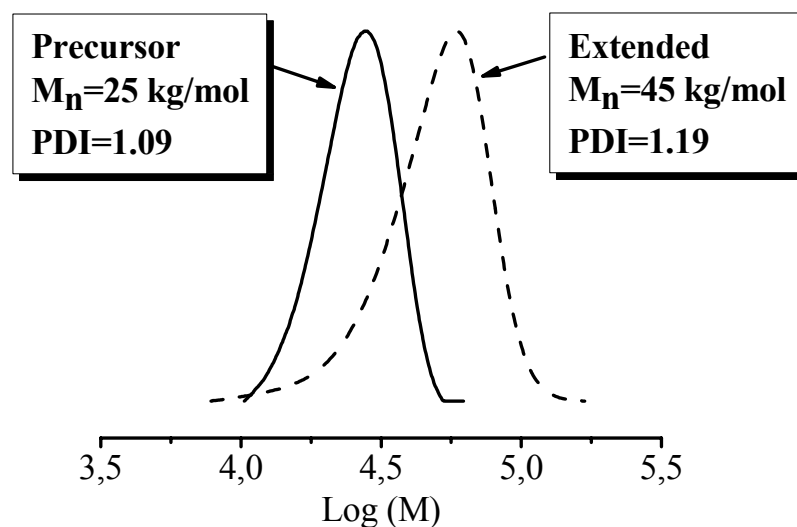


Figure 6.3: Molecular weight distribution for the chain extension of PNIPAAm by ATRP in water at 4 °C. $[M]_0=0.5$ M, $[M]_0/[PNIPAAm_{120}-Cl]_0 = 300$. (–) precursor, (– –) extension after 40 % conversion.

Fig. 6.3 depicts the MWDs during the process. As for the homopolymerization, even for a full conversion of the first block, throughout chain extension, there is no appearance of a shoulder due to the termination by recombination. However, a small tailing can be observed which might be due to a loss of terminal chloride of the precursor. Nevertheless, such evidence combined with a low PDI suggests that the large majority of the PNIPAAm precursor retained the functionality and was available for subsequent chain extension.

6.4 Conclusion

For the first time it had been demonstrated that ATRP of NIPAAm can be carried out in water at low temperature by using a low molecular weight water-soluble initiator. It was also shown that by choosing an appropriate ligand and catalyst system a well-controlled polymerization can be achieved. Under these conditions, the controlled/living characteristics were proven when BIBA, CuBr/CuBr₂ and Me₆TREN were used for a large range of monomer/initiator ratios. Moreover, even at full conversion the polymerization control is maintained. The living character of the generated PNIPAAm was confirmed by subsequent chain extension di-

rectly by addition of a second portion of the degassed monomer solution. During chain extension, no side reactions were observed and the polydispersity remained low throughout the polymerization. Finally, due to the terminal carboxylic end group present on the polymer, post polymerization treatments like protein conjugation are possible. Given the environmental benefits associated with aqueous polymerizations at low temperature and the possibility to tailor a large variety of block lengths, it is believed that the method reported in the present study represents a significant advance in the ability to prepare complex architectures based on this smart polymer.

7 Synthesis & ATRP of the Photocrosslinker DMIAAm

7.1 Introduction

As the aim of this thesis is to build a polymer membrane, it is necessary to crosslink the polymer chains to create a matrix embedding the proteins. The chosen way is the copolymerization of NIPAAm and 2-(dimethyl maleimido)-*N*-ethylacrylamide (DMIAAm). Poly(DMIAAm) is a polymer-analogous to PNIPAAm but has a dimethyl maleimido function able to photocrosslink the polymer chains under UV light. The monomer was synthesized following the protocol of Vo *et al.* and the random ATRP of NIPAAm and DMIAAm was realized without ferritin in this chapter. This approach allows the formation of the film before crosslinking and a better control of the conjugates layer, and hence also of the future membrane. Kuckling *et al.* were able to synthesize photocrosslinked thermoresponsive PNIPAAm nanogels following this approach^{199,200}.

7.2 Experimental

7.2.1 Materials

Synthesis of DMIAAm. Diaminoethane (Fluka), dimethyl maleic anhydride (98%, Aldrich), acryloyl chloride (97%, Aldrich), di-*tert*-butyl dicarbonate (98%, Aldrich), trifluoroacetic acid (98%, Aldrich), and diethylamine (99,5%, Aldrich) were used as received.

ATRP. NIPAAm (99%, Acros) was purified by two successive recrystallizations in

a mixture of n-hexane and benzene (4:1 (v:v)). 2-bromo-isobutyric acid (BIBA) (98%, Aldrich) and CuBr₂ (98%, Aldrich) were used as purchased. CuBr (98%, Aldrich) was purified by stirring it with acetic acid overnight. After filtration, they were washed with ethanol and ether and then dried in vacuo. Tris(2-dimethylaminoethyl)amine (Me₆TREN) was prepared as described in the literature¹⁸⁷. Water was obtained from a Sartorius apparatus.

Crosslinking procedure. The polymer solution contained 20 wt% polymer and 2 wt% thioxanthone with respect to the polymer dissolved in butanone or chloroform. The polymer films were prepared by spin coating onto silicon wafers after plasma treatment. Films were placed under UV-light. The UV irradiation was carried out with a 400 W UV lamp (Panacol 400F), at a wavelength of $\lambda > 315$ nm at 20 cm from the sample.

7.2.2 Characterization

Polymers were characterized by size exclusion chromatography (SEC) using a solution at 0.05 M of LiBr in dimethylacetamide (DMAc) as eluent. PSS GRAM columns (300 mm·8 mm, 7 μ m): 10³, 10² Å (PSS, Mainz, Germany) were thermostated at 70 °C. A 0.4 wt% (20 μ L) polymer solution was injected at an elution rate of about 0.72 mL·min⁻¹. RI and UV (λ =270 nm) were used for detection. Polystyrene standards were used to calibrate the columns, and methyl benzoate was used as an internal standard.

¹H-NMR spectra were recorded on a Bruker AC-25 spectrometer in CDCl₃ (reference peak δ = 7.26 ppm) at room temperature and for conversion purpose DMSO was used as the internal standard (δ = 2.7 ppm).

DSC measurements were carried out with a DSC Q1000 instrument to determine the glass transition temperature (T_g) of the polymers (T_g at $\Delta Cp/2$) and the T_c of the polymer solutions. The T_g values were measured with a heating rate of 10 K·min⁻¹ from 20 to 200 °C and back, and the DSC thermograms of the polymer solutions were recorded at a heating rate of 5 °C·min⁻¹ from 20 to 200 °C and back. The polymer concentration was 50 mg·mL⁻¹ in deionized water, and the onset value of the transition was taken as T_c .

Turbidity was measured on 1 mg·mL⁻¹ polymer samples by UV-Vis spectroscopy at 600 nm.

MALDI-ToF mass spectra were recorded on a Bruker Reflex III spectrometer in a linear mode using a 337 nm nitrogen laser and an accelerating potential of 20 kV in positive ion mode. For P(NIPAAm-DMIAAm) characterization, 2,5-dihydroxyben-

zoic acid ($20 \text{ mg}\cdot\text{mL}^{-1}$) was used as a matrix, and polymer samples were dissolved in THF ($10 \text{ mg}\cdot\text{mL}^{-1}$). A droplet of $5 \mu\text{L}$ of this mixture was spotted on the steel target plate, and it was dried in vacuo.

FT-IR measurements were recorded on a Bruker Tensor 27 instrument with a range of 1000 to 700 cm^{-1} . Polymer solutions with 0, 3, 5, 7 mol% crosslinker were prepared in 1 mL of chloroform, with 2 wt% thioxanthone and 20 wt% polymer, respectively. Each solution was crosslinked under UV-light over the time and characterized by FT-IR.

A spectroscopic ellipsometer SE850 (SENTECH) with a UV-Vis lamp was used to measure the thickness of the different polymer films with different mol% of crosslinker to estimate the influence of the crosslinking (at an angle of 70°). The refractive index of the dry PNIPAAm films is assumed to be 1.46 for films less than 50 nm thick. For thicker films, ellipsometer measurements yield a refractive index of 1.47 to 1.49²⁰¹. Measurements were fitted using a model: silicon Jellison (100) wafer with a 2 nm layer of SiO_2 and the polymer layer is to estimate. Polymer solutions with 0, 3, 5, 7 mol% crosslinker were prepared in 1 mL butanone, with respectively 2 wt% thioxanthone and 20 wt% polymer. Large silicon wafers were cleaned with the help of CO_2 snowjet. $100 \mu\text{L}$ of polymer solution was spin coated on them. Silicon wafers were placed into a chamber under the ellipsometer in order to control the humidity and the temperature.

7.2.3 Synthesis and Characterization of DMIAAm

The DMIAAm monomer was prepared according to the literature, Vo *et al.*²⁰², following the Fig. 7.1.

7.23 g of the final product was obtained.

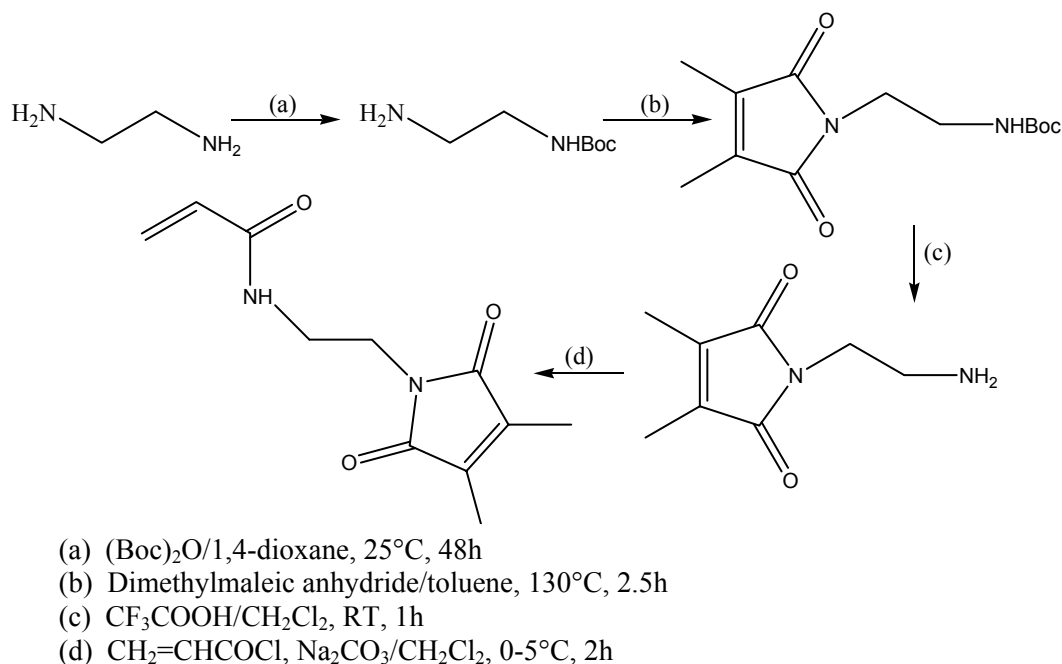


Figure 7.1: 2-(dimethyl maleimido)-N-ethylacrylamide (DMIAAm) synthesis scheme²⁰².

The characterization was achieved by ¹H-NMR and ¹³C-NMR in CDCl₃.

¹H NMR: δ (ppm) 1.85 (6H, 2CH₃), 3.39 (2H, CH₂NH), 3.58 (2H, CH₂N), 5.51 (1H, CH=), 6.00 (1H, CH=), 6.10 (2H, CH₂=), 6.64 (1H, NH).

¹³C NMR: δ (ppm) 8.46 (2CH₃), 37.12 (CH₂NH), 38.89 (CH₂N), 126.00 (CH₂=), 130.63 (CH=), 137.16 (2C=C), 165.80 (C=O, acrylic), 172.11 (2C=O, maleic).

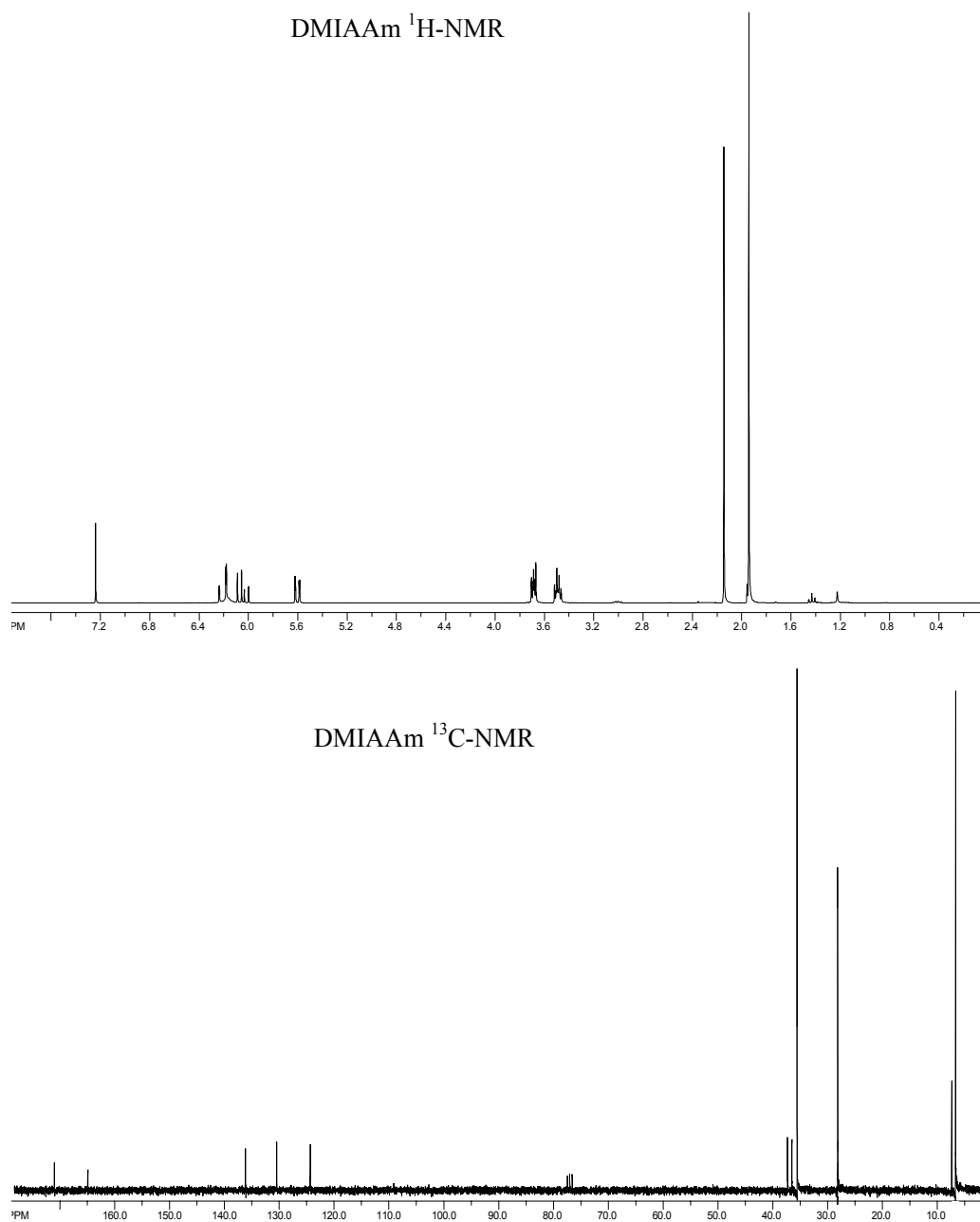


Figure 7.2: NMR measurements of 2-(dimethyl maleimido)-N-ethylacrylamide (DMIAAm). Top: ^1H NMR measurement. Bottom: ^{13}C NMR measurement.

7.2.4 Copolymerization of NIPAAm-DMIAAm

As described in Chapter 6, NIPAAm, DMIAAm and BIBA were dissolved in 19 mL of pure water, then 118.5 μL of DMSO was added as an internal standard. Finally, the CuBr/CuBr₂ system was added. Monomer concentrations and monomer/BIBA/Cu(I)/Cu(II)/Ligand ratios are given in Table 7.1. The vial was capped with a rubber stopper to allow the addition of the ligand and placed in an ice bath. In a second small flask, 2 mL of an aqueous ligand solution was prepared. Then both were deoxygenated by purging with nitrogen gas for 15 minutes. Afterwards 1 mL of ligand solution was withdrawn with a degassed syringe and placed in the polymerization flask to start the reaction. Polymerizations were run over night and quenched by air. P(NIPAAm-DMIAAm) samples were purified by dialysis against millipore water and then freeze-dried to remove the solvent. The whole process is performed in the dark to avoid crosslinking of the DMIAAm and to retain a good control of the polymerization-process.

7.3 Results and Discussion

7.3.1 Characterization of Polymer Properties

Polymerizations with different amounts of DMIAAm crosslinker were carried out with 0, 3, 5, 7 mol%, as shown in Table 7.1. The random ATRP process is shown in Fig. 7.3. The followed ATRP composition is resulting from Chapter 6 using as a catalyst system CuBr/CuBr₂ and Me₆TREN. It allows to foresee the crosslinker contribution to the polymer properties. The polymer chains distribution and hydrophilicity are affected by the content of DMIAAm.

By increasing the DMIAAm amount, both molecular weight, as well as the polydispersity index increased, probably due to the polystyrene calibration. MALDI-ToF MS measurements were performed to overcome the calibration disadvantage, but due to the polydispersity the only sample which flew was the one without crosslinker, having a molecular weight of 15.6 kg·mol⁻¹ and a polydispersity of $M_w/M_n=1.08$. But the maximum polydispersity is about $M_w/M_n=1.3$, so the distribution is acceptable for the application as can be seen from Table 7.1). Polymerizations reach a full conversion (>95%) except for 7 mol% DMIAAm content

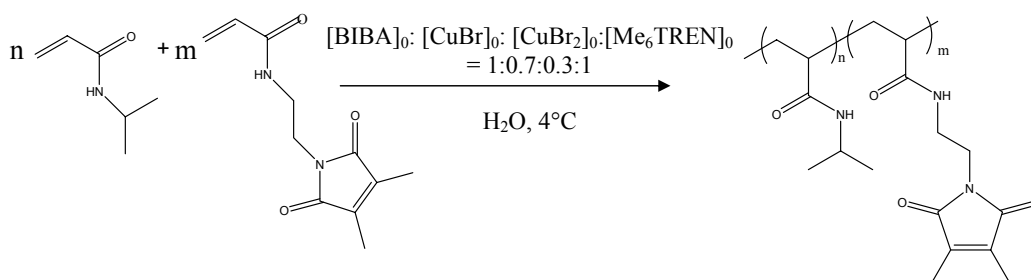


Figure 7.3: Synthesis of the photo-crosslinkable polymers P(NIPAAm-DMIAAm).

polymer. DMIAAm is slowing down the polymerization process. This may result from the increased hydrophobicity of DMIAAm compared to NIPAAm.

Table 7.1: ATRP of NIPAAm-DMIAAm with different mol% of crosslinker with $[M]_0 = 0.5 \text{ mol} \cdot \text{L}^{-1}$ with $[M]_0/[BIBA]_0/[CuCl]_0/[L]_0 = 100/1/0.7/0.3/2$.

Polymer	DMIAAm <i>feed</i> (mol%)	NIPAAm: DMIAAm	DMIAAm ^[1] <i>exp</i> (mol%)	M_n ^[2] ($\text{kg} \cdot \text{mol}^{-1}$)	PDI ^[2]	Conversion ^[3] (%)
A	0	100:0	0	23	1.16	97.4
B	3	97:3	4	28	1.27	96.2
C	5	95:5	5.5	32	1.31	99
D	7	93:7	7	32.5	1.32	76

[1] was estimated by $^1\text{H-NMR}$ in D_2O .

[2] were measured by size-exclusion chromatography (SEC) using polystyrene standards in dimethylacetamide (DMAc) as eluent.

[3] was estimated by $^1\text{H-NMR}$ in D_2O .

The polymer properties in solution are also changed because DMIAAm is more hydrophobic than PNIPAAm. Aqueous polymer solutions were investigated by UV absorption and DSC. As seen in Table 7.2, the cloud point T_c of the polymer is decreasing with the amount of crosslinker from 40.6 to 34.3 °C. The cloud point of a thermosensitive polymer is dependent of both the molecular weight as well as the polydispersity. Moreover in this study, the slightly different amount of DMIAAm tends to decrease T_c . As T_c represents the temperature where the polymer chains turn hydrophobic in aqueous solution, a higher hydrophobicity of the thermosensitive polymer will result in a decrease in T_c . The glass transition temperature was investigated because of the importance that the polymer stays in a glassy state under UV irradiation, as the crosslinking needs a finite motion of the polymer side groups. T_g is constant at about 138 °C for all samples, independently from the

amount of crosslinker, and as the UV irradiation is performed around 80 °C, the crosslinking process can be completed.

Table 7.2: ATRP of NIPAAm-DMIAAm with different mol% of crosslinker.

Polymer	DMIAAm <i>feed</i> (mol%)	$T_c^{[1]}$ (°C)	$T_c^{[2]}$ (°C)	$T_g^{[3]}$ (°C)
A	0	40.6	35.5	138.4
B	3	36.6	33.5	138.7
C	5	35.0	31.4	137.0
D	7	34.3	30.1	137.2

[1] was estimated by UV spectrometer at $\lambda=610$ nm ($1\text{ g}\cdot\text{L}^{-1}$) in millipore water.

[2] were measured by DSC with solution at $50\text{ g}\cdot\text{L}^{-1}$ with a cycle temperature at $10\text{ K}\cdot\text{min}^{-1}$.

[3] were measured by DSC with a cycle temperature at $10\text{ K}\cdot\text{min}^{-1}$.

7.3.2 Photocrosslinking of Copolymers under UV Irradiation

Photocrosslinking is carried out using the DMI-chromophore, because it is known for forming very stable dimers. Crosslinking is completed by cycloaddition between the double bonds of the DMI moieties, which destroys the conjugation of the carbonyl moieties and leads to the formation of cyclobutane rings as shown in Fig. 7.4). Thioxanthone is used as an efficient sensitizer: it is excited by UV irradiation and excites the maleimide groups by energy transfer inducing the cycloaddition. It is soluble in organic solvents compatible with the copolymer P(NIPAAm-DMIAAm) such as butanone, chloroform but also DMF and DMSO which are miscible with water. The [2+2] cyclodimerizations are not influenced by oxygen so it can be performed under laboratory conditions.

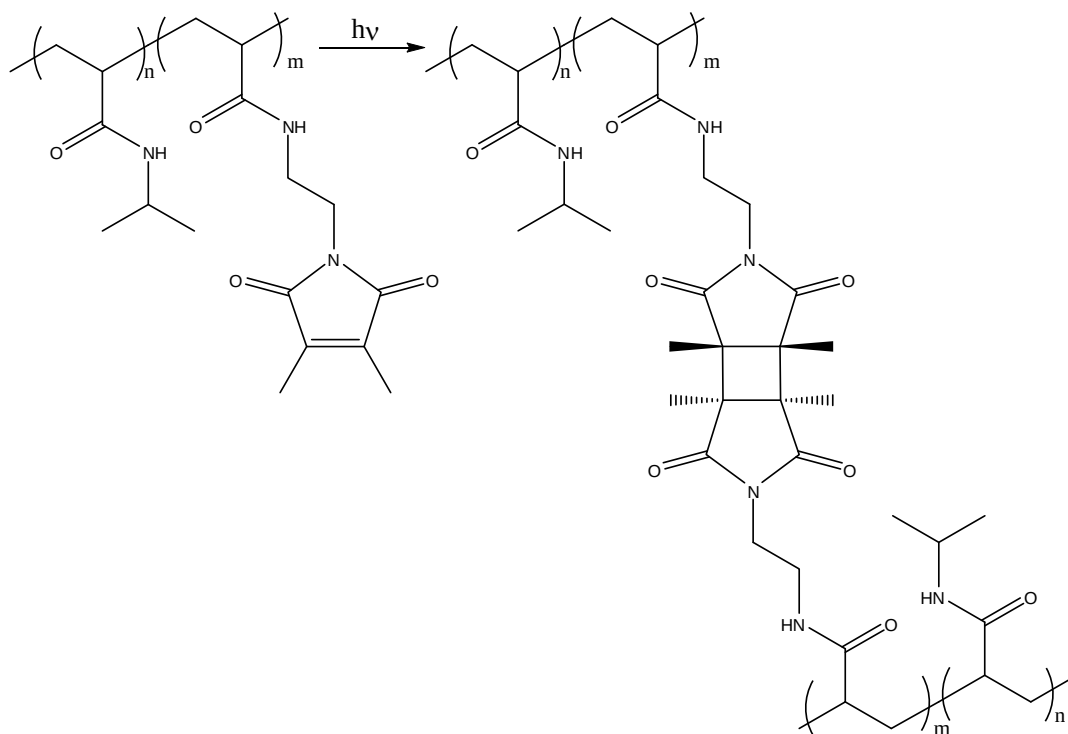


Figure 7.4: NIPAAm-DMIAAm copolymers crosslinking under UV irradiation.

7.3.2.1 Kinetics of the Crosslinking Process with FT-IR

Kinetics of the crosslinking under UV light were investigated by FT-IR to follow the change in structure of the crosslinker during derivatization.

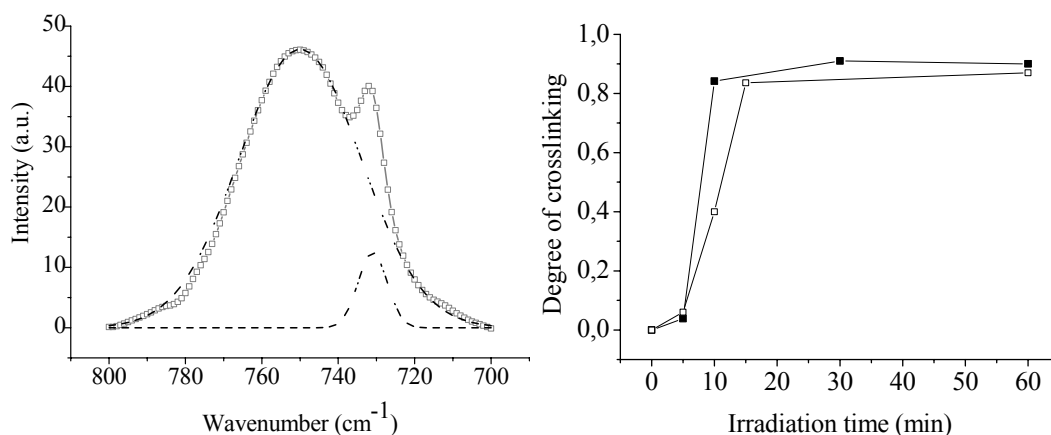


Figure 7.5: Left: Deconvolution of the absorption band of P(NIPAAm₉₃-DMIAAm₇) copolymer (higher energy band decreased with increasing time of irradiation, lower energy band remained constant). Right: kinetics of the photocrosslinking reaction for different copolymers: □ 3 mol% and ■ 7 mol% DMIAAm.

This method allows the calculation of the degree of crosslinking versus the time of irradiation. The absorbance peak at 750 cm^{-1} can be attributed to the conversion of the DMIAAm chromophore but it is overlapped by an absorbance peak at 732 cm^{-1} which belongs to the polymer and is constant over the time of irradiation²⁰⁰. This peak was fitted with two gaussian curves (cf. Fig. 7.5). The integral of the absorption band at 732 cm^{-1} remained constant during the decrease of the absorption band at 750 cm^{-1} allows the determination of the degree of crosslinking. With A the area of the absorption band at 750 cm^{-1} at different times, the degree of crosslinking χ can be calculated as followed:

$$\chi = 1 - A_t / A_0 \quad (7.1)$$

By plotting the degree of crosslinking over the irradiation time, the obtained kinetics show that for both copolymers containing 3 and 7 mol% of DMIAAm, the crosslinking is completed after 20 min.

7.3.2.2 Swelling and Shrinking of NIPAAm-DMIAAm Copolymer Films followed by In Situ-Ellipsometry

Ellipsometer measurements were performed in order to estimate the influence of the mol% of crosslinker on polymer film swelling and shrinking behavior as well as the crosslinking under UV light.

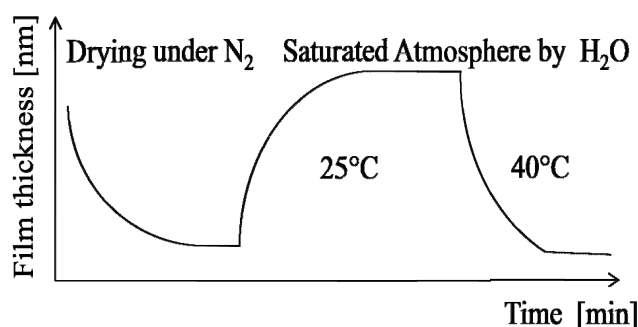


Figure 7.6: Scheme of swelling and shrinking experiment of (non) crosslinked NIPAAm-DMIAAm copolymer films followed by ellipsometry.

Spin-coated silicon wafers were placed into a chamber under the ellipsometer in order to control the humidity and the temperature. Cycles were followed as described Fig. 7.6: first drying of the film with a flow of N_2 , then saturation of the atmosphere with water at $25^\circ C$ until reaching the maximum swelling, and then increasing the temperature from $25^\circ C$ to $40^\circ C$ in order to estimate the shrinkage of the film. Finally, the temperature was set again to $25^\circ C$ to prove the reversibility of the thermoresponsiveness. After crosslinking of one hour under UV irradiation, the measurement was repeated.

Table 7.3: Shrinking ratio of NIPAAm-DMIAAm copolymer films under temperature before and after UV crosslinking by in-situ ellipsometry.

Polymer	DMIAAm ^{feed} (mol%)	% Shrinkage before UV radiation	% Shrinkage after UV radiation	Ratio
A	0	0.355	0.355	1
B	3	0.125	0.097	0.77
C	5	0.069	0.054	0.78
D	7	0.145	0.11	0.75

The maximum plateau of swelling and the minimum plateau after increase of the temperature are used to calculate the percentage of shrinkage. Measurements of polymer films with different mol% of crosslinker reveal, that the addition of crosslinker reduces both the swelling as well as the shrinkage of the polymer film in comparison to the film without any crosslinker. The results are summarized in Table 7.3. In detail, the crosslinking reduces the shrinking of each film by 20 %. This ratio seems to be independent of the amount of DMIAAm in the copolymer as used in this study (3 mol%-7 mol%).

8 Synthesis of Ferritin-P(NIPAAm-DMIAAm) Conjugates

This chapter focuses on the synthesis of ferritin-P(NIPAAm-DMIAAm) conjugates. The *grafting from* approach was investigated to attach polymer to ferritin particles, as its efficiency for other systems has been shown in the literature⁸⁵. *Grafting from* is divided into two steps: first the particles are modified into a macro-initiator, after which the polymerization is initiated and the polymer begins to grow from the particles. Horse spleen ferritin, once purified by SEC as monomeric particles, was modified as macro-initiator through its addressable ϵ -amino groups using first a zero length crosslinker and, as an alternative, an ATRP initiator which presents a high reactivity towards amino groups.

8.1 Modification of Horse Spleen Ferritin into a Macro-Initiator

Performing chemistry on proteins may affect their structure and properties. Therefore, it is necessary to adapt the pH, solvent, concentration and temperature to minimize these effects. For example, the approach of Lele *et al.* cannot be applied, as the initiator (2-bromoisobutyryl bromide) for conjugation is not water soluble and precipitates ferritin as soon as it is added to the protein solution⁶⁴. In this work, different approaches in water have been attempted to conjugate ferritin to an ATRP initiator while preserving its monomeric and unfolded state.

8.1.1 Conjugation of ATRP Initiator BIBA using a Zero Length Crosslinker Carbodiimide

Zero length crosslinkers are the smallest available reagent systems for bioconjugation. These compounds mediate the conjugation of two molecules by forming a bond containing no additional atoms. Carbodiimides¹⁴¹ are the most popular zero length crosslinkers to mediate the formation of amide linkages between a carboxylate and an amine or phosphoramidate linkages between a phosphate and an amine. EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride) is a water soluble carbodiimide and is often used for biochemical conjugation. It is used to form active ester functional groups with carboxylate groups using the water-soluble compound N-hydroxysulfosuccinimide (sulfo-NHS). Sulfo-NHS esters are hydrophilic active groups that react rapidly with amines on target molecules. Sulfo-NHS esters are water soluble, long lived and hydrolyze relatively slowly in water²⁰³. The advantage of adding sulfo-NHS to EDC is to increase the stability of the active intermediate, which ultimately reacts with the attacking amine. EDC/sulfo-NHS-coupled reactions are highly efficient and usually increase the yield of conjugation dramatically over that obtainable solely with EDC. A protein can be incubated in the presence of EDC/sulfo-NHS esters and amine containing molecules directly or off-line and the active ester form can be isolated and then mixed with a second protein or other amine-containing molecules for conjugation. Horse spleen ferritin is incubated with the ATRP initiator BIBA (Bromoisobutyric acid), EDC and sulfo-NHS to link BIBA to the addressable ϵ -amino groups through an amide bond formation (cf. Fig. 8.1).

8.1.1.1 Experimental Part and Results

Materials. Ferritin (Fluka) is purified by size exclusion chromatography to obtain monomeric particles. Bromoisobutyric acid (BIBA) (99%, Aldrich), EDC (99%, Fluka) and N-hydroxysulfosuccinimide (sulfo-NHS) (99%, Fluka) were used as purchased.

Batch reaction. After purification of horse spleen ferritin by SEC, the pH of the protein solution was adjusted to pH 8. The reaction can be accomplished in one step by mixing all reagents in the protein solution. Different molar ratios of EDC/sulfo-NHS/BIBA to monomeric ferritin have been tested from 10 to 1000. The

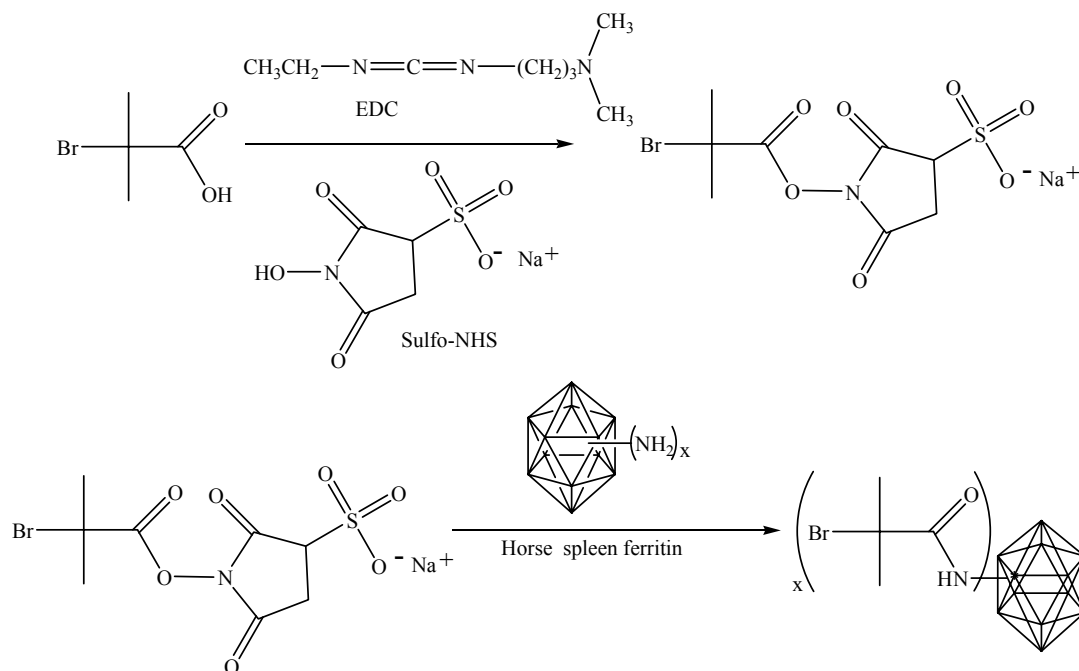


Figure 8.1: EDC/sulfo-NHS conjugation-Modification of horse spleen ferritin into a macro-initiator with BIBA.

result is an irreversible crosslinking of the ferritin particles, which does not allow the *grafting from* in a controlled manner. The presence of both carboxylate and amine groups on the protein shells to be conjugated with EDC may result in a self-polymerization, because the substance can then react with another protein instead of the ATRP initiator as illustrated Fig. 8.2.

Off-line reaction. Protein can be mixed with EDC/sulfo-NHS ester in a first step, isolated by dialysis or gel filtration and then mixed with the ATRP initiator. This two-step process allows the active species to form only on one protein, thus gaining greater control over conjugation. It still must be considered that the hydrolysis of the intermediate in buffer solution may not help the modification of ferritin. In the desired modification reaction, the ATRP initiator contains the carboxylic end group and it is then necessary to mix it first with EDC and sulfo-NHS, and then to add it to the protein. Different processes were tested but in order to avoid precipitation of EDC, it is necessary to first mix EDC, then BIBA and then sulfo-NHS with a large excess of BIBA compared to protein. Afterwards, the monomeric ferritin is added at pH 8.5 and the solution is shaken at 650 rpm at RT for 2 hours. Finally,

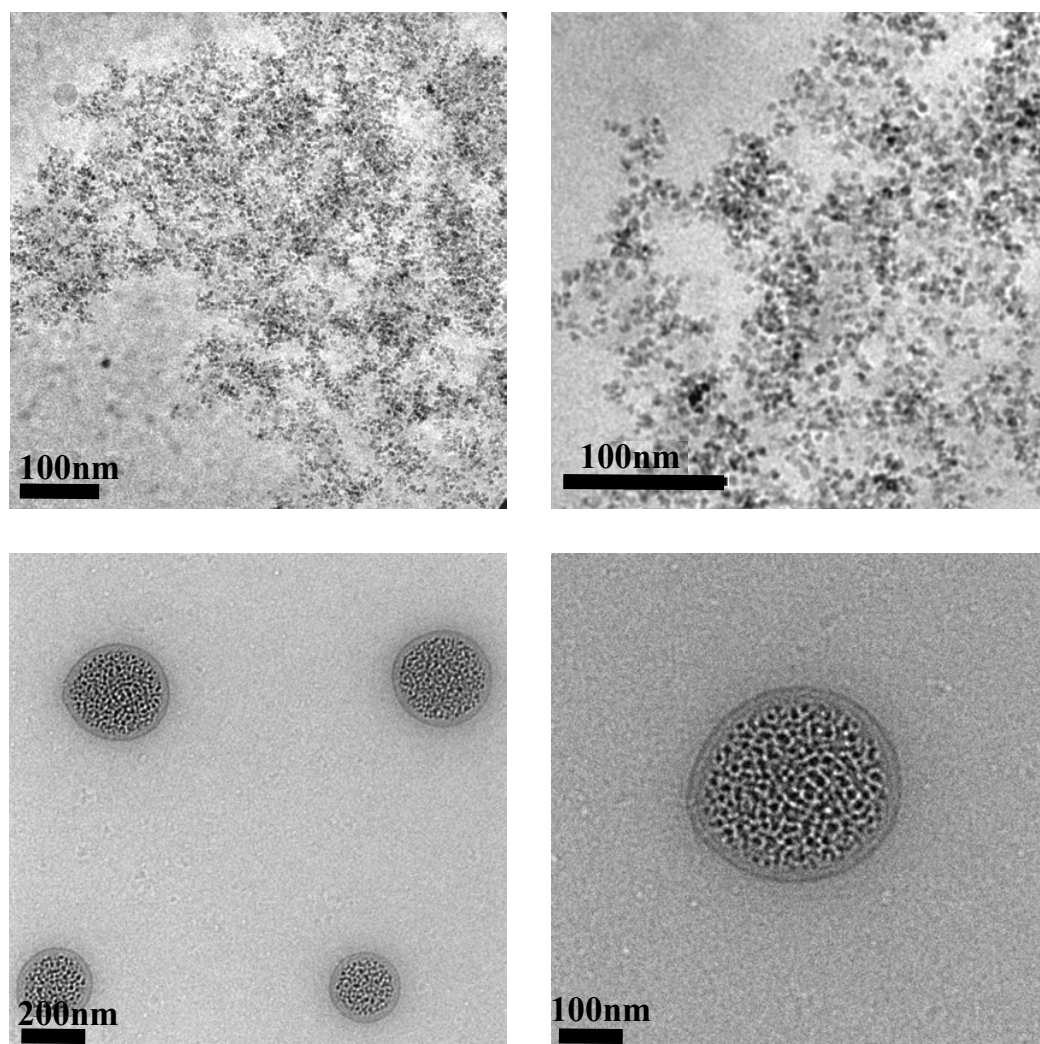


Figure 8.2: Crosslinked horse spleen ferritin with active ester chemistry after treatment with EDC/sulfo-NHS molecules.

the purification was performed by dialysis over night against sodium phosphate buffer at 0.1 M. It would have been necessary to purify the result of the off-line reaction without the protein by chromatographic separation, but the product would have been hydrolyzed. The result of this modification led to non crosslinked ferritin, whereas side reactions can not be excluded. The modification could not be quantified.

8.1.2 Protection of Carboxylate Groups of the Protein

Another approach is to block the addressable carboxylic groups of ferritin estimated at 168²¹ in order to avoid the crosslinking of ferritin particles due to the activation of carboxylate groups by EDC. One possibility to achieve this is to first protect the primary amine of the side chain ϵ -amino groups of lysine residues in a reversible manner, then to irreversibly block the carboxylate groups of the side chain of glutamic acid residues, and finally to deprotect the ϵ -amino groups in water. The conjugation is performed using the couple EDC/sulfo-NHS and BIBA. The drawbacks of this method are that it is carried out in four steps and the protein structure may suffer from all the chemistry and that the yield can be low.

8.1.2.1 Materials

Citraconic anhydride (99 %, Fluka), ethanolamine (99 %, Aldrich) and EDC (99 %, Fluka) were used as purchased. Trinitrobenzene sulfonate acid (TNBS) (Fluka, 99 %) was used following the protocol from Habeeb *et al.*¹⁴³.

8.1.2.2 Experimental Part

The first step is the reversible protection of NH₂ groups of ferritin. The pH of the monomeric ferritin solution is adjusted between 8 and 9 by addition of NaOH. It is necessary to use a sodium phosphate buffer or another non amine containing buffer (no Tris buffer). The citraconic anhydride is then added with a molar excess of 10 to 50. The solution is then shaken at 800 rpm for two hours. The solution was purified by dialysis over night. The following step is the irreversible blocking of the carboxylate groups of the protein using the carbodiimide conjugation. The pH was adjusted at 7.5, even if the optimum conditions are between pH 4.7 and 6, to avoid the hydrolysis of EDC. The ethanolamine was added at 0.1 mol·L⁻¹ followed by 10 mg of EDC per milliliter of the solution. The reaction was kept at RT for two to four hours. The purification and the next step were performed simultaneously. The solution was dialyzed at pH 3 to 4 in a sodium phosphate buffer. It allows abortion of the previous reaction by releasing the excess of EDC and the deprotection of the ϵ -amino groups, as illustrated Fig. 8.3. The modification of the

resulting protein is then carried out as previously described using EDC/sulfo-NHS and BIBA to obtain the macro-initiator.

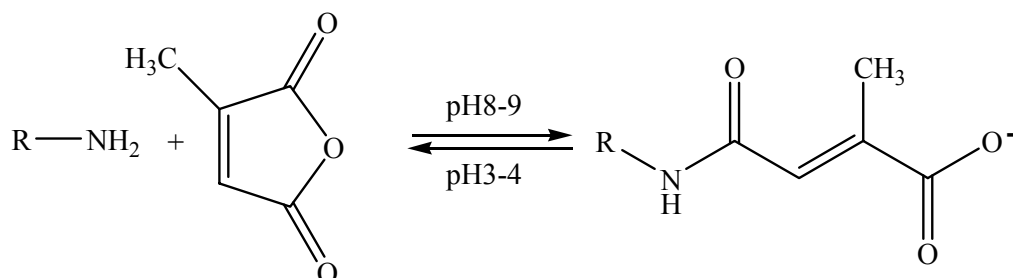


Figure 8.3: Protection and deprotection of ϵ -amino groups of lysine residues of ferritin particles with citraconic anhydride by switching of pH.

8.1.2.3 Results

In order to quantify the efficiency of the protection process, the TNBS assay was applied to specifically determine the number of lysine ϵ -amino groups in carrier proteins that were coupled to the citraconic anhydride. Ferritin protected with citraconic anhydride is compared to ferritin and ferritin assayed by TNBS. TNBS has a maximum peak at 340 nm ($\epsilon_{340}=6.5 \cdot 10^2 \text{ M}^{-1} \cdot \text{cm}^{-1}$). There is only 1/4 of the ϵ -amino groups which can be labeled with TNBS after the protection by citraconic anhydride. This shows that the protection of ϵ -amino groups is relatively efficient.

Table 8.1: Labeling of primary amino groups of horse spleen ferritin with picryl-sulfonate acid (TNBS) characterized by UV-Vis.

Protein	Degree of labeling of ϵ -NH ₂ (per particle) by TNBS
Ferritin	60-61
Ferritin protected by citraconic anhydride	16-17

After the protection of ϵ -amino groups, the blocking of carboxylate groups is achieved by the conjugation of EDC and ethanolamine. The aim is to obtain monomeric ferritin able to be modified into a monomeric macro-initiator. The protected

and blocked protein is measured by SEC (cf. Fig. 8.4). It can be seen that the protected and blocked protein is not affected by the aggregation and is monomeric after the use of EDC, which uses to crosslink the ferritin particles without protection of ϵ -amino groups and has a molecular weight of $4.745 (\pm 0.042) 10^5 \text{ g}\cdot\text{mol}^{-1}$. Ferritin is not being unfolded or denaturated by these chemical procedures. The protection is useful and the blocking is successful. The peak of protected and blocked protein is slightly shifted to lower elution times, meaning that it is eluted before the monomeric ferritin, because its molecular weight is higher due to the conjugation of citraconic anhydride and ethanolamine and is about $4.673 (\pm 0.041) 10^5 \text{ g}\cdot\text{mol}^{-1}$.

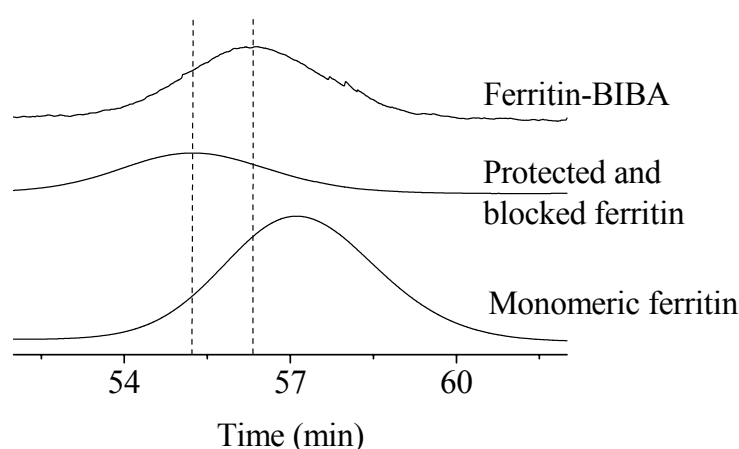


Figure 8.4: SEC measurement of monomeric protected and blocked ferritin (using LS detector).

The final chemical procedure is the deprotection of the ϵ -amino groups by tuning the pH of the solution from alkaline to acidic by dialysis. It is supposed to purify the sample by eliminating the excess of non-reacted EDC and to release the citraconic anhydride by hydrolysis. In order to modify the protein into a macro-initiator, the resulting protein was mixed with BIBA in large excess and with EDC/sulfo-NHS. The macro-initiator was analyzed by SEC (cf. Fig. 8.4). The macro-initiator ferritin-BIBA is still monomeric and has a higher molecular weight than native monomeric ferritin, but a lower one than the protected and blocked ferritins, which proves an effective deprotection. Nevertheless it may be that a part of protein solution may suffer from the long exposition to pH 3. In addition, the four step process leads to a low yield of the macro-initiator and therefore is not sufficiently efficient for our application.

8.1.3 Synthesis and Conjugation of the ATRP Initiator: N-Hydroxysuccinimide-2-bromo-2-methylpropionate

Lecolley *et al.*²⁰⁴ and Zhang *et al.*²⁰⁵ have developed a new approach to synthesize an ATRP initiator reactive towards the addressable groups of proteins. They describe two different routes to synthesize a N-succinimidyl-ester functionalized ATRP initiator, the N-hydroxysuccinimide-2-bromo-2-methylpropionate abbreviated NHS-BIBA, which can react with the ϵ -amino groups of lysine residues, modifying proteins or dendrimer initiators to achieve a well-defined macro-initiator. Zeng *et al.*²³ has already applied this to ferritin and managed to initiate a polymerization.

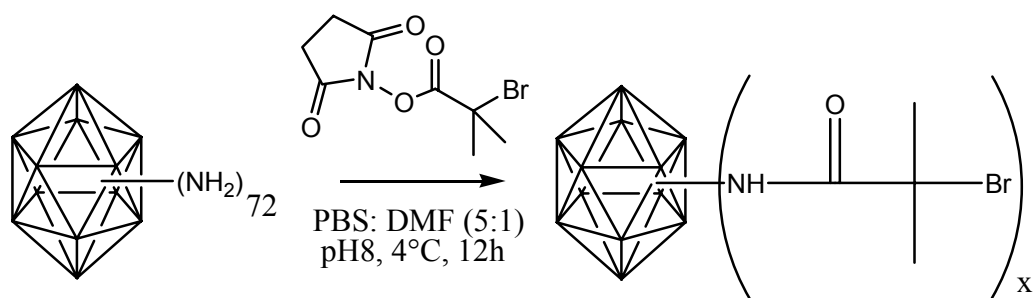


Figure 8.5: Modification of ferritin with N-hydroxysuccinimide-2-bromo-2-methylpropionate into a macro-initiator.

8.1.3.1 Materials

Dichloromethane was distilled over CaH_2 and stored under nitrogen. 2-bromoisobutyric acid (BIBA) (99 %, Aldrich), N-hydroxysuccinimide (HOSu) (Aldrich, 98 %), 1,3-dicyclohexyl carbodiimide (DCC) (Aldrich, 99 %) were used as purchased.

8.1.3.2 Experimental Part

Synthesis of NHS-BIBA. To a solution of 2-bromoisobutyric acid (15 g, 90 mmol) in dry DCM (40 mL) was added hydroxysuccinimide (HOSu) (12.4 g, 107.7 mmol)

at RT. Aside, DCC was melted at 34 °C to be handled. After 15 min, 1,3-dicyclohexal carbodiimide (DCC)(23.2 g, 112.5 mmol) was added at -30 °C. The temperature was controlled by adding carbonic ice to an isopropanol bath. The resulting mixture was warmed to RT and kept stirred for another 15 hours. After the solid was filtered off, chromatographic separation yielded the target substance (20.2 g, 84 %) as a white powder. After that the solvent was removed under pressure (silica gel, hexane/ethyl acetate 3:1 (v:v)).

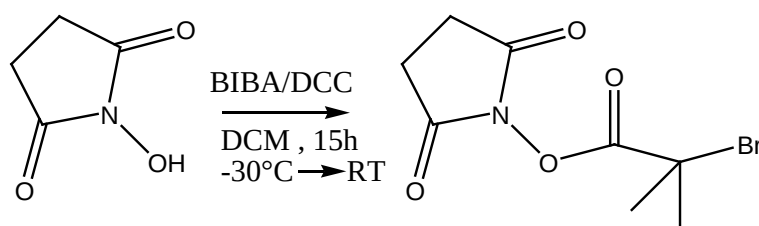


Figure 8.6: Synthesis of N-hydroxysuccinimide-2-bromo-2-methylpropionate.

The purity of the resulting product was verified by NMR measurements.

¹H NMR, in CDCl₃, δ (ppm) 2.04 (6H, CH₃), 2.82 (4H, CH₂) (cf. Fig. 8.7)

¹³C NMR, in CDCl₃, δ (ppm) 26.03 (2C, cycle), 31.09 (2C, C(CH₃)₂Br), 51.60 (1C, C(CH₃)₂Br), 167.89 (1C, C=O), 169.02 (2C, C(cycle)=O).

Ferritin modification. NHS-BIBA is not water soluble whereas BIBA is. It can however be dissolved in DMF, which is miscible with water. NHS-BIBA was incubated in a large molar excess with monomeric ferritin in a buffer at pH 8 (the purification of monomeric ferritin is achieved by SEC using a sodium phosphate buffer at pH 8) with a ratio buffer:DMF of 5:1 (v:v) and incubated for 24 hours at 4 °C or for 12 hours at RT. The protein solution is then purified by dialysis against buffer. N-hydroxysuccinimide is not water soluble but can also be hydrolyzed. NHS-esters have a half-life in the order of hours under physiological pH conditions. However, hydrolysis and amine reactivity both increase with increasing pH. At pH 9, NHS-esters have a half-life of only a few minutes, so the incubation is performed at pH 8 and 4 °C. No amine containing buffers should be used to avoid competitive reactions¹⁴¹.

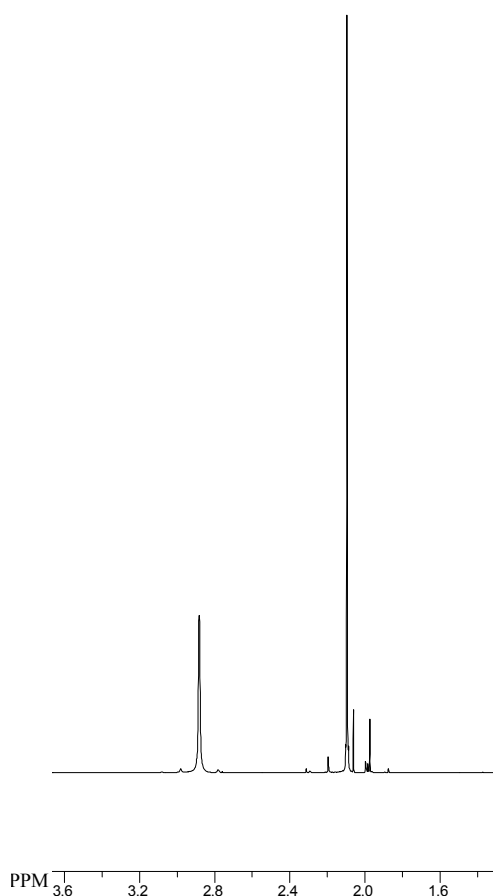


Figure 8.7: ^1H NMR measurement of NHS-BIBA final product.

8.1.3.3 Results

The molecular weight determined by SEC is $4.995 (\pm 0.331) 10^5 \text{ g}\cdot\text{mol}^{-1}$. By comparing it to the result of monomeric ferritin of $4.488 (\pm 0.041) 10^5 \text{ g}\cdot\text{mol}^{-1}$, it is possible to determine the number of potential initiating sites on each ferritin particle. The increase of $50.7 \text{ kg}\cdot\text{mol}^{-1}$ corresponds to 192 initiating sites and 8 sites per subunit. It is known that there are only three addressable lysine residues per subunit in the ferritin structure, so this result is not possible. However, after subtracting the margin of error of the instrument, the molecular weight can be determined to $4.664 10^5 \text{ g}\cdot\text{mol}^{-1}$, which corresponds to 66 initiating sites per ferritin particle and 2.7 sites per subunit. This result is more relevant and shows that the modification is highly efficient, and ϵ -amino groups are conjugated to the ATRP

initiator with a high yield. This one step reaction does not damage the protein structure and leads to a stable solution of monodisperse macro-initiator.

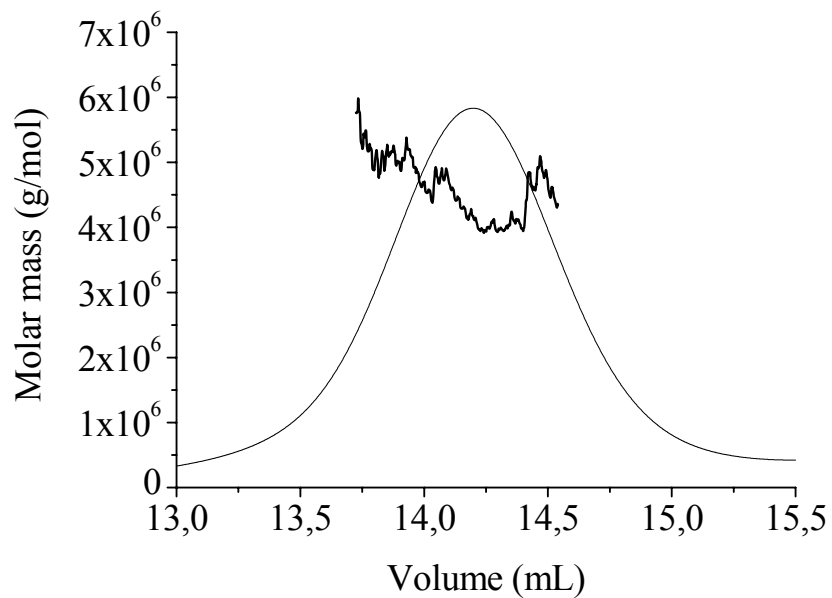


Figure 8.8: SEC measurement of ferritin-BIBA macro-initiator (LS detector) in 0.1 M sodium phosphate buffer at a flow of $0.25 \text{ mL} \cdot \text{min}^{-1}$.

8.2 Grafting from Horse Spleen Ferritin: ATRP of NIPAAm and OEGMA

After the successful modification of ferritin into a macro-initiator the *grafting from* can be achieved by ATRP of NIPAAm in water at a low temperature, following the protocol shown in Fig. 8.9.

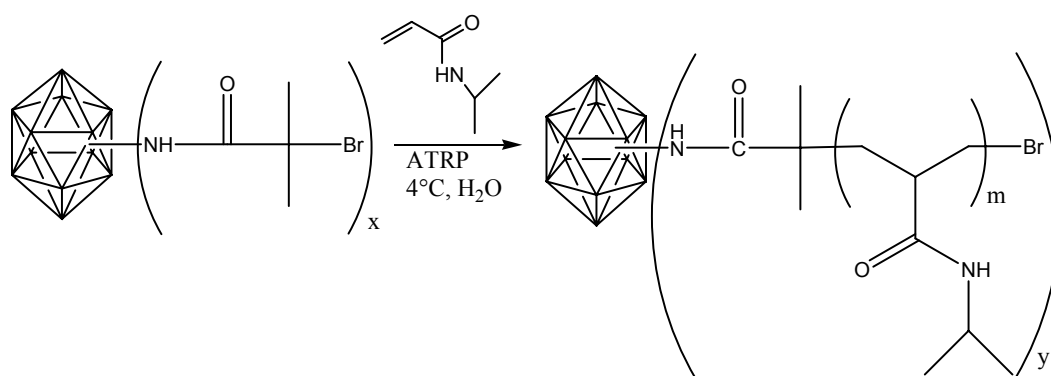


Figure 8.9: Reaction scheme for *grafting* PNIPAAm *from* ferritin via ATRP.

8.2.1 Materials

NIPAAm (99%, Acros) was purified by two recrystallization procedures in a mixture of *n*-hexane and benzene (4:1 (v:v)). OEGMA was purified by passing through a silicon column to remove the inhibitor. CuBr (98 %, Aldrich) was purified by stirring with acetic acid overnight. After filtration, it was washed with ethanol and ether and then dried in vacuo. Tris(2-dimethylaminoethyl)amine (Me₆TREN) was prepared as described in the literature¹⁸⁷. Millipore water was obtained from a Sartorius apparatus.

8.2.2 Experimental Part

ATRP of NIPAAm was conducted with a monomer/initiator/Cu(I)/Cu(II)/ligand ratios of 100/1/0.7/0.3/2. The number of moles of the total initiator is fixed at

$4 \cdot 10^{-6}$ mol, corresponding to the total amount of initiator conjugated to ferritin and the sacrificial initiator (BIBA). The dialyzed macro-initiator ferritin is shaken with NIPAAm and BIBA until complete dissolution. The sacrificial initiator was dissolved separately from a concentrated batch in water, and added to increase the concentration of initiator into the solution. This allows a faster and better controlled polymerization. In a second flask, CuBr, CuBr₂ and Me₆TREN were mixed, and dissolved in Millipore water. Both solutions were then sealed and degassed for 15 min in an ice bath. 0.5 mL of the copper/ligand solution was added to the ferritin solution under agitation. The polymerization was performed over 24 hours, as it is carried out in a highly diluted solution. The conjugates are extensively dialyzed against water to remove the catalyst (cut-off 1 kDa). Then the conjugates are dialyzed with a higher cut-off membrane (25 kDa-50 kDa) to remove the polymer formed by the sacrificial initiator. They were then freeze-dried to remove the solvent for further characterization. The solution of conjugates was kept at 4 °C and a part of it was freeze-dried for further analysis. Conjugates were stable in water, as no precipitation could be observed even after months. ATRP was also performed with OEGMA and/or by incorporating the photocrosslinker monomer DMIAAm at about 7 mol% to the solution batch.

Polymer characterization. PNIPAAm was characterized by gas phase chromatography (GPC) using a 0.05 M solution of LiBr in dimethylacetamide (DMAc) as eluent. PSS GRAM columns (300 mm·8 mm, 7 μm): 10³, 10² Å (PSS, Mainz, Germany) were thermostated at 70 °C. A 0.4 wt% (20 μL) polymer solution was injected at an elution rate of 0.72 mL·min⁻¹. RI and UV (λ=270 nm) were used for detection. Polystyrene standards were used to calibrate the columns, and methyl benzoate was used as an internal standard. PEGMA was characterized by size exclusion chromatography using as eluent a sodium phosphate buffer at pH 7.4. LS, RI and UV ((λ=280 nm) are used for detection without needing standards for calibration. Polymer solutions were injected at an elution rate of 0.25 mL·min⁻¹. The conversion of each sample was determined directly after quenching into liquid nitrogen. ¹H-NMR spectra were recorded on a Bruker AC-25 spectrometer in D₂O (reference peak δ = 7.26 ppm) at RT. For conversion purposes, DMSO was used as an internal standard (δ = 2.7 ppm). MALDI-ToF mass spectra were recorded on a Bruker Reflex III spectrometer in linear mode using a 337 nm nitrogen laser and an accelerating potential of 20 kV in positive ion and reflectron modes. For polymer chains, 2,5-dihydroxybenzoic acid (20 mg·mL⁻¹) was used as a matrix, and polymer samples were dissolved in THF (10 mg·mL⁻¹). A droplet of 5 μL of this mixture was spotted on the steel target plate, and it was dried in vacuo.

Conjugates characterization. The conjugates samples were characterized by turbidity measurement using a photospectrometer to determine their cloud point at one wavelength ($\lambda=600$ nm). Dynamic light scattering measurements were performed on filtrated and diluted solutions ($20\ \mu\text{L}$ nylon filter) to avoid turbidity, which could damage the detector, as the measurements were done as a function of temperature. TEM samples were prepared by glow discharge on formvar copper grids and spotting $2\ \mu\text{L}$ of solution and drying the droplet with a filter paper. AFM samples were prepared by spin coating or drop casting the solution onto a freshly cleaved mica sheet or silicon wafer with plasma cleaning as a pretreatment. To analyze the conjugates by MALDI-ToF MS and SDS-PAGE, the ferritin-polymer particles had to be denaturated in order to fly for MALDI-ToF MS and be comparative for the SDS-PAGE measurement. $100\ \mu\text{L}$ of concentrated ferritin-polymer solution was added to a $300\ \mu\text{L}$ solution of urea at 7 M and $10\ \mu\text{L}$ of 2-mercaptoethanol. Afterwards 5 mg of trypsin were added to the solution. The solution was then incubated for 12 hours at 37°C . The denaturated conjugates were characterized by MALDI-ToF MS (as described previously) and SDS-PAGE using a prestained marker from 10 to 220 kDa. For characterization of Ferritin-P(NIPAAm-DMIAAm) conjugates, sinapic acid (saturated solution in acetonitrile: H_2O (1:1 v:v)) was used as a matrix, sodium trifluoroacetate salt (NaTFA) was used as a cationicating agent at $10\ \text{mg}\cdot\text{mL}^{-1}$, and conjugates samples were prepared following the Ziptip C18 protocol.

8.2.3 Results

Polymer chain characterization. Every polymerization was followed by ^1H -NMR measurements using DMSO in D_2O as the internal standard. All polymerizations were completed to at least 90% within 24 hours. Under the conditions of the NMR-experiment, the protein peaks cannot be observed, whereas the disappearance of NIPAAm can be easily followed. For this, the intensity of the peaks corresponding to the vinyl group at $\delta=5.72\text{-}5.8$ ppm (dd, $\text{CH}(\text{H})=$) was investigated. The molecular characterization of the polymer-ferritin conjugate imposes many challenges because of their high molecular weight. Thus, the ferritin cage was denaturated in order to investigate the subunit conjugates by GPC and MALDI-ToF MS. GPC of the denaturated PNIPAAm conjugates could not be measured in aqueous solution because PNIPAAm absorbs on the acrylamide column. Therefore, it has to be measured in organic solvents, which alters the protein's properties and offers only non reproducible results. The characterization of ferritin-PEGMA conjugates was

possible using a water-SEC without denaturation, and enable the comparison of the result with monomeric ferritin. Fig. 8.10 shows the molar mass and the geometrical radius of the resulting conjugates. The main problem is the choice of the model to fit the data as the protein-polymer conjugates have different conformation and the Zimm model does not yield an appropriate fit result. Choosing the coated sphere model, the molar mass and the radius can be calculated. However, GPC and SEC characterization of the conjugates depend on the polymer concentration, and therefore the refractive index increment of the protein-polymer mixture (dn/dc) and the extinction coefficient ϵ .

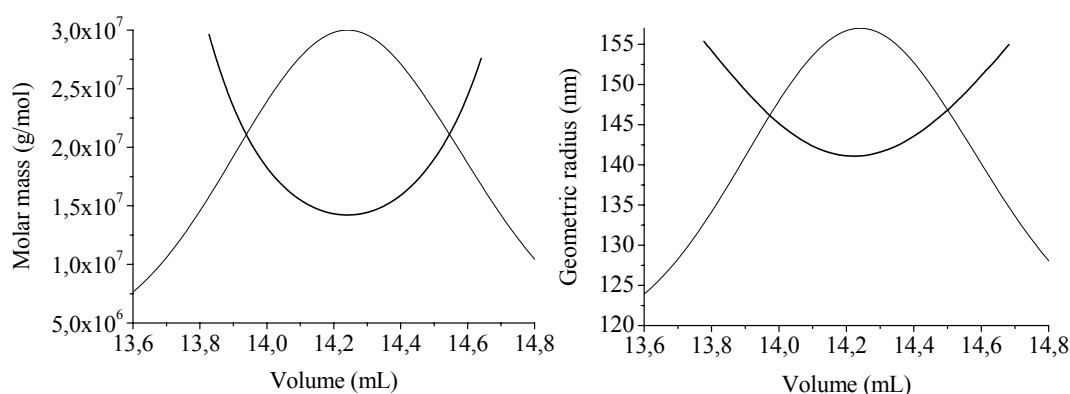


Figure 8.10: Left: Molar mass calculated from the coated sphere model of ferritin-PEGMA using UV detector to calculate the concentration and, LS detector at 90° : $M_w = (1.4)10^7 \text{ g}\cdot\text{mol}^{-1}$. Right: Geometrical radius calculated from the coated sphere model of ferritin-PEGMA using LS detector at 49° and 131° : $R_g = 141.1 \pm 0.1 \text{ nm}$.

It is quite difficult to evaluate the dn/dc and ϵ of the mixtures because it requires a high volume of conjugates, and so the determination of the exact molecular weight of the conjugates is not reachable. The result for ferritin-PEGMA conjugates calculated by the ASTRA software is not plausible as it is about $1.4 \cdot 10^7 \text{ g}\cdot\text{mol}^{-1}$ and does not correspond to the expected monomer/initiator ratio. The radius, which is about $141.1 \pm 0.1 \text{ nm}$, is also not realistic. Anyway, the conjugates are eluted before the monomeric ferritin meaning that they have a higher molecular weight, the polymerization is effective and the conjugates are homogeneous (cf. Fig. 8.11).

On the other hand, the free polymer chains from the sacrificial initiator which were added to achieve controlled polymerization reaction allows the determi-

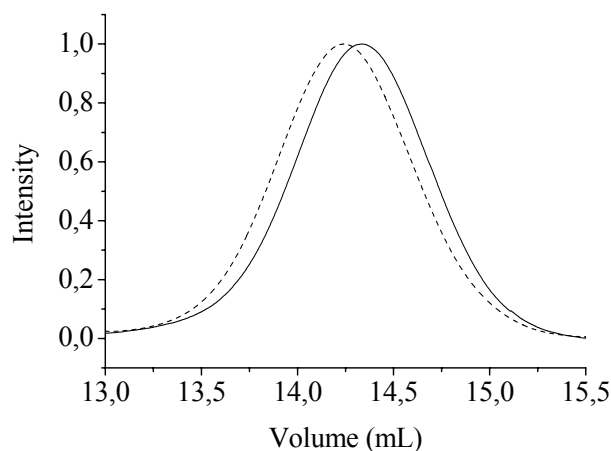


Figure 8.11: Comparison of the SEC elution of monomeric ferritin (···) with ferritin-PEGMA conjugates (—). Ferritin-PEGMA has a higher molecular weight than monomeric ferritin.

nation of polymer properties²⁰⁶. The characterization of free polymer chains is more accessible. Due to the small amount it was performed by MALDI-ToF MS, GPC requires too much materials.

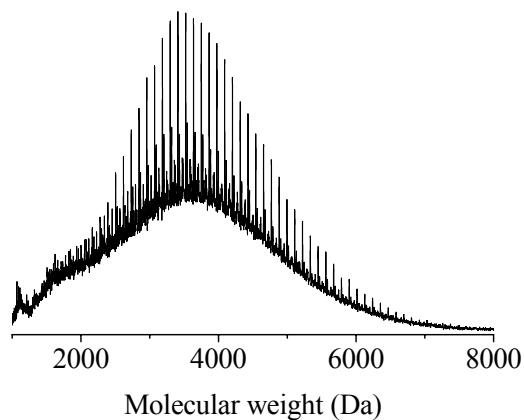


Figure 8.12: Free PNIPAAm chains from sacrificial initiator characterized by MALDI-ToF MS in reflectron mode. $M_n=5 \text{ kg}\cdot\text{mol}^{-1}$; PDI=1.06.

PNIPAAm with a low molecular weight was successfully analyzed by MALDI-ToF MS and had a number average molecular weight of 5 kDa with a low polydispersity of 1.06 (cf. Fig. 8.12). When PNIPAAm had a higher molecular weight it did not fly. It is assumed that both the polymer chains on the protein, as well as the free

polymer chains have the same properties.

Conjugates. The polymer-bionanoparticle conjugates were denatured with a 8 M solution of urea and 10 mM dithiothreitol and by heating the solution at 90 °C for three minutes. The polypeptide subunit of ferritin has a molecular weight of 19-21 kDa depending if it is part of the light or the heavy chain but the difference cannot be seen on the low resolving SDS-PAGE gel.

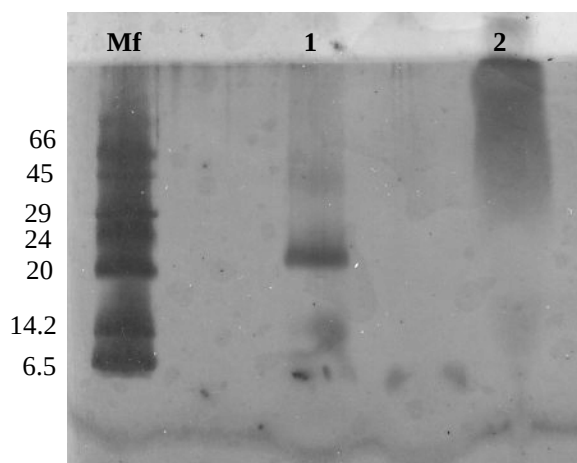


Figure 8.13: SDS-PAGE measurement of the macro-initiator Ferritin-BIBA (1) and the conjugate ferritin-P(NIPAAm₉₅-DMIAAm₅) (2) after denaturation, stained with silver solution, Mf being the reference marker.

SDS-PAGE measurement in Fig. 8.13 shows that after polymerization, no single subunit of monomeric ferritin is left from the modification which confirms that every subunit was modified by the ATRP initiator but a large smeared trace shows the subunit-polymer conjugates. The different hydrodynamic properties and adsorption processes of the polymer block do not allow the determination of the molecular weight.

The thermoresponsive properties of the conjugates were characterized in solution and on substrates. The turbidity was measured at the non specific absorbance of 600 nm (cf. Fig. 8.14). At the LCST, PNIPAAm undergoes an abrupt change in its dimension, followed by an aggregation of individual chains to larger particles resulting in an optically detectable phase separation, as a turbid solution. As the low molecular weight polymer derived from the sacrificial initiator was removed by dialysis, the conjugates solution turns turbid by the phase transition of the co-

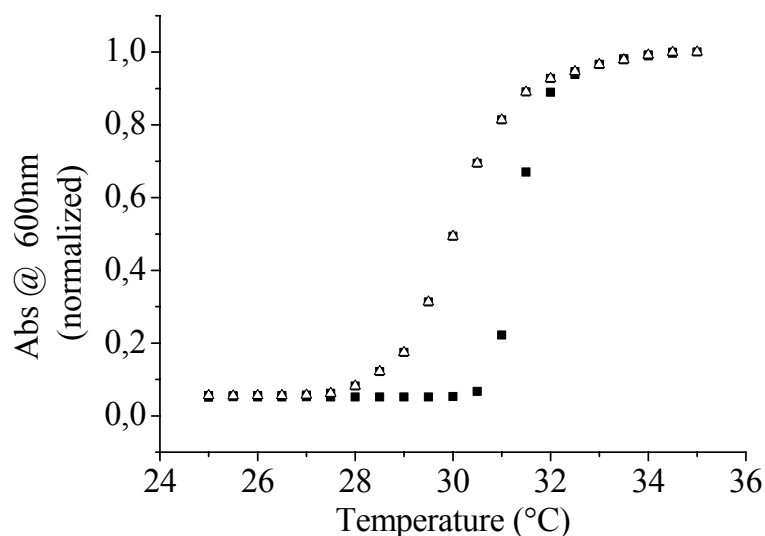


Figure 8.14: Turbidity measurement of the conjugates in function of temperature: ■ corresponds to the heating cycle; △ corresponds to the cooling cycle.

valently bound polymer. The detected cloud point is 31 ± 0.5 °C, which is lower than the theoretical value of PNIPAAm alone, but is influenced by the hydrophilicity of the end groups and the molecular weight of the polymer^{77,83}. The conjugates are thermo-responsive as the reverse transition can also be observed with a small hysteresis. The same property was detected for ferritin-PEGMA conjugates with a cloud point of 29.5 ± 0.5 °C.

The transmission electron microscopy allows the visualization of this phenomenon. Fig. 8.15 illustrates the successful achievement of single ferritin-PNIPAAm conjugates. The iron core of ferritin has a high contrast in the TEM micrographs and appears as a black dot of 6 nm. The corona around the ferritin is about 30 to 45 nm in diameter.

Dynamic light scattering measurements were also performed to observe the temperature transition of the conjugates in solution. The transition temperature, determined from the onset of strong scattering intensities due to the aggregation and phase separation of the bioconjugates was 31.5 ± 0.5 °C.

Fig. 8.16 shows a decrease of the hydrodynamic radius of the particles from 30 to 12 nm between 20 and 32 °C as the cloud point is approached (at 31.5 °C). After a further increase in temperature, the conjugates slowly start to aggregate.

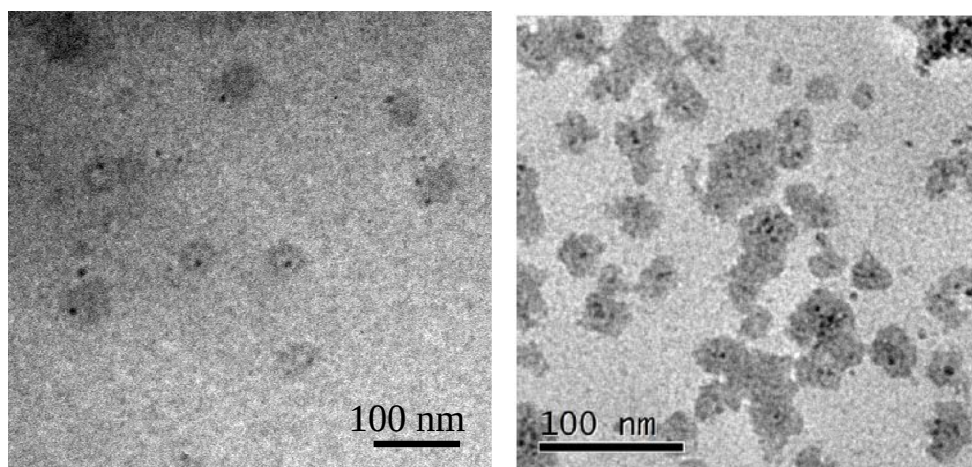


Figure 8.15: TEM micrographs of ferritin-PNIPAAm conjugates (on non treated formvar coated copper grid) and ferritin- P(NIPAAm₉₅-DMIAAm₅) (on hydrophilized formvar coated copper grid).

This may be explained by the fact that PNIPAAm is becoming hydrophobic and tends to reduce its interface with water. At around 36 °C, there are already large agglomerates of 250 nm. TEM micrographs in Fig. 8.16 also show an agglomeration with temperature. The size of the aggregates strongly depends on the polymer concentration in the solution, and the aggregation process is irreversible.

In order to directly image the bionanoconjugates, a solution of mixed with monomeric ferritin was spread by drop casting on a freshly cleaved mica sheet and dried at room temperature. At pH 7.5, mica sheet is hydrophilic and negatively charged, allowing a good adsorption of the proteins. By comparing height and phase images of the composite bioconjugates, it can be seen that ferritin is homogeneously surrounded by polymer. This is clearly visible in the phase image, which relates to the elastic response of the material. The hard iron oxide core of ferritin results in a large phase shift, indicated by white dots (cf. Fig. 8.17). Comparing the topography and phase images allows the visualization of the iron core of the horse spleen ferritin in the center of the conjugates. The conjugates are from 150 to 300 nm in diameter (for a polymerization of monomer:initiator of 500:1).

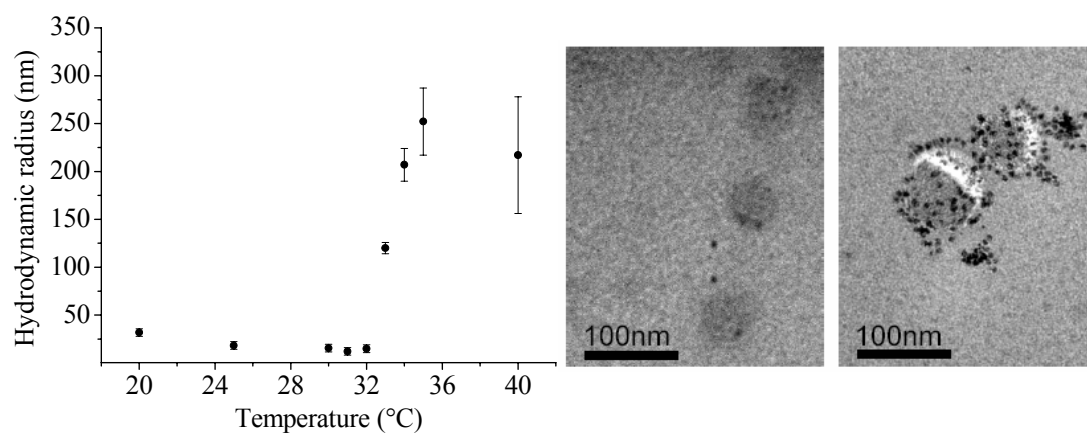


Figure 8.16: Effect of temperature on conjugate particles in solution. Left: DLS measurement of ferritin-PNIPAAm conjugate particles; Right: TEM micrographs of ferritin-polymer conjugates at room temperature and after heating to 40 °C.

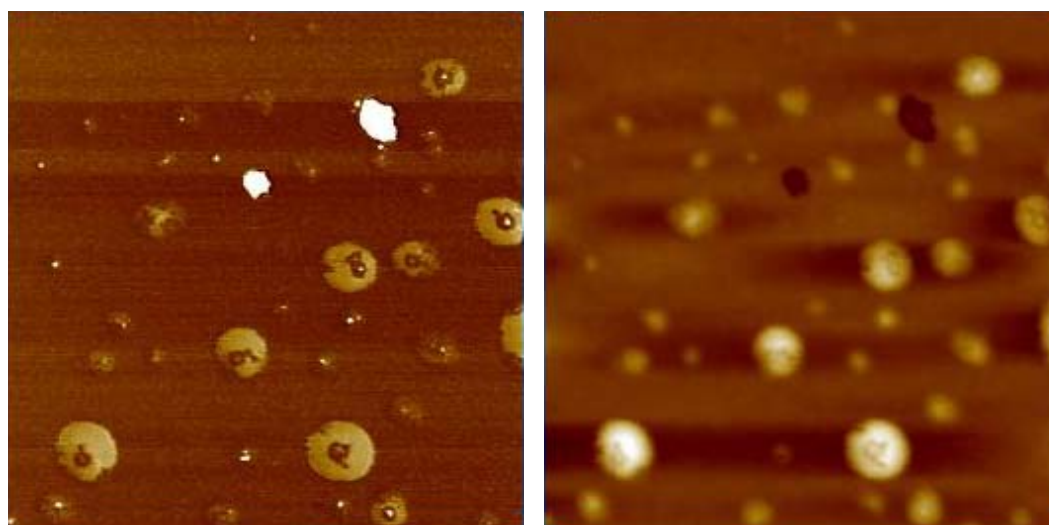


Figure 8.17: AFM phase ($z=30^\circ$) and topography ($z=30\text{ nm}$) images of horse spleen ferritin-PNIPAAm conjugates mixed with free horse spleen ferritin prepared on a freshly cleaved mica sheet, over a $9\text{ }\mu\text{m}^2$ scale. The iron oxide core of horse spleen ferritin appears as a bright dot in the phase image.

9 Towards the Building of the Membrane

This chapter is focused on the building of a thermoresponsive nanoporous membrane. The protein-polymer conjugates ferritin-P(NIPAAm-DMIAAm), described in Chapter 8, are able to adsorb at different solid-liquid and liquid-liquid interfaces. This feature is used to build supported membranes and 3D capsules. By assembly of the conjugates at the interface, it is possible to obtain a homogeneous protein-polymer film and allows the crosslinking through the maleimide sites of DMIAAm monomers, as shown in Chapter 7. Afterwards, the denaturation of the proteins and intensive washing will open the pores of the membrane. The 3D assembly of such protein-polymer conjugates can open further perspectives such as the creation of nanoporous capsules. For this purpose, the adsorption of protein-polymer conjugates at the liquid-liquid interfaces is investigated and the formation of Pickering emulsions is shown.

9.1 Adsorption at Solid-Liquid Interfaces & Supported Membrane Formation

Protein-polymer conjugates are able to assemble at the liquid-solid interfaces. Capillary and electrostatic forces are driving the conjugates adsorption. Hydrophilic and charged surfaces allow the spreading of the solution of protein-P(NIPAAm-DMIAAm) conjugates to reach a homogeneous polymer matrix. The crosslinking under UV irradiation should create the polymer matrix of the membrane. Finally, the denaturation of the proteins should allow the pores formation of the membrane.

9.1.1 Experimental Part

Ferritin-P(NIPAAm₉₅-DMIAAm₅) conjugates dialyzed at a cut-off 50 kDa (a solution at 1 mg·mL⁻¹ in 0.1 M sodium phosphate buffer solution at pH 7.4) are mixed with a 2 wt% sensitizer thioxanthone solution in DMF.

The resulting solution was spincoated (2000 rpm for 60s), or drop cast onto oxidized silicon wafers or freshly cleaved mica sheets. The salt was then washed several times with millipore water. For the crosslinking, the substrates were placed under UV light for 20 minutes, the UV irradiation was carried out with a 400 W UV lamp (Panacol 400F), with emitting light with a $\lambda > 315$ nm wavelength at 20 cm from the sample. The denaturation was achieved by adding a 300 μ L drop of a 8 M urea solution mixed with a 10 μ L drop at 10 mM of a dithreitol solution (DTT) covering the polymer film and placing the sample in the oven at 90 °C for 15 minutes. The sample was then extensively washed with millipore water and further investigated by AFM under dry conditions.

9.1.2 Results

Freshly cleaved mica sheets and oxidized silicon wafers are both hydrophilic and negatively charged at pH 7.5. The isoelectric point of ferritin is between 4 and 4.5. Both surfaces allow to obtain a homogeneous conjugates film before UV crosslinking as illustrated by Fig. 9.1. The protein particles are not hexagonally packed, which is not required for the desired application. The main interest is to introduce channels of well controlled size and with chemical functions into the polymer matrix, by means of denaturation of the proteins. Pore size control yields over membrane selectivity and chemical functions allow further modifications of the membrane channels.

The polymer film thickness average can be evaluated from the topographic image (cf. Fig. 9.2). The conjugates solution was drop cast on the mica sheet, and did not fully cover it. The proteins can be seen in the film on the phase image as bright dots and the mica sheet as a bright background (both iron core of ferritin and mica are harder than the polymer film). As the mica sheet is not fully covered by the conjugates film and it is possible to evaluate the thickness of the polymer film, which is of about 3 nm. The small thickness is foreseeable and de-

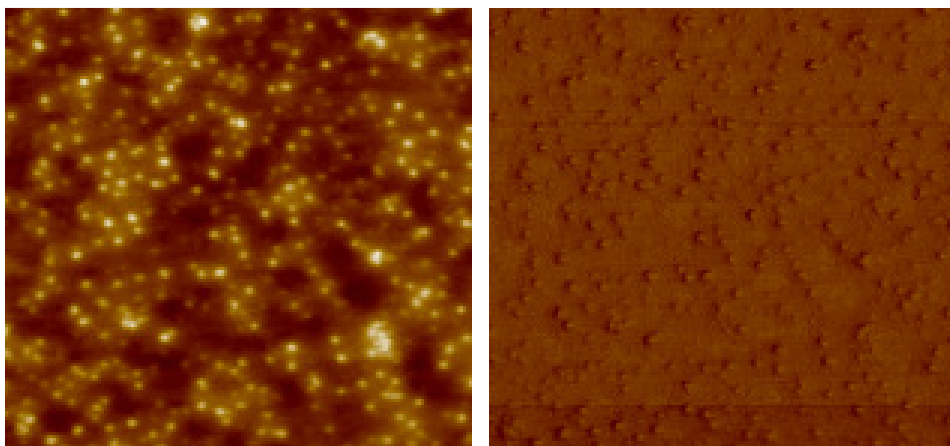


Figure 9.1: AFM topography and phase images of ferritin-P(NIPAAm₉₅-DMIAAm₅) conjugates film over 1 μm^2 on mica sheet. Left: z-range: 20 nm; right: z-range: 40 °.

sirable as the proteins have a diameter of 12 nm and cannot produce a thick film as P(NIPAAm-DMIAAm) is spreading on the hydrophilic substrate. If it would be thicker than 20 nm the proteins are covered by the polymer and are not accessible to denaturing agents and there will not be any holes (pores) in the polymer film after denaturation.

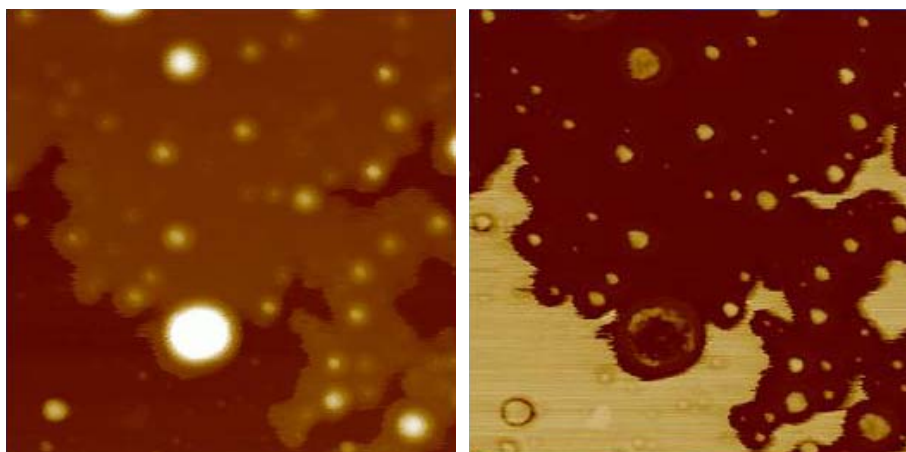


Figure 9.2: AFM topography (left) and phase (right) images of ferritin-P(NIPAAm₉₅-DMIAAm₅) conjugates forming a film over 1 μm^2 on mica sheet before crosslinking ; height z-range: 30 nm and phase z-range: 30 °.

UV irradiation crosslinked the polymer chains through the maleimide groups of DMIAAm in the statistical copolymers P(NIPAAm-DMIAAm). Ferritin is not altered by the UV irradiation even over a long period of time. However, the problem is that the required UV treatment heats the sample to about 80 °C and the polymer chains are shrinking around the protein particles disrupting the homogeneous polymer film letting appear the substrate, as seen in Fig. 9.3 (left). It is therefore required to cool the samples on an ice bath during irradiating to keep a homogeneous film, as illustrated in Fig. 9.3.

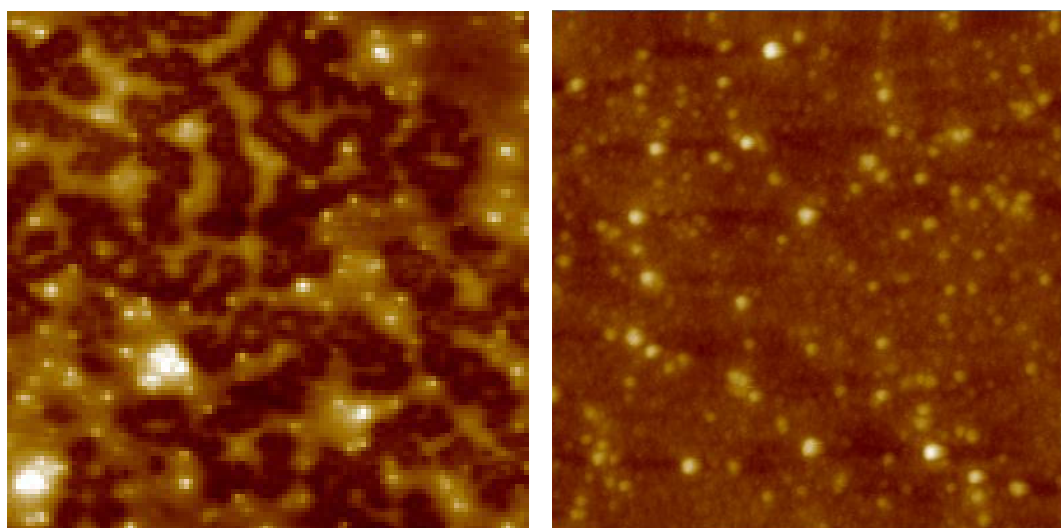


Figure 9.3: AFM topography images of ferritin-P(NIPAAm₉₅-DMIAAm₅) conjugates film on oxidized silicon wafer over 1 μm^2 crosslinked by UV irradiation. Left: z-range: 20 nm; Right: z-range: 20 nm with the sample cooled by an ice bath.

The denaturation was investigated on crosslinked and homogeneous films of ferritin conjugates, following the protocol established for ferritin in its native state. The films were covered with the denaturing agents solution and placed at 90 °C for just 15 minutes and were washed extensively with water. Ideally, the denaturing solution could reach the proteins and cleave the subunits which would be washed away with millipore water to create the pores of the membrane. After chemical denaturation, no more ferritin particles could be seen in the sample, that is why it is assumed that the proteins have been effectively denaturated and removed from the polymer matrix. Moreover, some round and homogeneous holes and/or pores appeared in the polymer film as illustrated in Fig. 9.4.

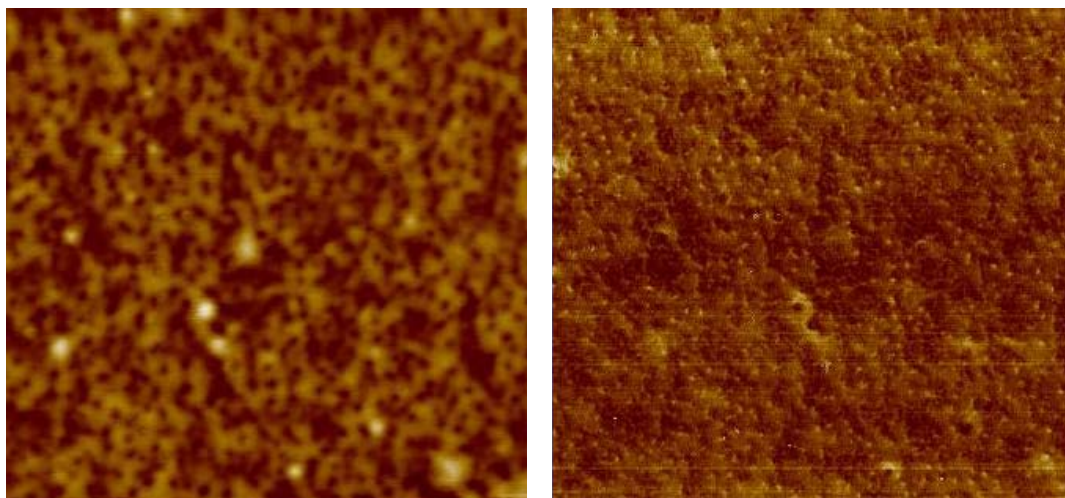


Figure 9.4: AFM topography (left) and phase (right) images of P(NIPAAm-DMIAAm) membrane after chemical denaturation of ferritin particles over $1 \mu\text{m}^2$; Left: z-range: 10 nm; Right: z-range: 20° .

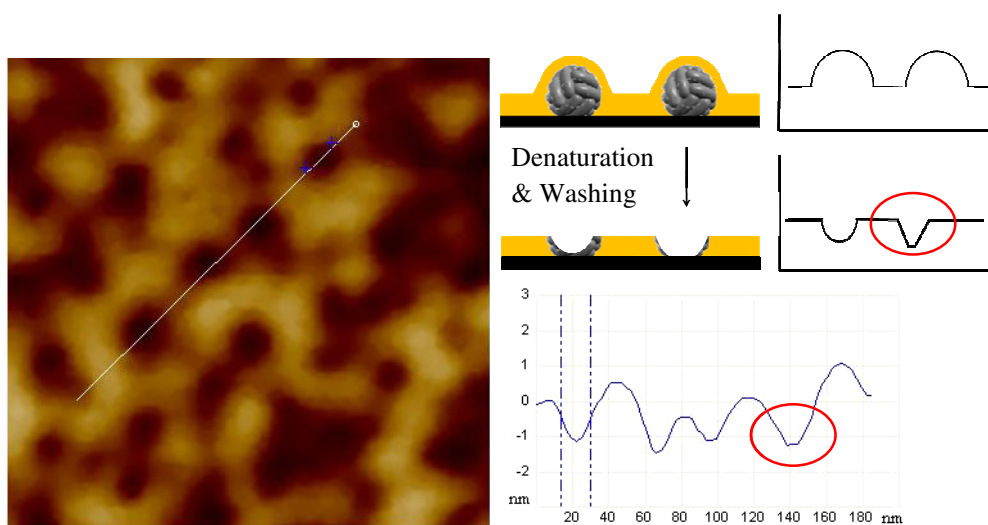


Figure 9.5: AFM zoom of topography image of P(NIPAAm-DMIAAm) membrane after denaturation of ferritin particles over $0.222 \mu\text{m} \cdot 0.222 \mu\text{m}$; Left: z-range: 5 nm; Right: cross-section measurement of the pores: diameter of about 16 nm and 1-2 nm depth. Sketch of partial and total denaturation of proteins and cross-sections.

They correspond to dark spots on the topography image and to very bright spots on the phase image. The pores are about 16 nm in diameter which is in the range of the protein diameter in the native state. The pores are about 2 nm deep which is in agreement with the polymer film thickness of 3 nm, as seen in Fig. 9.4 and Fig. 9.5. The cross-section does not exhibit a straight profile for the polymer matrix, as it seems that some polymer has been removed with the washing of the proteins. Moreover, the resulting images are depending on the sharpness of the AFM tip. It is important to determine if the channels/pores are penetrating the depth of the whole polymer matrix, as it is sketched in Fig. 9.5. There is some evidence showing that it is the case. The flat bottom of the holes may indicate that the protein has been fully removed, whereas a round-shape bottom may point to a protein left-over.

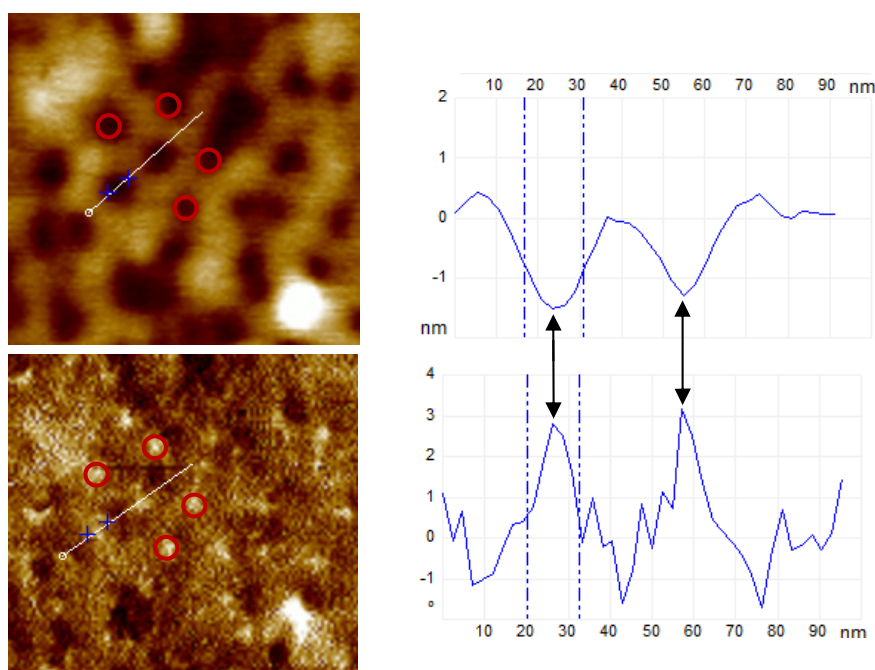


Figure 9.6: AFM zoom of topography (up) and phase (down) images of P(NIPAAm-DMIAAm) membrane after denaturation of ferritin particles over $0.367 \mu\text{m} \cdot 0.367 \mu\text{m}$ and their cross-section; Up: z-range: 5 nm; Down: z-range: 10° ; Right: cross-section measurements of the pores: diameter of about 16 nm and 2 nm depth.

Moreover, the cross-sections of the topography and phase zoom images (cf. Fig. 9.6) are showing that the minima of the height (corresponding to the bottom of the

channels) are corresponding to the maxima of the phase section, indicating that there is a different composition than the polymer in the holes. If the channels cross the whole polymer matrix, the AFM tip should reach the silicon substrate, and as the substrate is harder than the polymer, it appears brighter in the phase image. Some diffusion studies would be necessary to affirm this result.

This result is a proof of concept of the supported membrane. Indeed, it is shown here that ferritin can be used as a model protein for the building of a membrane by the bottom-up approach. Ferritin-polymer conjugates are building blocks able to assemble at the solid-liquid interfaces. After crosslinking under UV irradiation at low temperature, followed by chemical denaturation, the proteins create channels and/or pores in the polymer matrix. Many other proteins or non infectious viruses can be used in the same manner to control the pore size and shape of the membrane. Moreover, as the polymer matrix is made of the thermoresponsive copolymers P(NIPAAm-DMIAAm), this will allow triggering the pore size and selectivity with temperature.

9.2 Adsorption at Liquid-Liquid Interfaces & Capsule Formation

A further perspective is the assembly of ferritin-P(NIPAAm-DMIAAm) conjugates at the liquid-liquid interfaces and the formation of 3D capsules following the same process than the 2D membrane formed in the previous section. Pendant-drop tensiometric measurements were performed to follow the assembly process of the conjugates and to establish their role in the stabilization of an oil droplet. Pickering emulsions, based on oil-water emulsions, were prepared with the conjugates solution and 3,5 bis-(trisfluoromethyl) 1-bromobenzene). The latter is chosen due to its high boiling point of 146 °C and its density 1.699 g·cm⁻³. Different fluorescent dyes could be dissolved in this oil in order to visualize the capsules by laser scanning confocal microscopy.

9.2.1 Pendant Drop

9.2.1.1 Experimental Part

For the tensiometric measurements, a pendant oil droplet is placed in a cuvette containing the embedding phase being the solution of ferritin or ferritin-conjugates or pure water. The changes in the interfacial tension between the protein solution and the oil phase were determined at room temperature and monitored by a DSA100 pendant-drop tensiometer with a video camera for drop-image processing, which allows rapid drop-image acquisition, edge detection and fitting of the Young-Laplace equation. Small blunt-end canulas of 0.5 mm in diameter (NE44 Krüss) were used.

9.2.1.2 Results

Both ferritin and ferritin conjugates are investigated at the same concentration of particles of 0.65 mg·mL⁻¹. Tensiometric measurements are used to follow the adsorption kinetics of ferritin and ferritin conjugates at the oil-water interfaces.

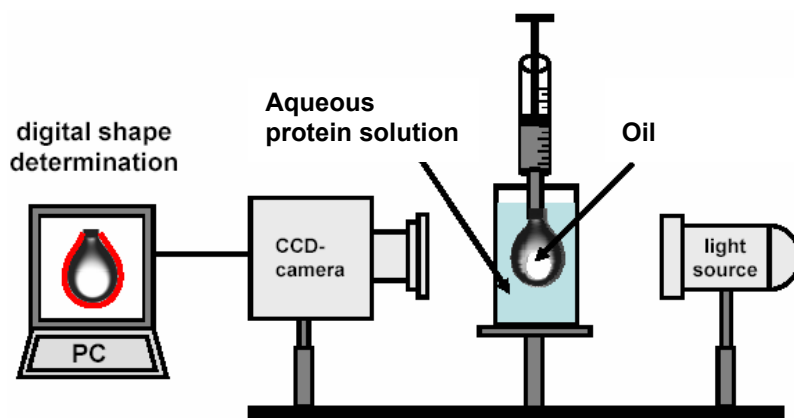


Figure 9.7: Schematic experimental setup for pendant drop tensiometry.

This phenomenon leads to a reduction of the interfacial energy between the two phases and stabilization of the oil-water dispersion²⁰⁷.

The baseline measurement of the interfacial tension of the oil droplet in water leads to a constant value of 36.3 mN/m in time. The addition of proteins to the solution decreases the interfacial tension and tends to stabilize the droplet.

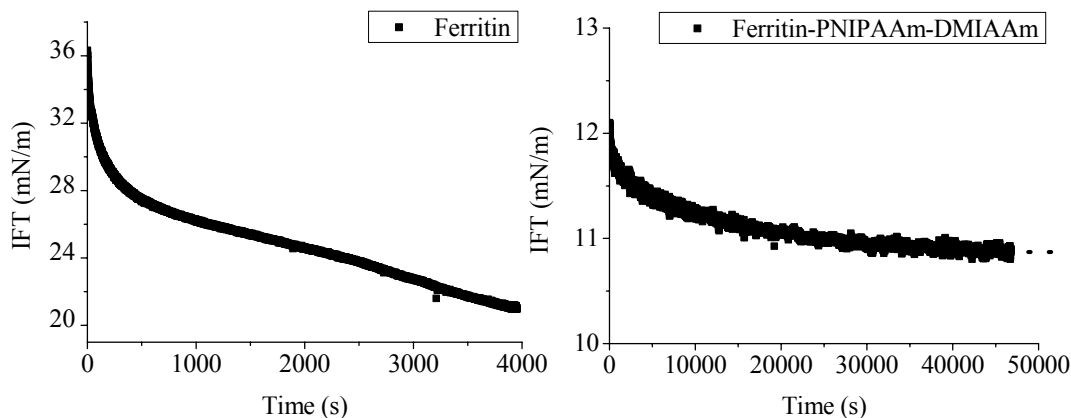


Figure 9.8: Dynamic interfacial tension measurements of ferritin and ferritin-P(NIPAAm-DMIAAm) conjugates suspensions at the 3,5 bis-(trifluoromethyl)1-bromobenzene)-water interfaces.

In this experiment, the chosen oil has a high boiling point to avoid the evaporation during the measurement. In the case of ferritin particles, the equilibrium was never reached. The assembly of ferritin at liquid-liquid interfaces is not fully

understood, but it seems to follow the theory of solid particles as ferritin particles are hard globular proteins. They undergo only minor conformational changes at the interface and the protein shell is not destroyed by the assembly, as explained in Günther Jutz's thesis²⁰⁸. In practice, the oil droplet easily detached from the canula after 4000 s, and the measurement could not be prolonged.

When the ferritin-P(NIPAAm-DMIAAm) conjugates solution was added to the aqueous solution, the interfacial tensions decreased rapidly and the equilibrium was reached after 1 hour and the interfacial tension was about 10.88 mN/m. The oil droplet was very stable and the measurement could be followed over 13 hours. The protein-polymer conjugates are still surface active and seem efficiently to stabilize the oil droplet. A possible explanation for this effect may be the difference of polarity of the two different monomers NIPAAm (more polar) and DMIAAm (more apolar), which is leading the stabilization of the droplet by the statistical copolymer as the polymer chains are organizing around the oil-water interfaces, as illustrated in Fig. 9.9. Earlier TEM investigations revealed a possible phase separation of the PNIPAAm shell around the ferritin leading to a Janus-like structure on a hydrophobic substrate (cf. Fig. 8.15 (left image)).

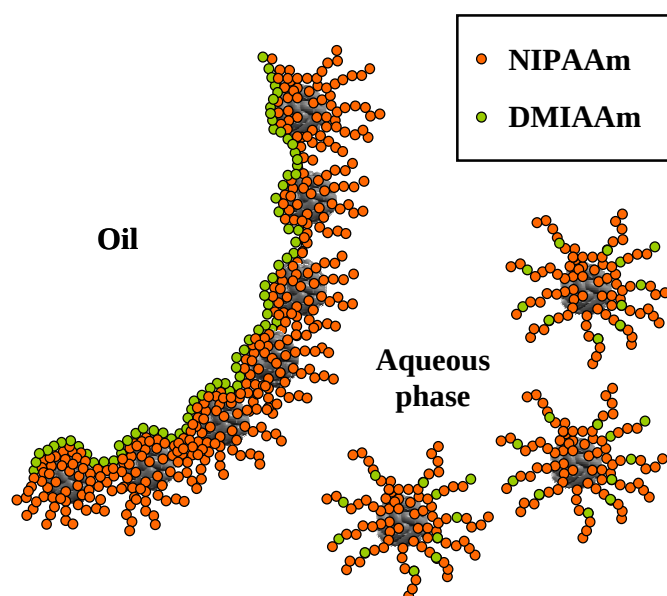


Figure 9.9: Sketch of ferritin-P(NIPAAm-DMIAAm) conjugates assembly at the oil-water interface.

9.2.2 Pickering Emulsions

9.2.2.1 Experimental Part

Pickering emulsions are prepared by adding 10 μL of fresh solution of sensitizer thioxanthone in DMF to 400 μL of the conjugates at $1\text{ mg}\cdot\text{mL}^{-1}$. Finally, 40 μL of oil are mixed to the aqueous solution and shake by hand to form the droplets and let them to stabilize by protein adsorption at the interface at room temperature. Perylene bisimide, a fluorescent dye synthesized by Andre Wicklein, is dissolved in the oil phase. It was chosen as it is not bleached by UV irradiation, necessary for the crosslinking step. The dye was added to the oil phase before the addition of the oil into the aqueous phase. The micro-capsules were observed by laser scanning confocal microscopy (LSCM) before and after crosslinking, with a Zeiss LSM 710 microscope with excitation by an Argon and a HeNe lasers and using an oil-emulsion objective Plan-Achroplan 63*/1.4 Oil DIC M27*. The fluorescence from the protein conjugates is shown by a color scale, in which brightness represents intensity.

9.2.2.2 Results

The pickering emulsions were visualized using confocal microscopy with and without crosslinking. A water-soluble dye, fluorescein isothiocyanate, is added to the aqueous phase of the non-crosslinked capsules, as shown in Fig. 9.10A.

Two lasers with different wavelengths at 488 and 543 nm allow the visualization of both dyes, the capsules appear in red while the aqueous phase appears in green. The capsules are not homogeneous in size but the capsules are not destroyed by UV irradiation. Moreover, the crosslinked capsules are stable in solution for several weeks.

In order to show the effect of crosslinking and prove the enhanced mechanical stability, the capsules were centrifuged into an oil phase. Fig. 9.10B shows that the crosslinked capsules did not disrupt, while the same experiment with non-crosslinked capsules lead to disruption of the capsules.

Ferritin-P(NIPAAm-DMIAAm) conjugates have been proven to lead to stabiliza-

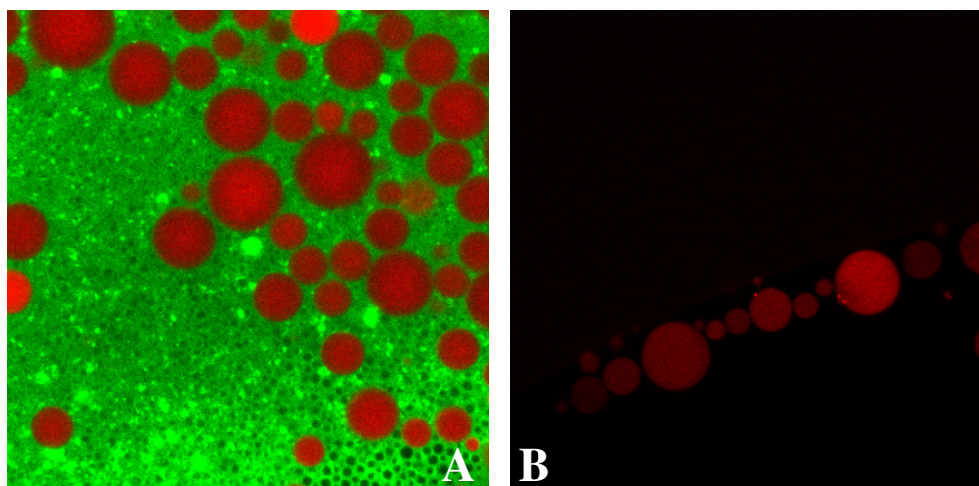


Figure 9.10: Laser scanning confocal microscopy images of ferritin-P(NIPAAm-DMIAAm) capsules. A: non crosslinked capsules with perylene bisimide dye dissolved into oil phase (red) and fluorescein dissolved in the aqueous phase (green); B: crosslinked capsules after UV irradiation with perylene bisimide dye dissolved in the oil phase and non colored free oil interface.

tion of oil droplets and allow the building of capsules. Moreover, they provide interesting mechanical properties. Further investigations can be made to estimate the porosity of the capsules, the organization of the protein-polymer layer. The denaturation of the proteins will lead to extra-porosity and change the mechanical properties. The first capsules are showing encouraging results through their stability and future crosslinked capsules can be treated to produce 3D nanoporous containers.

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Sonstiges

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