**Self-Assembled and Responsive
Supramolecular Nanoparticles
Mediated by Cucurbit[8]uril Host-
Guest Interactions**

Carmen Stoffelen

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The research described in this thesis was performed within the laboratories of the Molecular Nanofabrication (MnF) group, the MESA⁺ Institute for Nanotechnology and the Department of Science and Technology of the University of Twente. This research was supported by the Council for Chemical Sciences of the Netherlands Organization for Scientific Research (NWO-CW, Vici grant 700.58.443).

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ISBN: 978-90-365-3849-7
DOI: 10.3990/1.9789036538497
Cover art: Jan-Stefan Janssen and Carmen Stoffelen
Printed by: Gildeprint Drukkerijen - the Netherlands

SELF-ASSEMBLED AND RESPONSIVE
SUPRAMOLECULAR NANOPARTICLES MEDIATED
BY CUCURBIT[8]URIL HOST-GUEST
INTERACTIONS

DISSERTATION

to obtain
the degree of doctor at the University of Twente
on the authority of the rector magnificus,
Prof. dr. H. Brinksma,
on account of the decision of the graduation committee,
to be publicly defended
on Thursday March 26, 2015 at 14.45 h

by

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Born on June 10, 1983
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"Try to become not a man of success, but try rather to become a man of value."

Albert Einstein

This thesis is dedicated to my family.

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Chapter 1

General Introduction

Nanotechnology is an interdisciplinary field that describes the manipulation of objects in small dimensions to provide material properties that are significantly different from those observed in the bulk.¹⁻³ In particular, nanoparticles (NPs) of different compositions and designs have attracted much attention in science and society. Despite the concern about their potential toxicity, their new materials properties make them of high interest in electronics, catalysis, textile industry, renewable energy, and health.⁴⁻⁶ In particular the application of nanoparticles in biomedicine has provided new strategies for diagnosis and drug delivery.⁷⁻⁹

Supramolecular chemistry has gained in importance in chemistry and other disciplines over the past two decades.¹⁰ The self-assembly of small building blocks by non-covalent interactions, i.e. the process by which individual building blocks organize into ordered structures spontaneously, is the key principle in this area.¹¹ Inspired by nature, supramolecular chemistry enables the formation of functional materials in different dimensions from molecular building blocks, based on a toolbox of different non-covalent interactions with variable binding strengths and dynamics.^{12,13} The combination of multiple non-covalent interactions, called multivalency, leads to very stable assemblies in the absence of covalent bonds.¹⁴ At the same time, the non-covalent origin of the interactions, and its inherent reversibility, enables the design of responsive supramolecular materials.

Supramolecular nanoparticles (SNPs) are formed by multiple building blocks that are brought together by multiple non-covalent supramolecular interactions. SNPs can be divided into two major classes. Soft SNPs employ only organic and/or polymeric building blocks and are used, for example, for drug or gene delivery applications.^{15,16} Alternatively,

metallic or inorganic nanoparticles, modified with supramolecular binding motifs at the exterior, can be used as hard building blocks in SNPs and can, for example, be used as detection tool in various screening essays^{17,18} or for biomedical applications.¹⁹

The research described in this thesis aims to understand the design rules of SNPs, their self-assembly and triggered disassembly, and the dynamics of these processes. Therefore the heteroternary complexes of cucurbit[8]uril, methyl viologen and naphthol (CB[8]/MV/Np) and of CB[8], MV and azobenzene (CB[8]/MV/Azo) have been used for the formation of soft SNPs (Chapters 3-7) and for the clustering of hard SNPs employing MV-functionalized silica NPs as a building block (Chapter 8). Control over the SNP size, the assembly kinetics, and the disassembly pathway are major objectives of the work presented here.

Chapter 2 provides a literature overview of soft supramolecular nanoparticles. Emphasis is placed on the nature of the underlying supramolecular interaction, the valency of the stabilizing interactions and on the potential biomedical applications.

In Chapter 3, a novel strategy for the formation of SNPs is reported based on multiple CB[8]/MV/Np charge transfer complexes, thus employing CB[8] as a host instead of the typically used cyclodextrin. The self-assembly of the supramolecular building blocks into SNPs in aqueous solution and the size tuning of the SNPs by the stoichiometry of the components are studied. The dynamics of the assembly is investigated as well and is related to the dynamics of the individual ternary complex.

The same SNP formation strategy has been used in Chapter 4 to investigate the role of the guest-modified hydrophilic polymer assembling in the shell of the SNPs thus functioning as a stopper. Therefore naphthol was grafted onto oligo and poly(ethylene glycol)s with different lengths and SNP self-assembly was carried out. The colloidal stability and the size tuning are described, as well as the influence of the valency of the stopper on the SNP morphology and dynamics.

In Chapter 5, dual stimuli-responsive SNPs are presented, which are stabilized by multiple CB[8]/MV/Azo complexes. Photo-induced *trans-cis* isomerization of the Azo and chemical reduction of MV leads to disruption of the heteroternary complex going and subsequent SNP disassembly. By monitoring the particle size in time, the dynamics of the SNP disintegration is studied in detail.

SNP formation by a toolbox approach is described in Chapter 6. Hereto, SNPs are assembled by ternary CB[8]/MV/Np or CB[8]/MV/Azo interactions exclusively, or by a combination of the two. Photoisomerization of Azo enables disassembly of the CB[8]/MV/Azo moieties. Thereby, the SNP disassembly is studied as a function of the placement of the CB[8]/MV/Azo moieties in the SNP core, shell, or both.

The formation and responsive character of zwitterionic SNPs is reported in Chapter 7. Based on the supramolecular CB[8]/MV/Azo interactions, SNPs are formed by assembling a relatively short, non-fouling, Azo-functionalized zwitterionic molecule in the SNP shell. The colloidal stabilities of the zwitterionic SNPs in water, in phosphate-buffered saline at different salt concentrations, and at relatively high protein concentrations have been studied in detail. The effect of pH on the particle morphology was monitored as well.

Finally, the supramolecular assembly of MV-functionalized silica nanoparticles by Np-terminated building blocks in the presence of CB[8] is demonstrated in Chapter 8. Therein the self-assembly and size control of supramolecular NP clusters is compared for SNP preparation in bulk and in a microfluidic device. The effect of the controlled diffusion time of the supramolecular components in the microfluidic device, the stoichiometry of the host and guest binding partners, and the competition of the monovalent and multivalent Np-bearing building blocks on the size of the supramolecular NP clusters has been studied in detail.

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Chapter 2

Supramolecular Nanoparticles

Supramolecular chemistry provides a tool for the formation of highly ordered structures by means of non-covalent interactions. Soft supramolecular nanoparticles are self-assembled nanoassemblies, based on small building blocks and stabilized by basic non-covalent interactions, selective host-guest interactions or a combination of different interaction types. This chapter gives an overview of the existing approaches for the formation of supramolecular nanoparticles. The approaches are ordered based on the nature of the stabilizing supramolecular interaction while focusing on the aspects that determine the particle structure. When applicable, the use of these self-assembled nanostructures as vectors in molecular diagnostics and therapeutics is described as well. Generally, the stable yet reversible nature of supramolecular interactions and the chemical flexibility offers great prospects for the development of highly engineered nanoparticles which are compatible with the complexity of living systems.

2.1 Introduction

Nanoparticles (NPs) attract heavy interest in nanoscience, owing to their size-specific chemical and physical properties. Next to catalytic, optical, sensor and/or electronic applications, the use of NPs in biomedicine has gained intense scientific interest over the past two decades.^{1,2} Countless studies have been carried out for the implementation of nanoparticles in biologically and pharmaceutically relevant systems. These achievements have been summarized in numerous reviews, which are giving either a general perspective of the field²⁻⁵ or are focused on a particular application such as drug delivery,⁶⁻⁹ imaging technology^{10,11} or theranostics.¹²⁻¹⁵ Recently, the use of NPs as adhesive material for regenerative wound healing purpose has been reported as well.¹⁶ The combination of supramolecular chemistry and NP technology provides new prospects in the field of NPs for biomedical applications.¹⁷ The non-covalent origin and accompanying ability for building blocks to assemble and disassemble spontaneously is one of the main advantages that supramolecular chemistry contributes to this synergism.

2.2 Self-assembly and non-covalent interactions

Dynamic self-assembly rules biological processes *in vivo*.¹⁸ Cell formation, proliferation and replication are based on complex processes such as DNA (deoxyribonucleic acid) replication, mitosis, microtubule growth and shrinking, all in which selective assembly and disassembly are involved. The exceptional feature of non-covalent interactions is their reversibility; binding and unbinding occurs without rupture or alteration of the initial binding partners. Inspired by the control of matter at the smallest scale, nanotechnology in particular uses self-assembly and self-organization of molecules and bigger entities for the fabrication of desired structures and functional materials.^{18,19}

Supramolecular chemistry makes use of a toolbox of different non-covalent interactions. These interactions range from relatively weak interactions such as hydrogen bonding, π - π stacking, Van der Waals forces or hydrophobic interactions to stronger ionic and dipolar bonds. Next to these basic non-covalent interactions, host-guest chemistry provides a powerful paradigm to supramolecular chemistry. Host-guest formation, leading to the inclusion of a guest molecule into a host, is commonly based on a combination of some of these intermolecular forces. The choice of solvent is very important in synthetically self-assembled systems as it has a big impact on the affinity and selectivity of the formed complex. Naturally occurring self-assembled systems and nanometer-sized assemblies designed for biomedical applications are limited to aqueous media. Non-covalent supramolecular motifs capable of self-assembly in water have been the subject of several recent reviews.²⁰⁻²³

In all self-assembly processes, thermodynamics plays an important role in explaining the stability and properties of the resulting structure. The key challenge in synthetic self-assembly is to control multiple underlying non-covalent interactions to achieve an energetically more favorable state after assembly. This is achieved by the interplay between entropic and enthalpic factors. When zooming in solely on the interaction between a host and a guest, the interactions between these molecules are enthalpically favorable and have to overcome the loss in entropy caused by complexation.¹⁹ At the same time, entropically favorable solvent release and solvent rearrangement can contribute positively to the energy gain or even drive the formation of the supramolecular assemblies entirely. Contribution by the solvent is particularly important in polar solvents such as water, in which the interaction amongst solvent molecules and between solvent and solute is of comparable strength as the interaction between the binding partners.

As in covalent chemical reactions, the energy levels and the energy barriers determine whether the thermodynamic or a kinetic product is formed during molecular self-assembly. The thermodynamic product is the most stable product with the lowest achievable energy, irrespective of any intervening barrier. If the supramolecular product is in thermodynamic equilibrium, all intermediate states are reversible under the reaction conditions employed. This state ensures that the self-assembly is dynamic and an equilibrium between association and dissociation is present. In contrast, a kinetic product is associated with the lowest energy barrier to its formation so it is the fastest formed intermediate in the self-assembly process. Nature frequently uses such kinetically trapped states, for example in enzymatic catalysis, but developing artificial out-of-equilibrium self-assembled systems is challenging.²⁴

Multivalency, the interaction of multivalent receptors and multivalent ligands, plays an important role in biological processes and provides a simple strategy to increase the binding affinities in synthetic self-assembled structures.^{25,26} Multivalent binding events can occur between complementary functionalities on a single building block or between two complementary building blocks and leads to products bound by multiple inter and intramolecular connections.²⁷ For instance, single hydrogen bonds or Van der Waals interactions are relatively weak (0.4 to 20 kJ/mol) and do not differ strongly from the omnipresent thermal energy at room temperature (2.5 kJ/mol). Nevertheless, stable supramolecular structures have been reported that are exclusively formed by hydrogen bonding or Van der Waals interactions.²⁸ This could only be achieved by organizing the molecules using a multitude of these relatively weak interactions. A prominent example of multivalency in nature is the formation of the DNA double helix.

The multivalent character of the binding event bestows unique thermodynamic and kinetic characteristics onto the supramolecular complex.^{29,30} Modeling the thermodynamics of non-covalent binding events has shown that the association-dissociation mechanisms of monovalent and multivalent structures differ strongly from each other.³¹ Taking the effective molarity (EM , i.e. the probability of binding sites to meet depending on conformation length and flexibility)³² into consideration, a concentration dependent binding mode has been introduced for multivalent interactions. When the effective molarity of a guest moiety, already bound to the multivalent receptor in a preceding intermolecular binding step, is higher than the concentration of free guest in solution ($[G]_{sol}$), intramolecular, i.e. multivalent, binding is favored (Figure 2.1). However, when EM is lower than $[G]_{sol}$, intermolecular connections, i.e. supramolecular polymerization or cross-linking, take over.

Figure 2.1: Schematic representation of the effective molarity (EM) responsible for favored intramolecular multivalent binding instead of intermolecular binding with guest molecules from solution.²⁹

In kinetic terms, the dissociation of supramolecular assemblies connected via multivalent interactions is typically much slower than in assemblies stabilized by monovalent interactions of the same origin. Even though the individual dissociation rate of a single interaction in a multivalently connected supramolecular assembly does not differ from the dissociation rate of the corresponding monovalent interaction, the overall dissociation rate can decrease by orders of magnitude because the probability that all bonds are broken simultaneously dictates the overall dissociation rate.³¹ Thus, the overall dissociation rate of a multivalent supramolecular assembly is determined by the dissociation and concentration of an intermediate in which only one bond remains. As the population of this intermediate is suppressed by rebinding induced by the high effective concentration, extremely low dissociation rates are commonly observed for

multivalently linked assemblies. The use of multivalency as a chemical organization principle for the fabrication of nanomaterials, scaffold architectures and functional materials was reviewed by us²⁹ and, more recently, by Haag et al.³³

2.3 Supramolecular nanoparticles by basic non-covalent interactions

In general, the field of supramolecular NPs (SNPs) can be divided into two different classes. We focus here on soft SNPs which are formed and stabilized by non-covalent interactions of individual molecules. In contrast, hard inorganic or polymeric NPs that are decorated with supramolecular receptors at the shell of the NPs and provide so-called network aggregates, are not covered here. Numerous reviews have appeared which summarize the efforts made for the formation and application of organic-inorganic hybrid materials formed by hard supramolecular nanoparticles.³⁴⁻³⁷ More specifically, Rotello and coworkers have focused on the application of surface-engineered NPs for nanomedicine.^{3,38-40}

2.3.1 Supramolecular nanoparticles stabilized by hydrophobic interactions

Hydrophobic interactions constitute the driving force of apolar molecules to aggregate in aqueous solution. In nature, non-covalent hydrophobic interactions are important for protein folding, lipid bilayer formation and the insertion of membrane proteins into bilayers. The formation of such assemblies is driven by entropic and enthalpic gains. In the presence of an apolar molecule, water molecules are bound to the solute but with fewer H-bonds than in the bulk solution. By aggregation of the apolar molecules into higher assemblies, the total interface area between water and solute molecules is reduced and thereby the fraction of bound water molecules decreases, resulting in an overall favorable free energy change.

Scientists have already made use of this hydrophobic effect for a long time, and typically amphiphiles, molecules with hydrophilic and hydrophobic fragments, are used for the formation of distinct structures. In 1913 James W. McBain discovered that the conductivity of a soap solution changes with concentration. In the following century, countless studies have focused on the self-assembly of amphiphiles in aqueous environment.^{41,42} Next to the well-known micelle structure (Figure 2.2), other nanostructures such as liposomes,^{43,44} polymersomes,^{45,46} tubes,⁴⁷ or even helices⁴⁸ can be formed depending on the structure of the amphiphilic molecule. By tuning the amphiphilicity transformation of the formed assemblies can be achieved.⁴⁹

Figure 2.2: Schematic representation of micelle formation in an aqueous environment. The self-assembly of amphiphilic molecules reduces contacting of the hydrophobic segments with water.

2.3.2 Supramolecular nanoparticles stabilized by hydrogen bonding

Self-organization of molecular systems via multiple intra- and intermolecular hydrogen bonds is an established strategy for the formation of self-assembled structures.⁵⁰ Taking water molecules as an example, hydrogen bonding is based on the attractive interaction of a hydrogen atom with the electron pair-donating oxygen of another water molecule (Figure 2.3a). This leads to attractive forces that are relatively weak (5 to 30 kJ/mol) and depend on the heteroatoms on either side of the hydrogen atom, but multiplication of these interactions can lead to very stable self-assembled structures, for example, as shown for the formation of rosettes in Figure 2.3c.⁵¹

Figure 2.3: a) Model of a single hydrogen bond between two water molecules based on the interaction between hydrogen bond-donating and accepting water molecules. b) Complementary hydrogen bonding motifs of melamine and cyanuric acid. c) Formation of hydrogen-bonded macrocyclic structures called rosettes by complementary melamine and cyanuric acid molecules.

The use of multiple hydrogen bonding motifs (called dyads) in the formation of stabilized assemblies was applied in NP research for the clustering of dyad-functionalized (hard, inorganic) NPs,^{52,53} micellar structures^{54,55} and the formation of polymeric SNPs. Rotello and coworkers reported the formation of giant supramolecular vesicles by inter-chain hydrogen bonding between a diaminopyridine (DAP)-functionalized polymer and a thymine-based polymer.⁵⁶ A comparable type of vesicle structure was formed by the same dyad interaction using DAP and uracil-functionalized poly(norbornene).⁵⁷ Differential interference contrast measurements have shown that the instantaneously formed polymersomes are metastable and fuse into larger vesicles over time. Stable but temperature and competitor responsive microspheres were formed by dissolving DAP-functionalized poly(styrene) in chloroform at 20°C under the formation of a two-fold hydrogen bond.⁵⁸ By heating the microspheres to 50°C, dissociation was observed which was reversible upon cooling to room temperature. Furthermore, upon addition of thymine-functionalized poly(styrene) these microspheres transformed into vesicles, based on the more stable dyad interactions (Figure 2.4) of the hetero pair formation. This process was found to be reversible as the addition of an excess of DAP polymer reverted the vesicles to microspheres. Interestingly, in contrast to the microsphere structures, uncontrolled aggregation of the dyad-stabilized vesicles occurred upon heating. Another study reported the formation of size-tunable microspheres by mixing PEGylated DAP polymers with a complementary thymine polymer.⁵⁹ The size of the supramolecular structures depended strongly on the polymer length. In this case, an irreversible morphology change was shown by photo-chemical cycloaddition with UV light.

The layer-by-layer (LbL) assembly technique is a powerful strategy for the formation of self-assembled polymeric films and NPs. In the LbL approach, originally developed for polyelectrolytes,^{60,61} films are alternately assembled using complementary interactions. Besides films on flat surfaces, hollow supramolecular capsules have been formed by coating solid NPs with polyanions and polycations by LbL assembly based on hydrogen bonding prior to removal of the templating NP. Caruso and coworkers have provided an overview of hydrogen-bonded multilayer films and capsules, and their application in the biomedical field.⁶²

Figure 2.4: a) Schematic illustration of microsphere formation and reversible morphology control from microsphere to vesicle using specific non-covalent interactions. b) Chemical structures of diaminopyridine (DAP) and thymine polymers c) Two-fold hydrogen bonding between two DAP moieties and three-fold hydrogen bonding between DAP and thymine moieties.⁵⁸

2.3.3 Supramolecular nanoparticles stabilized by electrostatic interactions

SNPs mediated by electrostatic interactions are based on the attraction of oppositely charged species. Depending on the origin of these building blocks, the individual interaction strength can vary between 5-350 kJ/mol. The best known approach for the fabrication of SNPs by electrostatic interactions is the formation of non-viral gene delivery systems. These nucleic acid-based therapeutics are formed by the interaction of negatively charged nucleic acids with either cationic lipids forming lipoplexes or cationic polymers forming polyplexes (Figure 2.5).⁶³ As these types of SNPs are promising candidates for gene delivery, tremendous efforts have been made within this field, and these have been summarized in several reviews.⁶⁴⁻⁶⁶

Figure 2.5: Schematic representation of a) lipoplex and b) polyplex formation of negatively charged DNA in the presence of cationic lipids or polymers, respectively.

Chitosan-based polyelectrolyte complexes possess high potential as building material for drug delivery vesicles because of their biocompatibility, biodegradability and low toxicity.⁶⁷ In combination with anionic polysaccharides, such as alginate,⁶⁸ carrageenan,⁶⁹ hyaluronic acid,⁷⁰ pectin,^{71,72} xanthan,^{73,74} gum kondagogu⁷⁵ and gum ghatti,⁷⁶ the polycation can form SNPs based exclusively on electrostatic interactions. More specifically, core-shell SNPs resulted from a mixture of chitosan with dextran as the polyanion.⁷⁷⁻⁷⁹ Depending on the polymer used in excess, either positively or negatively charged SNPs were formed. The complexed polymer segments accumulate within the core of the particles whereas the excess polymer segments segregate in the shell of the SNPs. This enables selective administration of charges to the particles and ensures colloidal stability. Charge screening for negatively and positively charged particles showed that the stability of all SNPs decreases with the amount of salt added. This clearly proves that both types of SNPs are stabilized by electrostatic interactions. Furthermore, the resulting size of the SNPs could be tuned between 140-240 nm depending on the molecular weight of the constructing polymers. Modification of the chitosan polymer with *N*-(2-hydroxyl)propyl-3-trimethylammonium groups increased the water solubility and the stability of the SNPs at physiological pH.⁷⁹ Inclusion and release studies of bovine serum albumin (BSA) in chemically modified chitosan-dextran SNPs showed that the SNPs are internalized by cells, and that the release of BSA is pH dependent.

Electrostatically stabilized SNPs have also been formed by the interaction of two polyelectrolytes, one functionalized with protonated amines and the other with deprotonated carboxylic acids.⁸⁰ SNP formation was visualized by fluorescence resonance energy transfer (FRET) between fluorescein and tetramethyl rhodamine-functionalized

polymers. By incorporation of the recognition peptide selective for protein kinase A (PKA), the SNPs were used to detect PKA activity. In the presence of PKA, the SNPs were phosphorylated and subsequently dissociated. Thereby the distance between the FRET dye partners increased, and a fluorescence increase of fluorescein isothiocyanate was detected. Another strategy to detect the PKA activity with SNPs uses the same particle recipe, but has incorporated the near-infrared (NIR) fluorophore Cy5.5.⁸¹ The strong NIR fluorescence intensity of the Cy5.5 dye was quenched upon SNP formation, but was recovered upon SNP disassembly due to the interaction of PKA with the SNPs.

2.4 Supramolecular nanoparticles mediated by host-guest interactions

Host-guest chemistry describes the complexation of a guest molecule by a host molecule by intermolecular inclusion. Commonly, the host is a large molecule with convergent binding sites, having a central cavity to enable inclusion complexation.⁸² The guest molecule possesses divergent binding sites and its size can range from small cations or anions to elaborate organic molecules.⁸² Inclusion of the guest molecule into the host can be founded solely on hydrophobic interaction, electrostatic interaction, π - π stacking, hydrogen bonding, Van der Waals interactions or a combination of these.

2.4.1 Cyclodextrin-based supramolecular nanoparticles

Cyclodextrins (CDs) are cyclic oligosaccharides linked by α -1,4 glycosidic bonds. The best known examples are α , β and γ -CDs (Figure 2.6), which are composed of 6, 7 and 8 glucose units, respectively. The typical cone-shaped structure with the hydrophobic interior enables the inclusion complexation of various hydrophobic guests. Owing to their low toxicity and their excellent biocompatibility, cyclodextrins are the most used hosts for the formation of biomedically relevant assemblies.⁸³⁻⁸⁵ A broad variety of small molecules, ions, proteins or even drugs can be encapsulated into CD cavities. The size of the guest molecule is crucial for the host-guest interactions: too large guests cannot be encapsulated in the interior because of steric hindrance, whereas too small molecules cannot interact efficiently with the cavity of the host. In general, Van der Waals forces between the cyclodextrin cavity and the guest component stabilize encapsulation. However, depending on the guest molecule, other supramolecular interactions such as hydrogen bonds and dipole interactions can be involved. Additional entropically favorable release of water molecules from the CD interior can contribute positively to the relatively strong interactions.

Figure 2.6: Molecular structures of α -, β - and γ -cyclodextrin.

2.4.1.1 Monovalent cyclodextrin-stabilized SNPs

Non-viral gene delivery systems, solely stabilized by electrostatic interactions, have been modified with CD to allow for additional stability and functionalization. Davis and coworkers have synthesized linear β -CD-containing polycations by polymerization of difunctionalized β -CDs with complementary difunctionalized monomers.⁸⁶ These polymers form polyplexes in the presence of RNA based on electrostatic interactions. Modification of the polymeric structure had strong effects on the polyplex formation, the *in vitro* transfection efficiency, and the toxicity of the RNA delivery vectors.⁸⁷⁻⁹⁰ To overcome the poor stability of these polyplexes under *in vitro* salt conditions, and to make these gene delivery NPs applicable for therapy, monovalently interacting adamantane end-functionalized poly(ethylene glycol) (Ad-PEG) was added as stabilizer. Upon addition of the hydrophilic Ad-PEG to the preformed polyplex, the Ad moiety interacts with the β -cyclodextrin cavities, and consequently the Ad-PEG chains assemble at the outside of the polyplexes (Figure 2.7). This surrounding PEG shell leads to stabilization of the particles, decrease of aggregation at physiological ionic strength,⁹¹ and an increase in cellular uptake of the gene delivery vehicles.⁹²

Next to steric stabilization, the interaction of Ad-PEG to the β -CD-containing polyplex can be used to introduce cell targeting moieties at the shell of the SNPs (Figure 2.7). In general, the NP PEGylation decreases the interaction of the NP surface with the cell. Therefore, targeting moieties are used to bind the delivery vehicles selectively to cell surface receptors and with Ad at one side of the polymer backbone and with galactose, a moiety targeting mediate endocytosis. To introduce targeting moieties, the PEG chains were functionalized with heptacytes, at the other side.⁹¹ This concept was further expanded to transferrin (Tf),^{93,94} a glycoprotein targeting cancer cells with overexpressed Tf receptors. Generally, the composition of the shell can be tuned by the ratio of the two different stoppers (i.e. with and without targeting moiety). In the case of Tf targeting,

however, the content of Ad-PEG-Tf was not allowed to exceed 5 mol%, as intermolecular Tf-Tf interactions led to particle aggregation and destabilization.

Figure 2.7: a) SNP formation by polyplex formation followed by addition of Ad-PEG and Ad-PEG modified with a cell targeting moiety. b) Chemical structures involved in SNP formation.⁹⁵

In vitro and *in vivo* studies have been carried out to evaluate the therapeutic performance of these SNP gene delivery vesicles. Modification of the cationic polymer with imidazole groups resulted in a higher *in vitro* cell transfection efficiency.⁹⁶ Prior to clinical testing in humans, animal studies were carried out in mice⁹⁷ and non-human primates.⁹⁸ Therefore the SNPs were loaded with Gd^{3+} to evaluate the biodistribution within the animal. Comparative studies with SNPs containing only Ad-PEG or a mixture of Ad-PEG and Ad-PEG-Tf showed that the targeting moiety did not change the biodistribution of the particles in mice but increased the cellular uptake in tumor cells markedly.⁹⁷ Clinical trials, carried out in humans having solid cancer, showed the dose-dependent accumulation of SNPs in the tumor tissue which indicates that gene inhibition could be initiated.⁹⁹ Despite the non-immunogenic character of the Ad-PEG-stabilized gene delivery vehicles, studies have shown that the SNPs are rapidly eliminated from circulation. Contrary to normal polyplexes, these SNPs do not disassemble during blood circulation, but the particles accumulate next to the tumor tissue within the glomerular

basement membrane (GMP), a part of the *in vivo* renal filtration barrier.¹⁰⁰ Therein, the positively charged particles are attached to the negative glomerular membrane by electrostatic interactions and SNP disassembly is presumed.

Li and coworkers have reported a similar type of SNPs, in which the monovalent Ad-PEG is used to stabilize and selectively destabilize the DNA and siRNA delivery vehicle. The initial approach uses β -CD-functionalized chitosan to complex DNA and siRNA electrostatically and form stable gene-containing SNPs in the presence of Ad-PEG.¹⁰¹ Modification of this system enabled directed polyplex destabilization in certain cells.¹⁰² Therefore, SNPs were formed by electrostatic and host-guest interactions of β -CD-poly(ethylene imine) (CD-PEI), DNA, and a modified type of Ad-PEG. By including an acid-labile disulfide linkage between the host-binding Ad and the shielding PEG polymer, providing Ad-SS-PEG, significant enhancement of *in vitro* and *in vivo* gene expression was observed. The disulfide bonds break at lower pH values, usually observed within tumor cells. The Ad-SS-PEG provides sufficient stabilization of the polyplexes against extracellular salt or BSA-induced aggregation.

A different strategy for the formation of gene delivery vectors was reported by Thompson and coworkers. As seen in Figure 2.8, polyplexes were formed by Ad-modified poly(vinyl alcohol)-PEG (Ad-PVA-PEG),¹⁰³ monovalent cationic β -CD, and DNA using two different pathways.^{104,105} Either first host-guest interaction of the Ad-PVA-PEG with amino- β -CD followed by inclusion of the DNA, or precomplexation of DNA with CD followed by host-guest interaction with the guest polymer led to stable SNPs in solution. The acid-labile character of the benzylidene acetal-linked Ad groups on the PVA backbone enabled SNP degradation and DNA release in the acidic environment of the targeted cancer cells. By functionalization of the guest polymer with a FRET receptor and labeling of the DNA with a FRET donor, SNP assembly and disassembly could be monitored.¹⁰⁶ The results showed that the SNPs based on the highest host-guest binding affinities had the lowest polydispersity, higher stability, higher cellular uptake and higher transfection efficiency. The preparation method of such non-toxic DNA delivery vectors was further optimized using microfluidic-assisted assembly.¹⁰⁷ Thereby polyplexes were formed using single β -CD-modified poly(ethylene imine) (CD-PEI), Ad-conjugated hyaluronic acid (Ad-HA) and DNA. Contrary to bulk mixing, the microfluidic assembly of the SNPs showed a higher reproducibility and enabled flow-rate dependent fine-tuning of the SNP characteristics. This revealed that the SNP formation was not in thermodynamic equilibrium and the microfluidic technique enabled the formation of well-defined, kinetically trapped structures.

Figure 2.8:a) Formation of a gene delivery vector based on two different formation methods. In method A, amino- β -CD interacts first with multivalent Ad-functionalized PVA-PEG followed by siRNA condensation. In method B, first siRNA condensates with amino- β -CD followed by supramolecular interaction with the Ad-bearing polymer. b) Structure of Ad-modified PVA-PEG and amino- β -CD.¹⁰⁴

Amiel and coworkers used the interaction of a cationic surfactant, a neutral highly β -CD-modified polymer and DNA to prepare another type of gene-delivery SNPs.¹⁰⁸ By supramolecular interactions of n-dodecyltrimethylammonium chloride with the β -CDs grafted to the polymer chain, the hydrophobic tails of the amphiphiles were encapsulated within the cavity of the β -CD, exposing the cationic heads. These multiple cationic charges led to electrostatic interaction with the DNA going along with SNP formation. To investigate the effect of the amphiphilic head group on the stability of the forming DNA-SNPs, n-dodecyltrimethylammonium chloride was replaced by different Ad-modified amphiphiles.¹⁰⁹ Increasing the valency of cationic charges and integration of amide bonds in the hydrophilic tail enhanced the electrostatic interaction and promoted hydrogen bonding with the DNA. This led to an increased stability of the DNA-SNPs and prevented polyplex dissociation under physiological conditions.

2.4.1.2 Multivalent cyclodextrin-stabilized SNPs

Davis and coworkers have reported the formation of SNPs stabilized by multiple supramolecular host-guest interactions of a single polymer strand (Figure 2.9).¹¹⁰ Hereto, a linear, highly water soluble and non-biodegradable polymer containing alternating cyclodextrin and PEG segments was designed.¹¹¹ This polymer was further functionalized with camptothecin (CPT), a drug with anti-cancer activity. Self-assembly of this β -CD- and CPT-functionalized polymer in aqueous solution resulted in SNP formation with particle sizes of 30-40 nm. Due to the stability of the SNPs in water and DMSO and the selective SNP disassembly by addition of a competing guest molecule, the SNP self-assembly was attributed to host-guest complexation. Drug release experiments have shown that the CPT can be cleaved off from the polymer chain while deforming the SNPs. The supramolecular polymer was designed such that the CD-CPT-SNPs have long *in vivo* circulation times, whereas the CD polymer is renally cleared after the elimination of CPT.¹¹² These CPT-SNPs, called CRLX101 (previously IT-101), are currently in clinical trials for the treatment of patients with malignancies.¹¹³ The obtained CPT release kinetics, the overall pharmacokinetics and pharmacodynamic parameters have been correlated to the positive results obtained in animal studies.¹¹⁴ Owing to the flexibility of the polymer, the antiproliferative peptide tubulysin could be attached to the SNPs, which enhanced the maximum tolerable medication dose.¹¹⁵

Figure 2.9: a) Formation of a camptothecin (CPT) delivery vector based on intramolecular multivalent interaction of a single CPT- and CD-functionalized polymer chain. b) Structure of CD-CPT-PEG.¹¹⁴

Lu *et al.* have reported an analogous pathway to prepare CPT and gene co-delivering SNPs.¹¹⁶ Low molecular weight PEI was functionalized with β -CD and CPT, and intermolecular host-guest interactions led to the formation of SNPs with sizes of approx. 180 nm. The cationic nature of the PEI enabled condensation of p53, a tumor suppressor gene. The size of these co-delivering SNPs ranged from 220-400 nm depending on the content of CPT. Based on the hydrophobic character of CPT, the maximum loading of the polymer with CPT was determined to be 5%. Higher grafting densities resulted in poorly water soluble aggregates. *In vivo* studies in mice have shown that tumor growth could be suppressed by co-delivery of CPT and p53.

Supramolecular core-shell SNPs stable in aqueous solution are generally prepared by polymers with different hydrophobicities.¹¹⁷ To further stabilize such SNPs, Wang *et al.* have reported core-shell SNPs based on hydrophobic interactions and multiple inclusion complexes between β -CD and Ad moieties of β -CD-modified linear poly(glycidyl methacrylate) (PGMA) and Ad-containing poly(*tert*-butyl acrylate) (PtBA).¹¹⁸ As Ad-PtBA is relatively hydrophobic whereas CD-PGMA is hydrophilic, hydrophobic interactions led to the formation of core-shell SNPs, with Ad-PtBA assembled in the core and CD-PGMA in the shell. Residual free β -CD moieties in the shell enabled further supramolecular modification of the SNPs by monovalent Ad-containing molecules.

Ma and coworkers have reported the formation of supramolecular core-shell SNPs by multivalent host-guest interactions. They used the more hydrophobic guest polymer poly(β -benzyl L-aspartate) (PBLA) assembled in the core of the SNPs while undergoing multiple host-guest interactions with the hydrophilic host polymer β -CD-polyaspartamide-PEG diblock copolymer (Figure 2.10).^{119,120} In addition to the β -CD moieties also the PEG modification of the β -CD-diblock copolymer promotes the formation of the SNP shell. Size tunability of these SNPs was observed by varying the ratio of guest to host polymer, the composition of the PEG-CD polymer, and by increasing the molecular weight of the hydrophobic polymer backbone.^{119,121} Temperature-responsive SNPs were obtained by replacement of the poly(β -benzyl L-aspartate) with poly(*N*-isopropylacrylamide) (PNIPAM).¹²² The same β -CD-containing diblock copolymer was used as well to encapsulate and release hydrophobic drugs such as pyrene, ibuprofen, indomethacin or dexamethasone upon formation of smaller polymeric aggregates.^{119,123} In contrast, to prepare DNA delivery SNPs, CD-PEI instead of the CD polyaspartamide-PEG diblock copolymer was used in the presence of PBLA to enable electrostatic interactions. The hydrophobic PBLA assembled in the core of the SNPs and was found capable of the inclusion of lipophilic drugs. The hydrophilic CD-PEI assembled at the shell of the SNPs and condensed the DNA.

Figure 2.10: a) Formation of SNPs by multivalent interactions of β -CD and benzyl groups of a host- and a guest-functionalized polymer. The hydrophilic diblock copolymer contains a PEG block which assembles at the shell of the SNPs and a polyaspartamide block carrying the β -CD units which interact with poly(β -benzyl L-aspartate) stabilizing the core. b) Structure of poly(β -benzyl L-aspartate) and β -CD-polyaspartamide-PEG diblock copolymer.

More fundamental studies to understand the formation of multivalently connected SNP assemblies were reported by Couvreur and coworkers.¹²⁴ Here, alkyl chains were grafted onto dextran, and poly- β -CD was prepared by crosslinking β -CD with epichlorohydrin. Mixing these two polymers led to formation of so-called spherical nanogels. As the size of the formed assemblies was initially smaller than 200 nm, these structures could also be called SNPs. The concentration of the formed SNPs appeared to be crucial for their stability, because the probability of interparticle interaction increases with the overall concentration of building blocks. Furthermore the SNPs were not stable above 60°C; at this temperature only few alkyl chains were complexed in the β -CD cavity, and SNP disassembly occurred. In this study, small drugs were entrapped in the available β -CDs. This concept was further extended to a magnetic resonance imaging (MRI) contrast agent by integrating Gd^{3+} in the SNPs.¹²⁵ To this purpose, an Ad-bearing Gd-chelate

complex was synthesized and integrated into the SNPs by host-guest complexation with the free β -CDs.

The effects of the molecular structure and the valency of the host-guest interactions on the assembly and stability were studied by using differently modified dextran polymers. To study the effect of guest functionality and charges on the formation of multivalently interconnected SNPs, Amiel and coworkers synthesized β -CD-functionalized dextran and dextran modified with dodecyl, Ad and cyclohexanecarboxylic acid.¹²⁶ Isothermal titration calorimetry (ITC) experiments showed that, in addition to host-guest interactions of β -CD with the hydrophobic dodecyl and Ad groups, electrostatic interactions were responsible for SNP stabilization. Exclusively electrostatically stabilized SNPs were highly pH responsive, and the polymer-polymer association and dissociation mechanism was correlated to the charge densities of the dextran chains. A minimum number of multivalent interactions is apparently required to form stable SNPs, and the size of the SNPs decreases with the valency of the interaction.¹²⁷ By increasing the substitution degree of the host or guest polymer, a smaller number of polymeric chains was involved in the SNP formation, which caused a decrease in SNP size. Simultaneously, the enhanced amount of intermolecular connections led to an increase in SNP stability, which showed that the SNPs were stabilized by multivalent host-guest interactions.

Tseng and coworkers have reported a highly adaptable multi-component approach for the preparation of SNPs by the molecular recognition of Ad and β -CD moieties. Based on a three-component system consisting of a multivalent β -CD polymer (CD-PEI), Ad-functionalized dendrimer (Ad₈-PAMAM), and Ad-PEG, size-tunable SNPs were formed (Figure 2.11).¹²⁸ The SNPs assemble by multivalent interactions of CD-PEI with Ad₈-PAMAM in the core and an excess of monovalent interactions of CD-PEI with Ad-PEG in the shell. The monovalent hydrophilic polymer enables dispersion of the SNPs in aqueous solution, prevents uncontrolled aggregation, and enables functionalization of the SNP shell. Furthermore, the interplay between multivalent and monovalent Ad- β -CD interactions enables size tuning of the SNPs by varying the composition: the more monovalent Ad-PEG present in the SNP formulation the smaller the observed SNP size. By increasing the ratio of Ad₈-PAMAM to CD-PEI, while keeping the content of Ad-PEG constant, SNP sizes ranging from 30-450 nm were observed. To improve the size distribution of the SNPs, a digital microfluidic droplet generator was used in which the processing parameters are precisely controlled and multiple samples can be prepared in parallel.^{129,130} The high reproducibility and the narrow size distribution of the particles indicate that SNP formation can be kinetically controlled within the microreactor.

Figure 2.11: a) SNP formation mediated by host-guest interactions of Ad₈-PAMAM and Ad-PEG with CD-PEI. The interplay between multivalent vs. monovalent interactions enables size tuning of the SNPs depending on the ratio of guest building blocks. b) Structures of CD-PEI (average number of 10 CDs per polymer chain), Ad-PEG and Ad₈-PAMAM.¹²⁸

The toolbox versatility of the SNP approach reported by Tseng enabled the implementation of different functional molecules to make these SNPs applicable in biomedical diagnosis and therapy. Chemical modification of CD-PEI with DOTA molecules enabled the introduction of ⁶⁴Cu and the paramagnetic Gd³⁺ allowing for application in positron emission tomography¹²⁸ and magnetic resonance imaging,¹³¹ respectively. A collection of gene delivering SNPs with different sizes and tunable RGD ligand coverage were prepared by DNA condensating using the positively charged CD-PEI and RGD-modified Ad-PEG.¹³² For the delivery of intact transcription factors (TFs), pre-complexation of the TF with DNA plasmids containing five matching recognition

sequences was required to obtain an anionic TF-DNA complex which could be included inside the SNPs.¹³³ Electrostatic interactions were used additionally to integrate the negatively charged CPT-modified poly(L-glutamic acid) (CPT-PGA).¹³⁴ The design of CPT-PGA enabled controlled drug release by esterase-mediated hydrolysis under physiological conditions in tumor tissue.

The formulation developed for the formation of soft SNPs was extended to the inclusion of inorganic NPs for preparing optically and magnetothermally responsive SNPs.^{135,136} For photothermally responsive SNPs, Ad-grafted Au-NPs with an average size of 2 nm were prepared. These NPs were included in the SNPs by addition of CD-PEI and Ad-PEG. Similar to the soft SNPs, the size of the Au-SNP clusters is regulated by an equilibrium of monovalent propagation of Ad-PEG and aggregation of the multivalent Ad-Au-NPs. Using Au-SNPs with sizes of 120 nm, generation of microbubbles was observed at a relatively low laser energy. In contrast, no microbubble formation was observed for the individual Ad-Au-NPs at this laser power. The significant enhancement of the photothermal effect in Au-SNPs was attributed to the collective heating effect in the Au-SNPs. To make use of an external alternating magnetic field to selectively disassembly drug-containing SNPs, superparamagnetic $\text{Zn}_{0.4}\text{Fe}_{2.6}\text{O}_4$ NPs were modified with Ad and mixed with Ad₈-PAMAM, Ad-PEG, CD-PEI and doxorubicine (DOX).¹³⁶ *In vivo* mouse experiments showed on demand drug delivery and accelerated tumor suppression upon applying an alternating magnetic field.

Our group has recently reported an SNP study in which the function of the monovalently host-guest interacting Ad-PEG was evaluated in more detail.¹³⁷ SNPs were stabilized by multivalent host-guest interaction of *p-tert*-butylphenyl (TBP) and β -CD using β -CD and TBP-modified poly(isobutyl-*alt*-maleic acid) (PiBMA) (Figure 2.12). In water, the size and stability of the SNPs was independent of the addition of Ad-PEG, which was attributed to electrostatic repulsion of the polymer backbones preventing the addition of more polymer chains to the outer shell of the SNPs. In contrast, while increasing the ionic strength of the SNP medium, the SNPs destabilized and aggregated. This aggregation process was prevented if Ad-PEG was added to the SNP solution, while keeping the host-guest ratio constant. Thus, it can be concluded that the size and stability of SNPs are controlled by a balance of attractive and repulsive supramolecular host-guest interactions, and that the size tunability reported by Tseng and coworkers is only effective if repulsive electrostatic interactions are not dominating.

Figure 2.12: a) Schematic representation of SNP formation from poly(isobutyl-alt-maleic acid) (PiBMA) grafted with β -CD as the host polymer, PiBMA functionalized with *p*-tert-butylphenyl (TBP) as the guest polymer and Ad-PEG. b) Structures of TBP-PiBMA and CD-PiBMA.¹³⁷

Soft hollow microcapsules were formed by host-guest interaction of two electrostatically repulsive polymers.¹³⁸ Using an LbL approach, β -CD and ferrocene-modified poly(allylamine hydrochloride) (PAH) polymers were coated around CaCO_3 particles. To obtain hollow microcapsules, the CaCO_3 core was removed by ethylene diamine tetraacetate (EDTA). The thickness of the microcapsule shell was tuned between 5-50 nm depending on the number of assembly cycles. Based on the underlying electrostatic repulsion and host-guest interactions the formed hollow capsules showed capsule swelling induced by a decrease of pH and by the addition of a host competitor. To implement switching by light, α -CD and azobenzene (Azo) were used as host-guest recognition motif.¹³⁹ By irradiating the capsules with UV light, isomerization of *trans*-Azo into its *cis* isomer was induced and the host-guest complex was destroyed because of the resulting steric repulsion between *cis*-Azo and the CD cavity.

2.4.1.3 Cyclodextrin supramolecular nanoparticles stabilized by hydrophobic interactions

CD-containing SNPs, stabilized by hydrophobic interactions, are mostly based on covalent amphiphiles or non-covalent CD-based amphiphiles called pseudo-amphiphiles. As schematically shown in Figure 2.13, the CD cavity acts as the hydrophilic fragment and either covalently attached aliphatic chains or the guest molecule act as the hydrophobic fragment in CD amphiphiles. In water, the covalent amphiphile and the pseudo-amphiphile can form different supramolecular assemblies based on hydrophobic interactions.

Figure 2.13: Schematic representation of CD-based micelle formation by a) covalently modified CD amphiphiles and b) amphiphiles formed by host–guest inclusion complexation.

Chemical modification of the CD host molecules is not new and the synthesis of supramolecular amphiphiles by grafting one or more hydrophobic groups to the cyclodextrin ring was already reported in 1991.¹⁴⁰ Accordingly, the aggregation behavior of these structures was studied heavily over the past 20 years. Depending on the conformation of the CD-containing amphiphile, supramolecular micelles,^{141–143} supramolecular vesicles¹⁴⁴ or rod-like structure are formed in aqueous medium. These structures were used, for example, as drug delivering vesicles¹⁴⁵ or for the photoresponsive release of entrapped DNA.¹⁴⁶ In contrast, a pseudorotaxane formed by inclusion complexation can act as an amphiphile, if a considerable part of the

hydrophobic tail is exposed outside the cavity. Jiang and Chen summarized the efforts made in the field of CD-containing micelles, including systems requiring host-guest pre-complexation prior to self-assembly in water.^{147,148}

By focusing on responsive pseudorotaxane-based amphiphilic systems, polymer properties and the underlying host-guest mechanism, micelle and vesicle association and dissociation can be controlled selectively. Zeng *et al.* reported the formation of supramolecular micelles by the formation of host-guest complexes between β -CD-poly(4-vinylpyridine) (CD-P4VP30) and Ad-containing PNIPAM (Ad-PNIPAM).¹⁴⁹ The association of the formed amphiphiles leading to the supramolecular structure, was found to be strongly dependent on temperature and pH. At room temperature and pH 4.8, the amphiphilic complexes self-assembled into a vesicle structure under the formation of a supramolecular double layer. A decrease to pH 2 or an increase in temperature led to increase of the solubility of P4VP30 in water and thus transformation of the vesicles into micelles. According to this procedure, Liu *et al.* synthesized β -CD-PNIPAM and Ad-functionalized poly(2-(diethylamino)ethyl methacrylate) (Ad-PDEA), which formed micelles or vesicles depending on temperature and pH of the aqueous solution.¹⁵⁰ Variation of the host-guest motif was used to selectively disassemble micelles formed by host-guest interaction of β -CD-poly(styrene) (CD-PS) and ferrocene-poly(ethylene glycol) (Fc-PEG).¹⁵¹ The inclusion complex of Fc and β -CD is stable but can be easily dissociated by oxidation of Fc. Using this principle, reversible assembly and disassembly of supramolecular vesicles was shown by applying an alternating voltage. Furthermore, drug release profiles were tuned by the value of the oxidizing potential.

2.4.1.4 Cyclodextrin supramolecular nanoparticles stabilized by electrostatic interactions

Single CD molecules bearing cationic charges can be used for the so-called compaction of DNA as cationic polymers and lipids. To this purpose, CDs were modified with positively charged functional groups which form SNPs in the presence of negatively charged DNA.¹⁵² Reineke and coworkers reported the functionalization of β -CD with oligo(ethylene imine) moieties to form β -CD cationic molecules in which the number of charges can be tuned by varying the length of the ethylene imine chain.¹⁵³ Complexation of DNA with the different β -CD cations led to SNPs with an average diameter of 80-130 nm. Cell transfection studies have shown that the best transfection efficiency is obtained if the β -CD is modified with oligo(ethylene imine)s bearing four or five protonated amines per chain. This result shows that multiple charges are required to form stable and effective DNA delivery vectors. Next to amino-functionalized CDs, also guanine-functionalized α -, β - and γ -CDs were used to prepare electrostatically stabilized SNPs.^{154,155} Depending on

the structure of the guanine unit, enhanced SNP cell penetration was reported along with DNA transfection.

DNA and DOX co-delivering SNPs were prepared using PEI modified with multiple β -CD host molecules, a monovalent DOX-grafted Ad guest molecule (Ad-DOX) and DNA.^{156,157} The SNPs were prepared in two steps by host-guest interactions of Ad-DOX with CD-PEI, followed by condensation of the DNA, whereby the electrostatic interactions are the main driving force for stabilization of the SNPs. Although drug polymer conjugates with increased drug loading amounts could be prepared, DNA condensation and SNP formation required sufficient positive charges which are diminished by drug loading. The optimized SNP formulation was subsequently studied in order to monitor the drug and DNA co-delivery. Selective dissociation of the electrostatically stabilized SNPs could be monitored at reduced pH values. DNA release as well as host-guest bound DOX release was shown to be strongly dependent on the pH. *In vivo* experiments in mice indicated that these DOX-DNA-SNPs have a synergistic effect on tumor treatment, showing prolonged chances of survival.

2.4.2 Calix[n]arene and pillar[n]arene supramolecular nanoparticles

Calix[n]arenes are macrocyclic host molecules, which consist of a varying number of phenolic units ($n = 4, 5, 6$ or 8 repeating units). Their structure is composed of a hydrophobic upper and a hydrophilic lower rim which are surrounding the hydrophobic hollow cavity (Figure 2.14). Stabilized by hydrophobic effects, ion-dipole interactions and/or hydrogen bonding, ions or small molecules, such as sugars, amino acids, peptides, hormones, drugs¹⁵⁸ and proteins,¹⁵⁹ can form inclusion complexes with these supramolecular host molecules. A relatively new class of supramolecular structures are pillar[n]arenes which are structurally comparable to calix[n]arenes. The synthesis of the first pillar[n]arene, 1,4-dimethoxypillar[5]arene, was reported in 2008 by Ogoshi *et al.*¹⁶⁰ The hydroxyl functionality of the rims enables simple modification of this host and offers therefore a broad scope of host-guest complexes formed by these hosts.¹⁶¹

Figure 2.14: Molecular structures and cartoon illustrations of calix[n]arenes ($n = 4, 5, 6$) and pillar[5]arene

2.4.2.1 Multivalent calix[n]arene/pillar[n]arene-stabilized supramolecular nanoparticles

Recently, Xu and coworkers have reported the formation of SNPs for photodynamic therapy by multiple calix[n]arene and methyl viologen (MV) host-guest interactions.¹⁶² Tetrameric calix[4]arene porphyrin and three different MV guest molecules were synthesized and used to study the selective self-assembly. As schematically shown in Figure 2.15, the tetravalent host molecule forms SNPs in the presence of the divalent MV guest molecule by oligomerization of the building blocks initiated by supramolecular host-guest interaction. To induce SNP formation, the host and guest components were mixed in a 1:4 stoichiometry which led to an overall excess of guest molecules in the system.

Figure 2.15: a) Schematic representation of the formation of spherical SNPs by host-guest interaction of a tetravalent calix[4]arene-functionalized porphyrin derivative with different bis-viologen guest molecules. b) Structure of the tetra-calix[4]arene-substituted porphyrin and the divalent viologen derivatives with different lengths.¹⁶²

Irrespective of the stoichiometry and length of the MV guest molecule, size tunability of the SNPs was observed by using different concentrations of both host and guest components. In contrast to previously reported studies carried out with a pillar[5]arene trimer and bis-viologen,¹⁶³ the morphology of the self-assembled structure did not vary by changing the concentration. The covalent integration of porphyrin into the host bearing molecule enabled the application of these SNPs in photodynamic therapy. Generally, porphyrins strongly absorb light which is converted into heat in the illuminated areas. In *in vitro* cell internalization studies using HeLa cells, the SNPs showed a stronger photo-response than the free porphyrin derivative. Their increased cytotoxicity makes these SNPs a promising candidate for photodynamic therapy.

2.4.2.2 Calix[n]arene and pillar[n]arene supramolecular nanoparticles stabilized by hydrophobic interactions

The formation of amphiphilic structures by covalently modified calix[n]arenes is well known.¹⁶⁴⁻¹⁶⁶ For example, basic amphiphilic calix[n]arenes which self-aggregate in water into micelles were synthesized by the modification of the lower calix[n]arene rim with four undecyl chains and the upper rim with di- or oligosaccharides.¹⁶⁷ Also pillar[5]arene was covalently modified with amino groups at the top and alkyl groups at the bottom to form amphiphilic molecules that self-assemble in water into vesicles, sheets and microtubes.¹⁶⁸ The use of host-guest chemistry enabled thus an enhanced complexity and various applications of amphiphilic calix[n]arene-based SNPs, micelles and vesicles.

Amphiphilic supramolecular complexes were formed by host-guest interaction of the porphyrin-type photosensitizer Chlorin *e*₆ with PEG-modified calix[4]arene.¹⁶⁹ The formation of these host-guest-based amphiphilic structures is illustrated in Figure 2.16. After initial host-guest complexation, the amphiphilic complexes self-assemble into micelles with a size of 140 nm by hydrophobic interaction. The PEG chains of the modified calix[4]arene ensured water solubility and low toxicity of the micelles which was evaluated by a cell viability assay on HeLa cells. Furthermore, *in vitro* tests showed the successful uptake of supramolecular micelles by HeLa cells. Cell proliferation could be inhibited by photoirradiation, which makes these supramolecular micelles applicable as drug delivery vehicles for photodynamic therapy.

Figure 2.16: a) Schematic representation of the formation of supramolecular micelles by host-guest complexation of PEG-modified calix[4]arene and the porphyrin photosensitizer Chlorin e₆ followed by micelle formation driven by hydrophobic interactions between the formed supramolecular amphiphiles. b) Chemical structures of PEG-modified calix[4]arene and Chlorin e₆.

As described for CD-containing amphiphiles, host-guest chemistry enables the modification of calixarene-based supramolecular structures with stimuli-responsive properties. Different systems have been reported in the literature in which the disassembly of amphiphilic micelles and vesicles has been induced by enzymes^{170,171} or a change in temperature.¹⁷² More interestingly, multi-responsive, drug-encapsulated supramolecular calix[4]arene vesicles were reported by Wang *et al.*¹⁷³ The formation of these vesicles was induced by the host-guest interaction of *p*-sulfonatocalix[4]arene and decyl-modified viologen. An increase in temperature led to vesicle rupture as the encapsulation of viologen in sulfonatocalix[4]arene and the *π-π* stacking weaken upon heating. In contrast, the reduction of the viologen guest to its radical cation did not lead to disassembly of the vesicles but only to a decrease in vesicle size from 300 to 150 nm. The supramolecular host-guest interaction still persisted, whereas the weakened electrostatic repulsion led to a higher curvature and a concomitant decrease of the vesicle size. Upon a two-step reduction of the viologen unit to the neutral form, the host-guest interaction was completely destroyed and the vesicles fell apart. The same behavior was observed when an excess of calix[4]arene host or a competing α-, β-, or γ-CD host molecule was added to the system. The CD hosts interacted with the aliphatic chains of the viologen species, which blocked the hydrophobic interaction, essential for vesicle stabilization. This supramolecular approach shows potential for the formation of drug and dye-encapsulating vesicles, for which the release of the encapsulated molecules can be triggered by external stimuli.

The formation of supramolecular vesicles and fibers based on amphiphilic molecules composed of two different hosts was already reported in 1999.¹⁷⁴ Recently this concept was further developed to the formation of drug-binding core-shell SNPs based on strong hydrophobic interactions.¹⁷⁵ Hereto, an amphiphilic heterodimer with a hydrophobic calix[4]arene and a hydrophilic β -CD moiety was synthesized. In aqueous solution, the formation of the SNPs is driven by the strong hydrophobic interaction between the multiple carbon chains attached to the calix[4]arene fragment. The SNPs presented the hydrophilic β -CDs at the SNP shell, which was used for targeting by the interaction with a sugar-modified Ad guest molecule. NP precipitation, caused by a change of solvent, was used to form drug-loaded SNPs of the heterodimer in the presence of the antimitotic agent docetaxel (DXT). Drug release profiles were shown to depend on the length of the hydrophilic alkyl chain grafted onto the calix[4]arene moiety. As DXT was assembled in the SNPs by two different interactions, initial fast release was observed for drugs encapsulated in the shell by host-guest interactions, whereas core-encapsulated molecules were released more slowly.

Zhao and coworkers reported the formation of pillar[5]arene assemblies mediated by hydrophobic interactions of different amphiphilic pillararenes in aqueous solution.¹⁷⁶ Tadpole- and bola-like pillar[n]arenes were functionalized with hydrophilic ethylene glycol and hydrophobic alkyl groups. Depending on the pillar[n]arene design, the amphiphilic supramolecular hosts assembled in the absence or presence of a supramolecular guest molecule. Interestingly, the structure of the amphiphile also determined the encapsulation efficiency of different dyes and DOX in these vesicles. For example, all supramolecular assemblies could encapsulate rhodamine B, whereas only the tadpole-like amphiphilic pillar[n]arene-based assemblies included and delivered the hydrophobic dye fluorescein isothiocyanate to cells *in vitro*. To prove that these assemblies can be used for drug delivery applications, DOX was encapsulated and delivered to cells *in vitro*. Apparently, the structure of the amphiphilic pillar[n]arene has a strong impact on the successful formation and performance of supramolecular assemblies for bioimaging and drug delivery.

Recently, the formation of supramolecular amphiphiles stabilized by host-guest interaction of pillar[5]arene with an imidazole-containing rod-coil molecule was reported.¹⁷⁷ Due to electronic de-coupling of the quinquephenyl aromatic rings in the divalent imidazole-containing guest molecule, the fluorescence intensity increased drastically upon supramolecular recognition. Due to the stimulus-responsive character of the host-guest recognition motif, vesicle disassembly could be induced in three ways: by addition of the supramolecular competitor paraquat, by a decrease in pH, or by addition of silver oxide.¹⁷⁸

2.4.2.3 Calix[n]arene supramolecular nanoparticles stabilized by electrostatic interactions

The groups of Casnati and Ungaro have reported the synthesis of a series of guanidinium modified calix[n]arenes for the formation of SNPs in the presence of DNA, driven by hydrophobic and electrostatic interactions.^{179,180} Different calix[n]arene derivatives were synthesized and their DNA condensation and cell transfection efficiencies were tested depending on the calix[n]arene lipophilicity, size, and conformation. AFM studies showed that upper rim guanidinium-functionalized calix[4]arenes, functionalized with lipophilic hexyl or octyl chains at the lower rim, gave DNA condensation and promoted cell infection.¹⁸⁰ This process is driven by electrostatic interaction of the DNA with the guanidinium units and hydrophobic interaction of the lipophilic tails bound to the calix[4]arene. In contrast, larger guanidinium-calix[n]arene homologues with methoxy functionalization at the lower rim did not result in cell transfection, as these host molecules did not successfully compact the DNA. Only loosely self-assembled structures were formed by electrostatic interaction of the guanidinium groups with DNA filaments. Interestingly, the functionalization of the lower rim of the calix[4]arene with guanidinium instead of the upper rim yielded non-viral vectors with lower toxicity and high transfection efficiency.¹⁸¹ More recently, it was shown that DNA condensation, cell transfection and toxicity also depend on the modification of the upper rim of the guanidinium calix[4]arene.¹⁸² Recently, the same group has shown that a calix[4]arene modified with four arginines at the upper rim and four lipophilic tails at the lower rim enables high DNA condensation efficiency. Moreover, the enhanced transfection efficiency of these SNPs is caused by the host modification with a cell penetrating peptide.¹⁸³

A combination of hydrophobic and electrostatic interactions was used to induce the assembly of calix[4]arene-based, virus-sized DNA NPs.¹⁸⁴ Calix[4]arene was modified with alkyl chains at the lower rim and cationic groups at the upper rim. Due to hydrophobic interactions these amphiphilic host molecules assembled in water into very small micelles with an average diameter of 6 nm. Upon addition of DNA to these micelles, larger DNA-micelle complexes of approximately 50 nm were formed by electrostatic interactions of the DNA with multiple cationic groups at the micelle shells. These SNPs showed gene transfection efficiency in cell cultures and low cytotoxicity. As a control, a poor gene delivery composite was obtained for an amphiphilic calix[4]arene modified with short alkyl chains. These structures did not form micelles in water and consequently were not capable of condensing DNA since multivalent electrostatic interactions could not be formed.

2.4.3 Cucurbit[n]uril supramolecular nanoparticles

Relatively recently, the cucurbit[n]uril family (Figure 2.17) has attracted a lot of attention owing to its exceptional molecular recognition properties. The high binding affinities and the tunable association and dissociation properties make them of high interest for responsive SNP systems. Cucurbit[n]urils are pumpkin-shaped macropolycyclic compounds composed of *n*-glycouril units interconnected with methylene bridges. Their hydrophobic interior is surrounded by two equally sized, carbonyl-laced portals, which allows the inclusion of hydrophobic molecules, and of charged molecules such as various alkyl- and aryl-ammonium ions. In the series of CB[5] to CB[10], the internal cavity size increases from 4.4 Å to 10.7–12.6 Å as the cavity entrances increase from 2.4 Å to 9.0–11.0 Å.^{185,186} The host-guest chemistry of the different homologues, as reviewed by the groups of Kim and Isaacs,^{185,186} is strongly dependent on their internal cavity size. Whereas CB[6] forms stable inclusion complexes with a protonated aminoalkane, CB[8] and CB[10] are capable of including two guest molecules simultaneously, either in a homotopic (e.g. an amino acid) or heterotopic fashion. In the latter case, typically an electron-poor and electron-rich guest are co-included as a charge-transfer complex.¹⁸⁷ Due to its diversity, the cucurbit[n]uril family has emerged as a smart tool kit for the formation of supramolecular architectures in solution.¹⁸⁸

Figure 2.17: Molecular structures of cucurbit[n]urils ($n = 6, 7, 8, 10$)

2.4.3.1 Multivalent cucurbit[n]uril-stabilized supramolecular nanoparticles

SNPs based on CB[8] crosslinked single polymer chains were reported by the group of Scherman.¹⁸⁹ A polymer was functionalized with both naphthol (Np) and MV guest moieties, and the formation of multiple, intramolecular, heteroternary charge-transfer complexes induced by CB[8] led to the collapse of individual polymer molecules into NPs in aqueous solution (Figure 2.18). The size of the resulting SNPs was shown to depend on the molecular weight of the polymer and on the extent of intramolecular host-guest complexation. Similar behavior was shown for a polymer solely functionalized with MV in the presence of *nor-seco* CB[10].¹⁹⁰ This macrocycle contains two identical cavities and forms 2:1 homo-ternary complexes with two MV guest molecules, which lead to polymer folding going along with SNP assembly.

Figure 2.18: a) Single polymer chain SNPs based on multivalent ternary complexation of methyl viologen (MV) and naphthol (Np) moieties with cucurbit[8]uril. b) Polymer structure of methylviologen and naphthol functionalized poly(N-hydroxyethyl acrylamide).¹⁸⁹

2.4.3.2 Cucurbit[n]uril supramolecular nanoparticles stabilized by hydrophobic interactions

In 2006, Kim and coworkers reported the formation of vesicles using supramolecular complexes of CB[6].¹⁹¹ This required the covalent modification of (allyloxy)₁₂CB[6] with 2-(2-(2-methoxyethoxy)ethoxy)ethanethiol to form an amphiphilic host. In general, chemical modification of CB is not trivial, and heavy synthetic and purification efforts are required to obtain pure CB[n] homologues with functional groups at the periphery.¹⁹² Sonication of the modified CB[6] in aqueous environment led to vesicles of 30-1000 nm, as observed by light scattering and TEM. More monodisperse SNPs were prepared by chemical cross-linking of (allyloxy)₁₂CB[6] with 6-mercaptohexanol¹⁹³ and dithiols^{194,195} followed by sonication induced vesicle formation. The size of the covalently bound nanocapsules depended on the reaction medium and the length of the cross-linker. For

example, capsules prepared in methanol ranged between 50-110 nm depending on the length of the dithiol. These types of responsive nanocapsules have high potential in biomedical applications, as the hollow core and the supramolecular shell enables encapsulation of active compounds and decoration with different cell receptors, respectively.¹⁹⁶⁻¹⁹⁸

Zhou and coworkers have reported the formation of supramolecular vesicles by host-guest complexation of CB[6] and *N,N'*-hexamethylene-bis(1-octyl-4-carbamoyl pyridinium bromide) (HBPB-8) in water.¹⁹⁹ After host-guest complexation, the complexes formed vesicles, in which the hydrophilic CB[6] host molecules face the aqueous domains, whereas the hydrophobic tails of the pseudorotaxane are directed into the bilayer. Using a modified guest molecule and CB[7] to form a pseudorotaxane, supramolecular aggregate transformation from micelles to large vesicles was induced by subsequent addition of CB[6].²⁰⁰

The formation of CB-based supramolecular amphiphiles mediated by the ternary host-guest-guest interaction of CB[8], 2,6-dihydroxynaphthalene and alkylated MV was reported in 2002.²⁰¹ Initiated by the heteroternary complexation, the formation of supramolecular vesicles was induced by hydrophobic interactions between the alkyl chains. In the absence of CB[8] and 2,6-dihydroxynaphthalene, micelle formation of the MV derivative was observed. The structure of the MV derivative strongly affected the appearance of the host-guest stabilized vesicles. Relatively monodisperse vesicles of 20 nm were formed by MV functionalized with dodecyl, whereas vesicles ranging in size from 20 nm to 1.2 μm were observed for structures formed by MV-hexadecyl in the presence of CB[8] and 2,6-dihydroxynaphthalene.

Using the same CB-induced charge-transfer motif, Scherman and coworkers reported the formation of supramolecular block copolymers, which self-assemble in water into larger supramolecular aggregates.^{202,203} MV-modified poly(*N*-isopropylacrylamide) (PNIPAAm) and Np-modified poly(dimethylaminoethylmethacrylate) (PDMAEMA), provided thermo- and pH-responsive supramolecular guest molecules, respectively, which gave supramolecular micelles upon addition of CB[8].²⁰³ The hydrophobic core of the aggregates enabled the encapsulation of DOX. Triggered release of the anti-cancer drug was enabled upon addition of the competing Ad guest molecule, a decrease in temperature or a decrease in pH, thus inhibiting the proliferation of HeLa cells.

Peptide amphiphiles were formed using the ternary complex of pyrene, MV, and CB[8] as the interaction motif.^{204,205} Supramolecular peptide vesicles with sizes of about 200 nm were formed from a pyrene-imidazole conjugated peptide and CB[8] in the presence of MV-octadecyl²⁰⁴ or MV-PNIPAAm.²⁰⁵ Next to guest integration, the functionalization of the

hydrophilic peptide with pyrene-imidazole enabled the integration of a fluorescent marker which allowed visualization of the formation and rupture of the vesicles. In contrast to the vesicles formed by MV-octadecyl, the use of MV-PNIPAAm required heating to 37°C to render the polymer hydrophobic. Selective dissociation of the vesicles was achieved by addition of competing guest molecules, naphthalene and adamantantylamine. The peptide vesicle formed by MV-PNIPAAm enabled the encapsulation of basic fibroblast growth, which protected the protein against denaturation.

2.4.3.3 Cucurbit[n]uril supramolecular nanoparticles stabilized by electrostatic interactions

Kim, Park and coworkers have reported the formation of non-toxic self-assembled gene delivery vehicles based on electrostatic interactions and CB[6] inclusion chemistry.²⁰⁶ Hereto, different generations of cationic diaminobutane (DAB)-grafted poly(propyleneimine) dendrimers were synthesized. Two DAB head groups constitute a homoternary inclusion complex with CB[6]. The cationic character of the dendrimer led to polyplex formation in the presence of DNA. The size of the observed polyplexes depended strongly on the addition of the supramolecular host components. In the absence of CB[6], polyplexes of 160 nm were observed for the DAB dendrimer with of DNA. By addition of CB[6], the size of the electrostatically stabilized SNPs increased from 300 to 420 nm, depending on the amount of CB[6] inclusion cross-links. Furthermore, molecular dynamic simulation showed that the host-guest stabilized SNPs have a more rigid shell than the polyplexes.

2.5 Conclusions

Supramolecular nanoparticles represent a highly attractive platform for the formation of stable, yet reversible nanovectors. Induced by different non-covalent interactions, a broad variety of self-assembled structures has been reported in the literature over the last 20 years. The core and shell of the formed SNPs were modified to allow a variety of functional molecules to be integrated for biomolecular recognition, imaging, drug delivery, and photothermal treatment. The chemical flexibility of SNPs enables selective decoration of the particle shell by cell targeting receptors and the integration of responsive supramolecular clues for triggered SNP disassembly. Full realization of their potential requires better understanding of their assembly thermodynamics and assembly/disassembly mechanisms. Apart from their constituting supramolecular interaction motif, other structure determining parameters often remain obscure. Hence, understanding the formation, stability and disassembly of SNPs requires more efforts and will contribute to the design of functional and biomedically relevant nanodevices.

2.6 References

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Chapter 3

Size-Tunable Supramolecular Nanoparticles Mediated by Ternary Cucurbit[8]uril Host–Guest Interactions

Supramolecular nanoparticles (SNPs) stabilized by host-guest interactions are of great interest because of their potential for biomedical applications. The most prominent examples of SNPs in the literature use β -cyclodextrin-adamantyl host-guest interactions as the interaction motif. In this chapter, we show that cucurbit[8]uril (CB[8])-assisted charge-transfer complexes between naphthol (Np) and methyl viologen (MV) can be used for the formation of size-controlled SNPs. DLS and SEM analyses show that size control of the particles is achieved by tuning the ratio of the multivalent and monovalent Np derivatives. The formation of these size-tunable SNPs appear to be strongly time and temperature dependent, despite the fast ternary complex formation observed at all temperatures employed. This indicates that SNP formation requires dynamic disassembly and reassembly of the constituting ternary complex units.

3.1 Introduction

Supramolecular chemistry promises the assembly of molecular units into well-defined architectures, via specific non-covalent interactions.¹⁻³ Nanoparticles (NPs) possess unique size-dependent properties, that are not observable in bulk materials.⁴ The combination of both features is attractive, since stable, yet reversible hybrid assemblies hold high promises for biomedical applications.⁵⁻⁷ External triggers can induce the selective disassembly of supramolecular systems, which leads to responsive drug delivery formulations.^{8,9}

Supramolecular nanoparticles (SNPs) can be considered as particles in which multiple copies of different building blocks are brought together by specific noncovalent interactions, giving assemblies that are typically larger than the building blocks themselves. The most well studied SNP, a siRNA delivery system, has been developed by the group of Davis,^{10,11} in which DNA delivery vectors were formed owing to electrostatic interactions between a β -cyclodextrin (CD)-containing polycation in the presence of negatively charged DNA. Stabilization of these relatively weakly bound particles was achieved by supramolecular host-guest interactions of the CD-containing polycation with a monovalent adamantyl (Ad)-grafted poly(ethylene glycol).¹² In contrast, NP formation exclusively based on supramolecular CD-Ad interactions has been demonstrated by the group of Tseng, whereby the size of the formed NPs could be altered by varying the ratio of the monovalent and multivalent Ad derivatives.¹³

Although multivalency plays an important role in the dynamic molecular assembly and disassembly processes,¹⁴⁻¹⁶ the mechanism of the formation and size control of the SNPs remains elusive. Beyond the Ad-CD binding motif, a variety of molecular recognition units are available in supramolecular chemistry, however, none have been used for the preparation of size-tunable SNPs. Thus, a fundamental question arises whether SNPs can be formed using different interaction motifs. Additionally, the expected changes in interaction strength and exchange rate may have a profound influence on the assembly and disassembly process of the SNPs.

Cucurbit[n]urils (CB[n]s) constitute a class of macrocyclic molecules that form inclusion complexes through hydrogen bonds and by hydrophobic and ion-dipole interactions,^{17,18} e.g. when using positively charged guests such as methyl viologen (MV). Moreover, MV and naphthol (Np) can form a charge-transfer (CT) complex, which is included in the cavity of CB[8] to give a ternary complex with a micromolar affinity.¹⁹ Utilizing this or similar ternary supramolecular motifs, different nanoparticulate^{20,21} and microstructures²² have been reported by the groups of Scherman and Abell. In NP studies,^{20,21} however, the CBs have been used merely to crosslink intramolecularly connected guest moieties attached to a single polymer chain, thus smaller NPs are provided due to the collapse of

the starting guest-modified polymer. The strong binding affinity of this interaction motif, combined with a radically different kinetics compared to CD complexes, make it an interesting candidate to explore for its potential use for the formation of SNPs and to study their assembly kinetics.

Here, we report a novel strategy for the preparation of size-tunable SNPs in water using CB[8] as an intermolecular host for MV and Np-containing building blocks, under the formation of a host-guest-assisted CT complex. Scheme 3.1 shows a schematic overview of the proposed SNP assembly. The host-guest recognition between MV-functionalized poly(ethylene imine) (MV-PEI), Np-functionalized poly(amidoamine) dendrimer (Np₈-PAMAM) and Np-functionalized poly(ethylene glycol) (Np-PEG; $M_w \sim 5000$ g/mol) in the presence of CB[8] leads to SNPs composed of four building blocks and held together by multiple ternary complexes.

Scheme 3.1: a) Supramolecular nanoparticle (SNP) formation by heteroternary complex formation between cucurbit[8]uril (CB[8]), methyl viologen (MV) and naphthol (Np) moieties. b) The supramolecular building blocks involved in particle formation: methyl viologen-poly(ethylene imine) (MV-PEI), naphthol-poly(ethylene glycol) (Np-PEG), cucurbit[8]uril (CB[8]), and naphthol₈-poly(amidoamine) (Np₈-PAMAM). c) Ternary complex formation by inclusion of MV in CB[8], followed by inclusion of Np.

3.2 Results and Discussion

3.2.1 Synthesis of the supramolecular building blocks

The multivalent building blocks required for the formation of the SNPs are shown in Scheme 3.2. The Np-functionalized PAMAM G1 dendrimer was synthesized by first activating the terminating carboxylic acid group of 2-naphthol-1-hexanoate²³ with pentafluorophenol. Subsequent reaction of 2-naphthol-1-hexanoic acid pentafluorophenol ester with PAMAM dendrimer G1 in CH₂Cl₂ followed by repeated precipitation in diethyl ether led to Np₈-PAMAM. To prepare MV-PEI, poly(ethylene imine) (PEI, $M_w \sim 10000$ g/mol) was dissolved in dry DMSO and a solution of maleimido-hexanoic acid *N*-hydroxy succinimide ester was added slowly. Reaction with 1-(1-undecyl-11-thiol)-1-methyl-4,4'-bipyridinium²⁴ in water gave the polymer MV-PEI.

Scheme 3.2: Synthetic routes towards the multivalent building blocks Np₈-PAMAM (a) and MV-PEI (b) used in this study.

Characterization of the multivalent Np₈-PAMAM and the monovalent Np-PEG showed that the reactive binding sites of the parent amino-terminated dendrimer and PEG were fully functionalized with Np guest moieties. The degree of functionalization of the multivalent MV-PEI polymer (~ 10 kDa) was evaluated by UV/Vis spectroscopy, microcalorimetry and ¹H-NMR, and all techniques showed each polymer chain to be functionalized with, on average, four MV guest moieties.

3.2.2 Supramolecular nanoparticle formation in aqueous solution

Upon addition of aqueous MV-PEI to a previously prepared aqueous solution of CB[8], Np-PEG and Np₈-PAMAM, SNPs were formed using a 0.67 μ M concentration of each of the three molecular recognition groups (CB[8], MV and Np). The concentrations of Np-PEG and Np₈-PAMAM were varied in different ratios, while keeping the total Np concentration

constant, in order to study the influence of the multivalent-monovalent competition on the SNP size. Thereby, the resulting intrinsic ratio of the three functional host-guest recognition units CB[8]:MV:Np was kept constant and in a 1:1:1 ratio in accordance with the stoichiometry of the basic ternary complex motif. After 2 days, particle formation was confirmed by dynamic light scattering (DLS) and scanning electron microscopy (SEM). As visible in Figure 3.1a, utilizing 20% Np derived from Np₈-PAMAM, SNPs with an average hydrodynamic diameter of 85 ± 15 nm were observed by DLS. In good agreement, SEM (Figure 3.1b) showed particles with an average diameter of 74 ± 13 nm.

Figure 3.1: Characterization of CB-mediated SNPs prepared using CB[8]:MV:Np = 1:1:1, with 20% Np derived from Np₈-PAMAM: a) DLS data and b) SEM image.

To evaluate whether the SNP formation is based on the selective host-guest interaction of the different supramolecular building blocks, the formation of the CT complex was studied. An increase in UV/Vis absorbance (Figure 3.2a) as well as a decrease in fluorescence intensity (Figure 3.2b) indicates that the SNPs are assembled in aqueous solution as a result of formation of a ternary supramolecular complex. Congruently, no SNPs were formed in the absence of MV-PEI or CB[8], or upon addition of CB[7] instead of CB[8]. The dicationic MV moiety is required to enable inclusion of the neutral Np inside the cavity of the CB[8] host, hence no CT complex is formed in the absence of MV. Additionally, intermolecular crosslinking between the MV and Np derivatives cannot occur in the presence of CB[7]; its cavity is too small to host both guest moieties.

Figure 3.2: a) UV/Vis absorption spectra of an SNP solution in the presence of CB[8] (solid line), an analogous sample in the absence of CB[8] (dashed line) and solely CB[8] (dotted line). b) Fluorescence spectra of the SNP formulation before (dashed line) and after addition of CB[8] (solid line).

Remarkably, a fluorescence decrease was observed directly after mixing the different supramolecular building blocks at room temperature, whereas consistent SNP formation is only detectable after 48 h. This led to the presumption that the supramolecular host-guest interactions, forming the CB[8]-assisted ternary complexes, are established immediately after mixing the components. Initially, undefined, kinetically trapped structures are formed which slowly reassemble into stable SNPs by exchange of the guest moieties via (slow) dissociation and (fast) reassociation. In order to monitor the assembly behavior of the supramolecular building blocks in more detail, time-dependent DLS measurements were carried out at different temperatures. As seen in Figure 3.3, the apparent sizes observed by DLS using SNP formulations containing 25% Np, varied within time, and the extent of variations in SNP size depended on the temperature. At room temperature, the observed amplitude of the SNP size variation was very high and the measurements were not consistent. In contrast, SNP assembly occurred faster at elevated temperatures. Samples kept at 40°C showed distinct particle formation and consistent DLS measurements already 2 h after sample preparation, and a stable SNP size (96 ± 14 nm) was evident after 10 h.

Figure 3.3: a) Hydrodynamic diameters of SNPs, consisting of 25% Np from Np₈-PAMAM measured by time-dependent DLS as a function of temperature (data averaged over four measurements) and b) corresponding standard deviations.

The temperature-dependent particle formation is ascribed to the dynamic character of the supramolecular host-guest binding. After initial rapid ternary complex formation, disassembly and reassembly of the four building blocks is required for well-defined particle formation. At elevated temperatures this rearrangement is enhanced which leads to faster SNP formation. Such a time-dependent particle formation has so far not been reported for SNPs based on CD-Ad host-guest interactions. In all cases reported, SNP formation was observed directly after mixing the supramolecular components. We attribute the difference in dynamics to the stronger and more slowly exchanging complexes of CB vs. CD.

An important advantage of the use of supramolecular interactions for the formation of SNPs is their responsive character. The triggered disassembly of the SNPs reported here has been induced by the reductant $\text{Na}_2\text{S}_2\text{O}_4$ (Figure 3.4). After addition of the reducing agent, initially observed NPs could not be detected anymore by DLS and SEM. Apparently, the reduction of the MV moieties led to loss of affinity for the CB[8] cavity and concomitantly to dissociation of the SNPs.

Figure 3.4: DLS analysis of SNP sample prepared with 25% Np from Np_8 -PAMAM a) after assembly and b) after subsequent addition of $\text{Na}_2\text{S}_2\text{O}_4$.

3.2.3 Size tunability of supramolecular nanoparticles

SNP size control was achieved by varying the ratio of monovalent Np-PEG to multivalent Np_8 -PAMAM, while keeping the overall Np concentration constant and maintaining an equimolar CB[8]:MV:Np stoichiometry. SNPs were observed for all samples as shown by SEM and DLS (Figure 3.4a-g) and the size of the observed SNPs strongly depend on the origin of the Np derivative. In particular, by increasing the amount of Np derived from Np_8 -PAMAM from 10% to 35%, an increase in particle size from 57 ± 11 nm to 115 ± 13 nm and from 51 ± 13 nm to 137 ± 16 nm was observed by SEM and DLS, respectively. As shown in Figure 3.4h, the observed SNP size exhibits an apparent linear relationship to the relative amount of Np derived from Np_8 -PAMAM. For samples containing 50% or more of Np derived from Np_8 -PAMAM, DLS showed a hydrodynamic diameter beyond 1000 nm, as well as precipitation.

In general, the formation of SNPs is established by multivalent interactions in the core and monovalent interactions of Np-PEG at the outer surface of the particles. Thereby an equilibrium between capping and crosslinking of Np building blocks is established in time, as indicated by the time-dependent measurements. Stable particle formation requires termination of the multivalent interactions that are established between Np₈-PAMAM and MV-PEI in the presence of CB[8]. The results obtained for the size dependence show that more than 50% monovalent Np-PEG is required to stabilize the NPs. Distinct SNP size control is achieved by fine-tuning the balance of multivalent vs. monovalent interactions. As expected, increasing the concentration of the core forming multivalent dendrimer leads to the formation of larger SNPs.

Figure 3.4: Size determination of SNPs prepared with different formulations: SEM images (a-f) of the resulting SNPs, as a function of the Np content derived from Np₈-PAMAM dendrimer (a: 10%, b: 20%, c: 25%, d: 30%, e: 35%, f: 50%) used during supramolecular assembly (using CB[8]:MV:Np = 1:1:1, with Np coming from Np₈-PAMAM and Np-PEG). g) DLS curves for the different SNP formulations. h) SNP diameter by DLS (▲) and hrSEM (●).

3.3 Conclusions

In conclusion, we have developed a novel, versatile strategy for the preparation of SNPs, in which CB[8] acts as an intermolecular connector between MV-PEI, Np₈-PAMAM and Np-PEG upon formation of a ternary supramolecular CT-complex. Of particular importance are the observed assembly kinetics and the size-focusing in time which indicates progression towards thermodynamic equilibrium. The distinct size tunability as well as the stimulus-responsive particle disassembly make such NPs a potential candidate for biomedical application in drug delivery, peptide therapeutics as well as *in vivo* sensing.

3.4 Acknowledgements

Mark Smithers is acknowledged for the SEM images.

3.5 Experimental Section

3.5.1 Materials

Starting materials for organic synthesis were obtained from Sigma-Aldrich and used as received. Highly branched poly(ethylene imine) (PEI, $M_w = 10$ kDa) was purchased from Polysciences Inc. and contained primary, secondary, and tertiary amine groups in a ratio of 25/50/25. 1-(1-Undecyl-11-thiol)-1-methyl-4,4-bipyridinium and 2-naphthoxy-terminated poly(ethylene glycol) monomethyl ether were prepared according to literature procedures.^{24,25} Cucurbit[8]uril (CB[8]) was purchased from Strem Chemicals and its purity was assessed by microcalorimetric titration against paraquat. Deuterated solvents used for NMR spectroscopy were purchased from Cambridge Isotope and the water used for dialysis and SNP preparation was of MilliQ quality (Millipore, $R = 18.2$ M Ω cm⁻¹).

3.5.2 Synthetic procedures

Perfluorophenyl 6-(naphthalen-2-yloxy)hexanoate

Carboxylic acid activation of 2-naphthol-1-hexanoic acid was carried out by dissolving 2-naphthol-1-hexanoic acid (0.5 g, 2.05 mmol) in 230 mL THF. Afterwards pentafluorophenol

(0.509 g, 2.77 mmol) and DCC (0.570 g, 2.77 mmol) were added and the solution subsequently stirred for 2 days at RT. THF was removed in vacuum and the residue dissolved in CH₂Cl₂ and washed three times with saturated NaHCO₃ solution. The organic layer was dried with MgSO₄ and the solvent evaporated before the 2-naphthol-1-hexanoic acid pentafluorophenol ester was purified by flash column chromatography over silica gel with CH₂Cl₂ as eluent (RF=0.83). Yield: 0.198 g (25%). ¹H-NMR (400 MHz, CDCl₃): δ

(ppm) 7.78-7.70 (m, 3H), 7.46-7.29 (m, 2H), 7.21-7.13 (m, 2H), 4.15 (t, 2H), 2.80 (t, 2H), 2.01 (m, 4H). Mass: (ESI) calculated: 410.1 (M), found: 411.3 (M+H)⁺.

Octa-(E)-5-naphthol-pentanoyl-poly(amidoamine)-dendrimer (Generation 1) (Np₈-PAMAM)

To prepare the naphthol-terminated PAMAM dendrimer, 125 mg 20 wt% solution of PAMAM dendrimer in methanol, ethylenediamine core, generation 1.0 solution (0.017 mmol) was weighed and the MeOH was evaporated. The

residue was redissolved in 40 mL CH₂Cl₂ and 2-naphthol-1-hexanoic acid pentafluorophenol ester (0.074 g, 0.178 mmol) was added. The solution was stirred for 24 h at RT under nitrogen atmosphere. The solvent was evaporated and the product isolated by repeated precipitation with ether and hexane. Yield: 20 mg (35.3%). ¹H-NMR (400 MHz, DMSO): δ (ppm) 7.9-7.75 (m, 44H), 7.5-7.35 (m, 8), 7.35-7.25 (m, 16H), 7.2-7.1 (m, 8), 4.05 (t, 16H), 3.20-3.00 (m, 40H), 2.7-2.60 (m, 24H), 2.45-2.35 (m, 12H), 2.25-2.05 (m, 38H), 1.8-1.65 (m, 32H). Mass: (ESI) calculated: 3239.8 (M), found: 3241.2 (M+H)⁺ and calculated: 1620.9 (M+2)²⁺ found: 1621.4 (M+2H)²⁺.

N-Poly(ethylene imine)-7-(3-(1-(1-undecyl-11-thiol)-1-methyl-4,4-bipyridinium thio)-2,5-dioxo pyrrolidin-1-yl)heptanamide (MV-PEI)

Poly(ethylene imine) [$M_w \sim 10000$ g/mol] (132 mg, 13.2 μ mol) was dissolved in 35 mL dry DMSO and a solution of maleimidoheptanoic acid *N*-hydroxy succinimide ester (61 mg, 197.86 μ mol, 15 equiv) in 3 mL DMSO added under inert conditions. The obtained solution was stirred for 1 day at 50 °C. The second reaction step was carried out after cooling down the reaction mixture to RT. Phosphate buffer (30 mL) was added and the pH of the reaction mixture decreased to

6.9 (controlled via pH-meter). 1-(1-Undecyl-11-thiol)-1-methyl-4,4-bipyridinium (110 mg, 199.98 μ mol, 15.16 equiv) was dissolved and added to the reaction mixture. The solution was stirred overnight at RT and purification was carried out by dialysis (molecular cut-off

10.000 g/mol). The product was lyophilized and analyzed by ^1H -NMR and UV/Vis spectroscopy.

As is visible in Figure 3.5, the UV absorption spectrum of the MV-grafted polymer shows the characteristic UV absorbance band of 1-(1-undecyl-11-thiol)-1-methyl-4,4-bipyridinium at 268 nm. To determine how many MV were grafted onto the polymer, a calibration curve of 1-(1-undecyl-11-thiol)-1-methyl-4,4-bipyridinium in water was prepared. Thereby, on average 4 MV moieties were found to be grafted to a polymer chain.

Figure 3.5: UV absorbance of MV-PEI and PEI in aqueous solution.

3.5.3 Methods

Supramolecular nanoparticle assembly

For the preparation of size-tunable supramolecular NPs utilizing CB[8] as supramolecular host, various aqueous solutions of Np-PEG and Np-PAMAM in DMSO were prepared before mixing. Concentration of MV-PEI and CB[8] were kept the same regardless which size of NPs were formed. E.g. for preparing a solution having 20% Np entities derived from the aliphatic dendrimer, 1000 μL MV-PEI (0.336 μM) was added to a previously prepared solution of 500 μL Np-PEG (2.15 μM), 20 μL Np₈-PAMAM (1.65 μM) and 500 μL CB[8] (2.688 μM). The sample was mixed, combined and kept at RT for 2 days before DLS and SEM analysis.

SNP formation in time

The kinetics of the formation of supramolecular NPs was analyzed by carrying out continuous DLS measurements over 24 h at different temperatures. Therefore the required building block solutions were mixed as previously described and the SNP size analysis started directly after mixing. The samples measured at 30°C and 40°C were warmed up utilizing an oil bath. DLS data were observed directly after mixing, which

implies, that stabilization of the NP temperature was not yet reached for the starting measurements. In contrast to regular DLS measurements, single measurements were done for each reported data point. For samples prepared at 21°C and 30°C, distinct particle formation was confirmed by measuring the size of the NP sample 48 h after sample preparation.

Reductive SNP disassembly

SNP disassembly was carried out by adding 10 µL aqueous Na₂S₂O₄ (5 mmol) to the SNP sample under inert conditions. Particle size analysis was carried out via DLS at the following day.

3.5.4 Equipment

Dynamic light scattering (DLS)

DLS experiments were performed with a Zetatrac and Nanotrac by Anaspec operating with a Microtrac FLEX Operating Software at 25°C using a laser wavelength of 780 nm at a scattering angle of 90°. The observed sizes and standard derivations of the supramolecular NPs were calculated by taking an average of 5 measurements. In total, particles with sizes ranging from 0.8 to 6400 nm were detected. The DLS curves shown in this chapter are representative for the individual measurements.

Scanning electron microscopy (SEM)

All SEM images were taken with a Carl-Zeiss 1550 Gemini field emission scanning electron microscope. Therefore the aqueous SNP solutions were drop-cast on a Formvar coated copper TEM grid. The solution was dried and analyzed without further treatment of the samples.

UV/Vis spectroscopy

UV/Vis absorption spectra were recorded using a Perkin Elmer Lambda 850 UV-Vis spectrometer. To investigate the formation of a charge transfer band while SNP self-assembly, SNPs were prepared as described previously. The formation of particles was controlled using DLS. For UV/Vis analysis, the sample was concentrated 13.5 times to observe sufficient UV absorbance. As control experiments, CB[8] and a SNP formulation in absence of CB[8] was prepared in the same concentration, and the UV/Vis absorbance determined.

Fluorescence spectroscopy

Fluorescence spectra were recorded using a Perkin Elmer LS 55 fluorescence equipped with a high energy pulsed Xenon source for excitation. Emission and excitation slits were kept constant at 2.5 cm. To evaluate if SNP assembly is driven by the formation of the heteroternary complex between CB[8]:MV:Np, the SNP formulation was prepared in

presence and absence of CB[8]. And the fluorescence emission of both solution directly controlled after mixing using an excitation wavelength of 266 nm.

NMR spectroscopy

^1H and ^{13}C NMR spectra were recorded on Bruker 400 MHz and 600 MHz spectrometer. ^1H and ^{13}C chemical shift values, 400 MHz and 150 MHz, are reported as δ using the residual solvent signal as internal standard.

Mass spectrometry

Mass analysis was performed using the matrix-assisted laser desorption ionization (MALDI) and electrospray ionization using a Voyager DE-RP and a micromass LCT from Waters/Micromass, respectively.

3.6 References

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Chapter 4

Effects of Molecular Weight and Valency of Guest-modified Poly(ethylene glycol)s on the Stability, Size and Dynamics of Supramolecular Nanoparticles

The influence of polymer length and the valency of the guest-modified poly(ethylene glycol) (PEG) on the appearance, stability, size tunability and formation dynamics of supramolecular nanoparticles (SNPs) has been studied in this chapter. SNPs were formed by molecular recognition of supramolecular building blocks with host or guest moieties, providing ternary complexes of cucurbit[8]uril, methyl viologen and naphthol (Np). SNP assembly was carried out using monovalent Np-modified oligo(ethylene glycol)s and PEGs with molecular weights ranging between 133 and 20000 g/mol. SNP formation and stoichiometry controlled size tuning was observed for SNPs prepared with Np-modified PEGs of polymer lengths between 900 and 20000 g/mol, whereas no distinct assemblies were formed using the shorter Np-functionalized tri(ethylene glycol). Use of the divalent Np-functionalized PEG (5000 g/mol) slowed down the SNP assembly dynamics and prohibited the formation of distinct SNPs at RT within the first week after mixing of the components. In contrast, SNPs with distinct sizes were obtained after warming up the building blocks to 40°C for 12 h.

4.1 Introduction

Nanomaterials are synthetic materials with dimensions smaller than a few hundreds of nanometers and hold great promise in catalysis,¹ as analytical sensors,^{2,3} and for electronic⁴ or biomedical applications.^{5,6} Nanoparticles (NPs) in particular are often reported as multifunctional diagnostic and therapeutic tools to protect and deliver active compounds into targeted tissue *in vivo*, while preventing their degradation and minimizing undesired side-effects.⁷⁻⁹

NPs are generally not colloidally stable in biological fluids as the high surface energy of the NPs and the high ionic strength of the fluids lead to uncontrolled particle aggregation.¹⁰ Furthermore, physiological environments such as blood, interstitial fluids or cellular cytoplasm, contain mixtures of proteins which adsorb to the NP surface by non-specific interactions.^{11,12} These adsorbed proteins alter the size and interfacial composition of a nanomaterial and often act as opsonins, markers that enhance phagocytosis by the mononuclear phagocyte system (MPS).^{13,14} Therefore, polymer-based steric stabilization layers are required to prevent uncontrolled protein adsorption and rapid clearance of NPs from the bloodstream to increase the pharmacokinetics and biodistribution of NPs *in vivo*.¹⁵

Surface modification with oligo(ethylene glycol) or poly(ethylene glycol) (PEG) called "PEGylation" has been the most widely used method to create a steric barrier around NPs to prevent nonspecific adsorption from complex media.¹⁶ Using this strategy, a variety of NP systems has been reported in which the *in vivo* circulation time of PEGylated NPs was extended compared to the uncoated parent particles.^{17,18} Apparently, the PEG length^{19,20} and density²¹ have strong, inhibiting effects on the serum protein adsorption and macrophage uptake and therefore result in increased blood circulation times.

Supramolecular nanoparticles (SNPs) are formed by multiple monovalent and multivalent building blocks brought together by specific non-covalent interactions.²² The chemical versatility of the SNP assembly supports the inclusion of active compounds into the particles, and has been used for applications such as the delivery of RNA/DNA,²³⁻²⁵ drugs,²⁶ imaging agents²⁷ or transcription factors.²⁸ Assembled by multivalent electrostatic or host-guest interactions in the core, SNP formation strategies require additional stabilization by hydrophilic polymers assembling in the SNP shell. This colloidal SNP stabilization can be achieved, for example, by host-guest interactions of cyclodextrin and adamantane using a monovalent adamantyl-functionalized PEG interacting with the multivalent CD-functionalized polymer thus constituting the SNP shell.^{23,24,26,29,30} The shielding enabled by the PEG shell provided the stability of polycation-nucleic acid composites in biological fluids, and the long-term circulation of the SNPs *in vivo*.³¹

In Chapter 3, a novel SNP formation strategy was presented, in which the SNP assembly is promoted by multiple charge transfer complexes between cucurbit[8]uril (CB[8]), methyl viologen (MV) and naphthol (Np). The relatively high binding affinity of this ternary complex led to SNPs, the assembly dynamics of which appeared to be markedly slower than that observed for cyclodextrin assemblies. Nonetheless, the interplay between multivalent and monovalent Np-bearing guest molecules provided size tuning of the SNPs similar to the SNP formation strategy reported by Tseng and coworkers for the interaction of cyclodextrin and adamantyl-functionalized building blocks.³⁰ In the CB[8] and CD strategies, both the colloidal stability as well as the size tuning of the SNPs can be attributed to the shielding effect of the monovalent guest-modified PEG stopper. However, it is unclear to which extent the polymer length and the valency of the stopper affect the SNP shielding.

The aim of the work presented in this chapter is to correlate the structure of the guest-modified PEG stopper with the colloidal stability, size tuning and assembly dynamics of SNPs stabilized by CB[8]/MV/Np interactions. In particular, the effects of the length of the PEG chain and the number (valency) of guest moieties attached to the PEG end on the SNP stability are evaluated in detail.

4.2 Results and Discussion

4.2.1 SNP formation using different shell components

As schematically shown in Scheme 4.1, SNPs are formed by the self-assembly of MV-poly(ethylene imine) (MV-PEI), Np-poly(amido amine) dendrimer (Np₈-PAMAM), CB[8] and different Np-functionalized PEGs. In the presence of CB[8], the SNPs assemble by multiple interactions of MV-PEI and Np₈-PAMAM in the particle core and of Np-PEG in the SNP shell. Using monovalent Np-conjugated PEG with different lengths, the effect of the polymer length on the formation of SNPs is evaluated. Furthermore, the effect of the valency of the PEG stopper is tested by using a divalent Np-conjugated PEG with a polymer length comparable to that of the monovalent derivative described in Chapter 3.

Scheme 4.1: a) Supramolecular nanoparticle (SNP) formation by heteroternary complex formation between cucurbit(8)uril (CB[8]), methyl viologen (MV) and naphthol (Np) moieties using MV-functionalized poly(ethylene imine) (MV-PEI), Np-functionalized PAMAM G1 dendrimer (Np₈-PAMAM), CB[8] and different Np-functionalized poly(ethylene glycol)s (Np-PEGs). b) The supramolecular building blocks used for SNP formation: Np-tri(ethylene glycol) (Np-PEG₃), Np-PEG₁₈, Np-PEG₁₁₁, Np-PEG₄₆₄, Np₂-PEG₁₁₃, MV-PEI, Np₈-PAMAM and CB[8].

To study the effect of PEG length on SNP formation, various Np-conjugated PEG stopper molecules were synthesized. Four monovalent Np-conjugated PEG derivatives, with 3 or, on average, 18, 111, or 464 repeat units, and a divalent Np-grafted PEG, Np₂-PEG₁₁₃, were synthesized. As shown in Scheme 4.2, the short Np-PEG₃ was synthesized by the reaction of 2-naphthol with NaH followed by Williamson ether synthesis using chloroethoxy-ethoxy-ethanol. Np-PEG₁₈ was synthesized as reported for Np-PEG₁₁₁ in Chapter 3 by a substitution reaction of 2-naphthol with poly(ethylene glycol) methyl ether tosylate.

The longest monovalent Np-conjugated PEG, Np-PEG₄₆₄ was prepared by reaction of 2-naphthoylchloride with PEG methyl ether amine in dichloromethane. To prepare Np₂-PEG₁₁₃ (Scheme 4.2d), first the hydroxyl group of Np-PEG₃ was converted to the more reactive bromide using PBr₃ in toluene. This bromide (**4**) was reacted with 3,5-dihydroxy benzonitrile under reflux using K₂CO₃ and 18-crown-6 in acetone to obtain the divalent guest unit **5**. The nitrile functionality of **5** was converted to an amine by the hydrogenation with H₂ in the presence of Raney-nickel as the catalyst. Following, Np₂-PEG₁₁₃ (**6**) was obtained by reaction with methyl-poly(ethylene glycol) *N*-hydroxysuccinimide ester in CH₂Cl₂.

Scheme 4.2: Synthetic routes towards the monovalent Np-conjugated PEG derivatives (a-c) and the divalent Np₂-PEG₁₁₃ used in this study.

4.2.2 SNP formation with monovalent Np-PEG of varying length

The formation of SNPs was studied using a $0.67\ \mu\text{M}$ concentration of CB[8], MV and Np, while keeping the molecular recognition moieties in a stoichiometric ratio of 1:1:1. Therefore, Np₈-PAMAM was dissolved in DMSO and aqueous solutions of MV-PEI, CB[8] and Np-PEG_x ($x = 3, 18, 111, 464$) were prepared prior to mixing. Size tuning of the SNPs was assessed by varying the concentrations of the Np-PEG derivative and the Np₈-PAMAM dendrimer, while keeping the overall concentration of the Np moieties constant. As for the SNPs reported in Chapter 3 (prepared with Np-PEG₁₁₁), the Np-conjugated PEG_x was mixed with Np₈-PAMAM and CB[8] prior to addition of MV-PEI. The formation of SNPs was evaluated by DLS and SEM, either 2 or 7 days after mixing, respectively.

As shown in Figure 4.1, self-assembly experiments carried out with Np-PEG₃, Np₈-PAMAM, MV-PEI and CB[8] did not result in distinct SNP formation. Only few particle-shaped structures were observed, which were embedded in organic material. This observation is in accordance with DLS characterization carried out for this SNP formulation. For all concentrations of Np₈-PAMAM used during the self-assembly experiments, only irreproducible aggregation or failed measurements were observed.

Figure 4.1: SEM images observed for SNP formulations prepared with Np-PEG₃ as a function of Np content derived from Np₈-PAMAM dendrimer (a: 10%, b: 20%, c: 30%).

In contrast to Np-Peg₃, distinct SNPs were observed using Np-PEG₁₈ (whilst maintaining Np₈-PAMAM, MV-PEI and CB[8]). By increasing the amount of Np derived from Np₈-PAMAM, while decreasing the amount Np from Np-PEG₁₈, the observed SNP size increased from $37 \pm 7\ \text{nm}$ to $65 \pm 13\ \text{nm}$ as measured by SEM and from $50 \pm 3\ \text{nm}$ to $97 \pm 11\ \text{nm}$ as analyzed by DLS.

Figure 4.2: Size determination of SNPs prepared with Np-PEG₁₈: SEM images (a-c) as a function of the Np content derived from Np₈-PAMAM dendrimer (a: 10%, b: 20%, c: 30%) used during supramolecular assembly while keeping the concentration ratio of CB[8]:MV:Np = 1:1:1. d) SNP diameter as measured by SEM (■) and DLS (■).

As seen in Figure 4.3, distinct SNPs were formed as well for different formulations using Np-PEG₄₆₄, Np₈-PAMAM, MV-PEI and CB[8]. Also in this case, stoichiometric size control was observed by increasing the amount of Np from Np₈-PAMAM from 10% to 30%, while decreasing the amount of Np-PEG₄₆₄ from 90% to 70% correspondingly: an increase in SNP size was observed from 35 ± 4 nm to 61 ± 12 nm by SEM and from 47 ± 9 nm to 117 ± 15 nm by DLS (Figure 4.3d).

Figure 4.3: Size determinations of SNPs prepared with Np-PEG₄₆₄: SEM images (a-c) as a function of the Np content derived from Np₈-PAMAM dendrimer (a: 10%, b: 20%, c: 30%) used during supramolecular assembly while keeping the concentration ratio of CB[8]:MV:Np = 1:1:1. d) SNP diameter as measured by SEM (■) and DLS (■).

In summary, these results illustrate that distinct SNP formation is not possible using Np-PEG₃ as the stopper assembling in the shell of the SNPs. Most likely the short tri(ethylene glycol) moiety does not provide sufficient stability to the SNPs, because of its marginal steric shielding capacity. Including the results presented in Chapter 3 for Np-PEG₁₁₁, clear SNP formation was observed for SNPs formed using Np-PEG₁₈, Np-PEG₁₁₁ and Np-PEG₄₆₄. Remarkably, even the still fairly short Np-PEG derivative grafted with 18 repeat units is apparently capable of providing sufficient colloidal stability to the SNPs. Furthermore, for all SNPs, size tuning was achieved by stoichiometric control of the ratio between the multivalent and monovalent Np components, irrespective of the length of the Np-PEG.

Figure 4.4 shows a comparison of the particle sizes determined by DLS (Figure 4.4a) and SEM (Figure 4.4b). While the SEM diameters show no dependence on PEG length, the hydrodynamic diameters measured by DLS show a slightly upward trend for increasing chain lengths. Also the differences between DLS and SEM sizes increase with increasing percentage of multivalent Np dendrimer (Figure 4.4c). In all cases, the effect of PEG length on particle size seems to be statistically insignificant.

Figure 4.4: Size determination of SNPs prepared with Np-PEG₁₈ (■), Np-PEG₁₁₁ (■) and Np-PEG₄₆₄ (■): a) by DLS and b) by SEM. c) Difference in hydrodynamic diameter by DLS and SNP diameter by SEM.

It has been reported that the surface area occupied by PEG increases with the length of the polymer.³² When assuming that a PEG stopper is attached to the SNP surface in a mushroom conformation and that the particles are dispersed in aqueous solution, the

average diameter of the PEG coil (ξ_{mushroom}) assembled onto the SNP surface can be calculated by $\xi_{\text{mushroom}} = 0.76 \times (M_{\text{w(PEG)}})^{0.5}$ with $M_{\text{w(PEG)}}$ the molecular weight of the PEG chain and the area of SNP covered by the polymer with $A_{\text{mushroom}} = \pi \times (\xi_{\text{mushroom}}/2)^2$.³² Taking into account that the SNPs are surrounded by PEG, we estimate that the average SNP diameter in water, compared to SNPs made using Np-PEG₁₈, would increase by 6 nm for SNPs assembled with Np-PEG₁₁₁ and up to 17 nm for SNPs assembled with Np-PEG₄₆₄. Although the data shown in Figure 4.4 do not contradict, these calculations, the size distributions are too broad to give a firm experimental confirmation.

Table 4.1: Polymer diameter and area of PEG with different PEG length assembled in mushroom conformation onto the particles surfaces.

PEG derivative	M_{w} PEG chain (g/mol)	ξ_{mushroom} (nm)	A_{mushroom} (nm ²)
Np-PEG ₃	133	0.9	0.6
Np-PEG ₁₈	801	2.2	3.6
Np-PEG ₁₁₁	4884	5.3	22.2
Np-PEG ₄₆₄	20416	10.9	92.6

For the formation of SNPs with PEGs of longer chain lengths, the PEG chains require a larger surface area per chain, so less PEG chains are required to occupy the SNP surface and to stabilize the assembly. From this, it can be expected that an increasing fraction of stopper remains unused, and creates a thermodynamic driving force for the formation of smaller (by core) SNPs. To support this, it is estimated from the binding constant of Np with the preformed CB[8]/MV complex $((6.1 \pm 0.5) \times 10^5 \text{ M}^{-1})$ ³³ and the low building block concentration used for SNP formation (0.7 μM), that maximally only $\sim 42\%$ of the Np-PEG molecules are assembled onto the SNP surface and the residual PEG chains are free in solution. This percentage decreases upon increasing the PEG chain length, indicating a lower number of PEG stopper molecules are attached onto the SNP surface. This could explain the increasing difference between DLS and SEM sizes indicated in Figure 4.4c. The SNPs prepared with the longest PEG chains show the largest loss of water upon drying, which is related to a higher hydration of the SNPs, a larger content of PEG and possibly a lower content of stopper.

Overall, since we do not observe a clear size increase or decrease in the formation of SNPs with different PEG lengths, we assume that both mechanisms could be playing a combined and counteracting role in the determination of the SNP size by self-assembly. Although the mushroom conformation of the longer PEG chains attached onto the SNP surface is larger, the SNP core shrinkage due to the occupation of more space by PEG could lead to a negligible size difference between the SNPs prepared with different PEG lengths.

Upon close comparison between the composition and configuration of the SNPs, the often proclaimed effect of steric stabilization by monovalent Np-PEGs becomes obscure. Assuming that the SNPs are formed by 90% water and 10% organic material and that a single CB[8] has an intrinsic surface of 3 nm^2 ,³⁴ a single CB[8] molecule is assembled approximately onto a SNP surface of 30 nm^2 . Taking into account that only 42% of the Np-PEG interacts with the CB[8]/MV complexes, this corresponds to a single Np-PEG polymer chain assembled onto 71 nm^2 of SNP surface. By taking into account the mushroom conformational area occupied by the different Np-PEGs (Table 4.1), it is logical that only the SNPs prepared with Np-PEG₄₆₄ are formed by a shell in which the polymer chains feel each other and dense PEG coverage is ensured. Therefore only for these SNPs a lower number of PEG molecules might be assembled onto the SNP surface, whereas all other SNPs are formed by the same amount of Np-PEG chains, verifying the results shown in Figure 4.4. On the other extreme, Np-PEG₁₈ provides colloidal stability, but only about 5% of the SNP surface area is covered by PEG.

Overall, it can be assumed that the Np-PEGs with shorter PEG length provide only marginal polymer shielding of the SNPs. Therefore, we propose that the SNPs are additionally stabilized by a dynamic effect in which the Np-PEGs are hopping between the different positions available on the SNPs surface and thereby provide colloidal stabilization and prevent aggregation.

4.2.3 Supramolecular nanoparticle formation with stoppers of different valencies

To study the effect of the valency of the stopper on the SNP formation, the divalent Np₂-PEG₁₁₃ was synthesized and used for SNP formation. In accordance with the previous experiments, Np₂-PEG₁₁₃, Np₈-PAMAM, MV-PEI and CB[8] were mixed and SNP formation was evaluated by SEM and DLS after keeping the samples at room temperature for 2 and 7 days, respectively. It should be noted that, since two naphthol groups are grafted onto the divalent Np₂-PEG₁₁₃, only half of the molar concentration of the stopper was used for SNP formation, in comparison to the monovalent Np-PEGs, to keep the concentrations of host and guest moieties equimolar.

As observed by SEM (Figure 4.5), self-assembly of the supramolecular building blocks led to the formation of supramolecular particle-like assemblies for different dendrimer-stopper ratios, but the shape of these SNPs was not as spherical as observed for SNPs prepared with Np-PEG₁₁₃ (see Chapter 3). This is justified by the absence of stable and reproducible DLS readings for SNPs prepared with 10% Np from Np₈-PAMAM and the occurrence of heterogeneous structures by SEM for SNPs prepared with 20% and 30% Np from Np₈-PAMAM.

Figure 4.5: Size determination of SNPs prepared with Np_2 -PEG₁₁₃ at RT: (a-c) SEM images after 7 days as a function of the Np content derived from Np_8 -PAMAM dendrimer (a: 10%, b: 20%, c: 30%) used during supramolecular assembly whilst keeping the concentration ratio of CB[8]:MV:Np = 1:1:1. d) SNP diameter as measured by SEM (■) and DLS (■).

To evaluate whether the SNP heterogeneity is a result of slow dynamics, time-dependent DLS measurements were carried out at RT and at 40°C. As seen in Figure 4.6, the observed hydrodynamic diameter of the SNPs containing 25% Np from Np_8 -PAMAM varied with temperature and with the valency of the Np-functionalized PEG. As shown in Chapter 3, the size of the SNPs prepared with Np -PEG₁₁₁ is stabilized at 40°C within 4 to 6 h. However, prolonged heating (over 28 h), led to SNP aggregation. In contrast, SNPs formed with Np_2 -PEG₁₁₃ showed a reproducible size with low standard deviations after 12 to 14 h at 40°C, and were stable during the whole monitoring time (40 h). Interestingly, at RT, the samples prepared with Np_2 -PEG₁₁₃ were not as consistent as the samples prepared at 40°C and the standard deviations remained relatively high, even 35 h after mixing.

Figure 4.6: a) Hydrodynamic diameters of SNPs prepared with Np-PEG₁₁₁ or Np₂-PEG₁₁₃, consisting of 25% Np from Np₈-PAMAM measured by time-dependent DLS as a function of temperature (data averaged over four measurements) and b) corresponding standard deviations.

Subsequently, the size tunability of the SNPs formed with Np₂-PEG₁₁₃ was evaluated by preparing the SNP samples at 40°C instead of at RT. Hereto, the supramolecular building blocks were mixed and kept at 40°C for 10 h followed by 1 d at RT prior to characterization. As observed in Figure 4.7, more distinct and spherical SNPs were observed by SEM. Furthermore, SEM and DLS size determination show clearly the characteristic stoichiometric size control by varying the content of divalent and multivalent Np-bearing building blocks. In particular, by increasing the amount of Np from Np₈-PAMAM from 10% to 30%, while decreasing the amount of Np from Np₂-PEG₁₁₃ from 90% to 70% correspondingly, an increase in SNP size was observed from 60 ± 10 nm to 86 ± 16 nm by SEM and from 88 ± 9 nm to 144 ± 36 nm by DLS (Figure 4.7d).

Figure 4.7: Size determination of SNPs prepared with Np_2 -PEG₁₁₃ at 40°C: SEM images (a-c) as a function of the Np content derived from Np_8 -PAMAM dendrimer (a: 10%, b: 20%, c: 30%) used during supramolecular assembly while keeping the concentration ratio of CB[8]:MV:Np = 1:1:1. d) SNP diameter as measured by SEM (■) and DLS (■).

Taken together, the time and temperature-dependent DLS measurements and the stoichiometry controlled SNP assembly show that an elevated temperature is required to form SNPs with distinct sizes and homogeneous shapes for SNPs prepared with the divalent Np_2 -PEG₁₁₃ as the stopper. As described in Chapter 3, SNP self-assembly requires the dynamic disassembly and reassembly of the four supramolecular building blocks for well-defined SNP formation. By using a divalent instead of monovalent guest-modified PEG, this dynamic process is slowed down. This is in agreement with the observations and predictions that the overall dissociation rate of a guest molecule decreases by increasing its valency.³⁵⁻³⁸

For SNPs formed using the divalent stopper, the effective molarity (EM) – i.e. the probability of the second Np moiety of the divalent guest to find an available host molecule when it is already attached to the SNP surface via the first Np group – determines the thermodynamic and kinetic multivalent stopper effects. We estimate the effective molarity on a SNP to be about 1 mM, which is similar to the EM of a divalent adamantyl derivative binding to a cyclodextrin (CD) dimer. Although the size of CB[8] is comparable to CD, the value for a CD-coated surface (100 mM) is likely to be too high compared to SNPs, as the SNP surface is not expected to be close packed (10% packing volume seems more realistic) and not all MV moieties are complexed with CB[8].³⁷ Together with the binding constant of $((6.1 \pm 0.5) \times 10^5 \text{ M}^{-1})$ ³³ for a Np unit binding to MV-

CB[8], this leads to the estimation that the dissociation rate constant of the divalently bound stopper is 2-3 orders of magnitude lower than that of the monovalent one. This explains the slower formation observed at RT, although an exact timeframe for stable particle formation can currently not be established.

The time-dependent measurements at 40°C indicate that increasing the guest valency of the PEG stopper is slowing down of the dynamics with less than an order of magnitude at this temperature. It seems unlikely that such a small difference in temperature could cause a large change in binding affinity and effective molarity. Therefore, the limited change in the observed equilibration time probably indicates that the equilibration process is to a large extent dictated by the valency of the core dendrimer rather than the stopper, although the valency of the stopper has an unmistakable effect on the dynamics.

Notably, the SNPs formed by 10% to 30% Np from Np₈-PAMAM and stabilized by the divalent Np₂-PEG₁₁₃ stopper are larger (88 ± 9 nm to 144 ± 36 nm) than the SNPs assembled with the monovalent Np-PEG₁₁₁ (51 ± 13 nm to 137 ± 16 nm). Yet, the surface coverage of the PEGs with the different valency does not explain the difference in observed SNP size. Even assuming that Np₂-PEG₁₁₃ completely binds to all CB[8] hosts assembled in the SNP shell, the SNP surface area is not completely covered by PEG and the individual polymer chains does not interact with each other, which is comparable to the SNPs prepared with Np-PEG₁₁₁. More likely, the divalent character of Np₂-PEG₁₁₃ causes the divalent guest to take part, to some extent, in the crosslinking of the supramolecular building blocks. This behavior has also been observed for aggregation of CD-functionalized gold NPs with a bis-adamantyl guest molecule in solution.³⁹ In this way, use of the divalent stopper may lead to an increased SNP size.

4.3 Conclusions

SNPs stabilized by ternary charge transfer complexes between CB[8], MV and Np can be assembled using different Np-modified PEGs. No difference in formation and size tunability was observed for SNPs prepared with Np-PEG₁₈, Np-PEG₁₁₁ or Np-PEG₄₆₄, whereas no clear SNP formation was observed for SNPs prepared with the short Np-functionalized tri(ethylene glycol). From a comparison of the PEG size and the expected coverage of the SNP surface with ternary complexes, interaction of the individual PEG chains is only expected for the highest PEG length. Possibly, the SNPs are stabilized by the dynamic assembly and reassembly of the monovalent Np-functionalized PEGs, whereas steric effects provide some additional steric bulk, thereby leading to colloidal stability of the SNPs. In contrast to the monovalent Np-PEGs, the use of the divalent Np₂-PEG requires an increased temperature to form size-tunable SNPs with distinct sizes. The apparent slowing down of the formation kinetics is due to the slower dissociation rate constant of the stopper, but the overall formation dynamics is to a large extent controlled

by the multivalent core components. In a larger context, a better understanding of the colloidal stability and assembly dynamics of supramolecular nanoparticles stabilized by guest-functionalized PEGs will be beneficial for the development of functional delivery vectors that can be used in therapeutics.

4.4 Acknowledgements

Mark Smithers is acknowledged for the SEM images.

4.5 Experimental Section

4.5.1 Materials

Starting materials for organic synthesis were obtained from Sigma-Aldrich and Nanocs and used as received. Octa-(*E*)-5-naphthol-pentanoic-acid poly(amido amine) dendrimer (generation 1) (Np₈-PAMAM), methyl viologen-functionalized poly(ethylene imine) (MV-PEI) and naphthalene poly(ethylene glycol) methyl ether (Np-PEG₁₁₁) were synthesized as described in Chapter 3. Cucurbit[8]uril (CB[8]) was purchased from Strem Chemicals and its purity was assessed by microcalorimetric titration against paraquat. Deuterated solvents used for NMR spectroscopy were purchased from Cambridge Isotope and the water used for dialysis and SNP preparation was of MilliQ quality (Millipore, $R=18.2\text{ M}\Omega\text{cm}^{-1}$).

4.5.2 Synthetic procedures

2-(2-(2-(Naphthalen-2-yloxy)ethoxy)ethoxy)ethanol (Np-PEG₃) (**1**)

Under argon, 2-naphthol (0.52 g, 3.6 mmol) was dissolved in anhydrous THF (10 mL). At 0°C, NaH (60% in mineral oil, 140 mg, 3.6 mmol) was added and the solution was stirred 1 h, while warming to room temperature. Subsequently, the solution was transferred under argon to a second flask charged with chloro-ethoxy-ethoxy-ethanol (1.5 g, 8.95 mmol) in THF (40 mL). A trace of KI was added to promote Finkelstein halogen exchange and the solution was stirred at room temperature for 12 h under argon. The solvent was removed under reduce pressure and the residue dissolved in 30 mL CH₂Cl₂. Unreacted chloro-ethoxy-ethoxy-ethanol was removed by extraction of the organic solution with (6 × 50 mL) 2 M HCl followed by brine (30 mL). The organic phase was dried with MgSO₄ and the solvent removed under reduced pressure. Flash chromatography (SiO₂, gradient elution CH₂Cl₂ (100%) to CH₂Cl₂/MeOH (98:2)) gave Np-PEG₃ as a slightly yellow oil. Yield: 550 mg, 55%. ¹H-NMR (400 MHz, CDCl₃): δ 7.75-7.69 (q, 3H, 3ArH); 7.43-7.39 (t, 1H, 1ArH); 7.33-7.29 (t, 1H, 1ArH); 7.18-7.12 (m, 2H, 2ArH); 4.23 (t, 2H, CH₂); 3.91 (t, 2H, CH₂); 3.73-3.67 (m, 6H, 3CH₂); 3.6 (t, 2H, CH₂). ¹³C-NMR (100 MHz, CDCl₃): δ= 156.65, 134.49, 129.46, 129.08, 127.66, 126.78, 126.39,

123.72, 118.96, 106.77, 72.56, 70.85, 70.36, 69.73, 67.35, 61.76. ESI-MS (m/z): calculated for $[C_{16}H_{20}O_4]^+$: 276.14, found: 277.10($M+H$) $^+$.

Np-PEG₁₈ (2)

Under nitrogen, naphthol (0.8 g, 5.6 mmol), tosyl poly(ethylene glycol) methyl ether (M_w 900 g/mol) (1 g, 1.11 mmol) and K_2CO_3 (1.7 g, 5.6 mmol) were dissolved in anhydrous acetonitrile (40 mL) and stirred at reflux for 5 days. The solvent was evaporated and the residue dissolved in 10 mL CH_2Cl_2 . The excess of naphthol was removed by repeated precipitation in ice cold diethyl ether. The collected solid was dissolved in water, filtered and dialyzed against water for 5 days (M_w cut-off 550 g/mol). The aqueous solution was freeze-dried to obtain a dark brown solid. Yield: 550 mg, 55%. 1H -NMR (400 MHz, $CDCl_3$): δ = 7.70-7.68 (q, 3H, 3ArH); 7.38 (td, 1H, 1ArH); 7.30 (td, 1H, 1ArH); 7.13 (m, 2H, 2ArH); 4.22 (t, 2H, CH_2); 3.90 (t, 2H, CH_2); 3.73 (m, 2H, CH_2); 3.64- 3.52 (m, 64H, 32 CH_2) 3.35 (s, 3H, CH_3). ^{13}C -NMR (100 MHz, $CDCl_3$): δ = 156.74, 134.49, 129.36, 129.02, 127.63, 126.76, 126.33, 123.64, 119.02, 106.73, 71.93, 70.86, 70.55, 69.74, 67.42, 59.04. ESI-MS (m/z): calculated for $[C_{47}H_{82}O_{19}]^+$: 950.55, found: 945.57.

Np-PEG₄₈₄ (3)

Under argon, 2-naphthoylchloride (20 mg, 0.104 mmol), 130 mg amino-poly(ethylene glycol) methyl ether (M_n 20.000 g/mol) (130 mg, 0.013 mmol) and pyridine (8.3 μ L, 0.104 mmol) were stirred in CH_2Cl_2 (15 mL) at room temperature for 48 h. The solvent was evaporated under reduced pressure and the obtained solid precipitated in diethyl ether repeated times. The obtained solid was dissolved in 40 mL MilliQ water, filtered and dialyzed against water (disposable membrane molecular weight cut-off 5 kDa). Afterwards, the aqueous solution was filtered and a white fluffy solid was obtained after freeze-drying. Yield: 129 mg, 98%. 1H -NMR (400 MHz, D_2O): δ = 8.39 (m, 1H, 1ArH); 8.08-8.00 (m, 3H, ArCH); 7.88-7.82 (m, 1H, ArH); 7.62-7.70 (m, 2H, 2ArH); 4.0-3.0 (m, 940H, CH_2). ^{13}C -NMR (D_2O): δ = 69.56. Maldi-MS (m/z) calculated for $[C_{938}H_{1863}NO_{464}]^+$: 20581 g/mol, found: 20580 g/mol.

2-(2-(2-(2-Bromoethoxy)ethoxy)ethoxy)naphthalene (4)

A solution of phosphorus tribromide (0.40 g, 1.5 mmol) in toluene (50 mL) was added dropwise to a cooled (0 $^{\circ}C$) solution of compound **1** (1.02 g, 3.7 mmol) in toluene (50 mL). The mixture was stirred overnight at room temperature. The solvent was removed under reduced pressure and the residue was partitioned between CH_2Cl_2 (50 mL) and water (50 mL). The organic layer was washed with water (3 $\times$ 50

mL) and brine (1 × 50 mL) and dried over MgSO₄. The solvent was removed under reduced pressure and the residue was purified by column chromatography (SiO₂, CH₂Cl₂/MeOH = 99:1) to give 2-(2-(2-(2-bromoethoxy)ethoxy)ethoxy)naphthalene as a colorless oil. Yield: 920 mg, 73 %. ¹H-NMR (CDCl₃): δ= 7.75-7.69 (q, 3H, 3ArH); 7.44-7.39 (t, 1H, 1ArH); 7.33-7.29 (t, 1H, 1ArH); 7.18-7.12 (m, 2H, 2ArH); 4.25 (t, 2H, CH₂); 3.94 (t, 2H, CH₂); 3.77 (t, 2H, CH₂); 3.75 (m, 2H, CH₂); 3.70 (m, 2H, CH₂); 3.46 (t, 2H, CH₂). ¹³C-NMR (100 MHz, CDCl₃): δ= 156.73, 134.49, 129.40, 129.04, 127.65, 126.77, 126.37, 123.68, 119.02, 106.74, 71.28, 70.89, 70.63, 69.85, 67.43. ESI-MS (m/z): calculated for [C₁₆H₁₉BrO₃]: 338.05 found: 339.22 (M+H)⁺.

3,5-Bis(2-(2-(2-(naphthalen-2-yloxy)ethoxy)ethoxy)ethoxy)benzonitrile (**5**)

A suspension of **4** (350 mg, 1.26 mmol), 3,5-dihydroxy benzonitrile (59 mg, 0.57 mmol), dried potassium carbonate (210 mg, 1.52 mmol) and 18-crown-6 (70 mg, 0.27 mmol) in acetone (14 mL) was refluxed for 78 h. The solvent was evaporated and the residue was partitioned between water (30 mL) and diethyl ether (30 mL). The aqueous layer was extracted with diethyl ether (3 × 25 mL) and the combined extracts were dried over MgSO₄. The solvent was evaporated and the residue was purified by column

chromatography (CH₂Cl₂/ MeOH=98:3) to give 3,5-bis(2-(2-(2-(naphthalen-2-yloxy)ethoxy)ethoxy)ethoxy) benzonitrile as a colorless oil. Yield: 190 mg, 50.3%. ¹H-NMR (CDCl₃): δ= 7.70-7.63 (m, 6H, 6ArH); 7.36-7.34 (t, 2H, 2ArH); 7.29-7.2 (t, 2H, 2ArH); 7.10-7.06 (m, 4H, 4ArH); 6.67 (sd, 2H, 2ArH); 6.60 (sd, 1H, 1ArH); 4.19-4.17 (t, 4H, 2CH₂); 3.99-3.97 (t, 4H, 2CH₂); 3.87-3.85 (t, 4H, 2CH₂); 3.77-3.75 (m, 4H, 2CH₂); 3.73-3.68 (m, 8H, 4CH₂). ¹³C-NMR (CDCl₃): δ= 160.11, 156.71, 134.47, 129.40, 129.02, 127.65, 126.75, 126.38, 123.70, 118.98, 118.72, 113.76, 110.71, 106.80, 106.71, 70.95, 70.92, 69.84, 69.50, 67.91, 67.41. ESI-MS (m/z): calculated for [C₃₉H₄₁NO₈]: 651.28 found: 652.26 (M+H)⁺.

3,5-Bis(2-(2-(2-(naphthalen-2-yloxy)ethoxy)ethoxy)ethoxy)benzylamine

A suspension of compound **5** (136.2mg, 0.21mmol) and a catalytic amount of Raney-Ni was stirred under 10 bar H₂ (g) in 30 mL of 6 M NH₃ in ethanol for 48 h. The suspension was filtered over celite and washed with methanol. After evaporation of the solvent, the product was dissolved in chloroform. It was alkalinized with 50 mL of NaOH 0.1 M. The water fraction was washed with chloroform (3 x 50 mL). The organic fractions were combined and solvent evaporated under reduced pressure to give compound slightly greenish colored oil.

Yield: 113 mg, 81%, 80% pure. ¹H-NMR (CDCl₃): 7.67-7.63 (m, 6H, 6ArH); 7.36-7.34 (m, 2H, 2ArH); 7.29-7.22 (m, 2H, 2ArH); 7.10-7.06 (m, 4H, 4ArH); 6.50 (s, 2H, 2ArH); 6.37 (s, 1H, 1ArH); 6.26 (s, 1H, 1ArH); 4.17-4.12 (m, 5.6H, CH₂ 2CH₂); 3.96-3.90 (m, 4H, 2CH₂); 3.85-3.80 (m, 4H, 2CH₂); 3.71-3.61 (m, 12H, 4CH₂). ¹³C-NMR (CDCl₃): δ= 162.59, 156.74, 134.47, 129.41, 129.02, 127.63, 126.79, 126.37, 123.69, 119.47, 109.42, 107.33, 106.78, 70.84, 70.70, 69.9, 69.50, 67.81, 67.39, 51.19. ESI-MS (m/z): calculated for [C₃₉H₄₅NO₈]: 655.31 found: 656.68(M+H)⁺.

Np₂-PEG₁₁₃ (**6**)

3,5-bis(2-(2-(2-(naphthalen-2-yloxy)ethoxy)ethoxy)ethoxy)benzylamine (100 mg, 0.152 mmol), triethylamine (15 mg, 21 μL, 0.152 mmol) and methylpoly(ethylene glycol) *N*-hydroxysuccinimide ester (M_w ~ 5000 g/mol) (253 mg, 0.051 mmol) were dissolved in CH₂Cl₂ (30 mL) under argon. The solution was stirred for 72 hours at room temperature. The solvent was removed under reduced pressure and the product purified by precipitation with

diethyl ether and dialysis against water for 5 days. Yield: 265 mg, 93%. ¹H-NMR (D₂O): 7.72 (m, 6H, 6ArH); 6.99 (m, 2H, 2ArH); 6.87 (m, 2H, 2ArH); 6.74 (m, 2H, 2ArH); 6.61 (m, 2H, 2ArH); 6.22 (s, 2H, 2ArH), 5.98 (s, 1H, 1ArH), 3.98 (t, 4H, 2CH₂), 3.78- 3.10 (m,

394 H, 197 CH₂). ¹³C-NMR (D₂O): δ = 69.56. Maldi-MS (m/z) calculated for [C₂₆₅H₄₉₅NO₁₂₁]⁺: 5631 g/mol, found: 5626 g/mol.

4.5.3 Methods

Supramolecular nanoparticle assembly

For the preparation of size-tunable supramolecular NPs utilizing CB[8] as supramolecular host, various aqueous solutions of the Np-PEGs (Np-PEG₃, Np-PEG₁₈, Np-PEG₁₁₁, Np-PEG₄₆₄ and Np₂-PEG₁₁₃) and different concentrations of Azo₈-PAMAM in DMSO were prepared. Additionally, aqueous solutions of MV-PEI (0.336 μM) and CB[8] (2.688 μM) were prepared and used for all SNP compositions. E.g. for preparing a solution having 20% Np entities derived from the aliphatic dendrimer and 80% Np entities derived from Np-PEG₁₈, 1000 μL MV-PEI was added to a previously prepared solution of 500 μL Np-PEG₁₈ (2.15 μM), 20 μL Np₈-PAMAM (1.65 μM) and 500 μL CB[8] (2.688 μM). These aliquots were mixed and kept at RT for 2 days before DLS and SEM analysis.

SNP formation in time

To compare the kinetics of the formation of SNPs in the presence of Np-PEG₁₁₁ and Np₂-PEG₁₁₃, continuous DLS measurements were carried out for 40 h at RT and at 40°C. Therefore the supramolecular building blocks were mixed using a formulation containing 25% Np from Np₈-PAMAM, and DLS was started directly after mixing. The reported averaged SNP sizes and their standard deviations were obtained by averaging 4 data points measured over the course of 1 h.

4.5.4 Equipment

Dynamic light scattering (DLS)

DLS experiments were performed with a Zetatrac and Nanotrac by Anaspec operating with a Microtrac FLEX Operating Software at 25°C using a laser wavelength of 780 nm at a scattering angle of 90°. The observed sizes and standard derivations of the SNPs were calculated by taking an average of 4 or 5 measurements. In total, particles with sizes ranging from 0.8 to 6400 nm were detected.

Scanning electron microscopy (SEM)

All SEM images were taken with a Carl-Zeiss high 1500 resolution scanning electron microscope. Therefore the aqueous SNP solutions were drop-cast on a Formvar coated copper TEM grid. The solution was dried and analyzed without further treatment of the samples.

NMR spectroscopy

^1H and ^{13}C NMR spectra were recorded on Bruker 400 MHz spectrometer. ^1H and ^{13}C chemical shift values, 400 MHz and 100 MHz, are reported as δ using the residual solvent signal as internal standard.

Mass spectrometry

Mass analysis was done using the matrix-assisted laser desorption ionization (MALDI) and electrospray ionization using a Voyager DE-RP and a micromass LCT from Waters/Micromass, respectively.

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Chapter 5

Dual Stimuli-Responsive Self-Assembled Supramolecular Nanoparticles

Supramolecular nanoparticles (SNPs) encompass multiple copies of different building blocks brought together by specific noncovalent interactions. The inherently multivalent nature of these systems allows control of their size as well as their assembly and disassembly, thus offering promising potential as biomedical delivery vehicles. In this chapter, dual responsive SNPs have been based on the ternary host–guest complexation between cucurbit[8]uril (CB[8]), a methyl viologen (MV) polymer, and mono- and multivalent azobenzene (Azo)-functionalized molecules. UV switching of the Azo groups led to fast disruption of the ternary complexes, but to a relatively slow disintegration of the SNPs. Alternating UV and Vis photoisomerization of the Azo groups led to fully reversible SNP disassembly and reassembly. SNPs were only formed with the Azo moieties in the *trans* and the MV units in the oxidized states, respectively, thus constituting a supramolecular AND logic gate.

5.1 Introduction

The self-assembly of molecules into higher ordered supramolecular structures constitutes a powerful paradigm for the design of novel materials such as nanoparticles,¹⁻³ hydrogels,⁴⁻⁶ and polymers.⁷⁻⁹ These assemblies are held together by non-covalent weak interactions such as Van der Waals, hydrophobic, and electrostatic interactions. More robust host-guest interactions, for example, the inclusion of hydrophobic guests in cyclodextrins (CD)^{10,11} or the inclusion of amines and hydrophobic guest molecules into cucurbit[n]urils (CB[n]),¹² have been studied extensively in the last decade.

Among these systems, host-guest-based supramolecular nanoparticles (SNPs) currently receive significant interest.¹⁻³ The self-assembly of small building blocks offers size control and colloidal stability by an interplay between multivalent and monovalent interactions. Their drug encapsulation properties and the easy implementation of targeting ligands make these assemblies promising candidates as site-selective biomedical delivery vectors.^{1,3}

Although a variety of external controls has been used for the triggered release of proteins and cells from surfaces,^{13,14} RNA from micelles,¹⁵ and DNA from hydrogels¹⁶ and electrostatic delivery vectors,¹⁷ only chemical reduction¹⁸ as well as magnetic field¹⁹ have been used as specific triggers for the release of drugs encapsulated into SNPs.

Azobenzene (Azo) is known to bind to α and β -CD in the stable *trans* form. Upon irradiation with light at a wavelength shorter than 360 nm it undergoes a conversion to the unstable, bulkier *cis* form, which is released from the CD cavity. This phenomenon has been used to build photo-responsive supramolecular vesicles,²⁰ mesoporous silica nanoparticles^{21,22} and hydrogels,²³ which can bind and release DNA, drugs and proteins, respectively. In contrast, thermal *trans-cis* isomerization was used to fabricate an amine displacement assay driven by the complexation of Azo by cucurbit[7]uril.²⁴ Recently, the formation of a ternary light-responsive host-guest complex between Azo, methyl viologen (MV) and cucurbit[8]uril (CB[8]) has been reported.^{25,26} Upon UV light irradiation, the *cis*-Azo was formed, thus disrupting the heteroternary complex between CB[8], MV and Azo. Using this photo-induced mechanism, surface wettability changes²⁵ as well as the polymerization of supramolecular building blocks²⁶ could be triggered. Although so far uninvestigated, this system holds great promise as a reversible supramolecular glue for the controlled assembly and disassembly of supramolecular nanoparticles.

In Chapter 3, we have shown the formation of SNPs with controllable particle sizes, by employing a ternary charge-transfer complex between CB[8], MV and naphthol-functionalized components. Here, we report a fully reversible and dual responsive SNP system using Azo building blocks, in which SNP assembly and disassembly can be

switched reversibly in multiple cycles by photoswitching of Azo, and irreversibly by chemical reduction of the MV units. Based on the ternary host-guest complex formation between CB[8], MV-polymer and mono- and multivalent Azo-functionalized guest molecules, SNPs with controllable particle sizes are formed. As schematically shown in Scheme 1, the disintegration of the previously formed SNPs is studied upon the photochemical conversion from *trans* to *cis*-Azo induced by UV light irradiation ($\lambda < 400$ nm). Isomerization back to *trans*-Azo is induced by visible light and is expected to lead to full restoration of the SNPs. Additionally, the disassembly of the SNPs by chemical reduction has been investigated as well.

Scheme 5.1: a) Schematic presentation of the supramolecular nanoparticle (SNP) self-assembly and triggered disassembly mediated by formation and disruption of the ternary complex of cucurbit[8]uril (CB[8]), methyl viologen (MV) and azobenzene (Azo) moieties. b) Supramolecular host (CB[8]) and responsive guest molecules involved in SNP formation: azobenzene-poly(ethylene glycol) (Azo-PEG), methyl viologen-poly(ethylene imine) (MV-PEI) and Azo₈-poly(amidoamine) dendrimer (Azo₈-PAMAM).

5.2 Results and Discussion

5.2.1 Supramolecular nanoparticle formation in aqueous solution

SNP formation required the synthesis of the appropriate multi- and monovalent building blocks. To this end, Azo-terminated poly(amidoamine) dendrimer of generation 1 (Azo₈-PAMAM) was synthesized to act as the multivalent photoresponsive supramolecular crosslinker in the core of the particles. Therefore, methyl-6-bromohexanoate was grafted onto p-(phenylazo)phenol to prepare methyl (E)-5-(4-(phenyldiazenyl)phenoxy)pentanoate. After deprotection, the carboxylic acid was activated using pentafluorophenol and the product purified by column chromatography. The fully Azo-functionalized PAMAM dendrimer was obtained after reacting perfluorophenyl (E)-5-(4-(phenyldiazenyl)phenoxy)pentanoate with the amine-terminated poly(amidoamine) dendrimer in DMF. In the presence of the previously reported MV-substituted poly(ethylene imine) (MV-PEI, with a degree of substitution of 4 MV units per polymer chain) and CB[8], this Azo-dendrimer forms multiple ternary complexes in aqueous solution. To provide colloidal stability and size control of the formed SNPs, monovalent, Azo-functionalized poly(ethylene glycol) (Azo-PEG, M_w 5000 g/mol) was prepared by the condensation of poly(ethylene glycol) monomethyl ether tosylate with phenyldiazobenzene in acetonitrile. Comparable to the SNP formation strategy reported in Chapter 3, the monovalent Azo-PEG competes with the multivalent Azo₈-PAMAM for binding to CB[8], leading to termination of the assemblies and resulting in size-controlled SNP formation. The Azo-PEG also facilitates water solubility of the assembled SNPs by exposure of the PEG chains at the outside of the SNPs.

Size-controlled particle self-assembly was observed by mixing the four supramolecular blocks, i.e. mono- and multivalent Azo, multivalent MV-PEI, and CB[8], involved in ternary complex formation in varying ratios. To a mixture of Azo₈-PAMAM dissolved in DMSO, aqueous CB[8] and aqueous Azo-PEG, a solution of MV-PEI was added subsequently. Successful size tuning was observed (Figure 5.1) by varying the ratio of the two Azo compounds, keeping the overall stoichiometry of CB[8]:MV:Azo = 1:1:1, and the mixtures were analyzed after two days using scanning electron microscopy (SEM) and dynamic light scattering (DLS). As shown in Figure 5.1e, the observed SNP size depends strongly on the fraction of Azo₈-PAMAM used during particle self-assembly. By increasing the amount of Azo derived from Azo₈-PAMAM from 10% to 30%, while decreasing the amount Azo-PEG accordingly, the recorded particle size increased from 55 nm to 110 nm as measured by DLS and from 35 nm to 70 nm as observed by SEM. This discrepancy between the results from DLS and SEM is commonly observed and can be attributed to DLS reporting hydrodynamic diameters and to the loss of water during SEM sample preparation. Overall, the size increased monotonously and was approximately linearly dependent on the amount of Azo₈-PAMAM up to 30%.

Figure 5.1: Size determination of SNPs prepared with different formulations: SEM images (a-c) of the resulting SNPs, as a function of the Azo content derived from Azo₈-PAMAM dendrimer (a: 10%, b: 20%, c: 30%) used during supramolecular assembly (using CB[8]:MV:Azo = 1:1:1, with Azo deriving from Azo₈-PAMAM and Azo-PEG). d) DLS curves for the different SNP formulations. e) SNP diameter as measured by SEM (■) and DLS (■).

DLS analysis further revealed that addition beyond 35% Azo₈-PAMAM led to uncontrolled particle growth yielding large aggregates with a high polydispersity (Figure 5.1d). This observation can be ascribed to the supramolecular competition between the multivalent dendrimers that reside in the core of the SNPs while the monovalent Azo-PEG assembles at the exterior of the SNPs. An increasing amount of the multivalent component allows an extended growth of the SNPs, yet a minimum amount of monovalent supramolecular stopper is apparently required for the formation of stable SNPs.

To prove the supramolecular specificity of the SNP self-assembly, SNP assembly was carried out in the absence of CB[8] and in the presence of CB[7] instead of CB[8]. The smaller cucurbit[n]uril homologue does not sterically allow the formation of ternary complexes. As expected, no distinct particle formation was observed by DLS when MV-PEI, Azo-PEG and Azo₈-PAMAM (20% Azo from Azo₈-PAMAM) were mixed without CB[8] or when CB[7] was used (Figure 5.2). These observations show that ternary complex formation by CB[8] is essential for SNP formation.

Figure 5.2: DLS analysis of solutions containing MV-PEI, Azo-PEG and Azo₈-PAMAM (20% Azo from Azo₈-PAMAM) a) without CB[8] and b) in the presence of CB[7] instead of CB[8]

5.2.2 Dual responsive supramolecular nanoparticle disassembly

The light-induced disassembly and re-assembly of the particles was investigated making use of the photo-responsive heteroternary complex by conversion of *trans* to *cis*-Azo. UV-Vis spectroscopy (Figure 5.3) of the complex formed between CB[8], paraquat, and Azo-PEG, as well as ¹H-NMR spectroscopy (Figure 5.4) of Azo-PEG in D₂O confirmed that >90% of *trans*-Azo-PEG was converted into *cis*-Azo-PEG within 1 min after irradiating the aqueous solution with UV light ($\lambda < 400$ nm). Time-dependent UV/Vis measurements show that the absorption band at 345 nm was restored after exposing the sample to ambient light for 20 min (Figure 5.3), which is indicative of *cis-trans* isomerization and concomitant re-formation of the ternary complex.

Figure 5.3: UV-Vis absorbance of CB[8]:paraquat:Azo-PEG: (1:1:0.95) before and after 1 min UV light irradiation as well as the time-dependent conversion of *cis* to *trans*-Azo-PEG under ambient light.

Figure 5.4: Partial ^1H NMR spectra of Azo-PEG in D_2O after UV irradiation

Subsequently we irradiated previously prepared SNP solutions with UV light ($\lambda < 400$ nm) and followed the change in SNP size using DLS (Figure 5.5). After an initial UV exposure time of 1 h, aggregated particles with a high polydispersity were observed (Figure 5.5). In contrast, full disintegration of the SNPs was observed after prolonging the irradiation time to 14 h. This particle disassembly process is relatively slow in comparison to the 1 min switching time of Azo in the individual ternary complex as described above. This difference can be explained by i) an incomplete *trans*-to-*cis* isomerization of Azo and ii) a slow rearrangement of multivalently interacting building blocks. It has been reported, that the photostationary state of the UV irradiation of Azo contains up to 20% *trans*-Azo.²⁰ Presumably, upon UV illumination, the *cis*-Azo-PEG is immediately expelled from the SNP shell, leading to exposure of the core. At the same time, the photostationary state ensures that the multivalent Azo₈-PAMAM initially maintains some interactions through a few moieties inside the core, thus leading to significant clustering of the nanoparticles. As described in Chapter 3, the assembly of SNPs is slow- because of the slow rearrangement of the multivalently interacting components, therefore the disintegration of the remaining building blocks only takes place over the course of hours (Figure 5.5).

To verify the reversibility of the *cis*-to-*trans* switching that leads to the re-assembly of the individual components to nanoparticles, the disassembled SNP samples were kept in ambient light to induce the switching back to *trans*-Azo, thus enabling the re-formation of the ternary complex with MV and CB[8]. Complete SNP reassembly was observed after keeping the samples for 8 h under visible light (Figure 5.5). The SNP assembly-disassembly process was conducted several times by switching between UV and Vis light. Figure 5.6 shows the alternate switching for four complete cycles, and in all states a complete reformation (~ 116 nm $\pm$ 16 nm) or rupture (~ 2 -4 nm) of the SNPs was observed. These results confirm the successful development of a reversible, light-responsive supramolecular nanoparticle system.

Figure 5.5: DLS size determination of: as-prepared SNPs (30% Azo from Azo₈-PAMAM), and subsequently after 1 h UV light, after 2 h UV light, after 4 h UV light, after 6 h UV light, after 14 h UV light and after 8 h ambient light.

Figure 5.6: UV/Vis light-induced switching to trigger SNP assembly (SNPs prepared with 30% Azo from Azo₈-PAMAM) and disassembly for four consecutive cycles.

In contrast, no distinct particle reformation was observed for SNP samples kept in the dark for 6 h, 10 h and 14 h after UV irradiation (Figure 5.7). This shows, that the *cis*-to-*trans* isomerization is driven by visible light irradiation, and that the thermal process plays a marginal role.

Figure 5.7: DLS analysis of three SNP samples (all prepared with 30% Azo from Azo₈-PAMAM): a) as-assembled SNP sample 1 and b) and subsequently measured after 12 h UV irradiation followed by keeping in the dark for 6 h; c) as-assembled SNP sample 2 and d) subsequently measured after 12 h UV irradiation followed by keeping in the dark for 10 h; e) as-assembled SNP sample 3 and f) subsequently measured after 12 h UV irradiation followed by keeping in the dark for 14 h.

The constitutive ternary supramolecular complex containing MV also results in the SNPs being susceptible to changes induced by chemical reduction of the MV moieties. Chemically triggered particle degradation was observed after the addition of the reducing agent Na₂S₂O₄. DLS analysis demonstrates the disappearance of particles with a size of about 110 nm and formation of small aggregates of about 2-4 nm (Figure 5.8) after 12

h. This observation is in agreement with the supramolecular mechanism reported before. The addition of a chemical reductant leads to formation of MV^+ radical cations which are known to form stable homoternary complexes with CB[8],²⁷ thus causing the release of the Azo guest units (Scheme 5.1) and concomitant disassembly of the SNPs.²⁵

Figure 5.8: DLS analysis of SNPs prepared with 30% Azo from Azo₈-PAMAM a) as assembled, b) after Na₂S₂O₄ addition

To prove that particle reassembly requires both the *trans*-Azo as well as the dicationic MV species, the two particle disassembly processes were carried out consecutively. The formed SNPs (Figure 5.9a) were disassembled first by chemical reduction using Na₂S₂O₄, followed by UV irradiation. DLS studies show that the initially formed SNPs were neither observed after chemical reduction (Figure 5.9b), nor after UV light irradiation (Figure 5.9c). Furthermore, SNP reassembly was not observed after subsequent visible light treatment (Figure 5.9d), which can be ascribed to the still present homoternary complexes between MV^+ radical cations and CB[8].

In logic terms, the SNP system described here functions as an AND operator. Only when the Azo moiety is in the *trans* form and the MV moiety in the dicationic form, SNPs are formed (Figure 5.10). Switching either of them leads to inhibition of the particle formation process. Molecular systems representing logic operators have been shown and reviewed before.²⁸ To our knowledge, this is the first example in which the result of the operation is visualized in the formation of supramolecular particles.

Figure 5.9: DLS analysis of SNPs prepared with 30% Azo from Azo₈-PAMAM a) as assembled and b) after Na₂S₂O₄ addition, c) subsequently after 12 h UV light, d) subsequently after 8 h ambient light.

Figure 5.10 Molecular AND logic operator in dual-responsive SNPs: SNPs are only formed with trans-Azo and the MV dication; with Azo in the cis form (by photoirradiation) and/or MV in the radical cation form (by chemical reduction), only small building blocks are observed.

5.3 Conclusions

In conclusion, we have developed a supramolecular strategy for the self-assembly of size-controlled supramolecular nanoparticles based on the ternary interaction between a MV polymer, mono- and multivalent Azo building blocks and CB[8]. The heteroternary complex enables the particles to be assembled and disassembled, the latter triggered either by UV irradiation or by a chemical reductant. Reversible disassembly and re-assembly can be induced by illumination by UV and Vis light, respectively, whereas irreversible disassembly occurs following chemical reduction. The dual responsive character as well as its reversibility make this SNP system a promising candidate for advanced materials engineering.

5.4 Acknowledgements

Jens Voskuhl is acknowledged for the synthesis of Azo₈-PAMAM and fruitful discussions. Mark Smithers is acknowledged for taking the SEM images.

5.5 Experimental Section

5.5.1 Materials

Starting materials for organic synthesis were obtained from Sigma-Aldrich and used as received. MV-PEI was prepared as described in Chapter 3. Cucurbit[8]uril (CB[8]) was purchased from Strem Chemicals and its purity was assessed by microcalorimetric titration against paraquat. Deuterated solvents used for NMR spectroscopy were purchased from Cambridge Isotope and the water used for dialysis and SNP preparation was of MilliQ quality (Millipore, $R= 18.2 \text{ M}\Omega\text{cm}^{-1}$).

5.5.2 Synthetic procedures

Methyl (E)-5-(4-(phenyldiazenyl)phenoxy)pentanoate

To a solution of methyl 5-bromopentanoate in acetonitrile (1 g, 5.1 mmol, 1 eq) was added K_2CO_3 (6 g, 51 mmol, 10 eq.) and the suspension was stirred for 30 min at 80°C.

Thereafter, phenylazobenzene was added (1 g, 5.1 mmol, 1 eq.). Stirring was continued at 80°C for 18 h, followed by removal of the solvent. The residue was dissolved in 30 mL of CHCl_3 and extracted 3 times with distilled water (20 mL). The organic layer was separated and dried over MgSO_4 . The solvent was evaporated and the residue was subjected to silica gel column chromatography (CHCl_3). The product was obtained as bright orange solid. Yield: 1.31 g, 80%. $^1\text{H-NMR}$ (400 MHz, CDCl_3): $\delta = 7.90\text{--}7.85$ (m, 4H, 4CH); $7.50\text{--}7.46$ (m, 2H, 2CH); $7.43\text{--}7.41$ (m, 1H, CH);

6.99-6.97 (m, 2H, 2CH); 4.04-4.02 (m, 2H, CH₂); 3.67 (s, 3H, CH₃); 2.42-2.39 (m, 2H, CH₂); 1.85-1.83 (m, 4H, 2CH₂). ¹³C-NMR (100 MHz, CDCl₃): δ = 174.04, 161.77, 152.83, 147.06, 130.59, 129.25, 125.08, 122.76, 114.91, 67.94, 51.80, 33.85, 28.80, 21.81. ESI-MS (m/z): calculated for [C₁₈H₂₀N₂O₃H]⁺: 313.1, found: 313.0.

(E)-5-(4-(Phenyldiazenyl)phenoxy)pentanoic acid

To a stirred solution of methyl (E)-5-(4-(phenyldiazenyl)phenoxy)pentanoate (1.30 g, 4.1 mmol 1 eq.) in 30 mL of MeOH/THF (1:1) was added LiOH (2 g, 41 mmol, 10 eq.) dissolved in 15 mL distilled water. The reaction was stirred at room temperature for 3 h. After that time the solution was acidified with 1 M hydrochloric acid followed by dilution with 50 mL of CHCl₃. The suspension was extracted 3 times with 30 mL of distilled water. The organic layer was dried over MgSO₄ and the solvent was removed under reduced pressure. The product was obtained as brown-orange solid and was used without further purification. Yield: 1.15 g, 92%. ¹H-NMR (400 MHz, CDCl₃): δ = 7.90-7.85 (m, 4H, 4CH); 7.50-7.46 (m, 2H, 2CH); 7.44-7.41 (m, 1H, CH); 6.98-6.96 (m, 2H, 2CH); 4.06-4.04 (m, 2H, CH₂); 2.47-2.45 (m, 2H, CH₂); 1.87-1.85 (m, 4H, 2CH₂). ¹³C-NMR (100 MHz, CDCl₃): δ = 178.76, 161.79, 152.74, 147.02, 130.64, 129.27, 125.17, 122.77, 114.93, 67.92, 33.63, 28.71, 21.58. ESI-MS (m/z): calculated for [C₁₇H₁₈N₂O₃H]⁺: 299.1, found: 299.0.

Perfluorophenyl (E)-5-(4-(phenyldiazenyl)phenoxy)pentanoate

To a solution of (E)-5-(4-(phenyldiazenyl)phenoxy)pentanoic acid (1 g, 3.35 mmol, 1 eq.) in 30 mL of THF was added dicyclohexylcarbodiimide (1.1 g, 5 mmol, 1.5 eq.) and the mixture was stirred for 30 min at room temperature. After adding pentafluorophenol (1 g, 5 mmol, 1.5 eq.) stirring was continued for 18 h. The suspension was filtered and the solvent was removed under reduced pressure. The residue was subjected to silica gel column chromatography (CHCl₃). After removal of the solvent the product was obtained as a bright orange solid. Yield: 1.2 g, 77%. ¹H-NMR (400 MHz, CDCl₃): 7.92-7.85 (m, 4H, 4CH); 7.50-7.48 (m, 2H, 2CH); 7.44-7.40 (m, 1H, CH); 7.01-6.98 (m, 2H, 2CH); 4.11-4.08 (t, J = 5.4 Hz, 2H, CH₂); 2.79-2.76 (t, J = 7.0 Hz, 2H, CH₂); 2.01-1.95 (m, 4H, 2CH₂). ¹³C-NMR (100 MHz, CDCl₃): δ = 169.42, 161.60, 152.86, 147.18, 130.62, 129.25, 125.06, 122.77, 114.89, 67.69, 33.15, 28.51, 21.77. ¹⁹F-NMR (376 MHz, CDCl₃): δ = -152.72 (d, J = 18 Hz, 2CF); -157.94 (t, J = 22 Hz, 2CF); -162.25 (t, J = 22 Hz, 1CF), ESI-MS (m/z): calculated for [C₂₃H₁₇F₅N₂O₃H]⁺: 465.1, found: 465.0.

Octa-(E)-5-(4-phenyldiazenyl)phenoxy-pentanoyl poly(amidoamine) dendrimer (generation 1) (Azo₈-PAMAM)

To a solution of perfluorophenyl (E)-5-(4-(phenyldiazenyl)phenoxy)pentanoate (4) (300 mg, 0.65 mmol, 16 eq.) in a 1:1 mixture of 10 mL of CH₂Cl₂ and DMF (1:1) was added PAMAM dendrimer generation 1 (50 mg, 0.04 mmol, 1 eq.) dissolved in 1

mL of DMF. The reaction was stirred under an atmosphere of argon for 18 h. The resulting precipitate was filtered off and washed with CH₂Cl₂, MeOH and acetone. The residue was dried in vacuo yielding an orange solid. Yield: 33 mg, 24%. ¹H-NMR (400 MHz, DMSO-d₆): 7.95 (bs, 8H, 8NH); 7.89 (bs, 8H, 8NH), 7.87-7.81 (m, 32H, 32 CH), 7.57-7.50 (m, 24H, 24 CH); 7.10-7.05 (m, 16H, 16CH); 6.81 (bs, 4H, 4NH); 4.03 (t, *J* = 5.8 Hz, 16H, 8CH₂); 3.10-3.06 (m, 40H, 20CH₂); 2.67-2.62 (m, 24H, 12 CH₂); 2.44-2.40 (m, 12H, 6CH₂); 2.18-2.13 (m, 40H, 20CH₂); 1.70-1.66 (m, 32H, 16 CH₂); MALDI-MS (*m/z*): calculated for [C₁₉₈H₂₅₆N₄₂O₂₈Na]⁺: 3695.5 found: 3695.1.

Azobenzene-substituted poly(ethylene glycol) (Azo-PEG)

Polyethylene glycol monomethyl ether tosylate (average *M_w* 5000 g/mol; 2 g, 0.4 mmol), 4-hydroxyazobenzene (0.4 g, 2 mmol) and K₂CO₃ (0.28 g, 2 mmol) were

dissolved in 40 mL acetonitrile and stirred under reflux for 5 days. The solvent was evaporated and the residue redissolved in 20 mL CH₂Cl₂. The excess of 4-hydroxyazobenzene was removed by repeated precipitation in diethyl ether. The collected solid was dissolved in water and dialyzed against water for 5 days (*M_w* cut-off 2000 g/mol). The aqueous solution was freeze-dried to obtain a yellow product. Yield: 1.2 g, 60%, ¹H-NMR (600 MHz, D₂O): 7.96 (d, *J* = 8.46 Hz, 2H, 2 CH); 7.88 (d, *J* = 7.56 Hz, 2H, 2 CH); 7.65-7.61 (m, 3H, 3 CH); 7.25 (d, *J* = 7.25 Hz, 2H, 2 CH); 3.9-3.4 (m, 417H, 417 CH₂) MALDI-MS (*m/z*): calculated for [C₂₂₈H₄₃₈N₂O₁₀₇]⁺: 4919.8 found: 4928.0.

5.5.3 Methods

Supramolecular nanoparticle assembly

For the preparation of the supramolecular nanoparticles, aqueous solutions of CB[8], MV-PEI and different concentrations of Azo-PEG were prepared before mixing. DMSO was used to dissolve different concentrations of Azo₈-PAMAM. For example, for preparing a solution having 20% Azo moieties derived from Azo₈-PAMAM, 1000 μL of MV-PEI (0.31

μM) was added to a previously prepared solution of 500 μL Azo-PEG (2.24 μM), 10 μL Azo₈-PAMAM (1.75 μM) and 500 μL CB[8] (2.8 μM). The sample was mixed and kept at room temperature for 2 days before DLS and SEM analysis.

Control experiments for supramolecular nanoparticle formation

To evaluate whether SNP formation is triggered by the formation of the ternary complex between CB[8], MV and Azo, different control experiments were carried out. SNP formation using 20% Azo from Azo₈-PAMAM was carried out in the absence of CB[8] or in the presence of CB[7] instead of CB[8]. Hereto, solutions were prepared with 500 μL water or 500 μL CB[7] (2.8 μM) instead of 500 μL CB[8] (2.8 μM).

Light response of supramolecular nanoparticles

To evaluate the light responsiveness of the particles, SNPs containing 30% Azo from Azo₈-PAMAM, were prepared. After analysis by DLS, the samples were irradiated with UV light ($\lambda < 400\text{ nm}$) for different times. DLS analysis was performed immediately after exposure, while keeping the SNP samples in the dark. It has to be noted that the obtained hydrodynamic diameters of the UV-treated SNP samples (shown in Figure 5.5) are not absolute values since the polydispersity of the samples was too high.

Reversibility of SNP disassembly and re-assembly was evaluated by first formation and characterization of SNP with 30% Azo from Azo₈-PAMAM. Subsequently, the samples were irradiated overnight with UV light ($\lambda \leq 400\text{ nm}$) followed by DLS to prove particle disassembly. SNP re-assembly was induced by keeping the samples for 8-10 h under ambient light conditions. Particle reformation was evaluated by DLS. This procedure was repeated 4 times with the same particle solution.

Reductive supramolecular nanoparticle disassembly

To evaluate whether reduction of the MV species leads to disassembly of the SNPs, particles were prepared and analyzed by DLS. SNP disassembly was carried out by adding 10 μL aqueous $\text{Na}_2\text{S}_2\text{O}_4$ (5 mM) to the SNP sample under inert conditions. Particle size analysis was carried out by DLS after 8 h.

Combination of reductive and UV-triggered supramolecular nanoparticle disassembly

SNPs were prepared and analyzed by DLS. Thereafter, 10 μL aqueous $\text{Na}_2\text{S}_2\text{O}_4$ (5 mM) was added to the SNP sample under nitrogen. Particle size analysis was carried out by DLS after 8 h and after subsequent UV irradiation for 12 h. The reassembly ability of the reduced and UV-treated SNPs was tested after 8 h under ambient light conditions.

5.5.4 Equipment

DLS analysis (DLS)

Size tunability of the SNPs was assessed using a Malvern Zetasizer at 25°C. SNP disassembly and re-assembly experiments were performed with a Nanotracer wave from Anaspec operating with the Microtrac FLEX Operating Software. The observed sizes and standard deviations of the supramolecular NPs are based on the average number distributions of minimum five individual measurements per sample. Three samples were measured for each reported NP formulation. In total, particles with sizes ranging from 0.8 to 6400 nm were detected. The DLS curves shown in this chapter are representative for the individual measurements.

Scanning electron microscopy (SEM)

All SEM images were taken with a Carl Zeiss Merlin scanning electron microscope. Aqueous SNP solutions were drop-cast on Formvar coated copper TEM grids. The solutions were dried and analyzed without further treatment of the samples.

UV light irradiation

UV light irradiation experiments were carried out with a Hönle bluepoint 2 easycure using a shortpass filter $\lambda \leq 400$ nm. After formation, the SNPs were treated with UV light for different periods. Therefore the samples were filled in a quartz cuvette and exposed to light with $\lambda \leq 400$ nm. To analyze the ongoing disassembly process, the samples were analyzed by DLS in dark and UV-irradiation was continued.

UV/Vis spectroscopy

UV/Vis absorption spectra were recorded using a Perkin Elmer Lambda 850 UV-Vis spectrometer. To investigate the photoisomerization behavior of azobenzene in the heteroternary complex at room temperature, an aqueous solution of CB[8], paraquat and Azo-PEG (25 μ M, 25 μ M, and 23.75 μ M, respectively) was prepared and measured before and after UV irradiation. Furthermore, the time dependent *cis-trans* isomerization was controlled by measuring the UV absorbance after 1 min, 2 min, 5 min, 10 min, 15 min, 20 min and 30 min while keeping the same solution at ambient light.

NMR spectroscopy

^1H and ^{13}C NMR spectra were recorded on Bruker 400 MHz and 600 MHz spectrometers. ^1H and ^{13}C chemical shift values are reported as δ (ppm) using the residual solvent signal as internal standard. In addition, the ratio of *trans* and *cis* isomers of Azo in Azo-PEG before and after UV irradiation in D_2O was determined using ^1H -NMR.

Mass spectrometry

Mass analysis was done using the matrix-assisted laser desorption ionization (MALDI) and electrospray ionization using a Voyager DE-RP and a micromass LCT from Waters/Micromass, respectively.

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Chapter 6

Programmed Disassembly of Supramolecular Nanoparticles Stabilized by Heteroternary CB[8] Host-Guest Interactions

Controlled release is an important component for the *in vivo* performance of drug delivering nanoparticles (NPs). Therefore, the control over and understanding of the release mechanism, e.g. by disassembly or degradation of the carrier, is essential for the optimization of NP formulations. This chapter presents a supramolecular toolbox approach for the formation and UV-induced disassembly of supramolecular nanoparticles (SNPs) which are either exclusively stabilized by cucurbit[8]uril (CB[8])/methyl viologen (MV)/azobenzene (Azo) interactions or CB[8]/MV/naphthol (Np) interactions, or by a combination of both. Photoisomerization of the Azo units enables UV-triggered disassembly of the CB[8]/MV/Azo host-guest complex. Depending on the valency of the electron-rich guest moieties (Np or Azo), either SNPs with a UV-responsive shell or a UV-responsive core were formed by assembling SNPs using a mixture of Azo and Np bearing guest molecules. In contrast, non-responsive SNPs or SNPs which disintegrate at both the core and the shell were formed by using exclusively CB[8]/MV/Np or CB[8]/MV/Azo interactions, respectively.

6.1 Introduction

Inorganic and organic nanoparticles (NPs) of different compositions are versatile platforms for biomedical applications because they are able to carry small molecules or act as diagnostic tools.¹⁻⁷ Above all, selectively triggered nanoparticle (NP) disassembly is of substantial interest as encapsulated drugs or imaging agents can be released upon NP degradation.⁸⁻¹⁰ In particular, in drug delivery applications, control over the rate of degradation is of high importance to attain a constant and prolonged drug dose in the blood flow. When NP degradation occurs too fast, this can lead to a peak in drug concentration followed by a fast drop-off until re-administration, which is known to minimize the therapeutic performance of drug delivery vectors. Therefore, biodegradable polymer NPs and mesoporous silica nanoparticles have been developed to achieve prolonged drug release.^{11,12}

In oncology, most imaging and drug delivery NPs take advantage of the enhanced permeability and retention (EPR) effect, i.e. a preferential uptake of the NPs by the tumor caused by the higher permeability and porosity of tumor tissue in comparison to normal tissue.¹³ This passive targeting enables NPs with sizes below 100 nm to penetrate and release the active compounds in the desired tissue. For instance, Besenbacher and coworkers have shown that especially small NPs can re-enter the bloodstream after penetration.¹⁴ Accumulation of NPs by aggregation is an alternative method to avoid out-diffusion of NPs from the carcinogenic tissue. Different strategies to induce NP aggregation have been reported, however mostly restricted to inorganic NPs.¹⁵⁻¹⁸ Examples of triggered NP disassembly or degradation employ external stimuli such as pH,^{19,20} temperature,²¹ chemical reduction,²² light²³ or combinations thereof.^{24,25} Despite the interest in finding ways to control the degradation of NPs, or to trigger the release of small molecules from the NPs, very few studies elucidate the underlying decomposition mechanism in more detail.^{26,27}

Supramolecular nanoparticles (SNPs) established by multiple host-guest interactions form a specific category of responsive NPs.²⁸⁻³⁰ Their intrinsically stable yet reversible character and their chemical flexibility has been used to encapsulate DNA,^{31,32} drugs³³⁻³⁵ and imaging agents^{36,37} for the formation of biomedically relevant nanovectors. In the previous chapters, SNP formation was achieved by employing multivalent heteroternary host-guest interactions between cucurbit[8]uril (CB[8]), methyl viologen (MV) and either naphthol (Np) or *trans*-azobenzene (Azo) as the second, electron-rich guest molecule. Hereto, MV was grafted onto poly(ethylene imine) (PEI) while the multivalent poly(amido amine) (PAMAM) dendrimer and monovalent poly(ethylene glycol) (PEG) were both functionalized with Np or Azo. Irrespective of the stabilizing heteroternary complex, the electron-rich guest-bearing PAMAM dendrimers reside in the SNP core by multivalent interactions with MV-PEI and CB[8], whereas the monovalent guest-functionalized PEG

assembles at the exterior to ensure colloidal stability. The photoresponsive and reversible *trans-cis* isomerization of Azo has led to the selective disassembly of the heteroternary complex,³⁸ causing reversible SNP disassembly for the Azo-based SNPs. Interestingly, the disintegration of the Azo-based SNPs occurred via a transient aggregated state caused by the different valencies of the Azo-bearing components and the incomplete photoswitching of Azo (see Chapter 5).

In this chapter, we present a toolbox approach for the fabrication of SNPs, in which the UV-induced particle disassembly can be regulated by introducing photo-responsive Azo-bearing components selectively either in the SNP shell or in the core. This enables the formation of SNPs for which the UV-triggered disassembly can be pre-programmed by the composition of the SNPs.

6.2 Results and Discussion

6.2.1 SNP toolbox approach for programmable particle disassembly

As illustrated in Scheme 6.1, MV-PEI and CB[8] were assembled into SNPs by heteroternary complexes formed with either Np₈-PAMAM or Azo₈-PAMAM as multivalent core-forming crosslinker and with Np-PEG or Azo-PEG as monovalent stopper assembling in the SNP shell. SNPs were formed by solely CB[8]/MV/Np or CB[8]/MV/Azo complexes as reported in Chapters 3 and 5 or by a combination of both heteroternary complexes. As the binding constants of the two heteroternary complexes are similar^{38,39} and the Np and Azo guest moieties are similar in size, no difference in SNP formation and resulting SNP size is expected.

The placement of the responsive Azo moiety in shell, core, or both, enables the formation of SNPs which undergo different responses upon treatment with UV light. As shown in Scheme 6.1, the SNPs stabilized exclusively by CB[8]/MV/Np interactions should not undergo any change upon UV irradiation, while the SNPs formed by CB[8]/MV/Azo interactions in both shell and core show a time-differentiated response of the core and shell (see Chapter 5). As studied here, SNPs in which the core is assembled from CB[8]/MV/Np interactions and the shell from CB[8]/MV/Azo interactions are expected to aggregate by multiple CB[8]/MV/Np interactions in the core upon photoswitching of the SNP shell. Conversely, SNPs with Azo in the core and Np in the shell should shrink by the loss of the Azo building blocks from the SNP core upon photoswitching.

Scheme 6.1: a) Schematic representation of the assembly of supramolecular nanoparticles (SNPs) mediated by the heteroternary complexes between cucurbit[8]uril (CB[8]), methyl viologen (MV), and either naphthol (Np) or azobenzene (Azo), and the UV light-induced disassembly mechanisms of the different SNPs. b) The supramolecular building blocks involved in SNP assembly: Azo-PEG, Azo₈-PAMAM, Np-PEG, Np₈-PAMAM, MV-PEI and CB[8]. c) Photoisomerization of Azo moieties. d) Schematic representation of the UV-triggered, reversible dissociation of the heteroternary complex between CB[8], MV and Azo.

6.2.2 SNPs with a photoswitchable shell

Based on the concept described above, SNPs were formed using CB[8]/MV/Np interactions in the core and CB[8]/MV/Azo interactions in the shell. Therefore, the multivalent dendrimer Np₈-PAMAM and monovalent Azo-PEG were used for SNP preparation, in the presence of MV-PEI and CB[8]. To ensure comparability, all SNP formulations reported here were prepared using equimolar concentrations (0.7 μ M) of CB[8], MV, and the second guest molecule (total amount of Azo and/or Np), with 30% of the latter derived from the multivalent core-forming dendrimer (and 70% of the monovalent guest-PEG). After mixing the supramolecular building blocks, the SNP samples were kept for 2 days at RT and analyzed by scanning electron microscopy (SEM) and dynamic light scattering (DLS) prior to SNP disassembly via UV light.

SNPs with sizes of 129 ± 5 nm (DLS) were formed by self-assembly of Np₈-PAMAM, Azo-PEG, MV-PEI and CB[8] (Figure 6.1). This size compares well with sizes observed before for the systems using only Np (126 ± 16 nm, see Chapter 3) and only Azo (110 ± 19 nm, see Chapter 5). Upon irradiation with UV light, immediate aggregation (starting after 10 min) was observed by DLS, continuing for 3-4 h until only a peak at 1 nm persisted. This peak at 1 nm is an artifact indicating the presence of insufficient material dispersed in solution. These results indicate the UV-induced, uncontrolled aggregation of the supramolecular building blocks, and subsequent sedimentation of the polymeric materials. Remarkably, after keeping the SNP samples for 24 h at ambient light, the reformation of SNPs with a size of 122 ± 20 nm was observed by DLS. It should be noted that the aggregation behavior induced by UV switching observed here is different from the transient aggregation observed for the Azo-Azo system (Chapter 5). In the present system, the particles were found to increase in size monotonously, finally leading to invisibility by DLS, whereas in the previously studied Azo-Azo system, particles were found to get smaller after initial aggregation, finally leading to completely dissolved and dissociated building blocks.

Figure 6.1: DLS size determination of as-assembled SNPs (prepared from Azo-PEG, Np_8 -PAMAM (Azo:Np = 70:30), MV-PEI and CB[8] with CB[8]:MV:(Azo+Np) = 1:1:1), after UV irradiation for different amounts of time, and subsequently after 24 h ambient light.

SEM images confirm the observations made by DLS for the SNP samples prepared from Azo-PEG, Np_8 -PAMAM, MV-PEI and CB[8]. Clear spherical SNPs (73 ± 11 nm) were visible after self-assembly, whereas only aggregated polymeric material was detected after UV treatment (12 h). Similar to DLS, SEM shows SNP reformation with a size of 76 ± 23 nm after keeping the aggregated sample for 24 h at ambient light.

Figure 6.2: SEM images of SNPs (prepared from Azo-PEG, Np_8 -PAMAM (Azo:Np = 70:30), MV-PEI and CB[8] with CB[8]:MV:(Azo+Np) = 1:1:1) a) after self-assembly, b) after 14 h UV light, and c) subsequently after 24 h ambient light.

SEM images observed for SNPs formed exclusively from CB[8]/MV/Azo interactions showed initial aggregation after UV treatment (3 h) but complete SNP disintegration after further 8 h of UV irradiation (Figure 6.3). These results are in accordance with the DLS data presented in Chapter 5, and underline the difference in aggregation behavior

between these systems as described above. By comparing the extent of aggregation observed for UV-treated SNPs stabilized by CB[8]/MV/Azo interactions in the shell and CB[8]/MV/Np in the core (Figure 6.2b) with UV-treated SNPs stabilized by solely CB[8]/MV/Azo interactions (Figure 6.3 a and b), more clustering is evident for the SNPs with Np in the core. This observation is in agreement with the differences in disassembly mechanism. Shell disruption leads to uncontrolled aggregation in both types of SNPs, but the amount of crosslinks is lower in the solely CB[8]/MV/Azo stabilized SNPs as UV irradiation partially disrupts the multivalent interactions present in the SNP core immediately after short UV irradiation. Furthermore, UV treatment for 12 h led to complete SNP disintegration in these SNPs as visible in Figure 6.3b, which did not occur for the aggregated CB[8]/MV/Np-SNPs.

Figure 6.3: SEM images of SNPs (prepared from Azo-PEG, Azo₈-PAMAM (30% Azo from Azo₈-PAMAM), MV-PEI and CB[8] with CB[8]:MV:Azo = 1:1:1) a) after 3 h UV light, b) after an additional 8 h UV light treatment, and c) after subsequent 24 h at ambient light.

To evaluate whether SNPs stabilized by solely CB[8]/MV/Np interactions degrade upon UV irradiation, SNPs were formed by the self-assembly of Np₈-PAMAM, Np-PEG, MV-PEI and CB[8] and treated with UV light for 14 h followed by visible light for 24 h. As shown in Figure 6.4 the SNP size by DLS did not vary significantly before (112 ± 25 nm) and after 14 h UV irradiation (134 ± 23 nm), nor after subsequent visible light treatment (117 ± 15 nm).

Figure 6.4: DLS size determinations of SNPs (prepared from Np-PEG, Np₈-PAMAM (30% Np from Np₈-PAMAM), MV-PEI and CB[8] with CB[8]:MV:Np = 1:1:1) after self-assembly, after 14 h UV irradiation, and subsequently 24 h ambient light.

In accordance with these results, also SEM did not show significant differences between the as-assembled SNPs and the SNPs treated with UV light, nor upon prolonged exposure to ambient light. As seen in Figure 6.5, SNPs with sizes of 69 ± 11 nm, 63 ± 14 nm and 64 ± 15 nm were observed after assembly, UV light irradiation, and after keeping the samples at ambient light, respectively. This reveals that UV irradiation does not influence the heteroternary complex CB[8]/MV/Np on the molecular level and therefore does not affect the SNPs.

Figure 6.5: SEM images of SNPs (prepared from Np-PEG, Np₈-PAMAM (30% Np from Np₈-PAMAM), MV-PEI and CB[8] with CB[8]:MV:Np = 1:1:1) a) after assembly, b) after subsequent 14 h UV irradiation, and c) after keeping the samples for 24 h at ambient light.

Combined, the results presented here show that UV light can be used to trigger SNP disassembly for SNPs stabilized by CB[8]/MV/Azo interactions, whereas CB[8]/MV/Np-stabilized SNPs are not affected by UV treatment. Using the photolabile Azo in the shell and the photostable Np in the core of the SNPs, the shell of these SNPs can be photoswitched selectively leading to aggregation of the cores.

6.2.3 SNPs with a photoswitchable core

To make UV light-responsive SNPs with Azo in the core, SNP assembly was carried out using Azo₈-PAMAM as multivalent cross-linker and Np-PEG as monovalent stopping unit in the presence of MV-PEI and CB[8]. As shown in Scheme 6.1, it can be expected that these SNPs do not aggregate upon UV irradiation, since the SNP shell is not disrupted upon UV treatment. Moreover, UV treatment only affects the dendrimer molecules assembling in the SNP core because they are capable of *trans-cis* isomerization. To study the self-assembly and disassembly, and to guarantee comparability with the previously described SNPs, the core-forming Azo₈-PAMAM dendrimer constituted 30% electron-rich guest in the SNP formulation, while the Np-PEG formed the remaining 70%. As for the other SNPs, SNP disassembly was triggered by UV light after confirming SNP formation, and SNP reformation was evaluated after keeping the samples subsequently at ambient light.

DLS results shown in Figure 6.6 indicate SNP formation (117 ± 18 nm) after mixing of the supramolecular building blocks. By exposing the as-assembled SNPs to UV light, the

observed SNP size analyzed by DLS decreased gradually to 44 ± 22 nm after 14 h. Notably, this size is comparable to the size observed for SNPs prepared using 100% Np from Np-PEG in the absence of multivalent dendrimer.

Figure 6.6: DLS size determinations of as-assembled SNPs (prepared from Np-PEG, Azo₈-PAMAM (Np:Azo = 70:30), MV-PEI and CB[8] with CB[8]:MV:(Np+Azo) = 1:1:1), after UV treatment for various periods of time, and after subsequent 24 h of ambient light.

SEM images show distinct SNPs of 80 ± 15 nm after self-assembly of the four supramolecular building blocks (Figure 6.7). The size and appearance of the SNPs were changed strongly after UV light exposure. The SNPs showed a dense core surrounded by less dense organic material. The average SNP size was 47 ± 10 nm, which is significantly smaller than before irradiation. Keeping the samples at ambient light resulted in SNPs with an average size of 68 ± 19 nm, which indicates that the SNPs regained most of their original size but not completely. Furthermore, the SNP shell did not look as well-defined as observed for the SNPs prior to UV treatment, suggesting the samples may require more time for complete reintegration of all Azo₈-PAMAM.

Figure 6.7: SEM images of SNPs (prepared from Np-PEG, Azo₈-PAMAM (Np:Azo = 70:30), MV-PEI and CB[8] with CB[8]:MV:(Np+Azo) = 1:1:1) a) after self-assembly, b) after 14 h UV light, c) after subsequent 24 h ambient light.

Summarizing, SNPs stabilized by multivalent CB[8]/MV/Azo interactions in the core and by monovalent CB[8]/MV/Np interactions in the shell shrink gradually upon UV treatment. This SNP disintegration can be ascribed to the destabilization of CB[8]/MV/Azo interactions in the core by photoisomerization of the Azo going along with a release of photoswitched Azo₈-PAMAM, whereas the SNP shell with unaffected Np-containing ternary complexes persists. In comparison to the SNPs whereby photoswitching of the shell leads to aggregation, the disintegration process of the core-switching SNPs is rather slow: only a minor change was observed by DLS for samples treated for 15 min (111 ± 25 nm) and 1 h with UV light (102 ± 25 nm). This slow UV response can be explained by the incomplete *trans-cis* isomerization described in Chapter 5, whereby switching of the CB[8]/MV/Azo ternary complexes linked to the multivalent Azo₈-PAMAM provides dendrimers that still have a few non-switched and interacting Azo groups. This leads to a continuous, but rather slow release of dendrimer from the SNP core, while the SNPs remain continuously stabilized by the monovalent Np-PEG heteroternary complexes.

6.3 Conclusions

In summary, we have presented here a toolbox approach for the fabrication of SNPs stabilized by the heteroternary complexes CB[8]/MV/Np and CB[8]/MV/Azo, allowing the placement of photoresponsive Azo groups in the SNP shell, the core or both. SNP formation appeared to be independent of the use of Azo or Np guest moieties, and similar SNP sizes were observed for all SNP formulations. Depending on the choice of the mono and multivalent building blocks four different types of SNPs were prepared, all of which respond differently to UV light. Whereas non-responsive SNPs were formed by using CB[8]/MV/Np interactions exclusively, which SNPs may be useful as passive carrier in biomedical applications, photoisomerization of Azo going along with rupture of the host-guest complexes triggers different SNP disassembly pathways of the SNPs depending on the location of the switchable Azo moiety.

SNPs with a switchable shell aggregated relatively fast upon UV treatment, and could be useful as drug carriers in oncology as SNP aggregation could trap the SNPs in tumor tissue in an enhanced EPR effect. In contrast, SNPs with a switchable core shrank slowly upon UV treatment due to release of the multivalent dendrimer from the SNP core. This SNP formulation might be suited for drug delivery applications in which continuous drug release is required over the course of hours. A combination of both (transient) aggregation and disassembly was observed for the initially reported SNPs with photoresponsive Azo in both the shell and the core, which might be beneficial for drug release in tumor tissue. Overall, this study shows that the degradation rate and pathway of SNPs can be pre-programmed by selecting the correct SNP architecture.

6.4 Acknowledgements

Jens Voskuhl is gratefully acknowledged for the synthesis of Azo₈-PAMAM and for fruitful discussions.

6.5 Experimental Section

6.5.1 Materials

Cucurbit[8]uril (CB[8]) was purchased from Strem Chemicals and its purity was assessed by microcalorimetric titration against paraquat. The synthesis and characterization of Np-PEG (5000 g/mol), Np₈-PAMAM, MV-PEI, Azo-PEG (5000 g/mol) and Azo₈-PAMAM is described in Chapter 3 and Chapter 5. SNP preparation was carried out in water of MilliQ quality (Millipore, $R = 18.2 \text{ M}\Omega\text{cm}^{-1}$).

6.5.2 Methods

Supramolecular nanoparticle assembly

For the preparation of SNPs stabilized by heteroternary host-guest interactions, solutions of Azo-PEG (1.96 μM), Np-PEG (1.96 μM), MV-PEI (0.31 μM) and CB[8] (2.8 μM) in water and Azo₈-PAMAM (2.63 μM) and Np₈-PAMAM (2.63 μM) in DMSO were prepared. The SNPs were formed by mixing 500 μL MV-PEI, 250 μL CB[8], 250 μL Azo-PEG or Np-PEG and 10 μL Azo₈-PAMAM or Np₈-PAMAM. The samples were kept at RT for 2 days before DLS and SEM analysis.

UV irradiation of the SNPs

UV irradiation experiments were carried out with a Hönle bluepoint 2 easycure using a shortpass filter $\lambda \leq 400 \text{ nm}$. After formation, the SNPs were treated with UV light for different periods. Therefore the samples were filled in a quartz cuvette and exposed to light with $\lambda \leq 400 \text{ nm}$. To analyze the ongoing disassembly process, the samples were analyzed by DLS in dark and UV irradiation was continued.

The SNP reassembly was evaluated after keeping the UV treated SNP samples for 24 h at ambient light, prior to analysis by DLS and SEM.

6.5.3 Equipment

DLS analysis (DLS)

DLS experiments were performed with a Nanotracer Wave (Microtrac) at 25°C using a laser wavelength of 780 nm at a scattering angle of 90°. The reported SNP hydrodynamic diameters are averaged of at least three individual measurements. The DLS curves shown in this chapter are representative for the respective systems studied here. For UV-treated samples, the measurements were carried out in the dark directly after irradiation.

Scanning electron microscopy (SEM)

All SEM images were taken with a Carl Zeiss Merlin scanning electron microscope. For sample preparation, the aqueous SNP solutions were drop-cast on a Formvar coated copper TEM grid. The solution was dried and analyzed without further treatment of the samples. For the samples measured after UV irradiation, the drying process was carried out in the dark.

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Chapter 7

Zwitterionic Supramolecular Nanoparticles: Self-Assembly and Responsive Properties

Supramolecular nanoparticles (SNPs) are of high interest in both nanoscience and molecular diagnostics and therapeutics, because of their reversible and designable properties. To ensure colloidal stabilization and biocompatibility, most reported strategies require the use of hydrophilic long-chain polymers such as poly(ethylene glycol). Here, we show the formation of zwitterionic supramolecular nanoparticles (ZSNPs) from appropriately functionalized mono- and multivalent components, based on the heteroternary host–guest complexation between cucurbit[8]uril (CB[8]), methyl viologen (MV), and azobenzene (Azo), while using the monovalent, small-molecule, non-fouling Azo-carboxybetaine analogue (Azo-Zwit) as the shell-forming component. Even though steric shell stabilization is absent, the zwitterionic Azo-Zwit ensures stability of the ZSNPs in water, in PBS (pH 7.4) at ionic strengths ranging from 0–700 mM, and in PBS containing BSA. Size tuning by control over the stoichiometry of the components, as well as reversible assembly and disassembly by photoisomerization of the Azo moieties were observed. Surprisingly, the ZSNPs exhibited aggregation at the narrow pH range of 6.2–6.8, which holds promise for an enhanced EPR effect when considering the ZSNPs as potential cancer therapeutics.

7.1 Introduction

Nanoparticles (NPs) are of major interest because of their size-dependent chemical and physical properties. Next to catalytic, optical, sensor or electronic applications, the use of NPs in biomedicine has gained intense scientific interest over the past two decades.^{1,2} In oncology, for example, countless studies have been carried out on the targeted delivery of active compounds.³ Based on the enhanced permeability and retention (EPR) effect caused by vascular hyperpermeability and impaired lymphatic drainage, NPs have been used to accumulate drugs and imaging agents in the tumor tissue.^{4,5} The microenvironment of this tissue, in particular the more acidic extracellular pH (6.0-7.0) compared to healthy tissue, has enabled the selective release of active compounds using responsive drug delivery vehicles or has initiated NP aggregation to enhance their retention in tumor tissue.⁶

A general problem of the administration of NPs to the human body is the rapid clearance of the NPs from the blood stream by the reticuloendothelial system (RES) or mononuclear phagocytic system (MPS).⁷ This process is induced by opsonization, the non-specific binding of proteins to NP epitopes, resulting in NP aggregation and elimination of the NPs by the immune defense system.^{7,8} In general, these processes are strongly dependent on the surface properties of the NPs. To enhance the pharmacokinetics and biodistribution of NPs *in vivo*, a poly(ethylene glycol) (PEG) coating is commonly used.^{9,10} However, PEG degradation by oxidation can result in loss of anti-fouling properties upon prolonged usage or storage.^{11,12}

Recently, a new class of non-fouling materials based on zwitterions has been put forward as an alternative for PEG in the functionalization of biomedically relevant materials.^{13,14} Zwitterions are molecules carrying equimolar amounts of negative and positive charges guaranteeing overall charge neutrality. Due to their ionic character, zwitterion-bearing compounds are ultra-hydrophilic. Various naturally occurring membrane lipids are of zwitterionic origin and have shown to be strongly anti-fouling when assembled in a model bilayer.¹⁵ Jiang and coworkers have used zwitterionic compounds for the coating of different types of NPs.¹⁶⁻¹⁸ In these studies, it was shown that a carboxybetaine shell around the NPs prevents non-specific protein binding in undiluted blood serum.

Soft supramolecular nanoparticles (SNPs) are promising materials because their formation strategy results in stable, yet reversible nanovectors. A variety of biomedically relevant SNPs based on host-guest chemistry has been developed by including active compounds into the SNP formulation. Drug delivery SNPs have been formed by covalent modification of the supramolecular building blocks with chemotherapy drugs such as camptothecin¹⁹ and doxorubicin,²⁰ whereas gene delivery vehicles have been based on electrostatic interactions of positively charged building blocks and negatively charged

RNA.²¹ Despite the chemical flexibility of SNP approaches, almost all SNP formulations require a PEG coating to ensure colloidal stabilization^{22,23} in aqueous media or to inhibit aggregation by interactions with proteins in the blood flow.²¹ Not only the hydrophilic properties of PEG, but also steric stabilization by the long polymeric PEG chains are believed to be important for achieving these properties.

Here, we describe the formation, stability and responsive properties of zwitterionic supramolecular nanoparticles (ZSNPs). The zwitterionic motif proposed here, is an alternative, shell-forming moiety around the SNP core to provide colloidal stability and anti-fouling properties. Hereto, SNPs are designed based on the heteroternary host-guest interaction between cucurbit[8]uril (CB[8]), methyl viologen (MV) and azobenzene (Azo) (Scheme 7.1). The monovalent character of the non-fouling Azo-Zwit is required to avoid uncontrolled growth of the SNP core by assembly of the multivalent components. An important research question is whether the relatively short zwitterionic molecule, which is much shorter than the typically used polymeric PEG molecules, provides sufficient colloidal stability in aqueous solution, in the absence or presence of relevant concentrations of serum proteins. Furthermore, stoichiometry-based size control, UV-initiated rupture by switching of the Azo moieties, and the stability of the SNPs in different pH media are also described.

Scheme 7.1: a) Schematic illustration of the self-assembly of zwitterionic supramolecular nanoparticles (ZSNPs) mediated by ternary host-guest complexes between cucurbit[8]uril (CB[8]), methyl viologen (MV) and azobenzene (Azo), UV light-triggered SNP disassembly and pH-responsive SNP aggregation. b) Supramolecular components involved in ZSNP assembly: azobenzene-functionalized poly(amidoamine) dendrimer (Azo₈-PAMAM), a monovalent Azo-carboxybetaine analogue (Azo-Zwit), MV-functionalized poly(ethylene imine) (MV-PEI), and CB[8].

7.2 Results and Discussion

7.2.1 Supramolecular nanoparticle formation in different media

An Azo-carboxybetaine analogue (Azo-Zwit) was synthesized (see Scheme 7.2) and used as monovalent capping ligand in the formation of the ZSNPs. By mixing MV-substituted poly(ethylene imine) (MV-PEI, degree of substitution: 4.5 MV units per polymer chain, M_w 10 kDa, Chapter 3), Azo-terminated poly(amidoamine) dendrimer generation 1 (Azo₈-PAMAM, Chapter 5), Azo-Zwit and CB[8], SNPs are formed by multiple heteroternary complexes between CB[8], MV and *trans*-Azo. The core of these SNPs is assembled via multivalent intermolecular interactions between the MV-polymer and the Azo dendrimer in the presence of CB[8], whereas monovalent interactions involving Azo-Zwit constitute the shell.

Scheme 7.2: Synthetic route towards the azobenzene-functionalized zwitterion derivative Azo-Zwit used in this study.

The formation of ZSNPs was studied in water at an equimolar 1:1:1 ratio of the molecular recognition moieties CB[8], MV and Azo. Using 70% Azo derived from Azo-Zwit and 30% from Azo₈-PAMAM, ZSNPs with an average size of 78 ± 9 nm and an average hydrodynamic diameter of 133 ± 19 nm were observed by scanning electron microscopy (SEM) and dynamic light scattering (DLS), respectively (see Figure 7.1a-b). The ZSNP sizes observed by SEM are smaller than by DLS which is attributed to (i) ZSNP shrinkage during SEM sample preparation by the loss of water and (ii) DLS reflecting the hydrodynamic diameter of the ZSNPs.

Figure 7.1: Size determination by a) DLS and b) SEM of ZSNPs prepared with CB[8]:MV:Azo = 1:1:1, with 30% Azo from Azo₈-PAMAM prepared in water.

To verify that the ternary host-guest complexation is essential for stable ZSNP formation, different control experiments were carried out. Congruently, ZSNP formation was detectable neither for samples prepared in the absence of CB[8] or MV-PEI nor for samples prepared with CB[7] instead of CB[8] (see Figure 7.2). It is known, that the smaller CB[7] is too small to encapsulate both guest molecules. These results confirm that ZSNP formation is induced by ternary host-guest complexation between MV, Azo and CB[8].

Figure 7.2: DLS analysis of ZSNP solutions (30% Azo from Azo₈-PAMAM, in water): a) in the absence of CB[8], b) in the absence of MV-PEI, c) in the presence of CB[7] instead of CB[8].

The use of NPs for biomedically relevant applications requires NP stability at physiological ionic strength. Therefore, ZSNP self-assembly was evaluated in phosphate-buffered saline (PBS, 140 mM KCl pH 7.4). Using the same ZSNP composition, SEM and DLS showed negligible size and morphology differences in comparison to experiments carried out in water (see Figure 7.3). This observation clearly shows that stable ZSNPs have been formed in PBS. In contrast, SNP formation experiments carried out with MV-PEI, Azo₈-PAMAM, CB[8] and Azo-functionalized tri(ethylene glycol) (Azo-TEG) in PBS, showed irreproducible results and uncontrolled aggregation by DLS. Similarly, SEM (Figure 7.4) showed indistinct structures, which are incomparable to the ZSNP structures reported here or to previously observed SNPs prepared by polymeric Azo-PEG (M_w 5 kDa) as shown in Chapter 5. This illustrates that the monovalent Azo-Zwit provides sufficient colloidal stability to the ZSNPs in PBS, whereas Azo-TEG with a comparable molecular length does not. Most likely, the ionic character of the Azo-Zwit renders the ZSNP particle shell ultra-hydrophilic, obviating the steric stabilization known for PEG-modified SNPs.

Figure 7.3: Size determination by a) DLS and b) SEM of ZSNPs prepared with CB[8]:MV:Azo = 1:1:1, with 30% Azo from Azo₈-PAMAM prepared in PBS (140 mM KCl, pH 7.4).

Figure 7.4: Size determination by SEM of different SNP formulations prepared with Azo-tri(ethylene glycol): a) 10% Azo from Azo₈-PAMAM, b) 20% Azo from Azo₈-PAMAM, and c) 30% Azo from Azo₈-PAMAM.

To investigate the effect of ionic strength on ZSNP formation and stability, self-assembly of the four supramolecular building blocks was carried out in PBS with varying concentrations of KCl (50-700 mM) using 30% Azo from Azo₈-PAMAM. DLS and SEM revealed that ZSNPs are formed regardless of the ionic strength (see Figures 7.3 and 7.5). The ZSNPs were stable for at least 5 days after self-assembly. Nevertheless, the ZSNP size and size distribution observed for the samples prepared in PBS with 700 mM KCl were higher (237 ± 42 nm by DLS and 117 ± 41 nm by SEM) as compared to the ZSNPs at 140 mM KCl (154 ± 13 nm by DLS and 72 ± 17 nm by SEM). We have recently shown that SNP destabilization at high ionic strength can be inhibited by a monovalent capping PEG (M_w 5 kDa).²⁴ The results shown here indicate that Azo-Zwit is also capable of preserving colloidal stability at high ionic strength.

Figure 7.5: Size determination of ZSNPs prepared in PBS (pH 7.4) with different KCl concentrations: a-d) SEM images at ionic strengths of: 50 mM, b) 100 mM, c) 300 mM, d) 700 mM, e) DLS curves for the different SNP formulations. f) ZSNPs diameters as measured by SEM (■) and DLS (■).

Importantly, ZSNPs were also stable after mixing 250 μL ZSNPs solution with 500 μL of a 5 wt% dispersion of BSA in PBS, which is the common concentration of serum proteins in blood. DLS gave inconclusive results because of the high protein concentration, but SEM images unambiguously revealed the stability of the ZSNPs in this medium (Figure 7.6).

Figure 7.6: Size determination of ZSNPs prepared in PBS (pH 7.4 with 140 mM KCl) and BSA in PBS: a) DLS analysis of ZSNPs in PBS prepared with 14 μM of CB[8], MV, and Azo moieties, b) DLS analysis of 5 wt% BSA in PBS, c) DLS analysis of 1:2 v/v mixture of ZSNP and BSA solutions, d) SEM image of ZSNPs observed in BSA e) SEM image of ZSNPs observed at the drying edge of the SEM sample.

Size tunability of the self-assembled ZSNPs was evaluated in aqueous solution by altering the ratio of the monovalent Azo-Zwit and the multivalent Azo₈-PAMAM while keeping the overall CB[8]:MV:Azo stoichiometry at 1:1:1. By increasing the amount of Azo from Azo₈-PAMAM from 10% to 30%, while decreasing the corresponding amount of Azo-Zwit from 90% to 70%, an increase in ZSNP size was observed from 51 ± 11 nm to 78 ± 9 nm by SEM and from 62 ± 16 nm to 133 ± 19 nm by DLS (see Figures 7.1 and 7.7). This size tuning is attributed to the competition between monovalent and multivalent heteroternary interactions, similar to the SNP formulations stabilized with monovalent PEG reported in Chapter 3 and 5.

Figure 7.7: Size determination of ZSNPs prepared in water using different stoichiometries of the mono- and multivalent Azo components while maintaining CB[8]:MV:Azo at 1:1:1: a-b) SEM images of ZSNPs prepared in water with a) 10% and b) 20% Azo from Azo₈-PAMAM dendrimer. c) DLS graphs obtained for the ZSNPs prepared with different formulations in water. d) ZSNP diameters measured by SEM (■) and DLS (■).

7.2.2 Responsive properties of zwitterionic supramolecular nanoparticles

The reversibility of the CB[8]:MV:Azo host-guest complex, induced by photoswitching of the Azo moiety, has been used to trigger NP assembly and disassembly as shown in Chapter 5 and by Scherman and coworkers.²⁵ Photoisomerization of *trans* to *cis*-Azo by UV light causes disassembly of the ternary complex because the bulkier *cis*-Azo and MV do not fit in the cavity of CB[8] simultaneously. To evaluate whether the photoswitching of Azo can be used for the rupture of ZSNPs, the particles in aqueous solution were irradiated with UV light. The effect of UV irradiation on the size of the ZSNPs measured by DLS is shown in Figure 7.8. As observed for SNPs prepared with Azo-PEG (M_w 5 kDa) in Chapter 5, the ZSNPs started to aggregate after short UV irradiation. This effect continued until no ZSNPs were observed after prolonged UV exposure overnight. The relatively slow particle disassembly process is attributed to incomplete *trans*-isomerization (ca. 80%) and slow rearrangement of multivalently linked building blocks resulting in fast shell but slow core dissolution. In addition, ZSNP re-assembly was achieved upon *cis-trans* isomerization of the Azo-bearing building blocks by irradiation with visible light for 8 h.

Figure 7.8: DLS analysis of a ZSNP solution: a) as assembled, after UV irradiation for b) 10 min, c) 1 h, d) 2 h, e) 3.5 h, f) 6 h, g) 14 h, and h) after subsequent irradiation with visible light for 8 h.

Whether the *cis*-Azo or the MV moiety stays in the cavity of the CB[8] after photoswitching, depends on the molecular structure of the components. If the Azo derivative contains a positive charge in close proximity to the Azo moiety, the *cis*-Azo isomer is stabilized in the CB[8] cavity by the interaction of the positively charged guest with the ring of carbonyls surrounding the CB[8] cavity, thus leading to release of MV upon *trans-cis* isomerization.²⁶ In contrast, in the absence of a cationic charge close to the Azo moiety, the MV stays in the CB[8] cavity and the *cis*-Azo is expelled.²⁷ As the positive charge in Azo-Zwit is not located in the immediate vicinity of the Azo moiety and the charge is partially compensated by the carboxylate anion at the tail of the molecule, it was not clear a priori which moiety would remain bound upon UV-induced *trans-cis* isomerization. UV/Vis spectroscopy (Figure 7.9) of *cis*-Azo-Zwit in absence and presence of CB[8] showed that the *cis* isomer can be encapsulated in the cavity as witnessed by the decrease of the absorbance of *cis*-Azo-Zwit at $\lambda=432$ nm characteristic for inclusion in CB[8]. However, in case MV was present in the solution as well, the absorbance

decrease was only half. Therefore we conclude that an equilibrium between CB[8]-bound MV and *cis*-Azo-Zwit exists after *trans-cis* photoswitching.

Figure 7.9: UV-Vis absorbance of Azo-Zwit and Azo-Zwit and paraquat (PQ) in the presence of different concentrations of CB[8] a) under ambient light conditions and b) in dark, after UV irradiation.

To study the behavior of the ZSNPs as a function of pH, the supramolecular building blocks were dissolved in PBS at pH 5.6, 6.2, 6.8 and 7.4 prior to mixing. DLS and SEM images showed that individual ZSNPs were formed at pH 5.6 and 7.4 (see Figures 7.3 and 7.10). In contrast, both techniques indicated the formation and simultaneous aggregation of ZSNPs at the intermediate pH values 6.2 and 6.8 (Figure 7.10) with the stronger aggregation at pH 6.8. The aggregates clearly contain individual particles of similar size as formed at pH 7.4, which indicates that the aggregates are hierarchical assemblies of ZSNPs. Notably, the pH regime of 6.2-6.8 corresponds with the

extracellular pH observed in tumor tissue. Equally important to note, the pH regime corresponds also with the pK_w (13.7) of the water equilibrium, which indicates a balance between hydronium cations and hydroxide anions at pH 6.8. A similar aggregation effect in this pH range was reported by Liu et al. for gold NPs modified with mixed monolayers of 11-mercaptopundecanoic acid and (10-mercaptodecyl)-trimethyl ammonium bromide.²⁸ Only when using equimolar fractions of the carboxylic acid and quaternary ammonium adsorbates, was Au NP aggregation observed in a window between pH 6.0 and 7.0, whereas individual Au NPs were observed above and below this regime. We assume that, at all pH values used here, the ZSNPs are surrounded by overall charge-neutral Azo-Zwit bearing unprotonated carboxylate ions. We therefore attribute aggregation of the ZSNPs to interaction between the shells in a perfectly charge-balanced environment, while a slight imbalance between hydronium and hydroxide ions is apparently sufficient to prevent aggregation. At the same time, we note that this aggregation mechanism may be more universal, and can potentially be used to design self-assembling materials that will show an enhanced EPR effect.

Figure 7.10: SEM images of ZSNPs formed in PBS at: a) pH 5.6, b) pH 6.2, c) pH 6.8. d) DLS graphs obtained for the ZSNPs prepared with in PBS at different pH values. e) Average ZSNP diameters measured by DLS.

7.3 Conclusions

In conclusion, we have shown the formation of SNPs based on the ternary inclusion interaction between CB[8], MV and Azo, using the small-molecule zwitterionic Azo-Zwit as the monovalent capping ligand. In contrast to more conventional SNP formulations

requiring long PEGs for steric stabilization, colloidal stability of the ZSNPs without this steric effect, was observed in different, biologically relevant media. This supramolecular assembly strategy enables size tuning by stoichiometric control of the ratio of multivalent and monovalent Azo components as well as light-triggered SNP disassembly. The ZSNPs show a variety of properties that make them potentially interesting for biomedical applications. Firstly, the use of Azo-Zwit provides non-fouling properties also at high serum protein concentrations. Secondly, ZSNP aggregation is observed at pH values between 6.2 and 6.8, indicating a potentially enhanced EPR effect in tumor tissue. All these properties combined underline the promise of ZSNPs as nanovectors in cancer therapeutics.

7.4 Acknowledgements

Jens Voskuhl is acknowledged for the synthesis of Azo-terminated poly(amino amine) dendrimer generation 1 and Azo-tri(ethylene glycol). Mark Smithers is acknowledged for the SEM images.

7.5 Experimental Section

7.5.1 Materials

Starting materials for organic synthesis were obtained from Sigma-Aldrich and ABCR and used as received. Methyl-4,4-bipyridinium (methyl viologen, MV)-substituted poly(ethylene imine) (MV-PEI, degree of substitution: 4.5 MV units per polymer chain) and azobenzene (Azo)-terminated poly(amido amine) dendrimer of generation 1 (8 end groups) were synthesized as described in Chapters 3 and 5, respectively. Azobenzene-tri(ethylene glycol) was prepared according to the literature procedure.²⁹ Cucurbit[8]uril (CB[8]) was purchased from Strem Chemicals and the concentration of a stock solution was assessed by microcalorimetric titration (Microcal VP-ITC) against paraquat. Deuterated solvents used for NMR spectroscopy were purchased from Cambridge Isotope and the water used for dialysis and SNP preparation was of MilliQ quality (Millipore, $R=18.2 \text{ M}\Omega\text{cm}^{-1}$).

7.5.2 Synthetic procedures

N,N-Dimethyl-3-(4-(phenyldiazenyl)phenoxy)propan-1-amine

To a solution of 1-bromo-3-(*N,N*-diethyl)propane hydrobromide (200 mg, 0.405 mmol, 1 eq) in 10 mL acetonitrile, K_2CO_3 (170 g, 0.61 mmol, 1.5 eq) was added, and the suspension was stirred for 30 min at 70°C. After that time 4-phenylazophenol (160 mg, 0.405 mmol, 1 eq) was added and stirring was

continued at 80 °C for 12 h, followed by removal of the solvent. The residue was dissolved in 25 mL of CHCl_3 and extracted three times with distilled water (10 mL). The organic layer was separated and dried over MgSO_4 . The solvent was evaporated and the residue was subjected to silica gel column chromatography ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ gradient elution 100:1 to 98:2). The product 1 was obtained as a yellow solid. Yield: 143 mg, 62%. $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ = 7.86-7.79 (m, 4H, 4CH); 7.45-7.41 (m, 2H, 2CH); 7.39-7.35 (m, 1H, CH); 6.96-6.92 (m, 2H, 2CH); 4.07-4.04 (m, 2H, CH_2); 2.56 (t, 2H, CH_2); 2.32-2.27 (s, 6H, 2 CH_3); 2.05-1.99 (q, 2H, CH_2). $^{13}\text{C-NMR}$ (100 MHz, CD_2Cl_2): δ = 163.5, 154.6, 148.8, 132.3, 130.9, 126.5, 124.3, 116.6, 68.2, 57.8, 46.8, 31.6, 28.8. ESI-MS (m/z): calculated for $[\text{C}_{17}\text{H}_{21}\text{N}_3\text{OH}]^+$: 284.17, found: 284.20.

3-(Dimethyl-(3-(4-phenyldiazenyl)phenoxy)propyl)ammonio)propanoate (Azo-Zwit)

To an ice-cooled solution of 1 (60 mg, 0.21 mmol 1 eq) in 10 mL dry acetone, β -propiolactone (18 mg, 0.252 mmol, 1.2 eq) in 1 mL acetone was added slowly. The reaction mixture was stirred 2 h at 5°C and slowly warmed up to RT. The yellow precipitate was filtered and washed with anhydrous acetone and anhydrous ether. The product was dried under reduced pressure to obtain the final (E)-3-(dimethyl-(3-(4-phenyldiazenyl)phenoxy)propyl)ammonio) propanoate (Azo-Zwit). Yield: 27 mg, 36%. $^1\text{H-NMR}$ (400 MHz, D_2O): δ = 7.75-7.69 (dd, 4H, 4CH); 7.50-7.48 (d, 3H, 3CH); 7.03-7.01 (d, 2H, 2CH); 4.11 (t, 2H, CH_2); 3.54-3.50 (t, 2H, CH_2); 3.43 (t, 2H, CH_2); 3.02 (s, 6H, 2 CH_3); 2.70-2.66 (t, 2H, CH_2); 2.18-2.12 (t, 2H, CH_2). $^{13}\text{C-NMR}$ (150 MHz, DMSO-d_6): δ = 170.98, 161.43, 152.46, 146.95, 131.40, 129.89, 129.57, 125.08, 123.44, 122.74, 119.78, 115.63, 114.88, 65.69, 61.25, 60.81, 50.64, 30.28, 20.68. ESI-MS (m/z): calculated for $[\text{C}_{20}\text{H}_{25}\text{N}_3\text{O}_3\text{H}]^+$: 356.19, found: 356.19.

7.5.3 Methods

Zwitterionic supramolecular nanoparticle assembly

For the preparation of the zwitterionic supramolecular nanoparticles (ZSNPs), aqueous or PBS solutions of CB[8], MV-PEI and different concentrations of Azo-Zwit were prepared before mixing. DMSO was used to dissolve different concentrations of Azo8-PAMAM. For example, for preparing a solution having 30% Azo entities derived from the aliphatic Azo₈-PAMAM dendrimer, 1000 μL MV-PEI (0.31 μM) was added to a previously prepared solution of 500 μL Azo-Zwit (1.96 μM), 20 μL Azo₈-PAMAM (2.65 μM) and 500 μL CB[8] (2.8 μM). The sample was mixed and kept at room temperature for 2 days before DLS and SEM analysis.

Control experiments for zwitterionic supramolecular nanoparticle formation

To evaluate whether ZSNP formation is triggered by the formation of the ternary complex between CB[8], MV and Azo, different control experiments were carried out. ZSNP formation using 30% Azo from Azo₈-PAMAM was carried out without CB[8], without MV-PEI or in the presence of the cucurbit[n]uril homolog CB[7]. Hereto, the ZSNP solutions were prepared with 500 μ L water or 500 μ L CB[7] (2.8 μ M) instead of 500 μ L CB[8] (2.8 μ M). Additionally, ZSNP formation was evaluated in the absence of MV-PEI.

Supramolecular nanoparticle formation with Azo-tri(ethylene glycol)

To get a better understanding of the stabilizing role of Azo-Zwit in the formation of ZSNPs, SNP assembly was attempted in PBS using Azo-tri(ethylene glycol) as the shell-forming component. Therefore different formulations, containing 10%, 20% and 30% Azo from Azo₈-PAMAM, were tested according to the procedure described for ZSNP preparation and the formation of SNPs reported in Chapter 5.

Formation and stability of ZSNPs in PBS with different salt concentrations

In order to investigate the ZSNP formation in PBS at different ionic strengths, solutions of MV-PEI (0.31 μ M), Azo-Zwit (1.96 μ M) and CB[8] (2.8 μ M) were prepared in PBS (pH 7.4) containing 50 mM, 100 mM, 140 mM, 300 mM and 700 mM KCl. These solutions were mixed according the recipe given for the preparation of the ZSNPs in water (containing 30% Azo from Azo₈-PAMAM). ZSNP formation was analyzed by DLS after 2 days at RT and by SEM 7 days after preparation.

Stability of ZSNPs in PBS containing BSA to imitate protein concentration in human body

To verify the stability of the ZSNPs in the presence of proteins, ZSNPs were formed in PBS using an overall concentration of 14 μ M of the supramolecular host-guest binding partners and 30% Azo from Azo₈-PAMAM. This mixture was kept 2 d at room temperature to ensure stable ZSNP formation. In addition, a 5 wt% dispersion of BSA in water was prepared. Stability of the ZSNPs was controlled after mixing 500 μ L of the BSA solution with 250 μ L of the ZSNP solution. DLS analysis was carried out for the ZSNPs, BSA and a mixture of both. Additionally SEM was carried out 1d after mixing the two solutions.

Light response of supramolecular nanoparticles

To evaluate the photoresponsive properties of the particles, ZSNPs containing 30% Azo from Azo₈-PAMAM were prepared and analyzed. Samples were irradiated with UV light at $\lambda < 400$ nm for different times. DLS analysis was performed immediately after exposure, while keeping the SNP samples in the dark. Representative DLS scattering graphs are given in Figure 7.8, although the polydispersity of the irradiated samples were very high and these are not absolute values.

pH-induced aggregation of the ZSNPs

In order to investigate the ZSNP formation in PBS, solutions of MV-PEI (0.31 μM), Azo-Zwit (1.96 μM) and CB[8] (2.8 μM) were prepared in PBS at pH 5.6, 6.2, 6.8 and 7.4. ZSNP formation (30% Azo from Azo₈-PAMAM) at these pH values was carried out as described for ZSNP preparation in water by mixing the aqueous solutions with Azo₈-PAMAM in DMSO (2.62 μM). ZSNP formation was analyzed by DLS after 2 days at RT and by SEM 5 days after preparation.

7.5.4 Equipment

Dynamic light scattering (DLS)

DLS experiments were performed with a Nanotracer by Anaspec operating with a Microtrac FLEX Operating Software at 25°C using a laser wavelength of 780 nm at a scattering angle of 90°. The observed sizes and standard derivations of the ZSNPs were calculated by taking an average of 5 measurements. In total, particles with sizes ranging from 0.8 to 6400 nm were detected. The DLS curves shown in this chapter are representative for the individual measurements.

Scanning electron microscopy (SEM)

All SEM images were taken with a 1500 high resolution scanning electron microscope. Therefore the aqueous SNP solutions were drop-cast on a Formvar coated copper TEM grid. The solution was dried and analyzed without further treatment of the samples.

UV irradiation

UV light irradiation experiments were carried out with a Hönle bluepoint 2 easycure using a shortpass filter $\lambda \leq 400$ nm. After formation, the SNPs were treated with UV light for different periods. Therefore the samples were filled in a quartz cuvette and exposed to light with $\lambda \leq 400$ nm. To analyze the ongoing disassembly process, the samples were analyzed by DLS in dark and UV irradiation was continued.

To evaluate the SNP re-assembly, the SNP solution was kept at visible light for 8 h and analyzed afterwards.

UV/Vis spectroscopy

UV/Vis absorption spectra were recorded using a Perkin Elmer Lambda 850 UV-Vis spectrometer. Supramolecular encapsulation of Azo-Zwit in presence of CB[8] and in presence of paraquat (MV) and CB[8] was evaluated by UV/Vis spectroscopy at room temperature. Stock solutions of Azo-Zwit, CB[8] and paraquat were prepared in water. The stock solutions were mixed to result in solutions with Azo-Zwit (12.5 μM), Azo-Zwit/paraquat (12.5 μM /12.5 μM), Azo-Zwit/CB[8] (12.5 μM /6.25 μM), Azo-Zwit/paraquat/CB[8] (12.5 μM /12.5 μM /6.25 μM), Azo-Zwit/CB[8] (12.5 μM /12.5 μM), Azo-Zwit/paraquat/CB[8] (12.5 μM /12.5 μM /12.5 μM). The UV/Vis absorbance spectra of

these solutions were measured at visible light conditions and after irradiation with UV light to evaluate the encapsulation efficiencies of the two photoisomers of Azo-Zwit by CB[8] in the absence and presence of paraquat.

NMR spectroscopy

^1H and ^{13}C NMR spectra were recorded on Bruker 400 MHz and 600 MHz spectrometers. ^1H and ^{13}C chemical shift values, measured at 400 MHz and 150 MHz, respectively, are reported as δ using the residual solvent signal as internal standard.

Mass spectrometry

Mass analysis was performed by electrospray ionization using micromass LCT from Waters/Micromass.

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Chapter 8

Self-Assembly of Size-Tunable Supramolecular Nanoparticle Clusters in a Microfluidic Channel

Clusters of nanoparticles, also called network aggregates, form an important class of materials in the development of multifunctional materials using a toolbox approach. Microfluidics has emerged as a powerful technology in various fields such as chemical synthesis, medical diagnostics and high-throughput biological assays. Only few studies have described the merging of microfluidic technology with supramolecular host-guest chemistry for the fabrication of large self-assembled structures. In this chapter, supramolecular nanoparticle clusters (SNPCs) have been formed in a microfluidic device by controlling the diffusive mixing of the constituting supramolecular building blocks. Cluster formation between ligand-functionalized silica nanoparticles, dendrimers, and poly(ethylene glycol) (PEG) stopper molecules is induced by the ternary charge-transfer complex formation between cucurbit[8]uril, methyl viologen and naphthol. The resulting SNPC size depends strongly on the stoichiometry of the host and guest binding partners, the competition between multivalent and monovalent naphthol entities, and the microfluidic flow conditions. Variation of the PEG length leads to modulation of its diffusion rate and thus to an additional kinetic control parameter of the SNPC formation process.

8.1 Introduction

Self-assembly is the process by which individual components organize into ordered structures spontaneously, and it is a central theme of nanoscience and nanotechnology.^{1,2} Self-assembly enables the formation of functional materials in nano- or micrometer dimensions, based exclusively on non-covalent forces such as Van der Waals interactions, hydrogen bonding, electrostatic interactions, magnetic interactions and coordination chemistry.^{3,4} Supramolecular host-guest chemistry offers the ability to engineer desired structures based on a blend of different non-covalent interactions, which thus enables the formation of stable, yet reversible constructs. Inclusion complexes formed by the interaction of cucurbit[8]uril or cucurbit[10]uril with appropriately sized guest molecules are known to lead to high binding affinities, and their size allows the inclusion of two guests leading to ternary complexes.⁵ Besides supramolecular binding motifs, polymers such as poly(ethylene glycol) (PEG) can be used as structure-directing agents that support the formation of self-assembled functional materials.⁶ Using guest-modified PEG, we have demonstrated the formation of size-tunable (Chapter 3) and dual responsive (Chapter 8) supramolecular nanoparticles (SNPs) mediated by the heteroternary interaction of CB[8], methyl viologen (MV), and naphthol (Np), or of CB[8], MV, and azobenzene, respectively.

The modification of inorganic nanoparticles with host and guest moieties has been an established strategy to fabricate nanoparticle networks in aqueous solution.⁷⁻⁹ For example, the clustering of β -cyclodextrin-functionalized silver nanoparticles with aromatic guest molecules provides a sensitive detection method of different isomers by eye.¹⁰ The optical, magnetic and biological properties of clustered materials have been shown to depend strongly on their size and conformation.¹¹

The ultimate goal of chemists is to control the properties of a structure prior to synthesis. This requires superior control over the fabrication of the resulting structures, especially in self-assembly processes, because the formation of supramolecular host-guest structures is strongly dependent on thermodynamic and kinetic parameters. We have recently shown that the assembly kinetics of adamantane/cyclodextrin nanoparticle network aggregates can be varied by the flow conditions in a multi-inlet vortex mixer.¹² In contrast to the turbulent flow conditions in such a mixer, uniform and well-defined laminar flow is observed within microfluidic reactors.

Microfluidics deals with the manipulation of small volumes of liquids in a microscale channel, and its large applicability and versatility have allowed it to grow into a powerful interdisciplinary technology.¹³ The micrometer device dimensions provide a large surface-to-volume ratio, rapid heat transfer, low Reynolds numbers and unique mass transport properties which are beneficial in applications such as chemical synthesis,¹⁴ medical

diagnostics,¹⁵ high throughput biological assays,¹⁶ controlled synthesis of nano-materials¹⁷ and the controlled self-assembly of various nanostructures.¹⁸⁻²⁴ In microfluidic devices, liquid streams from different inlets flow in parallel, and the mixing of the interacting components in those streams is driven by lateral molecular diffusion. This diffusive mixing is strongly dependent on the diffusion properties of the building blocks and the residence time in the microfluidic device, and can therefore be directly regulated by the flow rates of the ingoing fluid streams. For electrostatic interactions, the formation of soft NPs was controlled using hydrodynamic flow focusing, by tuning the residence time, temperature and pressure.^{25,26} The assembly of gold and iron oxide nanoparticles onto silica nanoparticles has been controlled by varying the flow rates within a multistep microfluidic device.²⁷ The self-assembly of porphyrin architectures has been shown recently to be regulated by the flow field in a micro-flow environment.²⁸

Only few studies have been described in literature in which microfluidic technology is merged with the field of supramolecular host-guest chemistry for the fabrication of large self-assembled structures. Tseng and co-workers have used a digital microreactor to prepare an assortment of cell-targeting and DNA encapsulated SNPs based on the interaction of β -cyclodextrin and adamantane, providing a better dispersity and higher reproducibility than in batch processes.^{29,30} Using the same host-guest chemistry, Thompson and co-workers have described the variation of polymeric supramolecular nanoparticle properties (diameter, polydispersity and zeta-potential) by controlling the velocity of the two mixing inlet streams within a microfluidic reactor.³¹ The groups of Scherman and Abell have shown the formation of monodisperse supramolecular microcapsules self-assembled within microfluidic droplets using the ternary interaction between CB[8], and MV and Np building blocks.³² Nevertheless, the diffusive mixing omnipresent in microfluidics has not been used so far to control the formation of host-guest nanoparticle network aggregates.

In this chapter, we present the size-controlled self-assembly of aggregates of nanoparticles, so-called supramolecular nanoparticle clusters (SNPCs) (Scheme 8.1), mediated by multiple heteroternary host-guest interactions of CB[8], MV and Np. Cluster formation is induced by multivalent interactions forming between the MV-modified silica nanoparticles (SiO_2 -MV), a multivalent Np-modified dendrimer, and CB[8], whereas Np-PEG terminates the aggregation by monovalent interaction at the surrounding cluster shell. Aggregate formation was carried out comparing mixing in batch conditions and diffusive mixing within a microfluidic device. Only under diffusive mixing conditions, controlled SNCP formation and size tunability was observed by competition between the mono- and multivalent Np-bearing components, by changing the mixing time of the interacting host and guest binding partners in the microreactor.

Scheme 8.1: a) Schematic illustration of supramolecular network aggregation of silica nanoparticles, dendrimers and PEG stopper molecules, mediated by the ternary complex formation between MV, Np, and CB[8]. b) Supramolecular building blocks involved in SNPC formation: CB[8], Np-PEG (M_w 1000 or 5000 g/mol), Np₈-PAMAM, and SiO₂-MV. c) Ternary complex formation by inclusion of MV in CB[8], followed by inclusion of Np.

8.2 Results and Discussion

8.2.1 Supramolecular building blocks for SNPC formation

As interacting guest-modified building blocks, we used silica nanoparticles functionalized with MV (SiO₂-MV), Np-terminated poly(amido amine) G1 dendrimer (Np₈-PAMAM), and Np-terminated poly(ethylene glycol) monomethyl ether (Np-PEG) (Scheme 8.1b). The Np-bearing building blocks, Np-PEG (M_w = 5000/1000 g/mol) and Np₈-PAMAM, were prepared as described in Chapters 3 and 4. Silica nanoparticles functionalized with methyl viologen (SiO₂-MV) were synthesized by adapting a literature procedure for the functionalization of transferrin-decorated NPs (Scheme 8.2).³³ Bare silica NPs³⁴ were aminated using 3-(aminopropyl)-triethoxysilane (APTES), followed by reaction with an excess of bifunctional succinimide-maleimide hexa(ethylene glycol) linker. The terminal maleimide group was used to functionalize the particles with 1-(1-undecyl-11-thiol)-1-methyl-4,4'-bipyridinium,³⁵ followed by backfilling the unreacted maleimide groups with mercaptoethanol.

Scheme 8.2: Synthesis of methyl viologen-conjugated silica NPs ($\text{SiO}_2\text{-MV}$).

Dynamic light scattering (DLS) and scanning electron microscopy (SEM) showed an average NP size of 61 nm and 52 nm, respectively (Figure 8.1a and b). The density of guest moieties attached onto the particle surface, important for stoichiometry control during supramolecular assembly, was determined by UV/Vis spectroscopy to be $5.3 \mu\text{mol MV per g of NPs}$ ($\sim 900 \text{ MV/NP}$, approx. 1 MV per 12 nm^2) (Figure 8.1c). This coverage is 3.6 times higher than reported for the transferrin-functionalized silica nanoparticles, which might be attributed to the bulkier transferrin protein compared to MV. This density ensures that the guest moieties have sufficient space for supramolecular host-guest complexation with CB[8] and Np.

Figure 8.1: Characterization of $\text{SiO}_2\text{-MV}$ by a) DLS analysis. b) SEM analysis. c) UV/Vis spectroscopy.

8.2.2 Formation of SNPCs in bulk

The formation of SNPCs in solution was studied using a 2 μM concentration of CB[8], MV and Np, while keeping the molecular recognition moieties in an equimolar 1:1:1 ratio. The relative concentrations of the monovalent Np-PEG and the multivalent Np₈-PAMAM were varied during self-assembly, while keeping the overall concentration of Np moieties constant. As schematically shown in Figure 8.2, SNPC formation was tested in bulk by mixing an aqueous SiO₂-MV dispersion with a previously prepared aqueous solution of CB[8], Np-PEG ($M_w = 5000$ g/mol) and Np₈-PAMAM, using 25%, 37.5% or 50% Np from Np₈-PAMAM.

Figure 8.2: Schematic illustration of the formation of supramolecular nanoparticle clusters in bulk.

Instantaneous SNPC formation was observed 10 min after mixing as observed by a drastic increase of the average hydrodynamic cluster size by DLS (Figure 8.3). At the same time, the size distributions of the SNPCs were very broad (ranging from 143 nm to 441 nm and from 776 nm to 1813 nm for the 25% and 50% Np₈-PAMAM samples, respectively). As size focusing of soft SNPs formed by the same heteroternary supramolecular complex has been observed over the course of days (Chapter 3), the size of the SNPCs was monitored in time. In contrast to soft SNPs, the batch-formed SNPCs showed complete cluster sedimentation after 14 h. Apparently, the turbulence and fluid motion from stirring have led to uncontrolled and irreproducible NP aggregation.³⁶ We assume that, in contrast to the fully molecular SNPs, the SNPCs targeted here cannot

equilibrate to a thermodynamic minimum because of the higher valency and the slower diffusion of the SiO₂-MV NPs compared to the MV-polymer used in the soft SNPs. Therefore, kinetic control is deemed mandatory to achieve stable SNPCs.

Figure 8.3: Averaged DLS sizes of SNPCs prepared in batch at a constant ratio MV:CB[8]:Np of 1:1:1, as a function of Np derived from Np₈-PAMAM dendrimers used during SNPC self-assembly.

8.2.3 Formation of SNPCs in a microreactor

Subsequently, network aggregation of SiO₂-MV was evaluated in the confined environment of a microreactor. To this end, the dispersion of SiO₂-MV was injected in one of the two inlets of the microreactor, while a premixed solution of CB[8], Np-PEG, and Np₈-PAMAM was injected in the second inlet, and the two streams were put in laminar flow with an equal flow rate (Figure 8.4). The diffusion coefficients and the time required for the different SNPC components to diffuse over the distance of the microreactor were evaluated by the Stokes-Einstein ($D = \frac{kT}{6\pi\eta R}$) and the Einstein-Smoluchowski equations ($t = \frac{d^2}{2D}$), respectively (Table 8.1), assuming absence of complexation.

Figure 8.4: Schematic illustration of the microfluidic assisted supramolecular network aggregation of silica particles, dendrimers and PEG stopper molecules, mediated by the ternary interaction of MV and Np in the presence of CB[8].

Table 8.1: Diffusion coefficients and diffusion times of the supramolecular building blocks used in SNPC formation in a microfluidic device.

Supramolecular building block	R (m)	D (m ² /s) ^a	t (s) ^b
SiO ₂ -MV	2.5×10^{-8}	8.6×10^{-12}	911
CB[8] ³⁷	8.8×10^{-10}	2.5×10^{-10}	32
PAMAM G1 ³⁸	9.3×10^{-10}	2.3×10^{-10}	34
Np-PEG (1000 g/mol) ³⁹	9.7×10^{-10}	2.2×10^{-10}	35
Np-PEG (5000 g/mol) ⁴⁰	2.3×10^{-9}	9.4×10^{-11}	83

^a $D = \frac{kT}{6\pi\eta R}$, where, k is the Boltzmann constant (1.38×10^{-23} m² kg s⁻² K⁻¹), T is the temperature (293.15 K), η is the viscosity of water (1.002×10^{-3} kg s⁻¹ m⁻¹), R is the radius of the building blocks. ^b $t = \frac{d^2}{2D}$, where d indicates half of the microchannel width.

Given the dimensions of the device and the flow rates used here, the diffusion rate of SiO₂-MV is too small to expect appreciable penetration of the particles into the other stream. In contrast, the diffusion of Np₈-PAMAM, Np-PEG (M_w 1000 g/mol), and CB[8] are comparable, allowing these molecules to diffuse into the complete NP flow within approximately 35 s (Figure 8.5). The diffusion rate of Np-PEG (M_w 5000 g/mol) is calculated to be somewhat lower. The assembly formation in a microreactor is controlled by the diffusion of the molecular components into the nanoparticle stream, as exemplified by the diffusion profiles assumed in the absence of complexation. Based on these results, we decided to start clustering experiments with a residence time of 60 s.

Figure 8.5: Schematic illustration of the particle (SiO_2 -MV, red) and molecular (here shown for CB[8] in green, similar for Np-PEG and Np₈-PAMAM) diffusion profiles in time over the width of the microfluidic reactor (top view).

8.2.4 Size tuning of SNPCs by varying the stoichiometry

In contrast to the experiments performed in bulk conditions, distinct SNPCs were observed at a residence time of 60 s. DLS and SEM show clearly that the resulting cluster size can be tuned over a large dynamic range by varying the ratio of the two Np components while keeping the overall stoichiometry of CB[8]/MV/Np constant (Figure 8.6). By increasing the amount of Np from Np₈-PAMAM from 25% to 62.5%, while decreasing the amount of Np from Np-PEG (M_w 5000 g/mol) from 75% to 37.5%, an increase in SNPC size from 125 ± 10 nm to 370 ± 100 nm is observed by DLS, while SEM indicates a similar tuning range from 65 ± 20 nm to 256 ± 146 nm.

Figure 8.6: Size determination of SNPCs induced by ternary host-guest interactions ($2\ \mu\text{M}$ of interacting moieties, 60 s residence time). a) Average NP cluster diameter by DLS (light grey bars) and SEM (dark grey bars). SEM images (b-e) of the resulting SNPCs, as a function of the Np content derived from Np₈-PAMAM (b: 25%, c: 37.5%, d: 50%, e: 62.5%) used during microfluidic assisted NP network aggregation.

Furthermore, the ratio of the monovalent and multivalent binding partners does not significantly influence the uniformity of the formed aggregates: DLS shows polydispersity indices (PDI) of the observed SNPCs between 0.30 and 0.42 irrespective of the sample (Table 8.2).

Table 8.2: PDI observed by DLS of the SNPCs prepared with different concentrations of the Np-bearing guest components.

Np from Np ₈ -PAMAM	PDI
25.0%	0.30-0.40
37.5%	0.35-0.42
50.0%	0.30-0.42
62.5%	0.30-0.40

Generally, the clustering solution was collected from the outlet of the microreactor for 40 min to obtain sufficient sample for DLS studies. In contrast, all SEM samples were prepared by collecting the as-assembled SNPC solution directly from the microreactor onto the substrate. To assess the validity of the DLS results, SEM control experiments were carried out on samples collected for 40 min. These showed that the observed cluster sizes do not vary between the instantaneous measurements and the measurements prepared from collecting over 40 min (Figure 8.7). The good correlation between the DLS and SEM data, as well as this control, confirm therefore the strong kinetic trapping already mentioned above. In contrast, all SNPCs showed sedimentation from the suspension overnight. This is not unexpected as even the initial, non-aggregated SiO_2 -MV NPs start to sediment within a few hours.

Figure 8.7: SEM images of SNPC solution with 62.5% of the Np content derived from Np_8 -PAMAM: a) obtained directly from the microfluidic reactor and b) obtained 40 min after starting the supramolecular self-assembly. Residence time: 60 s.

To evaluate the effect of the Np-bearing components on microfluidic assisted self-assembly of SNPCs, SNPC formation was carried out using either 100% Np from Np-PEG or 100% Np from Np_8 -PAMAM. In contrast to the previously shown data, individual non-aggregated particles were observed by carrying out SNPC formation in the absence of Np_8 -PAMAM (Figure 8.8a and b), whereas large aggregates were observed by SEM (Figure 8.8c) upon mixing SiO_2 -MV, CB[8] and Np_8 -PAMAM in the absence of Np-PEG. These results show that Np_8 -PAMAM is required to crosslink the SiO_2 -MV, whereas uncontrolled aggregation is prohibited by competition with the monovalently interacting Np-PEG.

Figure 8.8: DLS and SEM images of self-assembly experiments: a) DLS and b) SEM of SiO_2 -MV, CB[8], and Np-PEG ($M_w = 5000$ g/mol) in the absence of Np_8 -PAMAM. c) SEM of SiO_2 -MV, CB[8], and Np_8 -PAMAM in the absence of Np-PEG. Residence time: 60 s.

In another control experiment, SiO₂-MV and both Np-bearing components (62.5% Np from Np₈-PAMAM) were used, but with CB[7] instead of CB[8]. The smaller CB[7] is too small to include both the MV and Np guest moieties in its cavity, and consequently the heteroternary complex cannot be formed. Indeed, no SNPC formation was observed by DLS or SEM (Figure 8.9a and b). A similar control was performed using bare silica nanoparticles instead of the MV-functionalized ones. Also here (Figure 8.9c and d), only non-aggregated NPs were observed.

Figure 8.9: DLS and SEM images of clustering experiments: a) and b) with SiO₂-MV, CB[7] (instead of CB[8]), 62.5% Np from Np₈-PAMAM, and 37.5% Np from Np-PEG; c) and d) with SiO₂-OH, CB[8], 62.5% Np from Np₈-PAMAM, and 37.5% Np from Np-PEG. Residence time: 60 s.

In summary, SNPCs can be formed within a microfluidic reactor in which diffusive mixing leads to controllable aggregation. Their formation is based on specific ternary complex formation, and the size of the resulting clusters is strongly dependent on the ratio between the mono- and multivalent Np guest species. The dependence of the resulting SNPC size on the fraction of Np₈-PAMAM in the Np-bearing building blocks can be ascribed to the competition between monovalent and multivalent host-guest interactions in the presence of CB[8] and SiO₂-MV. The amount of inter- and intramolecular binding events increases with the increased content of multivalent dendrimers, which leads to larger SNPC structures. The monovalent Np-PEG is essential to terminate the network formation and the accompanying uncontrolled aggregation as it blocks interaction sites and shields the crosslinked cores by the sterics of the PEG chains.

8.2.5 Size tuning of the SNPCs by varying the residence time

To investigate the effect of residence time and thus the diffusion time of the components on the size and kinetics of the assembly formation, clustering experiments were carried

out with SiO₂-MV, CB[8], Np₈-PAMAM, and Np-PEG ($M_w = 5000$ g/mol) at a constant 62.5% Np derived from Np₈-PAMAM. As seen in Figure 8.10, the cluster sizes observed by DLS varied strongly and in a non-trivial manner: a maximum in cluster size was observed at a 30 s residence time. The non-linear trend is tentatively explained by the calculated diffusion times of the different molecular building blocks. The diffusion time of Np-PEG ($M_w = 5000$ g/mol) is about 2.5 times slower than the diffusion time of Np₈-PAMAM and CB[8] (Table 8.1), which may result in less controlled cluster formation at the shorter residence times due to faster diffusion of the crosslinking Np₈-PAMAM compared to the cluster-stabilizing Np-PEG. For example, by using a residence time of 30 s, CB[8] and the dendrimer can diffuse over almost the complete width of the NP stream, whereas the monovalent Np-PEG ($M_w = 5000$ g/mol) can only penetrate approximately 25% into the other fluid stream. In contrast, at 60 s residence time, all components are able to penetrate the full NP stream, thereby explaining the observed size control at this residence time.

Figure 8.10: SNPC size by DLS upon assembly of SiO₂-MV, CB[8], Np₈-PAMAM, and Np-PEG ($M_w = 5000$ g/mol) at 2 μ M 1:1:1 CB[8]/MV/Np (62.5% Np from Np₈-PAMAM) as a function of residence time directly after SNPC assembly (light grey) or 60 min after starting SNPC formation (dark grey).

In order to eliminate this diffusion effect, we used Np-PEG with a M_w of 1000 instead of the earlier used 5000. The shorter Np-PEG polymer has a diffusion time that is very similar to Np₈-PAMAM and CB[8] (Table 8.1). Upon cluster formation using Np-PEG (1000 g/mol), Np₈-PAMAM, CB[8] and SiO₂-MV, a monotonous dependence of SNPC size with residence time is observed (Figure 8.11a and c-f), by both DLS and SEM. This confirms that the equal diffusion times of the molecular components govern the simultaneous assembly and termination of the clusters. Furthermore, DLS shows that the PDI strongly depends on the mixing time and decreases upon increase of the overall residence time of the components within the microreactor (Table 8.3). Nevertheless, the DLS size measurements only provide averages of cluster sizes, whereas the SEM images provide more detail on the size distribution. Many images show the presence of non-aggregated particles in the SNPC samples, which prompted us to evaluate their fraction as a function

of residence time (Figure 8.11b). At short residence times, the diffusion of the molecular components is insufficient to pass the whole width of the microfluidic channel. Consequently, not all SiO_2 -MV NPs are integrated in SNPCs. This is confirmed by the results shown in Figure 8.11b, whereby the fraction of non-aggregated NPs decreases strongly from 30 to 5% upon increasing the residence time from 15 to 60 s. By excluding the non-assembled NPs from the formed SNPCs, correction of the size determinations by SEM is possible. As shown in Figure 8.11a, a clear SNPC size increase and a decrease in the SNPC size distribution is observed by excluding the unbound SiO_2 -MV. These effects are more pronounced for the samples prepared with lower residence times, since the fractions of unbound NPs are higher in this case.

Figure 8.11: a) SNPC size and b) fraction of unbound SiO_2 -MV NPs, by assembly of SiO_2 -MV, CB[8], Np_8 -PAMAM, and Np -PEG ($M_w = 1000$ g/mol) at $2 \mu\text{M}$ 1:1:1 CB[8]/MV/Np (50% Np from Np_8 -PAMAM) as a function of residence time, as measured by DLS (light grey) and SEM (grey bars including all observed SiO_2 -MV NPs and dark grey bars excluding unbound SiO_2 -MV NPs). SEM images (c-f) of the resulting SNPCs prepared within a microfluidic device at different residence times of the two interacting streams (c: 15 s, d: 30 s, e: 45 s, f: 60 s).

Table 8.3: PDI observed by DLS of the SNPCs prepared at different residence times.

Residence time (s)	PDI
15	0.58-0.72
30	0.51-0.72
45	0.23-0.36
60	0.28-0.35

8.2.6 Size tuning of the SNPCs by varying the concentrations of the building blocks

Because SNPC formation is dependent on the diffusion of the molecular components into the NP stream, we decided to investigate the effect of the concentration of these components on the assembly process. Therefore, the stoichiometry of CB[8] and Np with respect to MV was varied under constant flow conditions (residence time 30 s), while keeping the content of Np from Np₈-PAMAM constant at 50%. By increasing the CB[8]/MV/Np ratio from 1:1:1 to 3:1:3, the overall observed size of the SNPCs increased from 180 ± 46 nm to 354 ± 60 nm and from 123 ± 58 nm to 205 ± 70 nm by DLS (Figure 8.12a) and SEM (Figure 8.12b-d), respectively. Further increase of the CB[8]/MV/Np ratio to 5:1:5 led to large SNPCs and visible precipitation (Figure 8.12e). The PDI of the samples increased upon increasing ratio of CB[8] and Np with respect to MV (Table 8.4). Irrespective of the composition, the samples showed the presence of 10% of unbound SiO₂-MV NPs. The latter result confirms that the extent of non-aggregated NPs is solely dependent on the diffusion rates of the molecular components and thus on the penetration depth of the molecular components into the NP stream (Figure 8.13a). On the other hand, this result and the observed increase of SNPC size upon increasing concentrations of the molecular components (Figure 8.13b) indicate that the molecular components are not fully incorporated into the SNPCs, since complete use at 1:1:1 stoichiometry should have been accompanied by an independence of aggregate size upon further increase of the concentration and by a depletion of the molecular components and thus a lower penetration depth (and higher fraction of unbound SiO₂-MV NPs) at lower concentrations.

Figure 8.12: a) SNPC size by assembly of SiO_2 -MV, CB[8], Np_8 -PAMAM, and Np -PEG ($M_w = 1000$ g/mol) at different CB[8]/MV/Np ratios (50% Np from Np_8 -PAMAM) as measured by DLS (light grey bars) and SEM (grey bars including all observed SiO_2 -MV NPs and dark grey bars excluding unbound SiO_2 -MV NPs). SEM images (b-e) of the resulting SNPCs prepared within a microfluidic device at different CB[8]/MV/Np ratios (b: 1:1:1; c: 2:1:2; d: 3:1:3; e: 5:1:5). Residence time: 60 s.

Table 8.4: PDI observed by DLS of the SNPCs prepared with different host-guest stoichiometries.

CB[8]/MV/Np	PDI
1:1:1	0.53-0.68
2:1:2	0.54-0.89
3:1:3	0.80-0.97
5:1:5	1.25-1.54

Figure 8.13: a) Schematic illustration of SNPC formation inside the microfluidic reactor depending on the diffusion of Np₈-PAMAM, Np-PEG, and CB[8] within the SiO₂-MV stream. (b) Dependence of SNPC size and of the fraction of unbound SiO₂-MV on the residence time, the relative fraction of multivalent Np₈-PAMAM, and the concentrations of the molecular components.

8.3 Conclusions

In conclusion, the formation of SNPCs driven by ternary host-guest interactions of CB[8], MV, and Np has been demonstrated within a microfluidic device. As schematically shown in Figure 8.13a, the diffusive mixing is strongly determined by the diffusion profile of the molecular components CB[8], Np₈-PAMAM, and Np-PEG into the SiO₂-MV NP stream. The size of the resulting SNPCs is strongly depending on the content of multivalently interacting Np dendrimers, the residence time of the interacting building blocks within the microchannel, and the stoichiometry of the ternary host-guest binding partners (Figure 8.13b). The residence time variation as well as the discrepancy between clustering experiments carried out in batch and in the microfluidic reactor show that the supramolecular host-guest assembly, is to a large extent, kinetically controlled. Interestingly, the molecular weight of the PEG stopper provides an additional kinetic control parameter to the assembly process because a longer length leads to slower diffusion into the NP stream and a concomitantly later termination of the clusters.

Overall, the combination of microfluidic technology with the design and control parameters provided by supramolecular chemistry provides an advanced platform for the tunable assembly of NP network aggregates, which may prove beneficial for the preparation of functional nanostructures for materials engineering.

8.4 Acknowledgements

Rajesh Munirathinam is gratefully acknowledged for the collaboration on the assembly experiments in the microfluidic devices. Jan Eijkel is gratefully acknowledged for fruitful discussions. Mark Smithers is acknowledged for the SEM images.

8.5 Experimental Section

8.5.1 Materials

Starting materials for particle synthesis were obtained from Sigma-Aldrich and used as received. The bifunctional linker NHS-(PEG)₆-maleimide was purchased from Fisher Scientific. 1-(1-Undecyl-11-thiol)-1-methyl-4,4-bipyridinium was prepared according to a literature procedure.³⁵ Cucurbit[8]uril (CB[8]) was purchased from Strem Chemicals and its purity was assessed by microcalorimetric titration against paraquat. In all experiments, the solutions required for SNPC formation were prepared in distilled water purified by MilliQ Advantage A10, Millipore, $R = 18.2 \text{ M}\Omega\text{cm}^{-1}$, before usage. Single-side polished silicon(100) wafers required for the preparation of scanning electron microscopy (SEM) samples were purchased from OKMETIC.

8.5.2 Synthetic procedures

Preparation of methyl viologen-functionalized silica nanoparticles (SiO₂-MV)

In accordance to a literature procedure,⁴⁰ bare silicon nanoparticles were prepared by adding 3.8 mL tetraethyl orthosilicate to a mixture of 5.7 mL NH₄OH in 114 mL ethanol under stirring. This resulted in nanoparticles of approximately 60 nm. Under argon, 2.3 mL (3-aminopropyl)triethoxysilane (APTES) was added under vigorous stirring to 120 mL of the previously prepared silica nanoparticles in ethanol. The dispersion was stirred overnight and the excess of APTES removed by repeated NP centrifugation and redispersion in 60 mL ethanol. The particles were dried under reduced pressure and stored at 4 °C as white solid. The amino-functionalized SiO₂ NPs were dispersed in degassed HEPES 20 mM buffer (pH 7.4) to prepare a dispersion with a concentration of 10 mg/mL. 10 mL of the particle solution was added to an equal volume of 23 mM of NHS-PEG₆-maleimide in 20 mM HEPES buffer (pH 7.4). The dispersion was sonicated for 5 min and further shaken overnight. The opaque dispersion was purified by centrifugation and subsequent redispersion of the NPs, twice in HEPES buffer (pH 7.4) and twice in HEPES buffer (pH 6.8) to remove the bivalent linker. Subsequently, the SiO₂-maleimide

NPs were dispersed in degassed HEPES buffer (pH 6.8) to obtain a concentration of 5 mg/mL. 1-(1-Undecyl-11-thiol)-1-methyl-4,4-bipyridinium dibromide (1.7 mg, 3.1 μmol) was dissolved in 3 mL degassed HEPES buffer (pH 6.8) and added to 15 mL of the SiO_2 -maleimide dispersion while stirring. After shaking the dispersion for 2 h, the unfunctionalized terminal maleimide groups were capped by reaction with 10 μL of 2-mercaptoethanol for 5 min. The resulting SiO_2 -MV NPs were purified by repeated centrifugation, once in HEPES buffer (pH 6.8), twice in ethanol and twice in water. Finally, the nanoparticles were freeze-dried and kept as a white solid in the fridge.

8.5.3 Self-assembly procedures

Supramolecular cluster formation in batch reaction

For the preparation of size-tunable supramolecular nanoparticle clusters (SNPCs), the SiO_2 -MV nanoparticles were dispersed in MilliQ water to obtain a concentration of 0.8 mg NP/mL. The solution was sonicated until only individual NPs were observed by DLS. In addition, 8 μM solution of CB[8] and various ratios of aqueous solutions of Np-PEG (5000 g/mol) and Np₈-PAMAM in DMSO were prepared, maintaining the MV:CB[8]:Np ratio at 1:1:1. For example, to form the SNPCs containing 50% Np derived from dendrimer, 500 μL SiO_2 -MV was added to a previously prepared solution of 250 μL Np-PEG (4 μM), 10 μL Np₈-PAMAM (12.5 μM) and 250 μL CB[8] (8 μM). The sample was mixed using a vortex mixer and analyzed by DLS at 10 min and 14 h after mixing.

Supramolecular cluster formation in a microreactor

A dispersion of SiO_2 -MV in water and a solution containing CB[8], Np-PEG ($M_w = 5000$ or 1000 g mol^{-1}), and Np₈-PAMAM in water were prepared as mentioned above. The concentrations of the different components were set such that all three binding motifs CB[8], MV, and Np were present in equimolar amounts and at a 2 μM concentration after mixing. For different residence times, the flow rates were adjusted accordingly.

Formation of SNPCs by varying the ratio of Np-functionalized components derived from Np₈-PAMAM and Np-PEG

Different concentrations of Nps derived from Np₈-PAMAM (25, 37.5, 50 and 62.5%) and Np-PEG ($M_w = 5000 \text{ g/mol}$; 75, 62.5, 50 and 37.5%), respectively, were prepared before mixing, maintaining the MV:CB[8]:Np ratio at 1:1:1. The dispersion of SiO_2 -MV was injected in one of the two inlets of a microchannel, while the premixed solution of CB[8] and Np-bearing components was injected in the second inlet. The two fluid streams were flowed in parallel with an equal flow rate of 4 $\mu\text{L/min}$, which corresponds to a residence time of 60 s. The SNPC solution was collected until a volume of 350 μL required for DLS analysis was obtained. SEM samples were prepared by collecting the SNPC solution directly from the outlet of the microreactor.

Formation of SNPCs by varying the residence time

The experiments carried out with variable residence times of the mixing solutions inside the microreactor, were carried out using a steady-state origin of the Np-bearing components (either 50% or 62.5% Np from Np₈-PAMAM). Therefore, the SiO₂-MV dispersion and the mixture of CB[8], Np-PEG (5000 g/mol and 1000 g/mol) and Np₈-PAMAM were prepared and mixed within the microfluidic reactor. The flow rates of two inlets were equal and were varied from 16.0, 8.0, 5.2 to 4.0 $\mu\text{L}/\text{min}$, which resulted in residence times of 15, 30, 45, and 60 s, respectively.

Formation of SNPCs by varying the stoichiometry of the building blocks

A constant content of MV and 50% of Np derived from Np₈-PAMAM dendrimer were used in all the experiments, while increasing the amount of CB[8] and Np stoichiometrically. For example, to obtain an overall CB[8]/MV/Np ratio of 2:1:2, a 0.8 mg/mL dispersion of SiO₂-MV in water was used in one inlet, and a premixed solution of CB[8] (8 μM), Np-PEG ($M_w = 1000$ g/mol, 4 μM), and Np₈-PAMAM (0.5 μM) in water was used in the other inlet. The other ratios (3:1:3 and 5:1:5) were made similarly, using accordingly higher concentrations of CB[8] and the Np components. All experiments were carried out with a residence time of 60 s.

SNPC formation in the absence of multivalent Np₈-PAMAM

A dispersion of SiO₂-MV (0.8 mg/mL) in water was injected into one of the inlets and a mixture of CB[8] (4 μM) and Np-PEG (4 μM) in water was injected into the second inlet using an overall residence time of 60s.

SNPC formation in the absence of Np-PEG

A dispersion of SiO₂-MV (0.8 mg/mL) in water was injected into one of the inlets and a mixture of CB[8] (4 μM) and Np₈-PAMAM (0.5 μM) in water was injected into the other inlet. The clustering was carried out using an overall residence time of 60s.

SNPC formation in the presence of CB[7] instead of CB[8]

A dispersion of SiO₂-MV (0.8 mg/mL) in water was injected into one of the inlets and a mixture of CB[7] (4 μM), Np-PEG (1.5 μM), and Np₈-PAMAM (0.313 μM) in water was injected into the second inlet using an overall residence time of 60s.

SNPC formation in presence of SiO₂-OH instead of SiO₂-MV

A dispersion of SiO₂-OH (0.8 mg/mL) in water was injected into one of the inlets and a pre-mixed solution of Np-PEG (1.5 μM), CB[8] (4 μM) and Np₈-PAMAM (0.313 μM) in water was injected into the second inlet with an overall residence time of 60s

8.5.4 Equipment

DLS analysis (DLS)

DLS experiments were performed with a Nanotracer wave by Anaspec operating with a Microtrac FLEX Operating Software at 25°C using a laser wavelength of 780 nm at a scattering angle of 90°. The observed sizes and standard deviations of the SNPCs were based on the average number distributions of minimum five individual measurements per sample. Three samples were measured for each reported NP formulation. In total, particles and SNPCs with sizes ranging from 0.8 to 6400 nm were detected.

Scanning electron microscopy (SEM)

All SEM images were taken with a Carl Zeiss Merlin scanning electron microscope. Samples were prepared by drop-casting an approximately 10 times diluted SNPC dispersion onto a cleaned silicon wafer. This was done by adding 2 µL of water followed by collecting a small droplet directly from the outlet of the microreactor. The dispersion was completely removed 10 s after addition and analyzed without further treatment. The sizes of at least 150 individual clusters were used to determine the average size of the SNPCs. Additionally, another average SNPC size was determined by excluding all unbound NPs.

UV/Vis spectroscopy

UV/Vis absorption spectra were recorded using a Perkin Elmer Lambda 850 UV-Vis spectrometer. The content of MV on the SiO₂-MV NPs was determined using the characteristic UV adsorption band of MV at 259 nm. Therefore a dispersion of SiO₂-MV in water (0.5 mg/mL) was prepared and analyzed by UV spectroscopy (Figure 8.1c). The content of MV was determined using a calibration curve of 1-(1-undecyl-11-thiol)-1-methyl-4,4-bipyridinium dibromide in water, after subtracting the enhanced background signal induced by NP scattering.⁴¹

Microreactor set-up

In all microreactor experiments, the sample solutions were mobilized by means of a PHD 22/2000 series syringe pump (Harvard Apparatus, United Kingdom) equipped with 250 µL flat tip syringes (Hamilton). Syringes were connected to fused silica capillaries (100 µm i.d., 362 µm o.d., Polymicro Technologies) by means of Upchurch Nanoport™ assembly parts (i.e., Nano-Tight™ unions and fittings, Upchurch Scientific Inc. USA). During the experiments the microreactor was placed in a home-built chip holder designed for fitting fused silica fibers into the inlet/outlet chip reservoirs by means of commercially available Upchurch Nanoport™ assembly parts. A glass microreactor with dimensions of 645.6 mm length, 52 µm depth, 254 µm channel top width, and 150 µm channel bottom width, and a residual volume of 13 µL were purchased from Micronit Microfluidics (Enschede, The Netherlands).

8.6 References

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Summary

Supramolecular nanoparticles (SNPs) have gained significant interest in nanoscience because stable, but reversible three-dimensional assemblies can be used for a variety of applications, ranging from stimuli-responsive and self-healing materials to biomedical applications. In particular, soft SNPs are promising candidates in biomedicine for drug delivery and diagnosis. The versatility of the SNP toolbox concept enables the easy decoration of SNPs with specific cell-targeting molecules and the incorporation of several small molecules, such as drugs or imaging agents, into the SNPs. Based on this strategy, drug and gene delivery vehicles have been developed, some of which are currently being tested in clinical studies as cancer therapeutics. At the same time, many of the underlying self-assembly and colloidal properties of these particles remain unclear, as well as the integration of stimulus-responsive properties for controlled disassembly and release. The work described in this thesis aims at the development of novel strategies for the formation of soft SNPs, while specific attention is paid to the understanding of SNP formation, stabilization, size tunability and triggered disassembly.

In the first part of this thesis (Chapters 3 and 4), a novel fabrication method for soft SNPs has been reported. Stabilized by multiple host-guest interactions between cucurbit[8]uril, methyl viologen and naphthol, thermodynamically controlled and time and temperature dependent SNP assembly was observed. These SNPs showed size tuning by the interplay between multivalent core and monovalent or divalent (Chapter 4) shell-forming stopper molecules. The length of the hydrophilic shell-forming polymer does not have a significant effect on the SNP size, as long the polymer is long enough to provide colloidal stability. Using a divalent stopper instead of a monovalent one slowed down the assembly kinetics significantly.

In the second part of this thesis (Chapters 5-7) stimuli-responsive SNPs have been developed based on the photoswitchable, heteroternary complex of cucurbit[8]uril, methyl viologen and azobenzene. Photoisomerization of azobenzene led to rupture of the supramolecular complex and thereby to UV-triggered SNP disintegration. In combination with the naphthol building blocks reported in Chapter 3, the SNP assembly strategy allowed SNP assembly in a toolbox approach, whereby the photoswitchable Azo was placed selectively in the core, the shell or both. Furthermore, by using a non-fouling azobenzene-conjugated zwitterion as the stopper, long PEG

stoppers could be avoided and colloidal stability was observed at high salt and high protein concentrations. A remarkable pH-dependent and reversible SNP aggregation was observed.

Finally, Chapter 8 describes the kinetically controlled self-assembly of supramolecular nanoparticle clusters (SNPCs) mediated by heteroternary host-guest interaction of methyl viologen-functionalized silica nanoparticles, cucurbit[8]uril and naphthol building blocks by diffusive mixing in a microreactor. The resulting SNPC size was shown to depend strongly on the residence time, the competition between multivalent and monovalent naphthol entities, and the stoichiometry of the host and guest binding partners.

In summary, the work described in this thesis provides a general strategy for the development of supramolecular nanoparticles mediated by cucurbit[8]uril host-guest interactions. Based on the heteroternary inclusion of guest molecules into cucurbit[8]uril, the formation of SNPs with highly controllable sizes, diverse shell functionalities and different responses to stimuli have been reported. The time and temperature dependent SNP self-assembly, as well as the different disassembly pathways give new insight towards the formation and disintegration of SNPs and their dynamics. This is essential for the development of effective carriers that can potentially be used in biomedical applications and meet the stringent and multifaceted demands of living systems. We expect that, upon inclusion of active compounds, these SNPs can provide a new generation of functional, biomedically relevant nanodevices.

Samenvatting

Supramoleculaire nanodeeltjes (SND's) zijn van groot belang in de nanowetenschap omdat deze als stabiele en reversibele driedimensionale structuren gebruikt kunnen worden voor verschillende toepassingen zoals in schakelbare en zelf-helende materialen en in biomedische toepassingen. Met name zachte SND's zijn veelbelovende kandidaten voor medicijnafgifte en diagnostiek. De veelzijdigheid van het concept van SND's maakt de functionalisatie mogelijk van SND's met celherkende moleculen of het insluiten van verschillende kleine moleculen, zoals medicijnen of contrastreagentia. Met behulp van deze strategie zijn verschillende systemen ontwikkeld voor medicinale en gentherapie, waarvan sommige op dit moment klinisch getest worden voor toepassing in het behandelen van kanker. Tegelijkertijd zijn vele van de onderliggende zelf-assemblerende en colloïdale eigenschappen van SND's onopgehelderd, evenals het implementeren van schakelbare eigenschappen voor het gestuurd afbreken van SND's en het daarmee vrijlaten van hun lading. Het werk beschreven in dit proefschrift richt zich op de ontwikkeling van nieuwe strategieën voor de vorming van zachte SND's, terwijl vooral aandacht is geschonken aan het begrip van de zelf-assemblage van de SND's, hun stabilisatie, het sturen van hun grootte en de gestuurde desintegratie.

In het eerste deel van dit proefschrift (hoofdstukken 3 en 4) is een nieuwe methode voor de vorming van zachte SND's beschreven. De vorming van SND's, die gestabiliseerd worden door meervoudige gastheer-gast-interacties tussen cucurbit[8]uril, methylviologeen en naftol, bleek thermodynamisch gecontroleerd te zijn en tevens afhankelijk van de temperatuur. De grootte van deze SND's kon gestuurd worden door middel van een samenspel tussen de multivalente kern en de monovalente of divalente (hoofdstuk 4) schilvormende stoppermoleculen. De lengte van het hydrofiele stopper-polymeer heeft geen significant effect op de grootte van de SND's, zolang het polymeer voldoende lengte heeft om voor colloïdale stabiliteit te zorgen. Het gebruik van de divalente in plaats van de monovalente stopper vertraagde de assemblagesnelheid aanzienlijk.

In het tweede gedeelte van dit proefschrift (hoofdstukken 5-7) zijn schakelbare SNDs ontwikkeld, gebaseerd op de lichtgevoelige, heteroternaire complexen van cucurbit[8]uril, methylviologeen en azobenzeen. Fotoisomerisatie van het azobenzeen onder UV-licht leidde tot

de afbraak van het supramoleculaire complex en daarmee tot de desintegratie van de SND's. In combinatie met de naftolbouwstenen beschreven in hoofdstuk 3 leidde het SND-concept tot een veelzijdige SND-assemblage, waarbij het lichtgevoelige azobenzeen selectief in de kern, in de schil of in beide ingebouwd kon worden. Bovendien konden lange PEG-stopppers vermeden worden indien gebruik werd gemaakt van een anti-fouling azobenzeen-geconjugeerd zwitterion als stoppermolecuul, waarbij colloïdale stabiliteit waargenomen werd bij hoge zout- en eiwitconcentraties. Tevens is er een opmerkelijke pH-afhankelijke en reversibele SND-aggregatie waargenomen.

Hoofdstuk 8 tenslotte beschrijft de kinetisch gecontroleerde zelf-assemblage van supramoleculaire clusters van nanodeeltjes (SCND's) gedreven door heteroternaire gastheer-gast-interacties tussen methylviologeen-gefunctionaliseerde silica-nanodeeltjes, cucurbit[8]uril en naftol-bouwstenen bij het diffusief mengen in een microreactor. De resulterende grootte van de SCND's bleek sterk afhankelijk te zijn van de verblijftijd in de microreactor, de competitie tussen de multivalente en monovalente naftol-moleculen en de stoichiometrie van de complementaire gastheer- en gast-eenheden.

Samenvattend biedt het werk beschreven in dit proefschrift een algemene strategie voor de ontwikkeling van SND's gedreven door gastheer-gast-interacties met cucurbit[8]uril. De vorming van SND's, gebaseerd op de heteroternaire interactie van gastmoleculen met cucurbit[8]uril, heeft geleid tot met een goed controleerbare grootte, tot verschillende functionalisaties van de schil en tot verschillende manieren van schakelen van de assemblage. De tijd- en temperatuur-afhankelijke assemblage van SND's en de verschillende routes voor hun desintegratie geven nieuwe inzichten in de vorming en afbraak van SND's en hun dynamiek. Dit is essentieel voor de ontwikkeling van effectieve medicijnafgiftesystemen voor mogelijke biomedische toepassingen, die moeten voldoen aan de strenge en veelzijdige eisen van levende systemen. We verwachten dat deze SND's, bij het insluiten van actieve componenten, verder ontwikkeld kunnen worden tot een nieuwe generatie van functionele en biomedisch relevante nanosystemen.

Acknowledgements

Last but not least, it is time to acknowledge all the people who have supported me not just during my PhD studies but also during the whole time I have spent in Enschede. During this time I have met so many people, which were not just scientifically but also personally very important for me.

First and foremost, I would like to express my gratitude and appreciation to my promotor Jurriaan. Thanks for convincing me to start my PhD. After four years I am sure that this was the right decision, the perfect project and you have been the best supervisor I could have imagined for me. The open and free atmosphere gave me the opportunity to work on my own ideas and become an independent researcher. I will never forget when you were saying: "Ik ben niet getrouwd met CB, dat was jouw keuze!" I truly appreciate how you taught me to ask the right questions (and hope I do so now) and guide me as smoothly as possible throughout all the challenges passing along the way. I really enjoyed our inspiring and mostly funny work meetings and your calmness to get me back on the track when I was again a bit overreacting. Finally I have to thank you particularly for the last year. I was not that productive as normal and struggling with my not favourite task– THE WRITING, but you kept motivating me with your full guidance and never put me under pressure. Jurriaan, hartelijk bedankt!

But many more people were involved to finish this thesis, successfully. Pascal, I did my Master project with you and you have been involved in the dual-responsive SNP project, but besides, your door was always open for any other question I had. Wim, after my Bachelor project I didn't want to work with microfluidics anymore, but in the end I could not get rid of it completely as it is visible in Chapter 8. Thanks for all the funny chats we had and the countless times you opened me the door to the office/lab. Melissa, thanks for all the nice (not only baby) chats and for carefully correcting my thesis. I hope you will enjoy your new job and get your family together in Mainz, as soon as possible. Although not directly involved in the research presented in this book, I would like to express my gratitude to the other scientific Sans staff members Tibor, Jeroen and Nathalie.

Furthermore, in my opinion the groups would not run without our technicians. Richard, Marcel, Regine, Bianca and Tieme-thanks for all the help and support during the daily laboratory life and all your struggles to provide "clean water". Especially I have to name Richard, for all the technical and scientific support starting from my Bachelor project on. I'm sure you have been one reason why I was continuing with my Master after finishing my Bachelor degree. I have to thank as well Clemens Padberg, who was always willing to help me with any request I had. Additionally, I would like to express my gratitude to the Sans secretaries Nicole and Izabel.

During my PhD period, I had the opportunity to work with great people on exciting projects. Jens, massive thanks for synthesizing the Azobenzene dendrimer. This molecule was the start for a fruitful collaboration with great scientific discussions. I'm sure your motivation and encouragement will help you to find a good position. I would like to thank Rajesh, for his patience and hard work to perform the microfluidic experiments presented in Chapter 8. Without your endurance, this project would have never been successful. Thank you Mark Smithers for all SEM images provided and the nice chats we had during our countless SEM measurements.

During my PhD, I had the pleasure to supervise a couple of students. Diana, Karabed and Eugenio thanks for your efforts and contributions you have put into my assignments.

I have to thank my paranympths Wies and Sarah. It's great to have both of you next to me at the end of this journey. Wies, we have had two great years together and became really good friends: Drinking thousand cappuccinos, having countless funny chats, running a few kilometers and had just 3 Tequillas ☺. Our WhatsApp chats are legendary and should never stop. I'm happy that you didn't went to Singapore and wish you so much getting "your" appropriate job soon. Sarah, vielen Dank für die unzähligen Gespräche, die wir geführt haben. Durch deine offene Art hast Du mir oft dabei geholfen, meine eigene Sichtweise zu überprüfen, zu überdenken oder gar zu ändern. Dein Arbeitseifer und dein unbändiges Bedürfnis, den Sachen auf den Grund zu gehen, machen aus Dir die perfekte Wissenschaftlerin. Bleibe immer positiv, denn es wartet etwas ganz wundervolles auf Dich. Auch wenn du manchmal glauben magst, es geht nicht weiter, ich bin mir sicher, Du wirst es schaffen!

A lot of great moments and crazy travels I have had with Raluca. Sharing is caring- that's your way of living. You have been a real friend for me, listening to all my problems and kept motivating me in all situations. I hope you can settle down in Spain for real and we will enjoy many more MUSE moments together.

I would like to thank my Lab 3 mates Raquel, Carlo, Laura, Alejandro, Maarten, Timon, Ina, Gavin, Anna, Janine, Pengkun and Jiguang for all the good moments we had. Raquel, struggling with similar challenges- we have had great times together. Mimi, your Colombian temperament brought another spirit into our group. We could laugh and even cry together. I believe you will as well finish soon and enjoy life with Thomas. Carlo - paparazzi, thanks for your support, synthetic discussions and for keeping the lab organized. I really enjoyed working with you. I wish

you and Mariska all the best in life... I still have to laugh about the fact, that you are getting married ☺. Alejandro and Laura, thanks for all the great scientific discussions we had. All the best for your future. Maarten, your humor and German song translation skills are beyond words!

I would like to thank the Dutch community (Pieter, Rick, Jasper, Sven, Roald, Janneke, Wouter, Mark, Mark, Stan, Rik, Rianne and Tom) for all the nice and funny chats we have had. Pieter- ich weiß eigentlich nicht, ob Du hier eingruppiert gehörst. Allein die Tatsache, dass ich jetzt schon wieder Deutsch schreibe, spricht Bände... Wir haben über viele Jahre sehr viel Spaß und manchmal auch Leid miteinander geteilt. Ich wünsche Dir und Aike für die Zukunft vom Herzen alles erdenklich Gute. Rick, thanks for correcting my Dutch summary. I really look up to your positive and relaxed way to see life and the PhD. I hope you will finish soon and have a great time with Alex. Jasper, we have started together and I really enjoyed our funny chats and the time we spent in San Francisco. Svenneke, thanks for the inspiring scientific talks and all the fun we have had together. Roald, thanks for driving us through the north of Spain during our legendary road trip.

I would like to thank all the other people of the MnF and BNT groups that shared with me so many great moments and helped me during my daily lab life: Aijie, Alexander, Andrea, Andreas, Angel, Anne, Ben, Bettina, Chengfeng, Dodo, Erhan, Gülistan, He, Liang, Maïke, Martijn, Nickolai, Nicole, Oya, Peter, Piotr, Rindia, Supaporn, Supitchaya, Tushar, Vijay, Xuemei, Yujie. I would like to especially name some people: Emanuela, with whom I really enjoyed working. We have had so many nice talks personally and scientifically. I wish you all the best with Mark and your PhD. Ben, thanks for all the fun you have brought into our labs. We have had countless great moments and conversations. I hope we will visit you (not just your apartment) in Paris quite soon. Jenny and Shirish, you are both such amiable persons struggling as well with the CB chemistry. I wish you all the best for your future and hope your dreams become true. Roberto, after a pretty bad start in Thailand, we did very well in the end. I really admire how you managed to finish your PhD, working with these incredible microreactors. Wilfried, we just shared the office in the last months, but I will always remember you singing at the computer and your face turning red when we told you crazy stories.

There are still a few people from the good old Langezijds period, who definitely have to be named here. Ok, some of them had physically moved to Carre, but for me they are still part of the Langezijds family. Jordi, thank you for the countless funny, happy but as well serious moments. You have been a great Master supervisor. I wish you and Danny all the best, hopefully in the Netherlands. Arancha, we were just living together for few months, never saw each other at home, but managed to become friends. I hope our small families will meet soon. Jealemy and Albert both great colleagues, with a lot of temperament and sometimes too little patience. All the best in living your dreams. Kim, let's call you the master of disaster... We have "shared" our lab items from the beginning, and after a couple of years we could even communicate. Shu-Han, it was such a pleasure to meet you. A little woman discovering and shaping the world. I will never

forget your wedding in Thailand. Thanks for all the funny moments we have had together. Melanie and Alberto we have had great dialogues... enjoy your life in Austria. And last but not least Muddasir, my dear Mr Pough. After such a bad start (my goodness you told me I'm stupid) we became perfect colleagues with a lot of humor. Your "helicopter" managed to fly always safe and finished her PhD.

Zu guter letzt möchte ich meiner ganzen Familie für den Beistand und Ihre kontinuierliche Unterstützung auf eine ganz besondere Weise danken. Auch wenn Ihr manchmal nicht ganz nachvollziehen konntet, warum ich mich all diesen herausfordernden Strapazen ausgesetzt habe, so habt Ihr immer sehr viel Verständnis für mich gehabt und mich in meinem unbrennsbaren Arbeitseifer sogar noch in vielfältiger Weise unterstützt. Ihr sollt wissen, wie unendlich wichtig Ihr für mich ward und seid. Ganz speziell möchte ich natürlich auch Dir, Jan, danken. Ich weiß, daß es für dich nicht der einfachste Moment war, als ich mir nach meinem Master nun noch meine Promotion als Ziel gesetzt habe. Ich erinnere mich noch sehr gut an unser gemeinsames „Entscheidungsgespräch“ zurück. Danach hast Du mich aber immer bedingungslos unterstützt- mehr noch, ohne Dich wäre es mir unmöglich gewesen, die Reise meines Dokortitels bis zu Ende zu machen. Du hast es immer wieder geschafft mich aufzubauen, besonders an den sehr schwierigen Tagen. Jetzt endlich und nach einer „kleinen aber wertvollen Verzögerung“ können wir unser gemeinsames Glück Liesbeth ebenso in den Händen halten, wie auch diese Arbeit.

Curriculum Vitae

Carmen Stoffelen was born on the 10th of June 1983, in Kleve, Germany. After finishing her professional education (Chemielaborant) at Bayer in Uerdingen in 2005 she was working as research scientist synthesizing fine chemicals. In 2006 she started studying Chemistry at Saxion-University of Applied Sciences, the Netherlands, where she received her bachelor degree in July 2008 (cum laude). During her internship and bachelor project she was working on the development of a durable acid catalyst inside a microfluidic chip. This work was carried out in the Molecular Nanofabrication group at the University of Twente under the supervision of Dr. Wim Verboom and Prof. dr. ir. Jurriaan Huskens.

In August 2010 she finished her master Chemical Engineering at the University of Twente, the Netherlands. During her master project she was working on the supramolecular immobilization of bifunctional peptides. This work was carried out under the supervision of Prof. dr. ir. Pascal Jonkheijm and Prof. dr. ir. Jurriaan Huskens in the Molecular Nanofabrication group at the University of Twente.

Since October 2010, she was Ph.D candidate under the supervision of Prof. dr. ir. Jurriaan Huskens. The aim of the project was the formation of size-tunable supramolecular nanoparticles. The results of the research are described in this thesis.