Cyclodextrin-Based Supramolecular Nanoparticles Mediated by Host-Guest and Electrostatic Interactions

From self-Assembly Properties to Biomedical Applications

Laura Graña-Suárez

**CYCLODEXTRIN-BASED SUPRAMOLECULAR
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ELECTROSTATIC INTERACTIONS:
FROM SELF-ASSEMBLY PROPERTIES TO BIOMEDICAL
APPLICATIONS**

Laura Graña-Suárez

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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 September 8, 2016 at 16.45 h

by

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"Imagination is the highest form of research"

Albert Einstein

"Only those who attempt the absurd can achieve the impossible"

Miguel de Unamuno

This thesis is dedicated to my family, friends, and JuanRi

Table of Contents

Chapter 1: General Introduction	1
1.1 References	3
Chapter 2: Cyclodextrin-Based Supramolecular Nanoparticles for Biomedical Applications	5
2.1 Introduction	6
2.2 SNP formation	8
2.2.1 Soft SNPs solely driven by host-guest interactions	8
2.2.2 Soft SNPs formed by host-guest interactions in combination with other interactions	15
2.2.3 SNPs with inorganic nanoparticle components	24
2.3 Biomedical applications of SNP systems	28
2.3.1 Therapeutics: encapsulation and release of drugs and photodynamic therapy	28
2.3.2 Diagnostics: imaging	36
2.4 Conclusions and outlook	38
2.5 List of abbreviations	39
2.6 References	41
Chapter 3: Cyclodextrin-Based Supramolecular Nanoparticles Stabilized by Balancing Attractive Host-Guest and Repulsive Electrostatic Interaction	45
3.1 Introduction	46
3.2 Results and discussion	48
3.2.1 SNP preparation	48
3.2.2 Influence of Ad-PEG	50
3.2.3 Influence of temperature on the SNP stability	51
3.2.4 Influence of the electrostatic interactions on SNP formation and stability	53
3.3 Conclusions and outlook	54
3.4 Acknowledgements	55
3.5 Experimental	55
3.5.1 Materials and equipment	55
3.5.2 Synthetic procedures	55
3.5.3 Methods	56
3.6 References	57

Chapter 4: Fluorescent Supramolecular Nanoparticles Signal the Loading of Electrostatically Charged Cargo

4.1	Introduction	60
4.2	Results and discussion	61
4.2.1	SNP preparation and synthesis of the components	61
4.2.2	Influence of the cargo on the size and charge of the SNPs	63
4.2.3	Optical properties of the SNPs upon addition of PEI	65
4.2.4	FRET studies	68
4.3	Conclusions and outlook	69
4.4	Acknowledgements	70
4.5	Experimental	70
4.5.1	Materials and equipment	70
4.5.2	Synthetic procedures	71
4.5.3	Methods	72
4.5.4	Calculation of the concentrations of charged groups induced by the SNP components and the cargo	73
4.6	References	75

Chapter 5: Loading and Release of Fluorescent Oligoarginine Peptides into pH-Responsive Anionic Supramolecular Nanoparticles

5.1	Introduction	78
5.2	Results and discussion	79
5.2.1	Peptide uptake and release into/from supramolecular nanoparticles in solution	79
5.2.2	Cell studies	88
5.3	Conclusions and outlook	90
5.4	Acknowledgements	90
5.5	Experimental	90
5.5.1	Materials and equipment	90
5.5.2	Methods	91
5.5.3	Calculation of the number of charges in the system	92
5.5.4	Cell experiments	93
5.6	References	93

Chapter 6: Versatile, Fast, and Easy One-Step Method for the Synthesis of Hydrophilic Lanthanide-Doped Nanoparticles

6.1	Introduction	96
6.2	Results and discussion	100
6.2.1	Synthesis and characterization of down-converting nanoparticles bearing different ligands	100
6.2.2	Synthesis and characterization of up-converting nanoparticles bearing different ligands	105

6.3	Conclusions and outlook	109
6.4	Acknowledgements	110
6.5	Experimental	111
6.5.1	Materials	111
6.5.2	Synthetic procedures	111
6.5.3	Methods	112
6.5.4	Calculation of the particle size from the powder XRD patterns	113
6.5.5	Calculation of the number of CDs per particle	113
6.5.6	ICSD PDF card of the standard tetragonal phase	114
6.6	References	114

Chapter 7: Host-Guest and Electrostatic Interactions in Supramolecular Nanoparticle Clusters **117**

7.1	Introduction	118
7.2	Results and discussion	120
7.2.1	Synthesis and characterization of the components	120
7.2.2	Cluster formation with the linear polymer TBP-PiBMA by host-guest interactions	121
7.2.3	Cluster formation with the cationic polymer PEI by electrostatic interactions	124
7.2.4	Cluster formation with the branched dendrimer Ad ₈ -PAMAM by host-guest and electrostatic interactions	126
7.3	Conclusions and outlook	128
7.4	Acknowledgements	128
7.5	Experimental	129
7.5.1	Materials and equipment	129
7.5.2	Methods	129
7.5.3	Calculation of the number of charges in the system	130
7.6	References	130

Summary **133**

Samenvatting **137**

Acknowledgements **139**

About the Author **145**

Chapter 1

General Introduction

Nanotechnology has emerged in recent years as a powerful tool for the design of new materials, in particular nanoparticles (NPs), for biomedical applications such as anticancer drug delivery and imaging.¹⁻³ Biomedical applications benefit from important characteristics of NPs: among others, they offer the proper size to enter cells, they have a large surface-to-volume ratio, allow easily functionalization for targeting applications and finally, they can be composed of smart materials which can respond to small changes, such as pH changes, in the environment.^{1,2,4-8}

Although there are concerns about the toxicity of NPs,⁹⁻¹² their interesting and useful properties provide a strong potential for their use in biomedical applications. To this end, it is important to study and control several aspects, such as particle size and morphology, surface charge, and behavior in a biological environment.^{13,14} Different materials can thus be used, such as polymers, liposomes, and inorganic particles.³ However, the preparation of NPs using these approaches, and in particular their tailoring for biomedical applications, can be tedious and time consuming. To a large extent this can be attributed to the need for optimization of NPs by a semi-empirical approach, which requires the design and investigation of the whole system from the beginning each time, including the synthesis of the components to their toxicological effects.³

Supramolecular chemistry employs non-covalent interactions to obtain larger self-assembled structures from small components, such as (bio)molecules and NPs.¹⁵ A special type of these interactions is the interaction between a host and a guest, which has found its inspiration in the biological world, in particular in the interaction between a receptor and a ligand. On a larger scale, several characteristics are similar in biological and synthetic supramolecular

systems, such as dynamicity, reversibility, topology and multivalency.¹⁶ These similarities have inspired the use of supramolecular chemistry in diverse biomedical systems.^{3,7,17,18}

Supramolecular nanoparticles (SNPs) are a special type of assemblies, in which NPs are made from multiple molecular components by the use of supramolecular interactions.^{3,19,20} The components of SNPs are joint together primarily by host-guest interactions, although other forces such as electrostatic or hydrophobic interactions can also be involved. This approach offers an important advantage: the modular character. It is relatively straightforward to use a single SNP system for several applications simply by changing or adding components without having to redesign the whole system, which is a very convenient approach for new trends in nanomedicine and personalized medicine.²¹⁻²⁶ In this way, SNPs can incorporate targeting moieties, imaging agents, drugs, or genetic materials simply by attached host or guest moieties (or other non-covalent interactions) to the desired building blocks.^{3,19,27}

The research described in this thesis aims to understand the forces involved in the formation of charged soft and hybrid SNPs based on cyclodextrin (CD). The guest groups chosen to build these SNPs are the *tert*-butylphenyl (TBP) and the adamantly (Ad) groups. The negatively charged polymer poly(isobutyl-*alt*-maleic acid) has been used for both the host and the guest components of the SNPs in Chapters 3, 4, and 5, on the one hand for the biocompatibility of this polymer and on the other hand to ensure that host-guest interactions are the primary force for self-assembly. In Chapters 6 and 7, hydrophilic, anionic, lanthanide-doped inorganic NPs containing CDs on their surface were used as the host materials. The main focus in all cases was to study the influence of attractive and repulsive electrostatic interactions on the formation and stability of the SNPs aimed for drug delivery bioimaging applications.

Chapter 2 provides a general overview about CD-based SNPs, with a special focus on the mechanism of particle formation and the biomedical applications of these SNPs.

In Chapter 3, the basic soft, multicomponent SNP system is described. The particles have been formed by multivalent host-guest interactions between CD and TBP, without the need of a monovalent capping agent. The importance of the balance between attractive supramolecular and repulsive electrostatic forces has been explored by studying the assembly behavior as a function of the ionic strength.

In Chapter 4, the encapsulation of a heavily charged cationic polymer (polyethylene imine, PEI) into these SNPs has been studied by means of fluorescence and FRET. To this end, we grafted PEI with the FRET donor fluorescein (FL) and the host component of the SNPs with the FRET acceptor rhodamine B (RhB). The study of the fluorescence properties of the assemblies helped to explore the effects of the addition of a cargo to the SNPs.

The same strategy has been applied in Chapter 5, however, using positively charged oligo-arginine peptides grafted with a cyanine dye instead of PEI. The possibility of using these anionic SNPs as a pH-responsive system has been investigated. The energy transfer between the FRET couple implemented at the peptide cargo and the SNP host component has been used as a responsive platform to study the encapsulation and release of the peptide. The release of the peptide *in vitro* has been studied as well.

The last two chapters of this thesis deal with lanthanide-doped NPs. In Chapter 6, the synthesis of hydrophilic down- and up-converting NPs (DCNPs, UCNPs) wearing different capping ligands is presented, using a one-step hydrothermal method. The influence of the ligand and the fluoride source on the particle size and luminescence properties has been explored. Furthermore, the preparation of UCNPs with CD groups on the surface (CD-UCNPs) by this one-step method has been investigated.

Finally, in Chapter 7, the formation of network aggregates of gold NPs functionalized with CDs (CD-AuNPs) and of the CD-UCNPs described in Chapter 6 is described using several polymeric materials to tune the interaction between the components. The influence of the charge, structure and host-guest interactions of the polymeric component in the formation and properties of the SNP clusters has been evaluated.

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Chapter 2

Cyclodextrin-Based Supramolecular Nanoparticles for Biomedical Applications

Supramolecular chemistry presents characteristics that are suited for biomedical systems, such as dynamicity, reversibility, topology, and multivalency. Supramolecular interactions involve non-covalent bonds between a host and a guest and are ideal for engineering supramolecular nanoparticles (SNPs), because their modular character offers the possibility of using the same basic SNPs in several applications. The most widely used host is cyclodextrin (CD), therefore, this Chapter will focus on SNPs involving CD as the host entity. In the first part, the focus is on the particle formation, that is, which forces induce the assembly between the different components and therefore, result in the formation of stable nanoparticles. In the second part, the use of CD-based SNPs for diagnostics and therapeutics is described. Here, the focus will be on how the therapeutic agent/imaging component is included in the system, how it is released, and how it enters into the site of action (in vitro and in vivo). This type of systems brings great possibilities in the formulation of nanoparticles for biomedical applications since they offer highly flexible but stable systems, easy modifiability for different applications, and biocompatibility.

2.1 Introduction

The area of biomedicine is shifting from conventional treatment based only on the disease to a more personalized treatment based on the combination of disease and patient, especially in the field of oncology.^{1,2} This change in perspective promotes new advances in the area of therapeutics and diagnostics, such as the engineering of novel molecules which offer special challenges for their delivery to the site of action.^{3,4} This has resulted in an increase of the investigations of new drug delivery and diagnostic systems, mainly because of the need of delivery platforms for the new pharmaceutical systems (proteins, oligonucleotides, hydrophobic anticancer drugs, imaging molecules) that have been produced.^{5,6} These therapeutics cannot be delivered in the traditional oral form, for example because of their size, instability, and/or hydrophobicity, which makes their delivery only possible via parenteral administration. This type of administration has inherent disadvantages such as poor patient compliance. Alternatives to the parenteral route are the buccal, sublingual, vaginal, nasal, and pulmonary routes, which have been extensively investigated for the design of new delivery strategies as well.⁵

Nanoparticles (NPs) have been considered as drug delivery agents, components thereof, and for imaging and therapeutic applications.⁷⁻⁹ They can be made of soft materials, such as polymers, dendrimers, and lipids, or of hard inorganic materials, as for example metals or silicon. NPs present several characteristics which make them interesting for biomedical applications such as the delivery of drugs. First of all, their size is similar to the size of biological entities; second, they can have a metallic core, which makes them useful for imaging applications. Furthermore, they have a large surface area, which can be functionalized with various ligands allowing the interaction of the particles with cells.^{8,10-15} Moreover, they can encapsulate diverse therapeutic and/or diagnostic molecules. Finally, some nanoparticles can remain in the blood circulation for prolonged times by including passivation ligands, and can be delivered specifically to the site of action by making use of, for instance, targeting ligands.¹²

Supramolecular chemistry can be defined as “the chemistry beyond the individual molecule”.¹⁶ It has found its inspiration in biology: the activity of biological species, such as

enzymes, nucleic acids, membrane receptors, and proteins, for example, is governed by supramolecular interactions, which can be turned on and off depending on the conditions. Therefore, several characteristics are similar for both biological and synthetic supramolecular systems, such as dynamicity, reversibility, topology, and multivalency.¹⁷ This relation between biological and synthetic supramolecular complexes makes the last ones ideal candidates in biomedicine. Indeed, in the last years, various supramolecular systems have found their way into drug delivery, imaging, signaling, and oncological (cancer imaging, targeting and therapy) applications.

Supramolecular host-guest chemistry is based on non-covalent interactions between a host and a guest. The supramolecular host entity that has been the most employed by far is cyclodextrin. Cyclodextrins (CDs) are cyclic oligosaccharides composed of 6 to 8 D-glucose units, where α -CD has 6, β -CD 7, and γ -CD 8 (Fig. 2.1). CDs have a toroidal shape, with a hydrophilic exterior and a relatively hydrophobic cavity, which can encapsulate a variety of guests with different binding affinities, thus providing a high versatility.^{12,18-22} The external hydroxyl groups of CDs can be functionalized to improve their water solubility and to reduce their self-aggregation.¹⁹ CDs are considered biocompatible at low concentrations, so they have been employed extensively in biomedical systems.¹² By far, the most commonly applied CD is β -CD, in particular to solubilize and stabilize drugs, and to regulate the drug release rate.²³⁻²⁷

Fig. 2.1. Structure of cyclodextrins.

CDs can host different low molecular weight lipophilic guests or macromolecules (such as different polymers) which can be totally or partially included in the cavity.^{25,28,29} The process of guest inclusion involves different factors, such as the displacement of water molecules from the cavity when an appropriate hydrophobic guest is included in the cavity. The interactions involved in the CD-guest complex are Van der Waals, hydrophobic interactions, hydrogen bonding, change in solvent surface tension, and even the release of strain energy from the CD ring.³⁰⁻³²

Supramolecular nanoparticles (SNPs) bring the field of supramolecular chemistry into the nanoparticle world. In the formation of SNPs, non-covalent interactions between host and guest components are involved. Therefore, they offer the high versatility of host-guest systems combined with a modular character: by exploiting the host-guest and multivalency concepts, SNPs are composed of several components such as different multivalent host and guest components, passivating coating agents, and targeting moieties^{19,33,34} Biomedically relevant molecules such as drugs, imaging agents, and genetic material, can be incorporated by host-guest or other non-covalent (e.g., electrostatic) interactions.^{33, 34} This modular character of SNPs makes them ideal candidates to adopt the new trends in biomedicine, as their multifunctional nature allows easy exchange of components without the need of redesigning the whole system, which is very adequate for moving towards personalized medicine.^{3, 4,35-38}

In this Chapter, we will focus on soft and hybrid organic-inorganic SNPs based on CDs, formed by host-guest and electrostatic interactions for therapeutic and diagnostic applications.^{19,33,39-41} For other types of SNPs involving other hosts (such as cucurbiturils and calixarenes) and other forces (such as merely electrostatic or hydrogen bonding), numerous reviews already exist in the literature.⁴²⁻⁵²

2.2 SNP formation

2.2.1 Soft SNPs solely driven by host-guest interactions

In many SNP systems, the main driving force involved in the formation of the SNPs is the host-guest interaction between CD and suitable guest moieties in a multivalent fashion. In

some cases, a guest with a relatively high binding constant, such as adamantane, is being used; in other cases, a guest with a medium binding strength such as polymers, leading to the formation of (pseudo)rotaxanes; also guests with a weak binding constant such as drugs (for example, camptothecin or doxorubicin).^{52,53} In the following paragraphs, these systems and their assembly to form nanoparticles is described in detail.

Tseng and coworkers developed highly modular supramolecular nanoparticles based on the host-guest interaction between β -CD and adamantane, by mixing three main components: a hyperbranched poly(ethylene imine) functionalized with β -CD (CD-PEI), a first generation poly(amido amine) (PAMAM) dendrimer functionalized with adamantyl (Ad) groups (Ad-PAMAM), and an excess of a monovalent capping agent functionalized with adamantane (Ad-PEG) (Fig. 2.2).⁵⁴

Fig. 2.2. Assembly a) and molecular structures b) of the components of the basic system developed by Tseng and coworkers.⁵⁴

By controlling the ratio between multivalent and monovalent guest units (of Ad-PAMAM vs. Ad-PEG), the authors obtained a set of SNPs of different sizes varying from 30 to 450 nm: the smaller the ratio, the smaller the particles were. The excess of Ad-PEG was used to limit the growth of the multivalent core nanogel network to avoid the formation of large aggregates which could precipitate from solution. As the multivalent host (CD-PEI) and guest (Ad-PAMAM) components are both positively charged, the core of these SNPs is cationic. The modular character of this system allowed the easy replacement of one or more of the components, or addition of extra components, and thus to use the same system for different applications. For example, Ad-PAMAM has been replaced by Au NPs⁵⁵ or magnetic nanoparticles functionalized with Ad.⁵⁶ Furthermore, as these particles are positively charged, they could easily encapsulate negatively charged components, such as peptides,⁵⁷ transcription factors,^{58, 59} and DNA.⁵⁹⁻⁶²

Zhao and coworkers prepared SNPs based on multivalent host-guest interactions between Ad grafted onto poly(acrylic acid) (Ad-PAA) and CD grafted onto PAA (CD-PAA).⁶³ They also used Ad-PEG to avoid the continuous growth of the crosslinked supramolecular network. An excess of Ad was used with respect to CD (Ad-PEG : Ad-PAA : CD-PAA was 8 : 8 : 5). The nanoassemblies had a size of approx. 35 nm and a slightly negative ζ -potential (-0.38 mV). This system was used for the targeted drug delivery application of doxorubicin (Dox).

Also based on multivalent interactions between CD and Ad, but using neutral polymers instead of charged components, Larsen and coworkers have developed SNPs based on the neutral polymer dextran grafted with β -CD (CD-Dextran) (see Fig. 2.3).^{64,65} As guest polymer they used Ad-grafted dextran polymers (Ad-Dextran). By using dextrans with different degrees of grafting, some degree of control over the size of the assemblies was achieved. In particular, the size of the assemblies was reduced upon increasing the degree of grafting of either the CD or Ad moiety in the dextran polymers. An increase of the number of crosslinks per polymer chain that can be formed through inclusion complexation, results in the compression of the nanogel (higher crosslinking density) and therefore to a decrease of the size of the nanoassemblies. However, increasing just the number of Ad or CD moieties without increasing the grafting density did not have an influence on the particle size or

stability. However, the SNPs increased almost two-fold in size in less than one week, most probably due to the absence of a monovalent capping agent. As dextrans are neutral polymers, these SNPs are neutral as well. Chen *et al.* also prepared SNPs based on dextran, however using the CD-benzimidazole couple as the host-guest interaction.⁶⁶

Fig. 2.3. Dextran-based SNPs prepared by Larsen and coworkers. a) Self-assembly of the Ad-Dextran and CD-Dextran to form the assemblies; b) components of the system.⁶⁴

Zhang and coworkers have prepared pH-responsive degradable microcapsules by assembling host and guest polymers via multivalent interactions onto the surface of CaCO_3 microparticles with previously entrapped materials inside (Fig. 2.4).⁶⁷⁻⁶⁹ Adding ethylene diamine-tetraacetic acid (EDTA) to the medium destroyed the CaCO_3 core, letting intact the entrapped material and the supramolecular shell of the microparticle. Different systems based on this concept were developed: in one system a dextran-CD polymer was used as the host, and adamantyl groups were grafted onto poly(aspartic acid) as the guest, whereas RhB was encapsulated into the CaCO_3 microparticle.⁶⁸ In another system, both the host and the guest polymers were based on dextran polymers, notably polyaldehyde dextran-grafted-

adamantane (Ad-Dextran) and carboxymethyl dextran-grafted- β -CD (CD-Dextran). This system was used for drug and imaging applications, encapsulating doxorubicin grafted with an adamantyl group (Ad-Dox) and dextran grafted with fluorescein groups (FL-Dextran) as the drug and imaging components, respectively.⁶⁷ Photoswitchable microcapsules were designed based on host-guest interactions between dextran-grafted- α -CD and poly(acrylic acid)-grafted-azobenzene which also were assembled layer by layer onto CaCO_3 particles.⁶⁹ Here, they encapsulated α -CD-rhodamine B (α -CD-RhB) by monovalent host-guest interactions with the guest polymer. The photosensitivity of the interaction between α -CD and azobenzene allowed the UV light-driven disassembly of the host-guest interactions and thereby the dissociation of the microcapsules.

Fig. 2.4. Supramolecular microcapsules using CaCO_3 as template prepared by Zhang and coworkers. a) Assembly of the components on the surface of CaCO_3 microparticles followed by the destruction of the microparticle by EDTA; b) components of the system.⁶⁸

CD can form inclusion complexes not only with small molecules which fit inside the hydrophobic cavity, but also with large linear polymers which pass through the cavity,

resulting in rotaxanes and pseudorotaxanes. In both cases, the CD can slide and rotate along the linear polymer axis. The difference between both derives from the reversibility of the interaction: in the case of rotaxanes it is not possible that the CD slides off without breaking any covalent bond (normally achieved by attaching a molecular stopper at the end of the polymer backbone), so the assembly is irreversible. In the case of the pseudorotaxanes, the CD can slide off again, rendering the assembly reversible.²⁸ Several groups have developed different SNP systems based on this host-guest interaction. For example, Gref and coworkers made SNPs based on dextran-grafted cyclodextrins in which the guest was a linear polymer grafted with dodecyl chains (Dod-Dextran).⁷⁰⁻⁷² The components and their assembly are shown in Fig. 2.5. The main interaction which holds the assemblies together is the inclusion of the appended alkyl moieties of the polymer into the α -CD cavity providing pseudorotaxanes. In this case, the assemblies were only stable when an excess of CDs with respect to alkyl chains was used. This allowed the encapsulation of benzophenone by host-guest interactions with the free CDs after particle formation, and this system was used for drug delivery applications.

Fig. 2.5. Dextran SNPs based on pseudorotaxanes by Gref and coworkers. a) Assembly of the components, b) inclusion interaction between α -CD and an alkyl group, and c) components of the system.⁷⁰⁻⁷²

A different approach was taken by Davis and coworkers: instead of a multi-component system, they developed SNPs based on only one component. A cyclodextrin-containing linear polymer conjugate of camptothecin (CPT) was developed, called IT101.⁷³⁻⁷⁶ The polymer and its assembly are shown in Fig. 2.6. This polymer was engineered to be linear, highly biocompatible, and non-degradable. It contains CDs in the PEG-based backbone, so it is highly water-soluble. CPT is grafted to the polymer through a glycine linker via the carboxylic groups, and is kept in the lactone form. The molecular weight of this polymer is ca 70 kDa. In PBS, they assembled forming SNPs of around 30 nm and PDI 0.277, with a ζ potential of -2 mV (at 10% wt of CPT grafting).

Fig. 2.6. a) self-assembly of multiple IT101 polymer chains into SNPs. b) Structure of the polymer.⁷⁶

Each SNP was composed of several polymer chains assembled by multiple interactions, mainly multivalent interstrand $\text{CPT} \leftrightarrow \beta\text{-CD}$ inclusion complexes, although this monovalent interaction is quite weak (K ca 100 M^{-1}). This was proven by addition of Ad-PEG, which led to the disassembly of the particles. Yet, CPT-CPT interactions may also occur. Apart from CPT, other molecules could also be grafted onto the same polymer backbone. For example,

Tubulysin A (a short peptide) was linked via a disulfide linkage,⁷⁷ and α -methylprednisolone has also been conjugated to the CD polymer forming particles of around 30 nm.⁷⁸ Finally, incorporating the 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) macrocycle into the polymer allowed the addition of ⁶⁴Cu or Gd for imaging purposes.⁷⁹

Based on multivalent weak interactions between CD and small drug or linear polymer molecules, Zhang and coworkers formed SNPs by self-assembly of a CD-containing star polymer and hydrophobic guests such as Dox and poly(lactic acid) (PLL) via host-guest interactions.^{80,81} The star polymer was synthesized by atom transfer radical polymerization using a mono- and multi-methacrylate substituted CD together with 2-(dimethylamino ethyl methacrylate) (DMAEMA) and a PEG macroinitiator. The star polymer assembled already by itself, forming nanoparticles with a size of 50 nm and a high polydispersity (0.5). When Dox or PLL was added to the system, the size increased to more than double (depending on the amount of guest added), but the polydispersity was lower (0.13). The morphology of the assemblies could be tuned from spherical to rod-like or vesicle-like by varying the guest molecule (Dox or PLL), the hydrophobic/hydrophilic ratio, and the pH of the solution. Increasing the pH of the solution from 3 to 10 resulted in a more hydrophobic microenvironment formed by the star polymers, caused by the deprotonated PDMAEMA chains.

2.2.2 Soft SNPs formed by host-guest interactions in combination with other interactions

In several SNP systems, the host-guest interactions are supplemented by other forces such as electrostatic or hydrophobic interactions. In the case of polyplexes, for example, in which the main driving force that induces SNP formation is the electrostatic interaction between a positively charged polymer and a negatively charged polynucleotide (DNA or RNA), the host-guest interaction is merely used to stabilize the particles or to encapsulate different drugs. In the next paragraphs, an overview of the main examples of this category will be presented.

Fig. 2.7. a) Self-assembly of the different components to form SNPs loaded with a negatively charged cargo for DNA or CPT delivery. b) Structure of the different components of both systems for (i) DNA or (ii) CPT delivery.^{57,60}

The basic system of Tseng and coworkers described in the previous section, can be extended to include negatively charged components which are incorporated in the SNP interior by attractive electrostatic interactions between the positively charged core of the SNPs and the cargo components (Fig. 2.7). For example, a library of SNPs with encapsulated DNA was created by adding negatively charged DNA to the mixture of building blocks while forming SNPs.^{60,82} Similarly, SNPs were loaded with a transcription factor (a protein), which was coupled to plasmid DNA and incorporated into the SNPs by electrostatic interactions.⁵⁸ In

another study, CPT was encapsulated by grafting it to the negatively charged polymer poly(glutamic acid) (PGA).⁵⁷ In a different approach, the authors prepared SNPs by only using the electrostatic interactions between a CD-PEI scaffold and plasmid DNA, while entrapping two different proteins. The SNPs were stabilized by adding the monovalent capping agent AdPEG, with/without a targeting moiety, to control the particle growth.⁵⁹

Fig. 2.8. Assembly and disassembly a), and components b) of SNPs based on CD-PEI, Fc-PAMAM, and AdPEG. Ferrocene oxidation c) induced by Ce^{4+} .⁸³

The CD-PEI/guest-PAMAM/Ad-PEG system was also studied recently in our group.⁸³ SNPs were formed by supramolecular host-guest interactions between ferrocene (Fc) and β -CD using positively charged components: CD-PEI, Fc-PAMAM, and a neutral stabilizer (Ad-PEG), see Fig. 2.8. The particles were formed primarily by multivalent host-guest interactions between the CD and Fc moieties. However, electrostatic repulsive forces between CD-PEI and Fc-PAMAM were also shown to play an important role in the particle formation and

stabilization, as demonstrated by experiments at different ionic strengths in the absence of stabilizer. The monovalent stopper was necessary to stabilize the particles and to avoid unlimited particle growth. In this work, different stoppers were studied, and it was found that the most effective one should have a strong (Ad) guest and a relatively long PEG polymer chain to achieve SNP stability. These SNPs were stimuli-responsive: they were disassembled by oxidation of the Fc groups, upon which they lose their affinity for the CD cavities.

Zhang and Ma prepared core-shell nanoassemblies with hydrophilic-hydrophilic block copolymers containing CD (a PEG block and a polyaspartamide block grafted with β -CD (PCD), PEG-*b*-PCD) by host-guest interactions of CD with small hydrophobic molecules or polymers (pyrene, indomycin, coumarin, poly(β -benzyl L-aspartate) (PBLA)).⁸⁴ The formation of the assemblies is as follows: the host-guest interaction between CD and hydrophobic molecule/polymer induced a local hydrophobicity, thus obtaining a pseudo-amphiphilic block copolymer, which further self-assembled into NPs of about 30 nm (pyrene) or of about 120 nm (PBLA). In this system, the PEG segments of the block copolymer are situated at the shell. With the same polymer, the authors prepared polyion complex (PIC) micelles by choosing appropriately charged guests, such as adamantane carboxylate. As a result, a pseudo-polyelectrolyte copolymer with one negatively charged block was formed due to host-guest interactions between Ad and CD. PIC micelles were obtained after adding the cationic PEI, which interacted electrostatically with the pseudo-polyelectrolyte copolymer, resulting in assemblies of about 100 nm.

The group of Irache prepared NPs from poly(methyl vinyl ether-co-maleic anhydride) (PVM/MA or Gantrez®-AN) and various CD derivatives.⁸⁵⁻⁸⁸ The different β -CDs used in this study (2-hydroxypropyl-CD, native CD, 6-monodeoxy-6-amino- β -CD) did not participate in the particle formation, however, they reduced slightly the size of the NPs, increased their stability and provided the nanoparticles with drug delivery properties because they are available to encapsulate drugs by host-guest interactions. The particles were prepared by a solvent-replacement method by simply pouring a solution of the polymer in acetone and of the CD into a water : ethanol mixture and stirring it, followed by the elimination of the organic solvents at reduced pressure. The optimum conditions for

preparing the NPs were obtained when using a ratio of 0.25 by weight of CD with respect to PVM/MA.

A different approach to form supramolecular assemblies was taken by Ravoo and coworkers: they developed bilayer vesicles formed by the self-assembly of amphiphilic CDs (CDVs) in water.⁸⁹⁻⁹⁴ This type of vesicles combines the properties of both liposomes and host molecules. The CDs were functionalized at the primary side by thioalkyl chains, in particular dodecyl, which caused the molecules to form a thermotropic mesophase as well as forming monolayers on the air-water interface. On the secondary side, the CDs were functionalized with hydrophilic hydroxyethyl groups, which improved considerably the water solubility and conferred amphiphilic character to the CD (see Fig. 2.9). These CDs assembled in water into bilayer vesicles due to chain-chain interactions between the alkyl chains at the primary side. Host-guest interactions are therefore not essential for vesicle formation. Thus, the vesicles still offer the possibility of host-guest chemistry useful for different applications. These vesicles could bind the linear, negatively charged polymer PiBMA grafted with the guest *tert*-butyl phenyl group (TBP) or Ad (PiBMA-TBP and PiBMA-Ad) with different degrees of grafting.⁹² In the case of PiBMA-TBP, the binding with the CD vesicles was two orders of magnitudes stronger than with PiBMA-Ad, while the monovalent interaction showed the opposite trend. Also, the degree of TBP binding per CD accessible is much lower in the case of PiBMA-TBP than PiBMA-Ad. With this polymer, not all the TBPs are included into the CD cavities by host-guest interactions since the complete binding would restrict the conformational freedom of the polymer, imposing therefore a high entropic penalty. Furthermore, in this case there is competition between host-guest interactions (polymer-CDVs) and intramolecular association (polymer-polymer), resulting in little amount of the TBP groups bound to the CD vesicles. This explains why PiBMA-TBP with a lower degree of grafting binds more efficiently to the CD vesicles and why the polymer arranges itself into a mushroom-like structure around the CD-vesicles, since the lower degree of grafting decreases the tendency for intramolecular association and favors host-guest interactions with the CD-vesicles. In the case of PiBMA-Ad, independent of the degree of grafting, the polymer arranges itself into an extended brush conformation because there is competition between intramolecular and host-guest interactions, and the structures are stabilized

enthalpically by these intermolecular interactions. This behavior causes an entropic penalty and explains the difference in the binding affinity with PiBMA-TBP and PiBMA-Ad.

Fig. 2.9. CDV system from Ravoo and coworkers: a) schematic representation of a CDB; b) molecular structure of the components of the system; c) arrangement of (i) PiBMA-TBP and (ii) PiBMA-Ad on the surface of the CDVs via host-guest interactions.⁹²

In a different study from the same authors, a polypeptide functionalized with Ad groups was used as the guest of which the peptide exhibits pH sensitive β -sheet formation.⁹⁴ Upon self-assembly of the polypeptide on the surface of the CD vesicles which were pre-loaded with different contents, the peptide exhibited a random coil conformation at neutral pH. In

contrast, at acidic pH (pH 5.0), the peptide adopted a β -sheet conformation which enforced a morphological change of the amphiphilic CD assemblies from spherical to fiber-like. In another example, multivalent recognition was used to study intra- and inter-vesicular interactions with the help of orthogonal host-guest and metal-ligand complexation.⁹⁵ The difference in binding strength of Ni^{2+} or Cu^{2+} with the ethylene diamine ligand, functionalized on one end with adamantane, was the main cause for forming intra- or inter-vesicular host-guest interactions with the amphiphilic CD vesicles. Interventricular interactions were clearly witnessed by aggregation observed by the increasing turbidity of the solution.

Polyplexes are the typical example of SNPs formed primarily by electrostatic interactions. This type of complexes are formed owing to attractive electrostatic interactions between a cationic polymer and a polynucleotide. In these systems, host-guest interactions are mainly used to stabilize the particles or to encapsulate different materials to obtain a multifunctional nanoparticle.⁹⁶ For example, Davis and coworkers have prepared a four-component gene delivery system by mixing a two-vial formulation (Fig. 2.10).⁹⁷⁻¹⁰⁰ One vial contained the delivery system, composed of a CD polymer (CDP, linear, cationic polymer), Ad-PEG (neutral), and Ad-PEG-transferrin (Ad-PEG-Tf, neutral, targeting); the other vial contained siRNA (anionic). Mixing both vials resulted in the formation of nanoparticles of about 70 nm by electrostatic interactions between CDP and siRNA, providing the CD at the shell. The host shell allowed steric stabilization by particle pegylation together with binding of a targeting moiety by the addition of Ad-PEG and Ad-PEG-Tf, respectively, due to the supramolecular interaction between adamantane and CD at the shell of the particles. The formulation accepted up to 5% of Ad-PEG-Tf with respect to Ad-PEG (higher amounts induced aggregation by Tf-Tf interactions). Moreover, the pegylation did not affect the particle size nor the morphology of the particles, but prevented the aggregation of the nanoparticles and undesired interactions in biological fluids that contain salts and serum proteins, in which the unstabilized cationic/DNA composites were shown to be unstable. However, pegylation reduced the interaction of the NPs with cells. In the presence of the targeting ligand Tf, receptor-mediated endocytosis was observed.

Fig. 2.10. siRNA delivery system developed by Davis and coworkers: a) assembly of the four components to obtain the SNPs, and b) components of the system.¹⁰⁰

A different type of polyplex, without making use of pegylation, was developed by Li and coworkers for drug and gene co-delivery.¹⁰¹ They prepared a star-shaped polymer by functionalizing a γ -CD core both with multiple cationic oligo(ethylene imine) (OEI) arms and with folic acid linked via a disulfide bond. They first encapsulated the poorly soluble drug paclitaxel (PCTX) in the γ -CD cavity. In a second step, plasmid DNA (pDNA) was added, which interacted with the arms of multiple star-shaped polymers by electrostatic interactions forming nanoparticles with sizes between 70-110 nm and a ζ potential between +25 to +36 mV depending on the N/P ratio. By the use of the targeting ligand folic acid, the nanoparticles entered the cell by receptor-mediated endocytosis.¹⁰² Using a different approach,¹⁰³ a three-component system of PEI-CD, Ad-Dox, and DNA was developed. First the drug was included by mixing PEI-CD and Ad-Dox resulting in the self-assembly of Dox in the CD cavity. Afterwards, pDNA was added and SNPs with sizes between 140 and 200 nm were formed by

electrostatic interactions between the cationic PEI and the anionic pDNA. These SNPs were used successfully for the co-delivery of anticancer drugs and pDNA both *in vitro* and *in vivo*.

Amiel and coworkers presented a supramolecular approach for the preparation of cationic polymers to form polyplexes.¹⁰⁴⁻¹⁰⁶ A neutral epichlorohydrin-crosslinked- β -CD polymer was synthesized to which a cationic component functionalized with a guest with a high CD-binding affinity (Ad or cholesterol (Chol)) was added. A cationic supramolecular polymer was obtained as a result, with the ability of modulating the amount of positive charges by changing the stoichiometry of the host-guest interactions. Also, DNA compaction with Chol was obtained at a lower charge ratio than with Ad. Moreover, the cationic density governed the gene transfection efficiency of this ternary system, that is, at a low charge ratio ($Z^{+/-}(\text{cation/DNA}) \leq 1$), free adenyl residues (from DNA) could be detected, which implies that the DNA is partially accessible and therefore the condensation is incomplete. At larger charge ratios ($Z^{+/-} \geq 5$), no free adenyl was detected, and the DNA appeared to be fully condensed. These vectors were tested *in vitro* showing a higher efficiency than cationic chol-derived lipoplexes.¹⁰⁷ A higher transfection efficiency was obtained at a charge ratio of 2.5, which was beyond the neutrality point.

Related to CD-based rotaxanes, rotaCDplexes make use of the same concept by inclusion of a polymer chain into the CD cavity, but now using a cationic polymer that interacts at the same time with genetic material forming a new type of CDplexes.¹⁰⁸ Several groups have worked in this direction. For example, Kissel and coworkers have developed a linear polycationic block copolymer composed of PEG, poly- ϵ -caprolactone (PCL), and branched PEI (PEG-PCL-PEI) to complex plasmid DNA (pDNA), see Fig. 2.11.¹⁰⁹ However, solubility issues arose which put constraints on the assembly with PEI caused by hydrophobic interactions of the PCL polymer chains. After addition of α -CD, which is known to complex PCL forming rotaxanes, these hydrophobic interactions were greatly shielded, by which the solubility and complexation issues were overcome, thus obtaining rotaCDplexes with a size of about 200 nm and a neutral surface charge. Li and coworkers prepared cationic rotaxanes by interacting a neutral random copolymer chain composed of ethylene oxide (EO) and propylene oxide (PO) (pEO-*r*-PO) and a cationic α -CD (obtained after grafting linear or branched oligo(ethylene imine), OEI, to it).^{110, 111} The cationic α -CD formed rotaxanes selectively with

the EO segments, leaving the PO segments free. This enhanced the mobility and flexibility of the polyrotaxanes, which increased the interaction of the cationic CD with DNA, resulting in rotaCDplexes. The diameter of these SNPs ranged from 100 to 200 nm, and the ζ potential varied from +13 to +30 mV depending on the OEI part and on the N/P ratio. Yamashita *et al.* used linear PEI with a MW of 22 kDa as polymer block, and formed rotaxanes by addition of γ -CD.¹¹² Plasmid DNA gave the formation of polyplexes by electrostatic interactions with PEI at a higher N/P ratio than PEI alone, which was attributed to the steric interference of γ -CD with the polyplex formation.

Fig. 2.11. RotaCDplexes developed by Kissel and coworkers. a) Assembly of the PEG-PCL-PEI block copolymers (1st step) together with α -CD (2nd step) and DNA (3rd step). b) Components of the system.¹⁰⁹

2.2.3 SNPs with inorganic nanoparticle components

The functionalization of inorganic nanoparticles with supramolecular host or guest groups allows the use of these inorganic nanoparticles as the host and/or guest components in SNPs.¹¹³ Because such a component is a preformed nanoparticle, and thus does not possess the flexibility of a soft polymer, this type of systems assemble differently than those composed of only soft materials. By far, most of the work about SNPs involving inorganic nanoparticles employs gold nanoparticles (AuNPs).

The clustering of AuNPs surface-functionalized with β -CDs (CD-AuNPs) as induced by host-guest interactions was investigated in two different studies in our group. In one study,¹¹⁴ the cluster formation of the CD-AuNPs was studied with different Ad derivatives, by monitoring changes of the AuNP surface plasmon band in the UV spectra as a function of the geometry and the valency of the guest component. In case of using monovalent Ad, no cluster formation was observed at any Ad : CD stoichiometry used. When using the divalent Ad₂-triethylene glycol, weak signs of aggregation were observed, but in general intramolecular interactions onto the same AuNP were favored over intermolecular interactions. However, in the case of the multivalent poly(propylene imine) dendrimer guests (Ad₄-PPI and Ad₁₆-PPI), large aggregates were formed which resulted in the formation of an insoluble precipitate.

Fig. 2.12. Cluster formation of CD-AuNPs through host-guest interactions with Ad-PPI and Ad-PEG in a turbulent flow. a) Schematic illustration of the multi-inlet vortex mixer used to prepare the SNPs under a turbulent flow. b) Assembly and c) chemical structures of the components to obtain the SNPs (adapted from¹¹⁵ with permission of ©RSC).

In another study, size-controllable aggregates of CD-AuNPs with Ad₄-PPI were prepared in combination with the monovalent capping agent Ad-PEG using a turbulent flow reactor (Fig. 2.12).¹¹⁵ Supramolecular clusters with sizes between 20 and 1000 nm were obtained by specific multivalent host-guest interactions varying the CD : Ad ratio. Ad-PEG was used as a monovalent capping agent to avoid the uncontrolled growth of the multivalent network. Size control was achieved in this system by varying the Ad-PPI/CD ratio from 1-7 while keeping

the Ad-PEG/Ad-PPI constant. Most importantly, the size of the clusters was larger when prepared in the turbulent regime than in the laminar regime or manually. This proved kinetic control over the assembly size.

Rotello and coworkers investigated network aggregates of AuNPs by forming microcapsules by host-guest multivalent interactions of (water-soluble) CD-AuNPs and (water-insoluble) Ad-AuNPs at the oil-water interface in a water-in-oil emulsion.¹¹⁶ The AuNP core sizes of the host and guest components were 3 and 6 nm, respectively. Upon self-assembly, the size of the microcapsules reached up to $18.3 \pm 9.3 \mu\text{m}$, and the microcapsules were stable for several days. By adding an excess of external monovalent competitor, Ad-tetra(ethylene glycol) (Ad-TEG-OH), it was possible to tune the size of the microcapsules: Ad-TEG-OH interferes with the multivalent recognition between the CD-AuNPs and Ad-AuNPs disrupting the interfacial crosslinking, which was followed by the coalescence of several microcapsules together forming larger vesicles.

Ravoo and coworkers prepared assemblies of CD-AuNPs and a divalent and photoswitchable guest based on arylazopyrazole (AAP),¹¹⁷ resulting in photoresponsive assemblies. The conformation of AAP could be reversibly photoswitched from the *E* to the *Z* isomer. Irradiation of the *Z* isomer at 520 nm afforded the *E* isomer, while upon subsequent irradiation at 365 nm the molecule switched back to the *Z* isomer. Since the *E* isomer can form host-guest interactions with β -CD while the *Z* isomer cannot, the aggregates could be disassembled and reassembled by irradiation at 365 and 520 nm, respectively. By adding different amounts of the guest, the size of the SNPs could be controlled from 30 to 300 nm.

In an early study dealing with the assembly of AuNPs into network aggregates, Kaifer and coworkers studied the aggregation of γ -CD-AuNPs with the fullerene C_{60} in water.¹¹⁸ The host-guest interaction in this case is the inclusion of one C_{60} molecule by two γ -CD cavities (of different CD-AuNPs), so it acts as a “molecular glue” resulting in the crosslinking of the CD-AuNPs. Whereas the AuNPs had a size of 3.2 nm, the network aggregates reached sizes up to 300 nm. The size of the aggregates could be controlled and changed by preparing the nanoassemblies at different temperatures: increasing the temperature caused shrinkage of the aggregates and vice versa. The disassembly of the networks was achieved by adding an

excess of free γ -CD to the aggregates, thus competing with the CD-AuNPs and effectively breaking up the host-guest interactions.

The group of Liu made supramolecular assemblies of β -CD or L-tryptophan-functionalized β -CD (Try-CD) together with the polymeric guest poly(propylene glycol) (PPG) in water,¹¹⁹ resulting in polypseudorotaxanes (PPRs). In a second step, they added 20 nm citrate-capped AuNPs, which were embedded in the SNPs by ligand exchange of citrate by PPR on the surface of the AuNPs. The aggregation of the AuNPs was confirmed by a redshift of the surface plasmon resonance band causing a visual change of the color of the NP solution from red to violet or blue. The size of these network aggregates was about 450 nm. In the case of Try-CD, the aggregates were fluorescent. However, when C₆₀ was added, resulting in ternary aggregates, quenching of the fluorescence was observed caused by electron transfer from Try to the fullerene.

The basic multicomponent system of Tseng and coworkers presented in the previous sections, was used as well to form SNPs containing inorganic nanoparticles.^{55,56} In one study, 2 nm AuNPs functionalized with Ad (Ad-AuNPs) were used as the multivalent guest component, while CD-PEI was used as the host component, and the monovalent Ad-PEG as the capping agent.⁵⁵ The particles were prepared simply by mixing the components in phosphate buffered saline solution (PBS). By tuning the CD-PE/Ad-AuNPs ratio, particles with sizes varying from 40 to 118 nm were obtained. The SNPs were stable in the pH range of 5-10 and at temperatures of 7-40 °C. At temperatures > 50 °C, the particles disassembled into smaller fragments due to the weakening of the CD-Ad host-guest interaction, making this system suitable for use in photodynamic therapy. Similar to this system, but using 6 nm Ad-grafted Zn_{0.4}Fe_{2.6}O₄ superparamagnetic nanoparticles (Ad-MNP) as the multivalent guest component instead of the Ad-AuNPs,⁵⁶ SNPs with sizes between 70 and 160 nm, containing also the anticancer drug Dox, were obtained by varying the ratio of the components. Applying an external alternating magnetic field, caused the local evolution of heat by the Ad-MNPs, which disassembled the SNPs releasing DOX at the site of action, making these SNPs thermally responsive.

Ravoo and coworkers prepared magnetic nanomaterials by incorporating 9 nm oleic acid-stabilized iron oxide nanoparticles (MNPs) in the cyclodextrin vesicles (CDVs) described

before (Fig. 2.13).¹²⁰ These MNPs were included in the hydrophobic leaflet of the vesicle by hydrophobic interactions of the oleic acid tails of the MNPs with the oleic chains of the amphiphilic CDs. The size of the CDVs containing MNP in the bilayer (MNP-CDV) was around 500 nm. These MNP-CDVs could be assembled into linear aggregates of around 10 μm in the presence of an external magnetic field, but when the magnetic field was switched off, the aggregates dissociated again. When an azobenzene (Az) divalent crosslinker (Az_2) was also added to the system, the aggregates were stable after turning off the external magnetic field. Since Az is photoresponsive and the *trans*-isomer is a strong binding guest for β -CD, while the *cis*-isomer is not, it was possible to break these vesicular aggregates by irradiating them at 350 nm.

Fig. 2.13. Magnetic and light responsive MNP-CDVs of Ravoo and coworkers. a) Schematic illustration of the MNP-CDVs showing the encapsulation of the MNPs in the vesicles by hydrophobic-hydrophobic interactions. b) Structure of the components of the system and the divalent linker Az_2 . c) Reversible formation of the MNP-CDVs nanoassemblies in the presence/absence of a magnetic field. When the divalent guest Az_2 is added to the system, the nanoassemblies are maintained. However, under UV irradiation at 350 nm, the *trans*-isomer transform into the *cis*-isomer and the nanoassemblies dissociate.¹²⁰

2.3 Biomedical applications of SNP systems

2.3.1 Therapeutics: encapsulation and release of drugs and photodynamic therapy

In an ideal drug delivery system, the drug should be temporarily encapsulated but released at the site of action, preferably in a time-controlled fashion.⁵ Supramolecular systems based

on CDs show good potential for drug delivery, since CDs can encapsulate a large variety of hydrophobic drugs thus allowing their uptake in aqueous media.^{17-19,33,39-41,45,52,121,122} Furthermore, the size of CD-based SNPs is often appropriate to enter cells, for example by the endosomal pathway. Owing to the modular character of SNPs, it is possible to easily add a targeting moiety which will direct the system to the preferred site of action. The encapsulation of drugs into SNPs has been achieved mainly by two interaction types: host-guest and electrostatic. The first type is quite common, since several drugs have hydrophobic moieties and can thus act as a guest for CDs, making this established practice to solubilize and deliver drugs.²³⁻²⁷ The drug of choice can also be modified with a strong guest such as adamantane, which will then form host-guest interactions with CD. The encapsulation by electrostatics is relatively straightforward in the case of charged SNPs, and it is the main strategy for gene delivery systems. In this section, some drug delivery systems based on CD SNPs are presented.

The system IT101 of Davis and coworkers, composed of a β -CD polymer conjugate of CPT, is a clear example of encapsulation of a drug by host-guest interactions.^{73,79,123-125} Here, the drug is also the guest, and it is responsible of holding together the nanoparticle at the same time. CPT is a potential inhibitor of topoisomerase I (TOPO I, essential for DNA replication). However, it is active only in the lactone form in which it is very hydrophobic. Unfortunately, under normal physiological conditions it is rapidly transformed into the inactive and more hydrophilic carboxylate form. By conjugating CPT into the cyclodextrin-containing hydrophilic PEG polymer, forming NPs between 30 and 40 nm in size, the drug was preserved in its lactone form. Moreover, the PEG blocks improved the solubility and imparted stealth properties, which all together minimized immunogenicity and allowed to evade phagocytic uptake by the reticuloendothelial system. Furthermore, as the MW of the polymer is less than 48 kDa, and the NPs are larger than 10 nm, they are expected to pass the kidney barrier and have a long circulating time. The NPs entered tumor tissue through the enhanced permeability and retention (EPR) effect.¹²⁶ Cell studies demonstrated the localization of IT101 in lysosomes, which indicates that the NPs enter the tumor cells via the endosomal pathway. As the lysosomes have an acidic environment, they modulate the slow release of CPT into the cell by hydrolysis of the peptide bond which links it to the polymer. After CPT

was released, the particle disassembled and the single polymer strands were easily cleared through the kidneys because they are smaller than 10 nm (Fig. 2.14). IT101 presents several advantages compared to the two commercially available products of CPT: it has an enhanced efficacy, improved pharmacokinetics, and less secondary effects. IT101 as monotherapy has been approved by the FDA for the treatment of ovarian cancer. Currently, clinical trials in combinatory therapy are ongoing.

Fig. 2.14. CPT release from IT101 in the lysosomes of the cell. The acidic pH of the lysosomes causes the slow release of CPT into the tumor cells. After particle disassembly, the single polymer strands are easily cleared through the kidneys.⁷³

Zhang and coworkers used the host-guest interactions between a drug (Dox) and β -CD in the star polymer PEG-(CDP-co-PDMAEMA) to encapsulate the drug and stabilize the SNPs that are initiated by star polymer aggregation, as explained in the previous section.^{80,81} The incorporation of Dox in the nanoparticles is most probably achieved by both host-guest interactions with CD and hydrophobic-hydrophobic interactions with hydrophobic domains of the nanoassembly. This star polymer is pH sensitive, with a pK_a at about 7.1. Below this pH, the PDMAEMA part is soluble in acidic media due to the protonation of the amines. Above this pH, the PDMAEMA part is hydrophobic allowing the incorporation of the hydrophobic Dox in the core of the assemblies, which causes the stabilization of the SNPs by host-guest interactions with CD. By decreasing the pH, the drug is slowly released, because the cores of the NPs swell due to the protonation of the PDMAEMA chains. The *in vitro* release profiles at neutral (PBS, pH 7.4) and acid pH (ABS, pH 5) showed a triphasic behavior: fast release during the first 8 h (due to free drug adsorbed on the surface of the NPs), then a moderate release during the next 24 h, and finally a slow release over the next 192 h. The

release rate at acidic pH was faster than at neutral pH, demonstrating the pH-responsiveness of the system, also caused by the higher solubility of Dox in acidic media. The nanoparticles entered the cell through the endosomal path, ending up in the lysosomes, from where Dox was released into the cytosol due to the acidic conditions of the lysosomes (pH 4-6). Encapsulation of a drug by host-guest chemistry was also followed by Gref and coworkers to entrap the hydrophobic drug benzophenone (BZ) into dextran-based nanogels by complexation with β -CD from a CD-polymer ($K = 2000 \text{ L mol}^{-1}$). In this case, the entrapment of the drug is not involved in the particle formation. Only half of the β -CDs present in the system were used to form inclusion complexes with the alkyl chains and, therefore, the other half was free to encapsulate the drug molecules. The drug was stabilized in the assemblies not only by host-guest interactions with the free β -CD, but also by hydrophobic interactions within the hydrophobic domains inside the nanogels. The loading of the drug was achieved by two methods: by assembling with the host CD-polymer before nanogel formation, and by loading it into the preformed SNPs. However, the loading achieved with the first method was very poor ($< 1\%$), which may be caused by a dilution effect after adding the dextran-guest solution which may result in CD-BZ dissociation before forming the nanogels. With the second method, the loading was almost three times larger. The nanogels increased in size from 140 to 190 nm upon adding the drug. Release studies in water showed a fast release within the first 15 min followed by a plateau region (no release). Successive dilutions of the nanogels provoked the release of BZ, which confirmed that the release mechanism is caused by dissociation of the CD-BZ complexes after dilution.^{70-72,127} Irache and coworkers encapsulated the hydrophobic drug PCTX into SNPs of PVM/MA with β -CD (PVM/MA/CD) via host-guest complexation of PCTX into the β -CD cavities ($K = 600 \text{ M}^{-1}$).⁸⁶⁻⁸⁸ As previously described, β -CD does not intervene in the particle formation, but it stabilizes the particles and provides them with drug delivery capabilities, since they can encapsulate hydrophobic drugs, such as PCTX. PCTX is an antineoplastic anticancer drug (it is anti-proliferative: it acts by damaging the DNA, thus initiating apoptosis) in use for the treatment of several types of cancer such as ovarian and breast cancer. Its low absorption at the intestinal level presents the main problem of this drug. The inclusion of PCTX into the CDs of PVM/MA/CD SNPs increased its absorption 500 times with respect to the control without CDs. PCTX was released *in vitro* at neutral or basic conditions due to the erosion of PVM/MA/CD

nanoparticles caused by the hydrolysis of PVM/MA under these conditions. The NPs eroded faster at higher pH, causing faster release of PCTX. Similar to these systems, Zan *et al.* encapsulated Dox and indocyanine green (ICG) into SNPs formed by host-guest interactions between CD-PAMAM and an Ad-conjugated copolymer,¹²⁸ and Zhao and coworkers encapsulated Dox into SNPs based on PAA.⁶³

Another way to introduce a drug into SNPs by host-guest interactions is by functionalizing the drug with a high-affinity guest molecule for CD (such as Ad) to provide monovalent interactions between CD and Ad. This is the approach taken by Zhang and coworkers to encapsulate Dox into pH-responsive microcapsules formed by the self-assembly of Ad-Dextran and CD-Dextran over CaCO_3 microparticles by host-guest interactions with free CDs after assembly, incorporating also FL-dextran for imaging purposes (Fig. 2.15).^{67,68} These capsules were degraded under weakly acidic conditions, such as encountered in cancer tissue. At low pH the guest component Ad-Dextran disassembled due to the hydrolysis of the hydrazine bond between dextran and Ad. The drug was released the same way, since it was also grafted to the Ad moiety by a hydrazine bond. After 13 h at pH 5.5, 80% of the encapsulated drug was released.

Fig. 2.15. Supramolecular microcapsules using CaCO_3 as template prepared by Zhang and coworkers. Assembly of the components on the surface of CaCO_3 microparticles followed by the destruction of the microparticles by EDTA. At slightly acidic pH such as in tumor tissue, the microcapsules disassemble causing the release of Dox into the cancer cells (adapted from⁶⁷ with permission of ©2012 ACS).

The use of electrostatics to encapsulate a drug into SNPs is very common in the case of charged particles. For example, Tseng and coworkers have encapsulated CPT following this strategy by grafting CPT onto the anionic peptide PGA via an ester bond, and binding that conjugate into the cationic SNPs.⁵⁷ Furthermore, the ester bond could be cleaved by esterase-mediated hydrolysis, allowing the controlled release of CPT from the SNPs. The drug was encapsulated during SNP formation simply by mixing it together with the other three components during self-assembly. The authors prepared and studied two SNP sizes, 37 nm ($\zeta = -11$ mV) and 104 nm ($\zeta = -4$ mV). Drug release in PBS buffer showed 20% CPT release after 6 days without any burst release. The particles entered the tumor tissue by the EPR effect. *In vivo* studies of the biodistribution with microPET of ⁶⁴Cu-labeled SNPs showed that the smaller particles were two-fold more accumulated in the tumor than the larger ones. When encapsulating the fluorescent anticancer drug Dox into the magnetic nanoparticles prepared with Ad-MNP, the SNPs also entered the tumor tissue by the EPR effect.⁵⁶ This drug was encapsulated into the SNPs most probably by a combination of electrostatic and host-guest interactions, since Dox can form inclusion complexes with β -CD. Once encapsulated, the fluorescence of the drug was fully quenched due to π - π stacking. After applying an alternating magnetic field, the fluorescence was recovered, indicating that the release of the drug occurred due to disassembly of the SNPs into smaller parts caused by the heat released during the magnetic treatment.

CD-based polyplexes are the most typical examples of SNPs in which therapeutic material (DNA or RNA in this case) is encapsulated by electrostatics. The system developed by Davis and coworkers (CALAA01) for the delivery of small interfering RNA (siRNA), which is now under clinical studies, is one of the most widely known. siRNA is a man-made double stranded RNA analogue of microRNA (miRNA).⁹⁸⁻¹⁰⁰ It operates within the RNA interference pathway, interfering in the expression of specific genes which results in the degradation of messenger RNA (mRNA) after the transcription step and, therefore, it blocks the translation of those specific genes. In the formulation of CALAA01, an excess of positive charges (of the polymer) compared to negative charges (from siRNA) is used to ensure that all the genetic material is encapsulated. This system makes use of the targeting moiety transferrin (Ad-PEG-Tf), whose receptor (TfR) is overexpressed in certain types of tumor cells (see Fig. 2.16). The drug was

administered intravenously. Unpegylated NPs accumulated in the lungs, while the pegylated ones ended up in the kidneys and the liver. This result confirmed that pegylation of the NPs prevented interaction with proteins, which causes particle growth in the blood stream. Both targeted and untargeted SNPs accumulated to a similar extent into the tumor environment due to the EPR effect, however, the Tf-functionalized SNPs showed enhanced internalization of siRNA by the tumor cells, which was attributed to receptor-mediated endocytosis. Similar polyplex systems have been developed by other groups by making use of CD-modified cationic components, also for gene and drug co-delivery.^{101-106,129}

Fig. 2.16. Schematic representation of the functioning of CALAA01. a) Assembly of the four components to obtain the SNPs, b) infusion of the aqueous SNPs solutions into a patient, c) circulation of the SNPs in the blood stream of the patients and entry into the tumor tissue by the EPR effect, d) SNP penetration of the tumor cells via receptor-mediated endocytosis, e) multivalent Tf-receptor interactions at the cell membrane stimulating the entrance of CALAA01 into the cancer cell. (adapted from¹⁰⁰ with permission of ©2009 ACS).

The rotaCDplexes, a subtype of CDplexes, with PEI in the backbone of the components, have a mechanism of action that is similar to PEI-gene delivery systems.^{40, 109-111} Interestingly, the presence of CD seems to enhance the gene delivery, and also reduces the cytotoxicity of the system (in some cases 100 times lower than PEI25kDa-based polyplexes), most probably due to the lower density of amino groups caused by the complexation by the CDs. RotaCDplexes were internalized into tumor cells via the endosomal pathway. The genetic material escaped from the endosomes most probably due to the proton sponge effect of the PEI component of this system. The transfection efficiencies were in all cases similar to PEI25kDa, however, with a much lower cytotoxicity.

Photodynamic therapy (PDT) and photothermal therapy (PT) are special types of therapeutics, both using light in a therapeutic way (phototherapy).^{130, 131} In PDT, non-toxic light-sensitive materials are exposed to light, producing toxic, reactive oxygen species (free radicals). In every PDT system, three components are essential: a photosensitizer, a light source, and oxygen (present in the tissue). PT is similar to PDT in that it also makes use of a photosensitizer. This is excited by absorption of a photon, after which the photosensitizer relaxes back while emitting vibrational energy (= heat) which kills the cells. Therefore, PT does not require oxygen and it does not produce free radicals.

AuNPs are promising PT materials.^{132,133} Tseng and coworkers have used their basic SNPs for PT applications, using Ad-AuNPs instead of Ad-PAMAM.⁵⁵ They equipped their SNP system with RGD, which is a recognition sequence for the cell receptor integrin, by introducing Ad-PEG-RGD. The PT effect of 118 nm SNPs was studied and compared to that of the parent 2 nm AuNPs by monitoring the heat accumulated locally by the SNPs. This was followed by laser-induced microbubble generation experiments, to study the locally accumulated heat of individual SNPs upon irradiation with a 532 nm pulsed laser at different energy densities. While the AuNP-containing SNPs produced microbubbles using a laser irradiation of 32 mJ cm⁻², the parent AuNPs did not produce any microbubbles at the maximum laser power of 265 mJ cm⁻². This photothermal enhancement for the SNPs was attributed to a collective heating effect of the many AuNPs that are close together in the SNPs. The formation of microbubbles only happens at elevated temperatures, because it is a threshold process which occurs when the water layer around the particles reaches a temperature close to the

critical temperature of water (375 °C).¹³⁴ Such a high temperature induces the disassembly or breakdown of the SNPs into smaller parts. Indeed, after successive laser irradiation steps of the SNPs, a dramatic decrease in microbubble formation was observed, which corroborates the SNP destruction. The use of the targeting moiety RGD provided the selective uptake of the SNPs into $\alpha_n\beta_3$ -positive cancer cells, resulting in cell detachment from the culture chambers and consequently cell death upon laser irradiation. When using $\alpha_n\beta_3$ -negative cells, SNPs without the targeting agent, or only AuNPs, no cell detachment was observed, which confirms the target specificity conferred by RGD.

2.3.2 Diagnostics: imaging

Many imaging techniques are currently being used for biomedical applications: optical imaging (OI), magnetic resonance imaging (MRI), computed tomography (CT) and positron emission tomography (PET).^{8,135} Table 2.1 shows several aspects related to these techniques, such as the source of imaging, spatial resolution, penetration depth, sensitivity and type of probes. The modular character of SNPs makes them ideal to be used as biomedical imaging agents, since it is relatively straightforward to functionalize one of the components with an imaging probe, or to functionalize the probe with host or guest groups and include it as a new component in the SNP system.¹³⁶⁻¹³⁸ In the next paragraphs, some examples of these supramolecular systems for imaging applications are presented.

Table 2.1 Comparison between several imaging techniques. Taken from refs.^{135, 139}

Imaging technique	Source of imaging	Spatial resolution	Tissue penetration	Sensitivity	Type of probe
Magnetic resonance imaging (MRI)	Radiowave	25-100 μm	No limit	mM to μM (low)	Para-(Gd^{3+}) or superparamagnetic (Fe_3O_4) materials
Positron emission tomography (PET)	γ -ray	1-2 mm	No limit	pM (high)	Radionucleotides (^{18}F , ^{11}C , ^{13}N , ^{15}O , ^{124}I , ^{64}Cu)
Computed tomography (CT)	X-ray	50-200 μm	No limit	Not well characterized	High atomic number atoms (iodine, barium sulfate)
Optical fluorescence imaging	Visible or near-infrared light	<i>in vivo</i> , 2-3 mm; <i>in vitro</i> , sub- μm	<1 cm	nM to pM (medium)	Fluorescent dyes, quantum dots, UCNPs

Tseng and coworkers have used their basic system for imaging applications.^{54,137,140} They covalently attached the ligand DOTA to the PEI-CD component.^{54,137} DOTA is a chelator which can coordinate with a variety of components such as radioisotopes and paramagnetic metal ions to be used in PET or MRI, respectively. For example, ^{64}Cu was coordinated into DOTA obtaining ^{64}Cu -labeled SNPs.⁵⁴ The size-dependent distribution and the lymph node drainage of these SNPs were followed *in vivo* in mice by using microPET/CT imaging. The biodistribution studies showed rapid body clearance by the liver: 5 min after injection 30–50% of the particles accumulated in the liver. Much less accumulation was observed in the kidneys and the lungs. The 30 nm particles showed faster *in vivo* clearance. Lymph node drainage was investigated for future applications in immunomodulation, and accumulation of the 30 nm particles was observed at the injection site and in connected lymph nodes, which demonstrates the potential for immunotherapy application of these 30 nm SNPs. However, no accumulation in the lymph nodes of the 100 nm particles was observed. These results demonstrate the size dependence of the biodistribution and bioaccumulation of these SNPs. As a variation of this system, the authors prepared a multicomponent system for pretargeting PET imaging with transcyclooctene (TCO) for *in vivo* imaging applications.¹⁴⁰ Upon injection, the SNPs accumulated in the tumor tissue due to the EPR effect. In a second injection, a small molecule containing both tetrazine (Tz, a complementary bioorthogonal motif for TCO) and a positron-emitting radioisotope (^{64}Cu) were delivered and reacted irreversibly with the SNPs at the tumor site, allowing visualization of the tumor by PET. This system is intended for preclinical oncologic PET imaging.

The same authors also developed a small MRI contrast agent library of chelated gadolinium (Gd^{3+} -DOTA) using Gd^{3+} -DOTA-CD-PEI as one of the three components of this system (Fig. 2.17).¹³⁷ The library was obtained by varying the mixing ratios of the molecular building blocks, leading to a broad range of relaxation rates. The optimal formulation showed a 4 times larger longitudinal relaxation rate (which is a measure of the r_1 relaxivity, that is, the ability of a magnetic compound to increase the relaxation of the spins of the surrounding water protons) and an almost 4 times better signal-to-noise ratio than the clinically used Gd^{3+} -chelates. The optimal formulation was tested *in vivo* and *ex vivo* in mice to detect cancer metastasis by dynamic imaging of lymphatic drainage. The draining of the lymph

nodes was clearly observed for the SNPs, but not for the control (Gd^{3+} -DTPA complex), which confirms the applicability of this system to detect cancer metastasis *in vivo*. Similar to this system, Zhou *et al.* developed a Gd^{3+} MRI contrast agent by host-guest interactions of a CD-functionalized polyhedral silsesquioxane (CD-POSS) derivative together with Gd -Ad-DOTA and the monovalent capping agent Ad-PEG functionalized with the targeting moiety RGD.¹⁴¹

Fig. 2.17. MRI contrast agent SNP library developed by Tseng and coworkers. a) Components of the system and self-assembly. b) Mesh plot of the r_1 relaxivity with respect to both the ratio of Ad-PAMAM to CD-PEI and the loading of Gd^{3+} into the SNPs. The peak value corresponds with the highest relaxivity (adapted from¹³⁷. ©Elsevier).

2.4 Conclusions and outlook

In this Chapter, CD-based SNPs for biomedical applications were reviewed. In the first part, the focus was on the process of assembly of the different components to obtain SNPs, based on host-guest interactions and other types such as hydrophobic and electrostatic ones. The particle formation, size, and stability are governed by different processes such as the nature of the interactions, equilibrium between the different forces involved, ionic force of the media, and mono- versus multivalent competition.

In the second part, the use of CD-based SNPs for diagnostics and therapeutics was presented. The modular character of SNPs is meaningful since it offers the possibility of using one basic system for a variety of applications, and to bring several applications together into one single system. In therapeutic applications, the non-covalent nature of host-guest chemistry is ideal for the encapsulation, water-solubilization, and controlled release of a drug. In bioimaging applications, supramolecular interactions bring the possibility of

incorporating easily the imaging agent into the SNPs by grafting it in one of the components before the NP assembly.

For future applications and development of SNPs in the biomedical field, it is crucial to comprehend the way these SNPs assemble and behave in biological media. Therefore, more fundamental research like the one presented in this thesis is relevant to provide a deeper understanding of SNPs.

2.5 List of abbreviations

AAP	arylazopyrazole
Ad	adamantane
Ad-Dextran	dextran grafted with Ad
Ad-Dox	Dox grafted with Ad
Ad-MNP	magnetic NPs grafted with Ad
Ad-PAMAM	poly(amidoamine) dendrimer functionalized with Ad
Ad-PEG	poly(ethylene glycol) functionalized with Ad
Ad-PEG-Tf	Ad-PEG functionalized with Tf
Ad-PPI	poly(propylene imine) dendrimer grafted with Ad
AuNPs	gold nanoparticles
Ad-TEG-OH	Ad-tetra(ethylene glycol)
Az	azobenzene
BZ	benzophenone
CD	cyclodextrin
CD-AuNP	AuNPs functionalized with CD
CD-Dextran	dextran grafted with CD
CDP	CD polymer
CD-PAA	CD grafted poly(acrylic acid)
CD-PEI	poly(ethylene imine) functionalized with CD
CD-POSS	CD-functionalized polyhedral silsesquioxane
CDV	vesicles of amphiphilic CD
Chol	cholesterol
CPT	camptothecin
CPT-PGA	PGA grafted with CPT
CT	computed tomography
DMAEMA	2-(dimethylamino ethyl methacrylate)
DNA	deoxyribonucleic acid
Dod	dodecylamine
Dod-Dextran	dextran grafted with Dod chains
DOTA	1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid
Dox	doxorubicin
EDTA	Ethylene diamine-tetraacetic acid
EO	ethylene oxide
EPR	enhanced permeability and retention
Fc	ferrocene

Fc-PAMAM	PAMAM grafted with Fc
FDA	food and drug administration
Gd ³⁺ -DOTA	Gd ³⁺ chelated by DOTA
ICG	indocyanine green
miRNA	microRNA
MRI	magnetic resonance imaging
MW	molecular weight
NP	nanoparticle
OEI	oligo(ethylene imine)
OI	optical imaging
PAA	poly(acrylic acid)
PAMAM	poly(amido amine) dendrimer
PBLA	poly(benzyl-L-aspartate)
PBS	phosphate buffered saline
PDMAEMA	poly(dimethylamino ethyl methacrylate)
PCD	polyaspartamide grafted with CD
PCL	poly- ϵ -caprolactone
PCTX	paclitaxel
PDI	polydispersity
pDNA	plasmid DNA
PDT	photodynamic therapy
PEG	poly(ethylene glycol)
PEG- <i>b</i> -PCD	block copolymer of PEG and PCD
PEG-PCL-PEI	block copolymer of PEG, PCL, and PEI
PEI	poly(ethylene imine)
PEO- <i>r</i> -PO	random copolymer of PEO and PO
PET	positron emission tomography
PGA	poly(glutamic acid)
PIBMA	poly(isobutyl- <i>alt</i> -maleic acid)
PIBMA-Ad	PIBMA grafted with Ad
PIBMA-TBP	PIBMA grafted with TBP
PIC	polyion complex
PLL	poly(lactic acid)
PO	propylene oxide
PPG	poly(propylene glycol)
PT	photothermal therapy
PVM/MA	poly(methyl vinyl ether-co-maleic anhydride)
RGD	arginyl glycyl aspartic acid peptide
RhB	rhodamine B
RNA	ribonucleic acid
siRNA	small interfering RNA
SNP	supramolecular nanoparticle
TBP	<i>tert</i> -butylphenyl
Tf	transferrin
TfR	transferrin receptor
TOPO	topoisomerase I
Try	L-tryptophan
Try-CD	Try functionalized with CD

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Chapter 3

Cyclodextrin-Based Supramolecular Nanoparticles Stabilized by Balancing Attractive Host-Guest and Repulsive Electrostatic Interaction

Multicomponent, negatively charged supramolecular nanoparticles (SNPs) were formed by multivalent host-guest interactions between the host polymer poly(isobutyl-alt-maleic acid) (PiBMA) grafted with cyclodextrin (CD, CD-PiBMA) and the guest polymer PiBMA grafted with tert-butylphenyl groups (TBP, TBP-PiBMA), at a 1 : 1 host-guest ratio. The particles were stable without the need of a monovalent capping agent (Ad-PEG) in water and in PBS, and also at various temperatures. Only at high salt concentrations, the Ad-PEG stopper was needed to achieve stable SNPs. The ratio monovalent/multivalent (Ad/TBP) guest moieties did not have an effect on the particle size. Studies at different ionic strengths showed that the size and stability of these SNPs are controlled by a balance of forces between attractive supramolecular and repulsive electrostatic interactions: a high ionic strength provoked the destabilization of the SNPs, leading to the continuous growth of the supramolecularly cross-linked network, while at lower ionic strengths the particles remained sufficiently charged to prevent further attachment of host and guest components to the already formed SNPs.

3.1 Introduction

Supramolecular nanoparticles (SNPs) are formed by specific non-covalent multivalent supramolecular interactions in a multicomponent system.¹ These SNPs present several advantages such as size control, controlled assembly/disassembly, modular character, and easy incorporation of imaging agents or targeting ligands.¹ Three basic types of SNPs can be distinguished: a) SNPs for siRNA delivery by Davis and coworkers,² in which the particles are based on electrostatic interactions between negatively charged siRNA and a positively charged cyclodextrin-containing polymer, b) a supramolecular system by Tseng and coworkers,³ in which the particles are formed by multivalent host-guest interactions between a positively charged cyclodextrin-grafted polymer and positively charged PAMAM dendrimers containing adamantyl moieties and c) the dextran-based host-guest nanoparticles by Larsen and coworkers,⁴ based on host-guest interactions between cyclodextrin and adamantyl-grafted neutral dextran polymers. These systems have been used for different biomedical applications, such as imaging,⁵ photothermal therapy,⁶ drug delivery,^{3,7,8} and gene delivery.^{2,9}

The cyclodextrin-based SNPs make use of adamantyl-terminated poly(ethylene glycol) (Ad-PEG) as a monovalent capping agent (stopper), to terminate the multivalent supramolecular network, avoiding therefore the continuous growth of the particles and their aggregation due to the attractive multivalent interactions in Tseng's system and electrostatic forces in Davis' system.^{2,3,10} However, in the case of the dextran-based nanoparticles, the absence of a monovalent capping agent to compete with the multivalent interactions together with the neutral character of the two different building blocks, results in a near doubling of the size in less than five days.⁴ Despite the importance for SNPs, little is known about the interplay of electrostatics, host-guest interactions and steric influences in the control of particle size and stability. In Davis' system,² the electrostatic interactions are attractive; in Tseng's system,³ they are repulsive; and in Larsen's system, electrostatics is assumed to play a negligible role because of the absence of formal charges on the building blocks.⁴

Here, we aim to study the influence of electrostatics in host-guest SNP systems, in which the host and guest components are two negatively charged polymers. The main focus points are size control, the role of the monovalent capping agent (stopper), and the SNP stability. These

SNPs are formed by supramolecular multivalent interactions between β -cyclodextrin (CD) and the *p*-*tert*-butylphenyl group (TBP),¹¹ using a multicomponent system based on the linear, negatively charged polymer poly(isobutyl-*alt*-maleic acid) (PiBMA) and the monovalent capping agent Ad-PEG, as shown in Fig. 3.1. PiBMA is a biocompatible anionic polymer,^{12,13} being widely used as a coating polymer to make inorganic particles water soluble, and for the formation of polymeric nanoparticles for drug and gene delivery.¹²⁻¹⁵

Fig. 3.1. a) Schematic representation of the assembly and b) molecular structure of the components of the SNPs from the host polymer PiBMA grafted with β -cyclodextrins (CD-PiBMA), the guest polymer PiBMA grafted with *p*-*tert*-butylphenyl groups (TBP-PiBMA), and Ad-PEG.

3.2 Results and discussion

3.2.1 SNP preparation

The host polymer CD-PiBMA and the guest polymer TBP-PiBMA (Fig. 3.1b) were synthesized by amidation of poly(isobutyl-*alt*-maleic anhydride) (MW 6 kDa) with 6-monodeoxy-6-monoamino- β -cyclodextrin¹⁶⁻¹⁸ or *p*-*tert*-butylaniline, respectively, and subsequent ring-opening of the unreacted anhydrides with a sodium hydroxide solution.¹⁹ The crude products were purified by dialysis and neutralized before freeze-drying. ¹H-NMR analysis revealed that the polymers contained nine CD or nine TBP moieties per polymer backbone by comparing the methyl signals of the polymer with the H¹-CD signal of the CD moieties (CD-PiBMA) or the backbone signals of the polymer with the *tert*-butyl signal (TBP-PiBMA). Ad-PEG (MW 5 kDa) was synthesized as described in the literature.⁵

The particles were easily prepared by mixing the components maintaining a 1 : 1 ratio between host (CD) and guest (TBP and Ad) moieties at 15 μ M CD and varying the multivalent/monovalent (TBP/Ad) ratio between 0% and 100% Ad-PEG. Fig. 3.1 shows a schematic representation of the assembly of these SNPs from the host (CD-PiBMA) and the guest (TBP-PiBMA) polymers with/without Ad-PEG.

Fig. 3.2 a, b) DLS graph and c, d) SEM images) of the particles prepared without Ad-PEG (a, c) and with 10% Ad-PEG (b, d) in water.

DLS measurements of the particles obtained without Ad-PEG (142 nm; Fig. 3.2.a) and with 10% Ad-PEG (145 nm; Fig. 3.2b) in water showed nanoparticles of comparable hydrodynamic diameters. SEM images (Fig. 3.2c without Ad-PEG and Fig. 3.2d with 10% Ad-PEG) show amorphous nanoparticles with a size of 70 nm. The particle size by SEM is smaller than that measured by DLS in aqueous solution due to a) shrinking of the particles upon drying, and b) DLS reporting the hydrodynamic radius of the hydrated particles.

SEM images of the host or guest polymers individually, and of the mixtures of host or guest polymer together with unfunctionalized PiBMA (controls, Fig. 3.3) only revealed some big polymer aggregates due to hydrophobic-hydrophobic interactions between the polymers²⁰ but no nanoparticle formation. This confirms that specific multivalent supramolecular interactions occur between the components of the SNPs in case CD-PiBMA and TBP-PiBMA are used together.

Fig. 3.3. SEM images of the controls a) TBP-PiBMA (1.11 μM), b) CD-PiBMA (1.67 μM), c) Ad-PEG (7.5 μM), d) TBP-PiBMA + PiBMA ([TBP]= 15 μM , [PiBMA]= 1.67 μM), and e) CD-PiBMA + PiBMA ([CD]= 15 μM , [PiBMA]=1.67 μM).

The particles were also prepared in phosphate buffered saline (PBS, 138 mM NaCl, pH 7.4) as a mimic of the ionic strength under physiological conditions, resulting in particles with

comparable size and morphology as the particles in water as determined by DLS and SEM (Fig. 3.4).

Fig. 3.4. a) DLS particle size graph and b, c) SEM images of the particles prepared without b) and with a,c) 10% Ad-PEG in PBS (138 mM NaCl, pH 7.4).

3.2.2 Influence of Ad-PEG

In order to investigate further the function of Ad-PEG in this system, SNPs were formed at different Ad-PEG : TBP ratios while maintaining the host-guest 1 : 1 stoichiometry. Fig. 3.5 shows that the particle size is comparable over the whole range of 0-100% of Ad-PEG. This means that the competition between multivalent (TBP-PIBMA) and monovalent (Ad-PEG) guest moieties does not have an influence on the particle formation and its size. This is in contrast to other supramolecular nanoparticles systems, where size control is achieved by changing the ratio between monovalent and multivalent components.^{3,10,21} This is even more remarkable when one recognizes that the Ad-CD interaction is stronger than the TBP-CD interaction (by approx. a factor 5).¹¹

Fig. 3.5. Particle size as a function of the percentage of Ad-PEG at host-guest = 1 : 1.

Overall, these results indicate that multivalent CD-TBP interactions, which are stronger than monovalent CD-Ad interactions, cause SNP formation but that electrostatic repulsion prevents further PiBMA building blocks to add to the outer layer of the SNP upon a certain particle size has been reached.

3.2.3 Influence of temperature on the SNP stability

The stability of the nanoparticles in pure water over time at 25 °C and 36 °C without Ad-PEG (Fig. 3.6a) and with Ad-PEG (Fig. 3.6b) at 15 μ M CD was studied by DLS. The results show that these particles are very stable in water: the size of the NPs is maintained for at least one month at 25 °C and for at least 20 days at 36 °C. This also demonstrates that these particles are stable at physiologically relevant temperatures. Furthermore, it is striking that in the absence of Ad-PEG, the particle stability is not affected over time.^{2,3,21}

Fig. 3.6. Particle stability over time of the SNPs prepared a) without and b) with Ad-PEG at 25 °C and at 36 °C in water.

The particle stability was also studied in PBS (138 mM NaCl, pH 7.4), at room temperature and at 36 °C without Ad-PEG (Fig. 3.7a), with 10% (Fig. 3.7b) and with 80% Ad-PEG (Fig. 3.7c). The graphs show that the particle size is maintained for almost 8 hours, after which it starts to slowly increase leading to particle aggregation. The results obtained are comparable with those in water and not influenced by the presence of Ad-PEG: the particle stability at 36 °C without (Fig. 3.7a) and with 80% Ad-PEG (Fig. 3.7c) is alike. This 8 hr stability under these conditions make these SNPs suitable for biomedical applications.

Fig. 3.7. Particle stability over time of the SNPs prepared in PBS (138 mM NaCl, pH 7.4) a) without Ad-PEG at 25 °C and at 36 °C, b) with 10% Ad-PEG at 25 °C, and c) with 80% Ad-PEG at 36 °C.

We attribute the remarkable stability of the particles, even without the use of a stopper, to the existence of a balance of forces in these SNPs: attractive host-guest interactions and repulsive electrostatic forces between the negatively charged host and guest polymers co-exist, which apparently lead to a stable supramolecular nanoparticle system without the need for a monovalent stopper. Strikingly, in the Tseng system,³ repulsive interactions also exist between the positively charged poly(ethylene imine) polymer grafted with cyclodextrins and the PAMAM dendrimers functionalized with adamantyl groups, yet this system shows size control by Ad-PEG. Probably, the charge density in our system is larger, which results in a larger contribution of the electrostatic repulsive interactions. In the Davis system,² the electrostatic interactions are attractive, therefore, a monovalent capping agent is needed to stabilize the particles in biological media, avoiding their aggregation.

3.2.4 Influence of the electrostatic interactions on SNP formation and stability

In acetic acid buffer at pH 4.2, the carboxylic groups of the PiBMA backbone are partly being protonated leading to a decrease of the overall charge of the host and guest polymers. Use of this buffer resulted in immediate aggregation and precipitation of the SNPs prepared without Ad-PEG. Analogously, decrease of the electrostatic repulsions between host and guest polymers was achieved by forming the SNPs at increasing ionic strengths varying from 100 to 1000 mM KCl^{22,23} at a 1 : 1 host-guest ratio without Ad-PEG and 15 μ M CD at pH 7. Fig. 3.8a shows DLS results of these experiments which clearly demonstrate the destabilization of the particles with increasing ionic strength. While in the absence of KCl, the particles remained stable for over one month, at 100 mM KCl, the particle size was maintained for one

day, after which it started to increase slowly. At 300 mM KCl, the SNPs began to grow already from the first day onward and reached almost twice their original size after 2 days. At 1000 mM KCl, the particles were continuously increasing in size and they reached four times their original size after 2 days. These results demonstrate that the balance between the attractive host-guest and the repulsive electrostatic forces between the host and the guest polymers can be disturbed by lowering the pH and/or increasing the ionic strength giving rise to the continuous growth of the supramolecularly cross-linked network.

Fig. 3.8. a) Influence of ionic strength on particle stability in the absence of Ad-PEG. b) Influence of Ad-PEG on particle stability at 1000 mM KCl in water.

These results open up the possibility of particle stabilization induced by a monovalent capping agent (Ad-PEG) at high ionic strengths. To assess this, SNPs were prepared at 1000 mM KCl at a 1 : 1 host-guest ratio in the absence of Ad-PEG, with 10% Ad-PEG and 50% Ad-PEG and their stability was monitored over time. Fig. 3.8b clearly shows that, interestingly, Ad-PEG has a clear function in this case. Upon increasing the percentage of Ad-PEG in the SNPs, the particle stability improves: at 50% Ad-PEG the particle size was maintained for about 24 h, whereupon it started to increase. At 10% Ad-PEG, the particle stabilization is apparent but less pronounced. These results clearly prove that once the electrostatic repulsions between the host and guest polymers are disturbed, the stopper Ad-PEG starts to play a role in the particle stabilization and consequently stops the continuous growth of the supramolecular network.

3.3 Conclusions and outlook

In conclusion, we have developed a multicomponent supramolecular nanoparticle system using the negatively charged polymer PiBMA. The particles are formed by multivalent supramolecular interactions between the host CD and the guest TBP, and their size and stability are controlled owing to a balance of forces between attractive supramolecular host-guest interactions and repulsive electrostatic interactions between the host and the guest polymers. The understanding of the forces underlying SNP formation and stability will further the development of SNPs as carriers for biomedical applications such as imaging or drug delivery agents.

3.4 Acknowledgements

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3.5 Experimental

3.5.1 Materials and equipment

Starting materials and solvents for the synthesis were obtained from Sigma-Aldrich and used as received. *N,N*-Diisopropylethylamine (DIPEA) was purchased from Biosolve. Deuterated NMR solvents were purchased from Cambridge Isotope Laboratories Inc. Phosphate buffered saline (0.01 M, pH 7.4 at 25 °C, 0.138 M NaCl, 0.0027 M KCl) was prepared from Sigma Aldrich powder. 6-monodeoxy-6-monotosyl- β -cyclodextrin, 6-monodeoxy-6-azido- β -cyclodextrin, and 6-Monodeoxy-6-monoamino- β -cyclodextrin were synthesized according to literature procedures.²⁴⁻²⁶ Ad-PEG was synthesized as described before.⁴ MilliQ water with a resistivity of 18.2 M Ω cm at 25 °C was used in all experiments. ¹H-NMR spectroscopy was performed on a Bruker 400 MHz NMR spectrometer. FTIR (ATR) was carried out on a Nicolet™ iS™10 FT-IR spectrometer equipped with a Smart iTR™ Attenuated Total Reflectance (ATR) sampling accessory (diamond plate). Dynamic light scattering (DLS) measurements were performed on a Zetasizer NanoZS (Malvern Instrument Ltd, Malvern, United Kingdom), with a laser

wavelength of 633 nm and a scattering angle of 173°. Scanning electron microscopy (SEM) pictures were taken on a Carl-Zeiss 1500 SEM.

3.5.2 Synthetic procedures

Synthesis of CD-PiBMA

To a solution of PiBMA (40 mg, 6.66 μ mol) in dry DMSO (8 mL), was added a solution of 6-monodeoxy-6-monoamino- β -cyclodextrin (121 mg, 0.1067 mmol) and DIPEA (35 μ L, 0.213 mmol) in DMSO (2 mL). The mixture was reacted overnight at 80 °C. To the crude reaction mixture, water (10 mL) was added and the unreacted anhydride rings were opened with NaOH_{aq} (0.26 mL 1 M NaOH). Afterwards, the mixture was purified by dialysis (SpectraPor membrane, MWCO 6-8 kD) for 1 week. Pure CD-PiBMA was obtained by freeze-drying (45 mg, 42% yield). The degree of grafting was calculated from the ratio between the CH₃ protons of the polymer and the C₁H of β -CD. ¹H-NMR (400 MHz, D₂O, pH 11): δ 0.25-1.25 (br, 234 H, CH₃), 1.25-3.0 (br m, 136 H, PiBMA backbone), 3.01-4.18 (m, 337 H, C₂₋₆H of CD), 4.88-5.1 (br, 60 H, C₁H of CD). FTIR: 3355 (OH), 1700 (amide), 1665 (COO⁻), 1558 (amide), 1371 (COO⁻), 1155 (CO), 1079 (CO/CC), 1029 (CO/CC) cm⁻¹.

Synthesis of TBP-PiBMA

TBP-PiBMA was synthesized similarly to CD-PiBMA. To a solution of PiBMA (250 mg, 0.042 mmol) in dry DMSO (15 mL), was added a solution of 4-*tert*-butylaniline (64 mg, 0.43 mmol) in DMSO (5 mL). The mixture was reacted overnight at 60 °C. To the crude reaction mixture, water (20 mL) was added and the unreacted anhydride rings were opened with 1 M NaOH (1.64 mL). Afterwards, the mixture was purified by dialysis (Slide-A-Lyzer® dialysis cassette, MWCO 3.5 kD) for 1 week. Pure TBP-PiBMA was obtained by freeze-drying (195 mg, 63% yield). ¹H-NMR (400 MHz, D₂O, pH 11): δ 0.63-1.25 (br d, 317 H, CH₃), 1.30-1.58 (br, 39 H, CH₂), 1.60-2.06 (br, 60 H, CH₂), 2.18-2.83 (br, 79 H, CH), 7.23-7.60 (br m, 40 H, Ar - H). FTIR: 3388 (OH), 2961 (CH), 1665 (amide), 1556 (COO⁻), 1516 (amide), 1469 (CH), 1392 (CO), 1371 (CO), 836 (ArH) cm⁻¹.

3.5.3 Methods

Preparation of the supramolecular nanoparticles

In water or in PBS: The SNPs were prepared maintaining the host-guest ratio at 1 : 1 at 15 μM CD from stock solutions of CD-PiBMA (6.44 μM), TBP-PiBMA (5.50 μM), and Ad-PEG (62.50 μM) by mixing the components with a vortex and afterwards they were measured by DLS at different times.

In acetic acid buffer: The SNPs were prepared maintaining the host-guest ratio at 1 : 1 at 15 μM CD from stock solutions of CD-PiBMA (10 μM) and TBP-PiBMA (10 μM) in milliQ water. In order to prepare the particles, 125 μL of the CD-PiBMA and TBP-PiBMA stock solutions were added to 500 μL of acetic acidic buffer (pH 4.2, 0.2 M) containing also 200 mM KCl (final concentration of KCl in the particles: 100 mM). The samples were vortexed for 30 s and afterwards measured by DLS after 15 min and 4 h.

In KCl: The SNPs were prepared maintaining the host-guest ratio at 1 : 1 at 15 μM CD from stock solutions of CD-PiBMA (10 μM), TBP-PiBMA (10 μM), and Ad-PEG (90 μM) in milliQ water. In order to prepare the particles, 125 μL of the CD-PiBMA and different amounts of the TBP-PiBMA and Ad-PEG stock solutions were added to 500 μL of KCl of different concentrations to reach a final concentration of KCl of 100 mM, 300 mM, and 1000 mM. The samples were vortexed for 30 s and afterwards measured by DLS at different times (15 min, 4 h, 7 h, 24 h, and 48 h).

DLS measurements

To perform the DLS measurements, the refractive index of the material was fixed to 1.465 and the absorbance to 0.01. The parameters for the dispersant (water or PBS) were taken from the database of the machine. For each measurement, the sample was equilibrated for 2 min. The measurement was set to automatic. The temperature was set to 25 $^{\circ}\text{C}$ or 36 $^{\circ}\text{C}$.

SEM measurements

The samples were prepared by drop-casting 5 μL of a SNP solution onto a silicon wafer or a carbon-coated copper grid. After 30 s, excess of water was removed by filter paper. The samples were allowed to dry overnight in a desiccator containing silica gel beads. Original samples and 20x diluted ones were used.

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Chapter 4

Fluorescent Supramolecular Nanoparticles Signal the Loading of Electrostatically Charged Cargo

Cyclodextrin (CD)-based supramolecular nanoparticles (SNPs), negatively charged and fluorescently labelled with rhodamine B (RhB), were used to study the effects of their loading with oppositely charged cargo. Upon addition of the cationic poly(ethylene imine) (PEI), grafted or not with fluorescein (FL, FL-PEI), as the cargo, the SNPs shrank in size until the point of neutralization was reached, as visualized by RhB self-quenching caused by the closer proximity of the dyes inside the SNPs. The particle shrinkage was attributed to the reduction of repulsion between the backbones of the host and guest polymers. Beyond the neutralization point, the SNPs destabilized and aggregated as witnessed by dynamic light scattering (DLS) and visualized by scanning electron microscopy (SEM). Since FL and RhB form a FRET couple, an increasing FRET effect was observed between the RhB units of the SNPs and the FL-PEI cargo upon cargo loading, until reaching the neutralization point. The absence of further change of the FRET effect beyond this concentration indicated that no more FL-PEI was loaded into the SNPs, attributed to charge reversal of the SNPs. The fluorescence effects used here may allow the development of responsive imaging and drug delivery vehicles.

4.1 Introduction

The loading of drugs or nucleic acid derivatives into supramolecular nanoparticles (SNPs) can be achieved by different strategies, e.g., by host-guest chemistry¹⁻⁶ or electrostatics.^{3,7-11} The latter approach is used extensively in the case of electrostatically charged guests, such as charged drugs. SNPs for the delivery of RNA or DNA constitute a clear example of this approach: the genetic material is negatively charged, and for this reason it needs a positively charged scaffold to be encapsulated.^{1,2,8,12} The components of the SNPs can be charged as desired: negatively, as in the system explained in Chapter 3 which makes use of the biocompatible poly(isobutyl-*α*/*t*-maleic acid) (PiBMA) polymer,¹³ or positively, such as in the systems of Tseng¹⁴ and Davis,⁸ in which cyclodextrin (CD)-grafted poly(ethylene imine) (PEI) has been used. In Tseng's system, the SNP formation is governed by CD host-guest interactions, and the role of electrostatics remains often subdued. In contrast, in Davis' system, the electrostatics between the CD-PEI and the DNA/RNA payload is the main SNP driving force, and the CD host-guest interactions are merely used for the attachment of the monovalent stopper which provides SNP stabilization. Both systems have been studied extensively in terms of particle formation, release of the cargo, optimum ratio of scaffold/cargo, and toxicology.^{3,4,9,11,12,15,16} Although the effects of the charged cargo on the particle characteristics and stability are of utmost importance for biomedical applications, these aspects have hardly been explored so far.

Fluorescence spectroscopy is a powerful and sensitive technique, and has been used, amongst others, for the study of supramolecular assemblies for sensing and on-off switching.¹⁷⁻¹⁹ Forster Resonance Energy Transfer (FRET) describes the process by which the excitation energy is transferred from one dye to another that is in close proximity, and this process is widely used for the study of conformational changes in assemblies and biomolecular systems.²⁰⁻²⁴ Moreover, the quenching of fluorescent polymers (superquenching) has appeared as an emergent technique to study DNA hybridization, and to detect a variety of biological targets.^{20,25-28} Fluorescence techniques thus can offer unique ways to explore the changes occurring in SNPs upon addition of a guest. Using charged SNPs that have been equipped with a fluorescent dye, the addition of a complementary charged guest, with or without a dye that forms a FRET couple with the first dye, may alter the

characteristics of the particles and affect the fluorescence properties of the SNPs. Therefore, we envisage that fluorescence and FRET methods will allow the study of the loading of guests into SNPs.

Here, we aim to study the effects of loading a charged fluorescent cargo into charged fluorescent SNPs held together by cyclodextrin (CD) host-guest interactions, thus changing the electrostatic environment of the particles, on the SNP properties, such as size and stability. Also, the changes in the fluorescent properties of the assemblies, such as self-quenching and energy transfer of the FRET couple, will act as a tool for studying these assemblies. The SNPs are characterized by DLS and zeta potential, while cargo loading is studied by monitoring the changes in UV and fluorescence properties upon the addition of cargo.

4.2 Results and discussion

4.2.1 SNP preparation and synthesis of the components

The components used to form the fluorescent SNPs and their self-assembly are shown in Fig. 4.1. Guest loading into the SNPs (Fig. 4.1a) is studied by interacting a fluorescently labelled polymeric cationic guest with SNPs that contain a fluorescent dye-modified anionic polymeric building block. As a consequence, the overall particle charge is expected to change from negative to neutral and ultimately positive upon increase of the cationic guest concentration. These changes of the electrostatic properties are expected to affect the fluorescence characteristics of the particles (Fig. 4.1a,b). Furthermore, fluorescein (FL) and rhodamine B (RhB), the two dyes used to tag the cargo and the labelled SNP building block, form a FRET couple, which is used here to study proximity effects within the SNPs.

The host and the guest polymers (Fig. 1b,c), cyclodextrin-grafted poly(isobutyl-alt-maleic acid) (CD-PiBMA) and *p*-tert-butylphenyl-grafted PiBMA (TBP-PiBMA), were synthesized as described before,^{13,29-31} and contained, on average, 9 CD units and 9 TBP units per polymer backbone, respectively. The host polymer grafted with rhodamine (CD-RhB-PiBMA) was synthesized in a stepwise process by amidation of poly(isobutyl-alt-maleic anhydride) (MW

6 kDa) with lissamine™ rhodamine B ethylenediamine followed by 6-monodeoxy-6-monoamino- β -cyclodextrin.

Fig. 4.1. a) Self-assembly of the components to form the fluorescent SNPs and their loading with a charged cargo. b) Fluorescence and FRET processes used here. c) Components of the SNP system: host polymer grafted solely with CD (CD-PiBMA), host polymer grafted with CD and lissamine rhodamine B (CD-RhB-PiBMA), guest polymer grafted with *p*-tert-butylphenyl groups (TBP-PiBMA), and the cargo polymer poly(ethylene imine) grafted with fluorescein (FL-PEI).

The positively charged cargo polymer, poly(ethylene imine) (PEI) grafted with fluorescein (FL), was synthesized by thiourea bond formation by reaction of PEI with fluorescein isothiocyanate. The crude products were purified by dialysis and neutralized before freeze-drying. ^1H -NMR analysis of CD-RhB-PiBMA revealed that it contained, on average, 5 CD moieties per polymer backbone, as determined by comparing the methyl signals of the polymer with the H1-CD signal of the CD moieties. CD-RhB-PiBMA was grafted with, on average, 1.5 RhB per polymer backbone, and FL-PEI with 0.3 FL per polymer backbone as determined by UV analysis (see calibration curves in Fig. 4.8).

The host polymer with/without dye (CD-RhB-PiBMA, CD-PiBMA) and the guest polymer (TBP-PiBMA) formed fluorescent SNPs in phosphate buffer (5 mM, pH 7.4, Fig. 4.1a) stabilized by a balance between attractive supramolecular host-guest interactions between CD and TBP and repulsive electrostatic interactions between the polymer chains, without the need of a monovalent stopper, as studied before for the parent system without RhB label.²⁰ The ratio between CD-PiBMA and its dye-modified counterpart CD-RhB-PiBMA was used to control the dye concentration. The cargo PEI, unlabelled or labelled with FL, was added after SNP formation, and it was incorporated in the SNP core (Fig. 4.1a) by attractive electrostatic interactions with the host and guest polymers. The concentration of PEI was determined based on its molecular weight, and, therefore, on the number of polymer chains.

4.2.2 Influence of the cargo on the size and charge of the SNPs

The change of the SNP particle size upon addition of increasing amounts of (unlabelled) PEI was monitored by dynamic light scattering (DLS). As shown in Fig. 4.2a, the particles shrank upon the first addition to approx. half of their original size. This size was maintained until reaching a concentration of 2.3 μM PEI, at which further increase of the concentration led to the formation of large aggregates. Although a size decrease upon loading cargo might seem counterintuitive at first, it must be noted that the electrostatic repulsive interactions between the SNP building blocks provides the SNPs with an open, hydrogel-like structure. Loading with oppositely charged cargo therefore reduces the repulsion and thus causes shrinkage of the SNPs, with concurrent loss of water from the SNP core.

Fig. 4.2. a) Hydrodynamic diameters from DLS and b) zeta potential (ζ) data of the SNPs composed of CD-RhB-PiBMA (3.3 μM CD, 1 μM RhB), CD-PiBMA (11.7 μM CD), and TBP-PiBMA (15 μM TBP) upon addition of increasing amounts of PEI.

A similar transition was observed by zeta potential (ζ) measurements (Fig. 4.2b) which showed that, upon addition of PEI, the charge of the SNPs gradually became less negative. In the case of using FL-PEI as the cargo, comparable results were obtained (Fig. 4.3). At a concentration of 2.3 μM PEI, the point of neutralization was reached, and the particles became positively charged at higher amounts of cargo. However, the absolute value of the zeta potential remained below 20 mV, which is in agreement with the loss of colloidal stability as observed by DLS. Moreover, the maintained, low positive charge at high cargo concentrations indicates that guest uptake beyond the point of neutralization is increasingly hampered by electrostatic repulsion.

Fig. 4.3. a) Hydrodynamic diameters from DLS and b) zeta potential (ζ) data of the SNPs upon addition of increasing amounts of FL-PEI.

Comparing the amounts of positive and negative charges present in the SNPs upon addition of PEI/FL-PEI to the preformed particles indicates that the particles reach the point of neutralization at a concentration of 2.3 μM PEI/FL-PEI (the theoretical calculation of the neutralization point together with the assumptions made are given in section 4.5.4, Table 4.1). This agrees well with the experimental DLS and zeta potential data shown in Fig. 4.2. Lindman and coworkers have observed a similar aggregation behavior for particles formed by condensates of DNA and a polyelectrolyte.^{32,33} In Chapter 3,¹³ we observed that the negatively charged SNPs are stabilized by a balance between attractive supramolecular and repulsive electrostatic forces. Whenever this equilibrium is perturbed, for example by exposing the particles to high ionic strength, or by addition of a charged cargo as done here, the particles destabilize and aggregate. Thus, as indicated by the zeta potential and DLS measurements, the cargo-loaded SNPs lose their colloidal stability when reaching the point of neutralization.

4.2.3 Optical properties of the SNPs upon addition of PEI

The optical properties of the cargo-loaded SNPs were studied by UV-Vis absorption and fluorescence spectrophotometry (Fig. 4.4) using the RhB-labelled component CD-RhB-PiBMA in SNP formation (Fig. 4.1). In the UV-Vis spectra (Fig. 4.4a), a Mie scattering component started to appear upon reaching a cargo (PEI) concentration of 2.3 μM , suggesting the presence of large aggregates. As described above, this cargo concentration coincides with the point of neutralization, leading to loss of colloidal stability.

The fluorescence emission intensity of RhB upon excitation of RhB at 560 nm of SNPs containing CD-RhB-PiBMA upon addition of PEI or FL-PEI is shown in Fig. 4.4b (fluorescence emission spectra in Fig. 4.4c,d). The addition of the cargo to the SNPs caused a continuous decrease in the RhB emission, attributed to self-quenching of RhB, until the neutralization point was reached at a concentration of 2.5 μM PEI/FL-PEI, upon which the intensity started to level off. The increased quenching of the RhB emission can in part be attributed to shrinking of the SNPs, which brings the dyes closer together, as observed as well by DLS, although the particles shrink sharply upon addition of cargo, while the quenching happens gradually. Additionally, the increasing amounts of charges upon cargo loading may increase

the polar nature of the SNP core promoting aggregation of the dye moieties of both the SNP building blocks and the cargo. Upon reaching the point of neutralization, the influx of cargo is largely inhibited, as observed as well by the zeta potential measurements, and consequently the fluorescence intensity levels off.

Fig. 4.4. a) UV/Vis absorbance spectra, b) normalized fluorescence intensities (excitation of RhB at 560 nm, emission of RhB at 585 nm) upon addition of increasing amounts of PEI (b, ○) or FL-PEI (a and b, ■), normalized fluorescence spectra after excitation at 560 nm upon addition of increasing amounts of c) FL-PEI or d) PEI of SNPs composed of CD-RhB-PiBMA (3.3 μ M CD, 1 μ M RhB), CD-PiBMA (11.7 μ M CD), and TBP-PiBMA (15 μ M TBP). The standard deviation of the normalized fluorescence intensities was on average 0.02.

In the case of FL-PEI, the quenching of RhB appeared to be more effective (Fig. 4.4b) due to the presence of increasing amounts of FL into the SNPs, which contribute to the dye aggregation and, therefore, to the extent of self-quenching of RhB, and possibly FRET (see below). These results clearly indicate that the SNPs have become responsive to cargo loading upon the use of a fluorescent dye as a label at one of the SNP components.

The particles were imaged by high-resolution scanning electron microscopy (SEM). Fig. 4.5 shows images of SNPs before and after addition of FL-PEI, at concentrations before (Fig. 4.5b) or after (Fig. 4.5c) the point of neutralization. Fig. 4.5b displays round, well defined small particles of approx. 50 nm in size, whereas in Fig. 4.5c large, amorphous aggregates are clearly visible. The original particles before adding the cargo (Fig. 4.5a) were 70 nm, comparable to the size obtained for the parent SNPs without RhB.¹³ This shows that the particles shrink in size, when cargo is added before reaching the point of neutralization, as shown before by DLS, although the size difference by SEM is less evident than by DLS. Beyond the point of neutralization, the particles destabilize and form aggregates, probably by the merging of several particles.

Fig. 4.5. SEM images of the SNPs composed of CD-RhB-PiBMA (3.3 μ M CD, 1 μ M RhB), CD-PiBMA (11.7 μ M CD), and TBP-PiBMA (15 μ M TBP) a) before and after adding b) 0.67 or c) 5.33 μ M FL-PEI.

As a control, SNPs of unlabelled CD-PiBMA were prepared and free RhB was added at the same dye concentration as for the dye-labelled SNPs shown in Fig. 4.4, after which free fluorescein with or without PEI was added. In both cases, quenching of RhB was no longer observed (Fig. 4.6a). This indicates that RhB must be incorporated inside the SNPs, by covalent attachment to one of its constituents, to be able to observe quenching due to the loading of cargo. Similarly, when free FL was added to the RhB-labelled SNPs prepared with CD-RhB-PiBMA, quenching of RhB was also not observed (Fig. 4.6). However, quenching did take place once PEI was also added, which indicates that the quenching of RhB originates from the charge neutralization induced by the cationic cargo.

Fig. 4.6. Fluorescence emission intensity (excitation 560 nm, emission 585 nm) of the controls. The difference in the x axis with respect to previous graphs is caused by the fact that here it concerns the concentration of FL, which is 3 times higher than the concentration of PEI (Table 4.1). When using free FL together with PEI, a ratio of 3 : 1 = FL : PEI is maintained. a) SNPs prepared with free RhB maintaining the same concentration of RhB in the particles as with the labelled ones, and adding free FL or free FL and PEI. b) SNPs prepared with labelled RhB and adding free FL or free FL and PEI. For comparison, the values with FL-PEI are also shown (as in Fig. 4.7b). The SNPs with labelled RhB were composed of CD-RhB-PiBMA (3.3 μM CD, 1 μM RhB), CD-PiBMA (11.7 μM CD), and TBP-PiBMA (15 μM TBP). The ones with free RhB were composed of CD-PiBMA (15 μM CD) and TBP-PiBMA (15 μM TBP).

4.2.4 FRET studies

FRET occurring between the fluorescein and rhodamine B dyes was studied by monitoring the fluorescence emission of RhB at 585 nm of SNPs upon excitation of FL at 450 nm upon addition of FL-PEI or PEI, as is shown in Fig. 4.7a (fluorescence emission spectra in Fig. 4.7c,d). When the particles were loaded with FL-PEI, an increase of the fluorescence emission of RhB was observed, indicating FRET from FL to RhB. The effect occurred until the point of neutralization at 2.5 μM, which indicates that beyond this concentration no more FL-PEI was loaded into the SNPs. Once the charges are compensated, there is no driving force to encapsulate more FL-PEI, and, consequently, the increase of the FRET effect levels off. In the case of unlabelled PEI as the cargo, no emission of RhB was observed under the same conditions, indicating that FRET is only observed when FL is attached to the cargo. Fig. 4.7b shows the fluorescence emission of the FL-PEI cargo at 520 nm when administered to the SNPs or to the buffer solution without SNPs, upon excitation of FL at 450 nm. The intensity increase upon increasing amounts of FL-PEI in the presence of RhB-labelled SNPs was slightly lower than in the absence of SNPs, which confirms FRET from FL to RhB in the cargo-loaded SNPs. As a negative control, Fig. 4.7b also shows the intensities upon loading unlabelled PEI

as a cargo to RhB-labelled SNPs. The absence of fluorescence confirms that the emission observed in the former cases is fully attributable to FL emission. Overall, the fluorescence properties of both dyes clearly indicate that FRET occurs between the dye-labelled SNP components and the dye-labelled cargo.

Fig. 4.7. Fluorescence emission intensity of the SNPs composed of CD-RhB-PiBMA (3.3 μM CD, 1 μM RhB), CD-PiBMA (11.7 μM CD) and TBP-PiBMA (15 μM TBP) after adding FL-PEI or PEI. a) Excitation of FL at 450 nm, emission of RhB at 560 nm (normalized to the maximum intensity of the emission of RhB obtained after its direct excitation; see Fig. 4.4). b) Excitation of FL at 450 nm, emission of FL at 520 nm (normalized to the fluorescence intensity of FL with free FL-PEI at 2.7 μM PEI). Normalized fluorescence spectra after excitation at 450 nm of c) the SNPs composed of CD-RhB-PiBMA (3.3 μM CD, 1 μM RhB), CD-PiBMA (11.7 μM CD), and TBP-PiBMA (15 μM TBP) upon addition of FL-PEI and d) buffer upon addition of FL-PEI; ■ SNPs to which FL-PEI was added. ○ SNPs to which PEI was added. ◇ free FL-PEI. The standard deviation of the normalized fluorescence intensities was on average 0.001 in a) and 0.02 in b).

4.3 Conclusions and outlook

In conclusion, we have developed a fluorescent multicomponent supramolecular nanoparticle system that is responsive to the loading of cargo. Equipment of one the SNP

components with a fluorescent reporter dye allowed us to study the effect on the particle behavior and stability upon the introduction of oppositely charged cargo. Particle destabilization and aggregation occurred once the neutralization point was reached. Before the neutralization point, the cargo loading caused shrinkage of the SNPs. When both the SNPs and the cargo were labelled with a dye, FRET was observed upon loading the SNPs with cargo. These results further the basic understanding of the forces underlying SNP morphology and stability when loading SNPs with an electrostatically charged cargo, such as in the case of anticancer gene delivery. Moreover, the counteracting fluorescence intensities of the SNP components and the cargo, caused by FRET, may allow the development of drug delivery vehicles and imaging agents in which the ratiometric detection^{34,35} of the dye intensities can be used to signal the SNP assembly and disassembly, as well as the release of cargo.

4.4 Acknowledgements

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4.5 Experimental

4.5.1 Materials and equipment

Chemicals and solvents were obtained from Sigma-Aldrich and used as received. PEI was purchased from Polysciences. *N,N*-Diisopropylethylamine (DIPEA) was obtained from Biosolve. CD-PiBMA and TBP-PiBMA were prepared as described before.¹³ Deuterated NMR solvents were purchased from Cambridge Isotope Laboratories Inc. 6-Monodeoxy-6-monotosyl- β -cyclodextrin, 6-monodeoxy-6-azido- β -cyclodextrin, and 6-monodeoxy-6-monoamino- β -cyclodextrin were synthesized according to literature procedures.³⁶⁻³⁸ MilliQ water with a resistivity of 18.2 M Ω cm at 25 °C was used in all experiments. Dynamic light scattering (DLS) and ζ -potential measurements were performed on a Zetasizer NanoZS (Malvern Instrument Ltd, Malvern, United Kingdom), with a laser wavelength of 633 nm and a scattering angle of 173°. UV/VIS absorption spectra were recorded using a Perkin Elmer Lambda 850 UV-VIS spectrometer. Fluorescence spectra were performed using a Perkin Elmer LS 55 fluorescence spectrophotometer equipped with a high energy pulsed Xenon

source for excitation. For the ^1H -NMR and the scanning electron microscopy (SEM) equipment characteristics see Chapter 3.

4.5.2 Synthetic procedures

Synthesis of CD-RhB-PiBMA

To a solution of PiBMA (20 mg, 3.33 μmol) in dry DMSO (2 mL) was added a solution of Lissamine RhB ethylene diamine (2.5 mg, 0.005 mmol) in dry DMSO (2.5 mL). The mixture was allowed to react at 45 °C for 1 day, after which a solution of 6-monodeoxy-6-monoamino- β -cyclodextrin (60.5 mg, 0.053 mmol) and DIPEA (81 μL , 0.107 mmol) in DMSO (1.5 mL) was added. The mixture was reacted for 2 days at 60 °C. To the crude reaction mixture, water (10 mL) was added and the unreacted anhydride rings were opened with 0.1 M NaOH_{aq} (1.3 mL). Subsequently, the mixture was purified by dialysis (SpectraPor membrane, MWCO 6-8 kD) for 1 week. Pure product grafted with 5 cyclodextrins and 1.5 RhB (CD-RhB-PiBMA) units was obtained by freeze-drying (10 mg, 42% yield). The degree of grafting of RhB was calculated with a calibration curve (Fig. 4.8a). The degree of grafting of CD was calculated from the ratio between the CH_3 protons of the polymer and the C1-H of β -CD. ^1H -NMR (400 MHz, D_2O , pH 11): δ 0.25-1.4 (br, 239 H, CH_3), 3.01-4.0 (m, 207 H, C2-6H of CD), 4.88-5.1 (br, 39.6 H, C1-H of CD), 7.25 (br, RhB), 8.5 (br, RhB).

Synthesis of FL-PEI

To a solution of PEI10000 (138 mg, 0.01 mmol) in dry DMSO (10 mL) was added a solution of fluorescein isothiocyanate (55.9 mg, 1.104 mmol) in dry DMSO (10 mL). The mixture was allowed to react at room temperature for 2 days. To the crude reaction mixture, water (10 mL) was added. Afterwards, the mixture was purified by dialysis (SpectraPor membrane, MWCO 1000 kD) for 1 week. Pure product grafted with FL (FL-PEI) was recovered by freeze-drying (50 mg, 36% yield). The degree of grafting of FL was calculated with a calibration curve (Fig. 4.8b).

Fig. 4.8. UV-Vis calibration curves of a) RhB and b) FL.

4.5.3 Methods

Preparation of the supramolecular nanoparticles

The SNPs were prepared in phosphate buffer (5 mM, pH 7.4), maintaining the host-guest ratio at 1 : 1 at 15 μM CD, from stock solutions of CD-RhB-PiBMA (6.44 μM polymer), CD-PiBMA (6.44 μM polymer), and TBP-PiBMA (4.28 μM polymer) by mixing the components with a vortex. The final composition of these SNPs was: CD-RhB-PiBMA (3.3 μM CD, 1 μM RhB), CD-PiBMA (11.7 μM CD), and TBP-PiBMA (15 μM TBP). To the prepared SNPs, FL-PEI or PEI was added from stock solutions of 10 or 100 μM PEI. At each addition of PEI or FL-PEI, the samples were allowed to equilibrate for 10 min prior to DLS, UV-VIS or fluorescence measurements.

In the case of the controls, the SNPs were prepared maintaining the same concentration of host, guest, RhB, PEI, and FL.

DLS and ζ potential measurements

To perform the DLS and ζ potential measurements, the refractive index of the material was fixed to 1.465 and the absorbance to 0.01. The parameters for the dispersant (water) were taken from the database of the machine. The temperature was set to 25 °C. The measurements were automated. PEI or FL-PEI was added to the pre-prepared SNPs from concentrated stock solutions (10 or 100 μM PEI) and allowed to equilibrate for 10 min before measuring.

UV-Vis measurements

PEI or FL-PEI was added to the SNPs from concentrated stock solutions (10 or 100 μM PEI). At each concentration, the samples were allowed to equilibrate for 10 min prior to the UV-Vis measurements.

Fluorescence measurements

PEI or FL-PEI was added to the SNPs from concentrated stock solutions (10 or 100 μM PEI). At each concentration, the samples were allowed to equilibrate for 10 min prior to the fluorescence measurements. At each concentration, the emission of RhB (685 nm) after FL excitation (450 nm), and the direct RhB excitation (560 nm) were recorded. The emission of FL (520 nm) after FL excitation (450 nm) was also recorded.

SEM measurements

See Chapter 3.

4.5.4 Calculation of the concentrations of charged groups induced by the SNP components and the cargo

The amount of charges in the SNPs was calculated based on the concentration of the different species in the SNPs, which are composed of CD-RhB-PiBMA (3.3 μM CD, 1 μM RhB), CD-PiBMA (11.7 μM CD), and TBP-PiBMA (15 μM TBP).

- 1) The concentration of carboxylate groups (COO^-), which remains always constant, was calculated by addition of the carboxylic groups of the 3 components:

CD-RhB-PiBMA: The total concentration of COO^- in the polymer CD-RhB-PiBMA is 71.5 μM . Taking into account that there are 5 CDs per polymer backbone, the concentration of CD-RhB-PiBMA is then 0.67 Mm. Calculating, the concentration of COO^- in the particles is then 48 μM

CD-PiBMA: The total concentration of COO^- in the polymer CD-PiBMA is 69 μM . Taking into account that there are 9 CDs per polymer backbone, the concentration of CD-PiBMA is then 1.3 μM . Calculating, the concentration of COO^- in the particles from this component is then 90 μM .

TBP-PiBMA: The total concentration of COO^- in the polymer TBP-PiBMA is 69 μM . Since there are 9 TBPs per polymer backbone, the concentration of TBP-PiBMA is then 1.67 μM . Thus, the concentration of COO^- in the particles coming from this component is then 115 μM .

Therefore, the concentration of COO^- in the SNPs is then 253 μM .

- 2) The concentration of PEI after its addition to the SNPs was calculated from the concentration of FL taking into consideration that there are on average 3 fluoresceins per PEI polymer backbone. The concentration of FL at each data point was calculated from the calibration curve of FL by UV-Vis (Fig. 4.8b).
- 3) The concentration of amines at each data point was calculated from the concentration of PEI taking into account the stoichiometry of the polymer and by addition of the concentrations of the primary, secondary, and tertiary amines. The PEI of MW 10 kDa has a concentration of 55 μM of primary amines, 116 μM of secondary amines, and 58 μM of tertiary amines. Therefore, the total amount of amines in PEI is: 229 μM . Taking into account the proton sponge effect,^{39,40} it was assumed that half of the total amount of amines are protonated at physiological pH (Table 4.1).

Table 4.1. Changes in the amount of charges of the SNPs upon addition of PEI.

[PEI], μM	[AMINE], μM	[FL], μM
0.0	0	0.0
0.2	23	0.5
0.3	34	1.0
0.7	80	2.0
1.3	149	4.0
1.7	195	5.0
2	229	6.0
2.3	263	7.0
2.7	309	8.0
5.3	607	16.0
10.7	1225	32.0

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Chapter 5

Loading and Release of Fluorescent Oligoarginine Peptides into pH-Responsive Anionic Supramolecular Nanoparticles

Supramolecular nanoparticles (SNPs) based on negatively charged polymeric components can act as pH-responsive systems which allow the encapsulation and release of a positively charged cargo by electrostatic interactions. Fluorescent SNPs, based on the negatively charged poly(isobutyl-alt-maleic acid) (PiBMA) and labeled with rhodamine B (RhB), were used as carriers to encapsulate positively charged oligoarginine (Arg_n) peptides grafted with a cyanine dye (Cy5). Changes in the absorbance and fluorescence properties of the cyanine dye and energy transfer (FRET) between the Cy5 and RhB dyes residing in the SNPs was used to monitor the encapsulation and release of the peptide cargo into/from the SNPs. The number of charges in Arg_n influenced the peptide uptake: Arg_2 (3 charges) was incorporated in the SNPs to a lesser extent than Arg_4 (5 charges), due to a lower affinity for the SNPs in the former case. The Arg_4 release from the SNPs occurred at a pH that mimics lysosome conditions. Finally, in vitro cell experiments revealed that the SNP encapsulation had a beneficial effect on the uptake of Arg_4 peptides by PC3 prostate cancer cells.

5.1 Introduction

Supramolecular nanoparticles (SNPs) can be fabricated from charged components, the most common ones being anionic polymers with carboxylic or sulfonamide groups.^{1,2} This makes it relatively easy to use such systems to encapsulate drugs, proteins or genetic materials, simply by electrostatics.^{3,4} The same systems can be engineered in such a way that it allows specific release of the cargo upon lowering of the pH. For instance, when nanoparticles composed of such components are introduced into cells through endocytosis, they end up in lysosomes^{5,6} where they are digested and provide the release of the cargo due to the local pH drop.⁷⁻⁹ A wide range of external stimuli have been proposed for drug delivery. These vary from physical (e.g. temperature and light) and chemical stimuli (e.g. pH) to biological stimuli (e.g. enzymatic activity).¹ Of these release mechanisms, using the pH as stimulus provides the most generic application in oncology. Such an approach can be used to realize drug delivery or the delivery of a diagnostic payload at the site of interest, while reducing the systemic side effects.

Oligoarginines (Argn) are branched, positively charged peptides that raise special interest in biomedical applications, among others e.g. as cell-penetrating peptides.^{10,11} Compared to other charged polymers such as poly(ethylene imine) (PEI), Arg_n derivatives have several advantages, *viz.* their synthetic versatility¹²⁻¹⁴ and accessibility for tagging^{15,16}/labelling.^{17,18} Recently, cyanine dye-grafted Arg_n have been used as a model to study enzymatic signal activation in combination with lifetime imaging.¹⁷ However, to the best of our knowledge, Arg_n have never been employed as model drugs to study their encapsulation and release from pH-responsive nanoparticles, nor to investigate the uptake of cargo-encapsulated supramolecular nanoparticles (SNPs) by cells.

The UV/Vis absorbance and fluorescence spectral signatures of cyanine dyes change depending on the proximity between individual molecules due to the formation of dimers,¹⁹⁻²¹ which has found diverse applications in recent years.²²⁻²⁴ In addition, they have been often used in Forster Resonance Energy Transfer (FRET) studies.²⁵⁻²⁷ However, to our knowledge, the combination of FRET together with spectral changes of cyanine dyes due to their aggregation has never been used to study the encapsulation and release of different types of species from nanoparticles.

Here, we aim to use fluorescent cyanine-containing Arg_n of two different peptide lengths as a positively charged model cargo for studying the uptake and pH-responsive release properties of anionic fluorescent SNPs in buffer and in cells. We use here multicomponent SNPs that are based on the negatively charged polymer poly(isobutyl-*alt*-maleic acid) (PiBMA), as we have described previously.^{28,29} The SNPs and the oligo-Arg cargo are grafted with the rhodamine B-cyanine dye FRET couple for visualizing their interaction. The loading and pH-dependent release of the cargo is correlated to the aggregation of the cyanine dyes inside the SNPs, which is known to cause changes in the UV-Vis and fluorescent properties of the dye, such as H-dimer formation,¹⁹⁻²¹ self-quenching and energy transfer of the FRET couple.²⁵⁻²⁷ The binding of the charged cargo to the SNPs should not be too strong to allow its release. This can be achieved by controlling the degree of charging of the cargo, that is, controlling the exact amount of Arg units, in order to have sufficient charges to allow the binding and the incorporation of the cargo, but not too many to prohibit its release into the cell. Therefore, the peptide length is varied to tune the affinity of the peptide for the SNP in order to show the effect of the Arg_n as a tag for the uptake and release of peptides into/from SNPs.

5.2 Results and discussion

5.2.1 Peptide uptake and release into/from supramolecular nanoparticles in solution

The SNP system is composed of three different components and can be loaded with Arg_n cargo, as schematically depicted in Fig. 5.1a. These negatively charged SNPs are formed by host-guest interactions between the β -cyclodextrin (CD) and *p*-*tert*-butylphenyl (TBP) groups that are bound to the PiBMA polymer. The size and stability are governed by balancing attractive supramolecular and repulsive attractive interactions, as described in Chapter 3.²⁸ To study the influence of the number of charges in the system, two different lengths of peptide were used: Arg₂ and Arg₄. In addition, both the dye-modified and unmodified Arg_n were studied to fully characterize the SNPs. The chemical structures of the individual components are shown in Fig. 5.1b.

Fig. 5.1. a) Loading and unloading of unmodified or dye-modified Arg_n ($n = 2, 4$) into fluorescently labeled SNPs (here shown for Cy5- Arg_4). b) Components of the SNP system: host polymer grafted solely with CD (CD-PiBMA), host polymer grafted with CD and lissamine rhodamine B (CD-RhB-PiBMA), guest polymer grafted with *p*-tert-butylphenyl groups (TBP-PiBMA), and the cargo oligoarginines with/without Cy5 (Arg_4 , Cy5- Arg_4 , Arg_2 , Cy5- Arg_2).

The peptides are loaded into the SNPs by electrostatic interactions, similar to loading of dye-modified PEI, as described in Chapter 4.²⁹ Here, we use a short Arg_n , with a comparatively low number of charges (five or less), that can possibly function as a tag for larger, therapeutic peptides. The length of the Arg_n provides control over the charge of the cargo and thus over

the affinity for the anionic SNPs. The carboxylate groups of the SNP components, on the other hand, render the SNPs pH-responsive, because their protonation will decrease the charge of the SNPs and thus also the affinity of the cargo. The host polymer CD-PiBMA is grafted with rhodamine B (RhB) and the cargo Arg₄/Arg₂ with Cy5, which dyes serve as a tool to study in detail the assembly formation and the loading and unloading of the cargo into the assemblies (Fig. 5.1b), also because these dyes form a FRET couple.^{25,26}

The host and the guest polymers, cyclodextrin-grafted poly(isobutyl-*alt*-maleic acid) (CD-PiBMA), *p*-*tert*-butylphenyl-grafted PiBMA (TBP-PiBMA), and cyclodextrin and rhodamin-grafted-poly(isobutyl-*alt*-maleic acid) (CD-RhB-PiBMA) were synthesized as described in Chapters 3 and 4,^{28,29} and contained, on average, 9 CD units, 9 TBP, and 5 CD plus 1.5 RhB units per polymer backbone, respectively.

SNPs were prepared as described in Chapter 4.29 by mixing the SNP building blocks in phosphate buffered saline (0.01 M, pH 7.4). The self-assembled particles were colloidally stable by a balance between electrostatic repulsive (polymer backbones) and supramolecular attractive (host and guest groups grafted in the polymers) forces.^{28,29} Peptide cargo (Arg₄, Cy5-Arg₄, Arg₂, or Cy5-Arg₂) was introduced into the SNPs by attractive electrostatic forces between the positively charged peptide, which have 5 or 3 charges per molecule in the case of Arg₄/Cy5-Arg₄ or Arg₂/ Cy5-Arg₂, respectively, and the negatively charged polymer backbones of the components of the SNPs. In order to study the influence of the loading of the cargo into the SNPs, we employed dynamic light scattering (DLS) to measure the hydrodynamic diameter (d_h) and the zeta potential (ζ) to measure the charge. With these techniques, we had to use the unlabeled Arg₄ and Arg₂ instead of Cy5-Arg₄ or Cy5-Arg₂, because of interference of the Cy5 dye with the laser light employed here.

The change in the hydrodynamic diameter of the negatively charged particles upon addition of the positively charged Arg₄ is shown in Fig. 5.2a. The addition of the peptide into the SNPs initially caused their shrinking to almost a quarter of their original size until the equilibration point at around 50 μ M, from which the SNPs started to grow considerably due to the destabilization of the forces involved in the particle formation. A theoretical neutralization point of 50 μ M can also be estimated from the addition of peptide into the SNPs by

comparing the number of positive (peptide) and negative (SNPs) charges in the system (*vide infra*), and is consistent with the experimental aggregation point.

Fig. 5.2. a) Hydrodynamic diameters from DLS and b) ζ potential data of the SNPs composed of CD-RhB-PIBMA (3.3 μM CD, 1 μM RhB), CD-PIBMA (11.7 μM CD), and TBP-PIBMA (15 μM TBP) upon addition of increasing amounts of Arg4.

From ζ potential measurements (Fig. 5.2b), a similar trend is observed: the particles maintained the original negative value in the beginning until a concentration of around 20 μM , from which they started to slowly become less negatively charged. However, in this case a real point of neutrality was never achieved, in contrast to what has been observed before for the loading of these SNPs with PEI grafted with fluorescein (FL),²⁹ most probably because the affinity of the SNPs for the oligomeric Arg4 cargo is lower than for the polymeric PEI-FL cargo, and the affinity is decreasing during the loading process, because the overall negative charge inside the SNPs is decreased by the cargo that has been incorporated before.

SEM was used to image the SNPs. Fig. 5.3a shows the original particles without any loaded cargo, with a size of 79 ± 11 nm.^{28,29} After addition of Arg4 (8 μM ; Fig. 5.3b), before the calculated neutralization point, the well-defined round particles shrank to a size of 37 ± 6 nm. After addition of 76 μM of peptide (beyond the calculated neutralization point; Fig. 5.3c), aggregates are clearly visible. These images show the same trend of shrinking followed by aggregate formation upon addition of the peptide, as observed with DLS. The aggregation of the particles upon addition of more cargo is most probably caused by the merging of several particles.²⁹

Fig. 5.3. SEM images of the SNPs composed of CD-RhB-PiBMA (3.3 μM CD, 1 μM RhB), CD-PiBMA (11.7 μM CD), and TBP-PiBMA (15 μM TBP) before a) or after adding b) 8 or c) 76 μM Arg₄.

The different absorption and fluorescence processes which take place inside the SNPs are summarized in Fig. 5.4. These processes are used to analyze the photophysical properties of these particles and the release of the cargo. In the UV absorbance and fluorescence experiments in phosphate buffered saline (0.01 M, pH 7.4) described below, the concentrations of the host and guest groups of the SNPs were 21 times lower compared to those in the experiments (DLS, ζ , SEM) described above. Therefore, the calculated neutralization point is 3 μM of Arg_n in the UV and fluorescence experiments.

Fig. 5.4. Absorption, fluorescence, and FRET processes occurring in the SNP-peptide system.

The incorporation of the peptide Cy5-Arg₄ into the SNPs was first studied with UV-Vis spectroscopy (Fig. 5.5b). Clearly, the intensity of the shoulder at 600 nm is steadily increasing starting from a concentration of 0.5 μM Cy5 and it becomes a second peak, which together

with the shifting of the mean peak at 655 nm to longer wavelengths signifies that, from this concentration onward, the dye aggregates and forms dimeric species.^{16,17,24-27,29} Still, the shift is relatively small which indicates steric hindrance of the dye in the SNPs that prevents larger shifts. In the presence of RhB the Cy5 absorption exhibits the same behavior (Fig. 5.5c). However, the ratio of the intensity at 655 vs 600 nm with the concentration (Fig. 5.5d) is not changing, which indicates that the ratio is independent of the concentration. Conversely, in solution at the same concentrations, no dimeric species were formed (Fig. 5.5a). In fact, dimers of Cy5 in solution do not form below a concentration of 3.5 mM.^{21,30} Yet, in the presence of SNPs, dimerization is already evident at a concentration that is 4 orders of magnitude lower, which effect we attribute to the strongly enhanced local concentration of the dye upon immobilization inside the SNPs. Therefore, this dimerization is a clear proof of the confinement and effective encapsulation of the peptide inside the SNPs.

Fig. 5.5. UV/Vis absorbance spectra of increasing concentrations of a) free Cy5-Arg₄ and SNPs without b)/with RhB c) to which Cy5-Arg₄ was added. d) Ratio of the absorbance at 655/600 nm with the concentration. The SNPs were composed of b) CD-PiBMA (0.7 μ M CD) and TBP-PiBMA (0.7 μ M TBP), or c) CD-RhB-PiBMA (0.16 μ M CD, 0.05 μ M RhB), CD-PiBMA (0.54 μ M CD), and TBP-PiBMA (0.7 μ M TBP).

Fig. 5.6a shows the fluorescence emission of RhB after RhB excitation upon adding increasing amounts of Cy5-Arg₄ or Arg₄ to the SNPs. In the presence of Cy5, full quenching of RhB took place until the calculated neutralization point (3 μ M). On the other hand, when the unfunctionalized peptide Arg₄ was added to the SNPs, no quenching was observed. This indicates that FRET occurs; Cy5 and RhB are a FRET couple, where RhB is the donor and Cy5 the acceptor.^{21,22} However, no increase in the emission of Cy5 upon RhB (Fig. 5.6b) or Cy5 excitation (Fig. 5.6c) was observed after adding increasing amounts of Cy5-Arg₄ into the SNPs until the calculated neutralization point. This is explained by the presence of optically silent stacked/dimeric species of Cy5 in the SNPs, which causes quenching of the fluorescence emission.²⁰ At high concentrations of Cy5-Arg₄, the fluorescence does increase, indicating that additional peptide is no longer included into the SNPs (Fig. 5.6b,c).

Fig. 5.6. Fluorescence emission of a) RhB (585 nm) of SNPs or of b), c) Cy5 (660 nm) of free Cy5-Arg₄ and of SNPs containing either Cy5-Arg₄ or Arg₄ after excitation at 550 nm (RhB; a, b) or at 640 nm (Cy5; c). The standard deviation in all cases was on average 0.01. The SNPs were composed of CD-RhB-PiBMA (0.16 μ M CD, 0.05 μ M RhB), CD-PiBMA (0.54 μ M CD), and TBP-PiBMA (0.7 μ M TBP).

In case of using a peptide with less charges (Cy5-Arg₂ with 3 charges instead of Cy5-Arg₄ with 5), DLS data indicate neither the shrinkage of the particles before nor their aggregation after the calculated neutralization point (Fig. 5.7a). Interestingly, in the proximity of the calculated neutralization point, no size change is obvious. Yet, the dimerization of the dye due to confinement inside the SNPs remains (Fig. 5.7b) indicating that the dye interactions are only dictated in part by the charge on the peptide. Moreover, quenching of RhB is observed (Fig. 5.7c). Finally, the Cy5 dye can be directly excited (Fig. 5.7d), in contrast to the case of using Cy5-Arg₄; see also the corresponding fluorescence spectrum of the direct excitation of Cy5 (Fig. 5.6c).

Fig. 5.7. a) Hydrodynamic diameters from DLS, b) UV/Vis spectra, and c) and d) normalized fluorescence intensities of the SNPs to which Cy5-Arg₂ was added. Fluorescence intensities were measured c) at an emission wavelength of 585 nm (RhB) upon excitation at 550 nm (RhB) or d) at an emission wavelength of 660 nm (Cy5) upon excitation at 640 nm (Cy5). The SNPs were composed of a) CD-RhB-PiBMA (3.3 μ M CD, 1 μ M RhB), CD-PiBMA (11.7 μ M CD), and TBP-PiBMA (15 μ M TBP). In b, c, and d the concentration of all species was 20 times more dilute, while d also shows results for SNPs without RhB.

These results indicate that in this case, the inclusion of Cy5-Arg₂ inside the SNPs is less pronounced than when using Cy5-Arg₄, most probably due to an equilibration of peptide inside and outside the SNPs. Cy5-Arg₂ has half the number of charges than Cy5-Arg₄, and apparently, the affinity of the peptide for the SNPs is much weaker than in the case of Cy5-Arg₄. Consequently, an equilibrium occurs between bound and unbound peptide, most likely in fast exchange. For drug delivery applications, full encapsulation of the peptide inside the SNPs is desired, to avoid premature release of the cargo. These results demonstrate that 3 charges per peptide are not enough to have Arg_n fully incorporated into these SNPs. In contrast, the full RhB quenching observed in the case of Cy5-Arg₄ indicates that this peptide does have a sufficiently strong affinity to load practically fully at low loading concentrations.

Fig. 5.8. Release of Cy5-Arg₄ from SNPs upon lowering the pH: changes in the UV-Vis spectra of the SNPs a) lowering the pH from 7.3 to 3.3 after addition of 0.1 M HCl and equilibration for 15 min after each addition, over the total course of 2 h. b) Similar experiments while maintaining neutral pH, over time. c) Decrease of the absorbance of the peak at 600 nm as a function of pH 15 min after addition of HCl. The SNPs were composed of CD-RhB-PiBMA (0.16 μ M CD, 0.05 μ M RhB), CD-PiBMA (0.54 μ M CD), and TBP-PiBMA (0.7 μ M TBP).

Changes in the UV-Vis absorbance spectra of the SNPs were recorded upon lowering the pH from neutral to a pH of 3.3 by the stepwise addition of 0.1 M HCl followed by an equilibration time of 15 min after each addition over the course of in total 2.5 h. The spectra (Fig. 5.8a) show the decrease of the absorbance peak at 600 nm relative to the situation at neutral pH during 2 h (Fig. 5.8b). The effect upon acidification becomes clearer when the absorbance of the peak at 600 nm is plotted against pH (Fig. 5.8c): the intensity decreases with the pH from a value of 0.185 at pH 7.3 to 0.125 at pH 3.3. The data indicate that the degree of dimerization of Cy5 is lowered upon acidification suggesting release of the peptide from the SNPs. Apparently, the carboxylic acid groups of the host and guest polymers (CD-PiBMA, CD-RhB-PiBMA, and TBP-PiBMA) get protonated upon lowering the pH of the medium.^{1,11} This affects the encapsulated cationic material inside the SNPs, provoking its release, because the electrostatic forces which held the cargo inside the SNPs are weakened upon lowering the pH. Studying the release events in more detail (with more data points in time; data not shown) indicates that release is prolonged for about 2 h upon every pH change.

5.2.2 Cell studies

Preliminary cell studies with prostate cancer cells (PC3) were performed to investigate the cellular particle uptake and release of the peptides from the SNPs. Confocal images are provided for cells incubated with Cy5-Arg₄ alone (Fig. 5.9a,b) or with SNPs that were preloaded with Cy5-Arg₄ (Fig. 5.9c,d). Free Cy5-Arg₄ can be visualized in the medium and was initially accumulated in the cell membrane. This was followed by diffusion throughout the cytosol. Since this molecule is charged, it ends up most probably in the lysosomes (charge-mediated uptake) as followed from staining of the PC3 cells with Mito green and Hoechst. In contrast, The SNPs were optically silent in the incubation medium as a result of dye-dye quenching in the particles (see above). However, uptake of the SNPs did occur, most likely through endocytosis, resulting in local fluorescent foci in the cytosol.^{6,31} The same foci contained a fluorescent emission in the spectral region of RhB, indicating this is where the SNPs reside.

Fig. 5.9. Interaction and localization of Cy5-Arg₄ in PC3 prostate cancer cells after replacing the cell culture medium and incubating the cells in PBS a, b) with Cy5-Arg₄ alone or c, d) with SNPs to which Cy5-Arg₄ was added. Images taken shortly after incubation indicate Cy5 fluorescence (a,c; excitation at 633 nm and emission at 650-700 nm), RhB fluorescence (b,d; excitation at 514 nm and emission at 570-600 nm). The SNPs were composed of CD-RhB-PiBMA (0.16 μ M CD, 0.05 μ M RhB), CD-PiBMA (0.54 μ M CD), and TBP-PiBMA (0.7 μ M TBP). The concentration of Cy5-Arg₄ was 19 μ M.

These confocal experiments, in combination with the above mentioned, pH-dependent release profiles, suggest that the pH drop that occurs in lysosomes causes the release of encapsulated Cy5-Arg₄ within the cell. After this pH-mediated release, Cy5-Arg₄ can again diffuse through the cell, in a manner similar to that observed when cells were incubated with Cy5-Arg₄ alone. Upon release from the SNPs the Arg₄ recover their cell-penetrating properties and allow uptake in the cytosol. From these preliminary results, we may conclude that the SNPs mediate a pH-dependent delivery of the oligo-arginine peptides to cells. One could reason that the dye could easily be replaced by an otherwise non-specific cytotoxic drug such as e.g. doxorubicin, camptothecin, and cisplatin. It is obvious that the therapeutic label and its size and chemical properties should not interfere with the Arg-sequence-based particle internalization process. However, although not specifically investigated, but given

the nature and size of the SNPs, it is not likely that larger structures, such as proteins, can be easily included, but small therapeutic peptides should be possible.

5.3 Conclusions and outlook

In conclusion, we have developed a pH-sensitive system based on negatively charged fluorescent SNPs which encapsulate positively charged fluorescent oligoarginine peptides containing cyanine dyes. The presence of a FRET couple in the cargo-loaded SNPs showed energy transfer from donor to acceptor, which is useful in biomedical applications as a model sensor to study the encapsulation and release of charged cargo from SNPs. Furthermore, lowering the pH caused the release of the encapsulated Arg_n. The Arg₄ release from these SNPs occurred in a biomedically relevant pH range which mimics lysosome conditions. The *in vitro* cell experiments indicate a clear effect of SNP encapsulation on the uptake of the peptides and combined with the pH effect, these indicate release of the peptide from lysozymes upon uptake of peptide-loaded SNPs. These results hold promise that both the uptake and release of Arg_n into and from SNPs may work also *in vivo*.

5.4 Acknowledgements

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5.5 Experimental

5.5.1 Materials and equipment

Chemicals and solvents were obtained from Sigma-Aldrich and used as received. CD-PiBMA, CD-RhB-PiBMA, and TBP-PiBMA were prepared as described in Chapters 3 and 4.^{28,29} Cy5-Arg₄, Arg₄, Cy5-Arg₂, and Arg₂ were synthesized by M. T. M. Rood at LUMC (Leiden).¹⁷ Phosphate buffered saline (0.01 M, pH 7.4 at 25 °C, 0.138 M NaCl, 0.0027 M KCl) was prepared from Sigma Aldrich powder. MilliQ water with a resistivity of 18.2 MΩ cm at 25 °C was used in all experiments. For the equipment details, see Chapters 3 and 4.

5.5.2 Methods

Preparation of the supramolecular nanoparticles at pH 7.4 for DLS, ζ potential, SEM, and cell studies

See Chapter 4.

Preparation of the supramolecular nanoparticles at pH 7.4 for fluorescence and UV-Vis experiments

The SNPs were prepared in phosphate buffer (1 mM, pH 7.4), maintaining the host-guest ratio at 1 : 1 at 0.7 μ M CD, from stock solutions of CD-RhB-PiBMA (0.64 μ M polymer), CD-PiBMA (0.64 μ M polymer), and TBP-PiBMA (0.43 μ M polymer) by mixing the components with a vortex. The final composition of these SNPs was: CD-RhB-PiBMA (0.16 μ M CD, 0.05 μ M RhB), CD-PiBMA (0.54 μ M CD), and TBP-PiBMA (0.7 μ M TBP). To the prepared SNPs, Cy5-Arg₄ or Arg₄ was added from stock solutions of 90 μ M Cy5-Arg₄/Arg₄. At each addition of peptide, the samples were allowed to equilibrate for 10 min prior to the measurements. For the fluorescent measurements, at each concentration, the emission of Cy5 (660 nm) after RhB excitation (550 nm), and the direct Cy5 excitation (640 nm) were recorded. The emission of RhB (585 nm) after RhB excitation (550 nm) was also recorded.

Preparation of the supramolecular nanoparticles for pH driven release

The SNPs were prepared in PBS, maintaining the host-guest ratio at 1 : 1 at 15 μ M CD, from stock solutions of CD-RhB-PiBMA (6.44 μ M polymer), CD-PiBMA (6.44 μ M polymer), and TBP-PiBMA (4.28 μ M polymer) by mixing the components with a vortex. The final composition of these SNPs was: CD-RhB-PiBMA (3.3 μ M CD, 1 μ M RhB), CD-PiBMA (11.7 μ M CD), and TBP-PiBMA (15 μ M TBP). To these preformed SNPs, Cy5-Arg₄ from a stock solution (42 μ L, 100 μ M Cy5-Arg₄) was added to achieve a final concentration of peptide of 8 μ M.

To slowly change the pH of the medium, aliquots of HCl (5 μ L, 0.1 M) were added to the SNPs. The pH was controlled with pH paper. The pH was also measured in parallel with a pH-meter in blank samples containing only PBS and HCl. After each addition, the samples were measured by UV-Vis 0 min and 15 min after addition of HCl. UV-Vis was measured of SNPs prepared in PBS without changing the pH at the same time-frames.

5.5.3 Calculation of the number of charges in the system

The number of charges in the SNPs was calculated based on the concentration of the different species in the SNPs, in the same way as in Chapter 4. The concentration of negative charges, coming from the carboxylic acid groups, in the case of DLS and ζ potential experiments is 253 μM . In the case of fluorescence and UV-Vis the concentration of negative charges is 12 μM .

The number of positive charges is coming from the peptide. The concentration of Cy5-Arg4 after its addition to the SNPs was calculated from the concentration of Cy5 taking into consideration that there is on average 1 Cy5 per peptide. The concentration of Cy5 at each data point was calculated from the calibration curve of Cy5 by UV-Vis (see Fig. 5.10). The concentration of Arg₄ was calculated by weighing. The results are summarized in Table 5.1.

Fig. 5.10. Calibration curve Cy5.

Table 5.1. Number of charges in SNPs.

[peptide]=[dye], μM [CD]=[TBP]= 15 μM	[+], μM	[peptide]=[dye], μM [CD]=[TBP]= 0.7 μM	[+], μM
0	0	0	0
1	5	0.6	3
4	20	1.2	6
8	40	2.4	12
16	80	3.6	18
24	120	4.8	24
32	160	6.0	30
48	240	7.2	36
64	320	8.4	42
		9.6	48
		10.8	54

5.5.4 Cell experiments

Live cell images using the PC3 prostate cancer cells were taken on a Leica SP8 confocal microscope under 63x magnification at LUMC (Leiden). Rhodamine B fluorescence was measured using 514 nm excitation and emission was collected at 570-600 nm. Cy5 fluorescence was measured using excitation at 633 nm and emission was collected at 650-700 nm. Cells were thoroughly washed with PBS before taking images. In the case of release experiments of Cy5-Arg₄ in cell, the images were taken two minutes apart from each other. The value of the fluorescent intensity emission was obtained from the cell images measuring mean gray values using the Leica software.

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Chapter 6

Versatile, Fast, and Easy One-Step Method for the Synthesis of Hydrophilic Lanthanide-Doped Nanoparticles

Lanthanide-doped nanoparticles have interesting optical and magnetic properties which make them ideal for bioimaging applications. For the development of photoluminescent probes, obtaining water-soluble particles in a simple and rapid way with a high photoluminescence efficiency is a major challenge. In this chapter, the preparation of hydrophilic lanthanide-doped nanoparticles (NPs) following a versatile one-step colloidal method at low temperature and short reaction time with different doped lanthanides, capping ligands, and fluoride sources is presented. The photoluminescence of the particles was easily tuned from blue or green down-converting (DC) to up-converting (UC) by simply changing the lanthanides used. When using the same core type of particles, the size and the photoluminescence intensity were tuned by changing the fluoride source or the capping ligand used: the size of the DCNPs varied from 9 nm (PAA) to 24 nm (PEI) when changing the capping ligand, while if PAA was used as capping ligand, the nanoparticles presented the most intense emission bands. Supramolecular host groups were introduced on the surface of the UCNPs by grafting cyclodextrin (CD) groups onto the capping ligand PiBMA prior to particle synthesis, reaching a CD surface density of 0.28 CD nm^{-2} . In this case, a small particle size (11.7 nm) and strong UC emission bands were observed.

6.1 Introduction

Lanthanide (Ln^{3+})-doped nanoparticles (NPs) have received much attention in recent years due to their special characteristics such as thermal stability, chemical durability, large Stokes shift, sharp emission peaks, low photobleaching, long luminescence lifetimes, weak autofluorescence, and excitation outside of the biological window.¹⁻⁷ These special characteristics make them ideal for several applications like components in LEDs, monitors, lasers, and bioimaging applications.^{3,6,8-10} The luminescence characteristics of the Ln^{3+} ions arise from their electronic structure: a) the electron transitions between the partially filled $4f$ orbitals of the Ln^{3+} are Laporte forbidden (parity forbidden transitions), b) the J levels of Ln^{3+} are split by ligand-field effects into extra sub-levels (Stark levels). Both characteristics make that most of the Ln^{3+} are luminescent providing f - f emission lines.^{6,7} If combinations of different lanthanide ions are used within the same NP, energy transfer can occur between these ions upon excitation of the absorbing ion, which results in nanoparticle systems that can be excited in the UV/blue region while emitting in the visible range (down-converting, DC) and others that are excited in the near-infrared also emitting in the visible (up-converting, UC). In the UCNP systems, one Ln^{3+} works as the sensitizer, absorbing the excitation photons and transferring them to the other Ln^{3+} , the emitter. In DCNPs, a single Ln^{3+} can give absorption and emission at longer wavelengths, or the energy can be transferred to another Ln^{3+} which gives emission at its characteristic wavelengths, requiring the doping with one or two lanthanides, respectively.

DC and UC nanoparticles (DCNPs and UCNPs) contain lanthanides as dopants in a solid matrix (host), and require capping ligands on the surface to stabilize the core.¹¹ These capping ligands are coordinated to the matrix cations on the surface via their carboxylic acid (COOH) or amino (NH_2) groups.¹² It is crucial to choose an adequate host material to obtain NPs with the desired optical properties. Generally speaking, the host material should have lattice parameters that match the dopant ions, and at the same time have low phonon energies.¹¹⁻¹⁵ This last characteristic is important to reduce non-radiative losses and increase the radiative emission, thus leading to high quantum yields.^{13,14} Taking this into consideration, halides (chlorides, bromides, iodides) have low phonon energies (less than 300 cm^{-1}) but they are very hygroscopic, so they are hardly used. Oxides are chemically stable, but they possess

a high phonon energy (usually more than 500 cm^{-1}). Fluorides, on the other hand, are also chemically stable and have low phonon energies ($\approx 350\text{ cm}^{-1}$) as well, so they are considered the best host materials for Ln^{3+} -doped NPs.¹²⁻¹⁴ Ln^{3+} and alkaline earth cations (Ca^{2+} , Sr^{2+} , and Ba^{2+}) have similar ionic sizes, as well as yttrium (Y^{3+}) which is often encompassed in the Ln^{3+} series because of its high chemical similarity to Ln^{3+} . Thus, inorganic fluorides, oxides, or halides of Y^{3+} , Ln^{3+} or alkaline earth cations are often used as host precursors.¹³ The most widely used matrix for these particles is MYF_4 , although other stoichiometries like MY_2F_7 , MY_3F_{10} ($\text{M} = \text{Li}, \text{Na}, \text{K}$) or MYF_5 ($\text{M} = \text{Ca}, \text{Sr}, \text{Ba}$) are also often used.¹⁴

DCNPs and UCNPs are prepared by many different synthetic procedures, such as coprecipitation, thermal decomposition, sol-gel, colloidal, and solvothermal methods.^{1,14-16} The widely employed solvothermal methods are performed in a sealed environment under high pressure and temperature above the boiling point of the solvent.^{1,15-17} Therefore, when using organic solvents with high boiling temperatures, like oleic acid and 1-octadecene, high reaction temperatures can be achieved. When using the lower-boiling water as the solvent, the synthesis is performed at a lower temperature, and the process is termed “hydrothermal”.^{13-16,18} The solvothermal technique is very versatile and allows the preparation of particles with different characteristics by simply changing the synthesis parameters (temperature, pressure, reaction time).¹⁷ It offers several advantages as for example excellent control over particle size and shape, high yields, the use of cheap raw materials, and a post-treatment which does not require heat.¹³ Solvothermal methods also present several disadvantages such as: a) the use of high pressure and high temperature which does not allow the introduction *in situ* of several functionalities which will decompose under those conditions, b) long reaction times, c) formation of carbon-based side products which complicates the purification process.¹⁹ The hydrothermal method solves some of these issues, and can be seen as a green method as no toxic waste products are generated. The coprecipitation method on the other hand, offers milder conditions and the simplest experimental setup together with short reaction times for the synthesis of this type of NPs.^{12-14,16} However, a post-heat treatment (annealing) at high temperature is often required.^{12-14,16} The colloidal route (a variation of the coprecipitation method),¹⁹ performed at low ($< 100\text{ }^\circ\text{C}$) temperature, offers the mildest conditions, but this method has so far not been performed

in water. An inconvenience of using apolar solvents in preparing lanthanide-doped NPs is the hydrophobicity of the particles obtained, which results from the presence of hydrophobic capping ligands, often oleic acid, required for solubility in the reaction medium.¹⁶ Yet, the morphology and the size of these nanoparticles can be tuned by choosing different capping ligands or fluoride sources.^{13,18-22} Biomedical applications of these NPs therefore require the transfer of these NP into aqueous media. This can be achieved by several methods, for example: a) ligand exchange to replace the initial coating,^{1,13} b) surface silanization, upon which an amorphous silica shell is grown by hydrolysis and condensation via a sol-gel process of siloxane monomers around the nanocrystal core,¹³ c) using an amphiphilic polymer to form a polymer coating via hydrophobic-hydrophobic interactions with the capped ligand,^{15,23,24} d) chemical ligand oxidation, by direct oxidation of the oleic acid ligand by a strong oxidizing agent into azelaic acid, which provides free carboxylic acid groups on the surface of the NPs,²⁵ and e) supramolecular chemistry forming host-guest interactions.²⁶⁻²⁸ However, such a process can be time consuming, low-yielding, and often the water-solubilized particles show a lower luminescence efficiency in water than directly after synthesis.¹⁴ In contrast, the hydrothermal method allows the direct preparation of hydrophilic Ln^{3+} -doped NPs in a one-step fashion by choosing water-soluble capping ligands, such as poly(ethylene glycol) (PEG), poly(vinyl pyrrolidone) (PVP), poly(ethylene imine) (PEI), or ethylenediaminetetraacetic acid (EDTA).^{11,15} Yet, due to the relatively low reaction temperature, both variations of the core component and the ligand are normally not straightforward. Even when the reaction temperature can be regarded as relatively low in the hydrothermal process, it still commonly amounts to 200 °C, and the reaction time is at least 24 h. Therefore, although some of these polymers (PEI) can be further modified with reactive groups or molecules to be included on the surface of the NPs, this cannot be done during the particle synthesis process, and has to be done after particle preparation in a separate step.

Here, we aim to synthesize water-soluble, Ln^{3+} -doped NPs, both up-converting and down-converting (UCNPs and DCNPs), using various Ln^{3+} combinations to obtain different photoluminescence characteristics. Two different matrices are used, KY_3F_{10} and Sr_2GdF_7 , in an easy, fast, and versatile manner following a one-step simple colloidal procedure at low temperature, much lower than used normally in hydrothermal processes, employing three

different fluoride sources (KF, NaBF₄, and NaF) and with different hydrophilic capping ligands (see Fig. 6.1). We choose these matrices because fluoride nanomaterials containing alkaline earth metals lead to minimal crystal defects and lattice stress so they are the most convenient host materials.^{12,14} The presence of Gd allows the preparation of nanocrystals with a smaller size,²⁹ and these can automatically host several different Ln³⁺ dopant ions.³⁰ The ligands used are the small molecules adipic acid (AA) and L-cysteine (L-Cys), and the polymers poly(acrylic acid) (PAA), poly(isobutyl-*alt*-maleic acid) (PiBMA), and poly(ethylene imine) (PEI) (see Fig. 6.1b). AA, L-Cys, PAA, and PiBMA form a hydrophilic stabilizing capping layer around the NPs by coordination of the carboxylic acid groups (COOH) with the metal ions at the surface of the NPs, while PEI coordinates via the amino groups (NH₂).

Fig. 6.1. Synthesis a) of water-soluble DCNPs and UCNPs with different capping ligands. b) Chemical structure of the ligands used: PEI, AA, PAA, L-Cys, poly(isobutyl-*alt*-maleic acid) grafted with dodecyl chains and CDs (CD-PiBMA-DOD).

We address here the influence of the type of ligand and fluoride source on the particle size and the photoluminescence characteristics of the particles. Moreover, since PiBMA can be easily functionalized with extra molecules such as host or guest supramolecular groups (see Chapters 3, 4, and 5), and the reaction conditions are relatively mild, we present the direct preparation of UCNPs surface-grafted with β -cyclodextrins (CDs) and investigate the amount of β -CDs per particle. These NPs are characterized by X-ray diffraction (XRD), photoluminescence (PL), transmission electron microscopy (TEM), thermogravimetric analysis (TGA), and ζ potential measurements.

6.2 Results and discussion

DCNPs and UCNPs were synthesized following a novel colloidal procedure in water involving only one step in a relatively short time (2 h) and at low temperature (70 or 80 °C) using different capping ligands which can coordinate via NH_2 or COOH groups with the metal centers on the surface of the nanoparticles. These ligands have additional hydrophilic end groups or moieties which confer water-solubility to the nanoparticles. The method allows the use of different fluoride sources (KF , NaF , NaBF_4), lanthanides (cerium/terbium, $\text{Ce}^{3+}/\text{Tb}^{3+}$; cerium/dysprosium, $\text{Ce}^{3+}/\text{Dy}^{3+}$; ytterbium/thulium, $\text{Yb}^{3+}/\text{Tm}^{3+}$) and ligands (PEI, AA, PAA, PiBMA, L-Cys). By this versatility in the choice of components and ligands, it is possible to tune the fluorescent emission and the outer groups on the surface of the nanoparticles. This also allows the easy introduction of host or guest groups.

6.2.1 Synthesis and characterization of down-converting nanoparticles bearing different ligands

Green DCNPs with KY_3F_{10} as the matrix, using KF as the fluoride source, $\text{Ce}^{3+}/\text{Tb}^{3+}$ (15/5 mol%) as the lanthanide couple and PEI, AA, or PAA as capping ligands were prepared in ethanol/water ($\text{EtOH}/\text{H}_2\text{O}$) at 70 °C. First, the $\text{Ln}^{3+}(\text{NO}_3)_3$ salts were dissolved in distilled water, to which a hot mixture of KF and the ligand in ethanol/water was added. After just 2 hours, the NPs were isolated from the reaction mixture by centrifugation. The powder X-ray diffraction (XRD) patterns of the crystals and their luminescence (PL) spectra upon excitation at 290 nm are shown in Fig. 6.2. The powder XRD patterns of the particles for the three

different ligands show a pure phase corresponding to a tetragonal crystalline system (PDF card 00-53-0775, see Experimental, Fig. 6.11).

From these powder XRD patterns, the average crystallite size of the NPs was calculated using the Debye-Scherrer equation. Small crystallites with sizes of around 10 nm were obtained with AA (Fig. 6.2c) and PAA (Fig. 6.2e) as ligand, and 24 nm when using PEI (Fig. 6.2a). This difference in crystallite size is visible from the powder XRD patterns as the peaks using PEI are narrower than for PAA or AA. The large crystallite size for PEI can be attributed to the smaller bond strength of amino (PEI) groups interacting with the metal centers on the surface of the nanoparticles compared to the carboxylate groups in AA and PAA. With a weaker binding ligand such as PEI, the coordination with the metal sites can be reversible, and therefore further growth is possible resulting in larger crystals.³¹⁻³³

Fig. 6.2. Powder XRD (a, c, e) and luminescence (b, d, f; $\lambda_{\text{ex}} = 290 \text{ nm}$, $[\text{NP}] = 0.5 \text{ mg mL}^{-1}$) spectra of $\text{Ce}^{3+}(15\%)/\text{Tb}^{3+}(5\%)$ -doped KY_3F_{10} nanoparticles capped with PEI (a, b), AA (c, d), or PAA (e, f).

The luminescence experiments of all the DCNPs samples were done at comparable conditions and concentrations. The luminescence spectra after excitation at 290 nm are shown in Fig. 6.2b (PEI), d (AA), and f (PAA). In all three cases, the four characteristic emission bands at 488 nm ($^5D_4 \rightarrow ^7F_6$), 544 nm ($^5D_4 \rightarrow ^7F_5$), 584 nm ($^5D_4 \rightarrow ^7F_4$), and 620 nm ($^5D_4 \rightarrow ^7F_3$) were observed (see the Jablonski diagram in Fig. 6.3).³⁴ The down-conversion process is initiated by the Ce^{3+} sensitizer ions which absorb the photons at 290 nm. Thereafter, energy transfer occurs from Ce^{3+} to Tb^{3+} , followed by non-radiative relaxation and radiative downward transitions to lower energy levels, resulting in the emission bands. There are no significant differences between the relative intensities of the different peaks for all three ligands. Thus we conclude that, in the case of these green down-converting nanoparticles, the different ligands used do not influence the luminescence properties.

Fig. 6.3. Jablonski diagram of the down-conversion process in $Ce^{3+}(15\%)/Tb^{3+}(5\%)$ -doped KY_3F_{10} nanoparticles.

Similarly, blue DCNPs were prepared in EtOH/water at 80 °C. In this case the fluoride source was NaF, the lanthanide dopant was Dy^{3+} (2 mol%), and the matrix consisted of strontium gadolinium fluoride (Sr_2GdF_7). Here, only Dy^{3+} is necessary to obtain DC (single ion doping). The capping ligands used for these NPs were PAA, PiBMA grafted with CD and dodecyl chains (CD-PiBMA-DOD), and L-Cys. The powder XRD spectra of the crystals and the luminescence

spectra upon excitation at 350 nm are shown in Fig. 6.4. The powder XRD patterns of all three cases are characteristics of pure phases, also corresponding to the tetragonal crystalline system (PDF card 00-53-0775, see Experimental, Fig. 6.11).

Fig. 6.4. Powder XRD (a, c, e) and luminescence (b, d, f; $\lambda_{\text{ex}} = 350$ nm, $[\text{NP}] = 0.5$ mg mL⁻¹) spectra of Dy³⁺(2%)-doped Sr₂GdF₇ nanoparticles capped with of PAA (a, b), CD-PiBMA-DOD (c, d), and L-Cys (e, f).

As described before for the Ce³⁺/Tb³⁺-doped KY₃F₁₀ nanoparticles, the use of different ligands affected the crystallite size of the nanoparticles. The crystallites of the DCNPs prepared with L-Cys were the largest (Fig. 6.4e, 17.0 nm), probably because L-Cys binds the weakest to the metal centers of the surface since it can only bind through one carboxylic group per molecule. With PAA (Fig. 6.4a, 13.9 nm) and CD-PiBMA-DOD (Fig. 6.4c, 15.1 nm), there are multiple

carboxylic acid groups available and therefore, the binding is stronger leading to a smaller crystallite size.³¹

TEM imaging of the Dy³⁺-doped Sr₂GdF₇ DCNPs capped with PAA was performed (Fig. 6.5). These images show well-defined NPs with a particle size (13.0 ± 1.8 nm) comparable to the crystallite size determined by XRD (13.9 nm). Also, it is possible to observe the lattice fringes in an individual particle (inset Fig. 6.5b). ζ Potential measurements showed a negative value in the case of PAA (-24.2 mV), indicating the presence of free carboxylate groups on the surface. The small positive value for PiBMA (13.4 mV) indicates that most or all of the carboxylic acid groups on the backbone of the PiBMA polymer are bound to metal ions on the surface of the crystallites. In the case of L-Cys, the highly positive value (42.0 mV) demonstrates the binding of carboxylate groups to the surface, leaving protonated amino groups free at the surface.

Fig. 6.5. TEM images of Dy³⁺(2%)-doped Sr₂GdF₇ DCNPs capped with PAA. In the inset of b) a single particle with the lattice fringes is shown.

The luminescence spectra upon excitation at 350 nm are shown in Fig. 6.4b (PAA), d (CD-PiBMA-DOD), and f (L-Cys). In all three cases, the characteristic emission bands of Dy³⁺ at 475 nm (⁴F_{9/2} → ⁶H_{15/2}), and 575 nm (⁴F_{9/2} → ⁶H_{13/2}) were observed.³⁴

Fig. 6.6. Jablonski diagram of the down-conversion process in Dy^{3+} (2%)-doped Sr_2GdF_7 nanoparticles.

In these NPs, there is direct excitation of Dy^{3+} at 350 nm. After non-radiative relaxation, emission of Dy^{3+} provides the emission bands as shown in the Jablonsky diagram (Fig. 6.6). In this case, the different ligands cause differences in the luminescence spectra. In particular, the luminescence intensity with L-Cys as the ligand is much lower than that observed for the other ligands. The apparent quenching of the luminescence might be caused by the proximity of the unbound thiol moieties of L-Cys with the metal ions on the surface of the nanoparticle.

6.2.2 Synthesis and characterization of up-converting nanoparticles bearing different ligands

UCNPs were prepared in EtOH/water at 80 °C. The fluoride sources were in this case NaF and NaBF_4 , the lanthanide couple $\text{Yb}^{3+}/\text{Tm}^{3+}$ (15/0.5 mol%) was used, and the matrix was Sr_2GdF_7 . PAA, CD-PiBMA-DOD, and CD-PiBMA were used as the capping ligands. The powder XRD spectra of the crystals and their luminescence spectra upon NIR excitation at 980 nm are shown in Fig. 6.7. As discussed above for the DCNPs, the powder XRD patterns of the three cases indicate the presence of pure phases corresponding to the tetragonal crystalline system (PDF card 00-53-0775, see Experimental, Fig. 6.11).

Fig. 6.7. Powder XRD (a, c, e) and luminescence (b, d, f; $\lambda_{\text{ex}} = 980 \text{ nm}$; solid samples) spectra of Yb^{3+} (15%)/ Tm^{3+} (0.5%)-doped Sr_2GdF_7 UCNP samples capped with PAA (a, b) using NaBF_4 as the fluoride source, or with CD-PiBMA-DOD (c, d) or CD-PiBMA (e, f) using NaF as the fluoride source.

The average crystallite size was calculated from these powder XRD patterns for the used combinations of ligand and fluoride source. In case of NaBF_4 as the fluoride source and using PAA as the ligand, the crystallites were much larger (Fig. 6.7a, 40.9 nm) than when using NaF with CD-PiBMA-DOD and CD-PiBMA (Fig. 6.7c,e, 14.1 and 11.7 nm, respectively). This is seen in the powder XRD patterns as the peaks are broader when using NaF (Fig. 6.7c,e) than when using NaBF_4 (Fig. 6.7a). In case of the green and blue DCNPs reported above, the smallest crystallites were obtained when using PAA as the ligand, but in these cases the same fluoride source was used for all NPs. In case of the UCNP, the crystallites are much larger, which is therefore due to the use of NaBF_4 as the fluoride source. The larger particle size is attributed

to the slow controlled release of F^- ions from $NaBF_4$ compared to NaF and KF in the formation of the Sr_2GdF_7 matrix.^{21,22,35}

TEM imaging of the UCNPs bearing the three different capping ligands was performed (Fig. 6.8) showing well-defined particles. The sizes of the NPs made using NaF as the fluoride source (13.4 ± 0.4 nm for CD-PiBMA-DOD, Fig. 6.8b, and 12.1 ± 1.4 nm for CD-PiBMA, Fig. 6.8c) were comparable to the crystallite size by XRD. The particles prepared with PAA (Fig. 6.8a) were cubic with a diameter of 192 ± 5 nm, which is more than 4 times larger than the crystallite diameter. This indicates that each particle is composed of multiple crystallites. In Fig. 6.8b,c the lattice fringes are clearly visible within individual particles.

Fig. 6.8. TEM images of the $Yb^{3+}(15\%)/Tm^{3+}(0.5\%)$ -doped Sr_2GdF_7 UCNPs with the capping ligand a) PAA, b) CD-PiBMA-DOD, c) CD-PiBMA. In b, and insets in a and c, the lattice showing the crystal structure.

The photoluminescence experiments were done at comparable conditions in the three cases. The luminescence spectra upon NIR excitation at 980 nm (Fig. 6.7b (PAA), d (CD-PiBMA-DOD), and f (CD-PiBMA)), show the four characteristic Tm^{3+} emission bands at 480 nm ($^1G_{H4} \rightarrow ^3H_6$), 650 nm ($^1G_{H4} \rightarrow ^3F_4$), 700 nm ($^3F_{2,3} \rightarrow ^3F_4$), and 800 nm ($^3H_4 \rightarrow ^3H_6$), clearly indicative of up-conversion (see the Jablonski diagram in Fig. 6.9).³⁴ The process is initiated by the Yb^{3+} sensitizer ions which absorb the photons at 980 nm. Upon repeated energy transfer (up-conversion) from Yb^{3+} to the higher energy levels of the activator Tm^{3+} , followed by non-radiative relaxation, emission by Tm^{3+} occurs.

Fig. 6.9. Jablonski diagram of the up-conversion process in Yb³⁺(15%)/Tm³⁺(0.5%)-doped Sr₂GdF₇ nanoparticles.

Comparing the three luminescence spectra, the 480/800 nm ratio is larger (more than twice) in the case of using PiBMA-based ligands compared to PAA. This is tentatively attributed to the different fluoride sources used. With PAA, NaBF₄ was employed, while in the other two cases, NaF was used. The fluoride source influences the size and the morphology of the Ln³⁺-doped nanoparticles and thus their luminescence properties.^{21,22,35}

For future use of the CD-functionalized UCNPs in self-assembly, it is important to know the amount of CDs per particle. This was calculated, for both the NPs coated with CD-PiBMA-DOD and with CD-PiBMA, from the TGA graphs (Fig. 6.10), considering that the material loss between 200 °C and 500 °C originates from the coating polymer and below 125 °C only from evaporated water

Fig. 6.10. Thermogravimetric analysis (TGA) of a) CD-PiBMA-DOD and b) CD-PiBMA functionalized Yb³⁺/Tm³⁺-doped Sr₂GdF₇ UCNPs.

In the case of CD-PiBMA-DOD as the ligand (Fig. 6.10a), the amount of organic material ($w\%_{\text{org}}$) in the UCNPs was 7.5% and that of CD ($w\%_{\text{CD}}$) is then 4.6%, as calculated from the molecular weight of the polymer (22054 g mol⁻¹) and the number of grafted CDs (12.0), considering that the MW per CD is then 1838 g mol⁻¹. Similarly, in the case of CD-PiBMA (Fig. 6.10b, 18300 g mol⁻¹, 9.0 CDs, MW per CD: 2033 g mol⁻¹), $w\%_{\text{org}}$ is 10.0% and $w\%_{\text{CD}}$ in this case is 5.6 wt%. Taking into account the density of these nanoparticles (ρ_{NaYF_4} , 4.21 g cm⁻³ for NaYF₄ bulk),³⁶ it is possible to calculate the number of CD per particle ($N_{\text{CD,NP}}$) and the CD surface density (Γ_{CD}). In case of CD-PiBMA-DOD, $N_{\text{CD,NP}}$ is 168 CDs/particle and Γ_{CD} is 0.27 CD nm⁻², while for CD-PiBMA $N_{\text{CD,NP}}$ is 122 and Γ_{CD} is 0.28 CD nm⁻². In CD self-assembled monolayers (SAMs)³⁷ and in CD gold NPs (CD-AuNPs),³⁸ the Γ_{CD} is 0.4 CD nm⁻². The CD surface cover in CD-UCNPs is slightly smaller, which means that the UCNPs are not densely packed with CDs, but the similarity indicates that the usability in self-assembly should be comparable between CD-AuNPs and CD-UCNPs.

6.3 Conclusions and outlook

In this chapter, the one-step synthesis of lanthanide-doped down-converting and up-converting hydrophilic nanoparticles is described. With this mild and aqueous colloidal method, the particles are obtained at low temperature and at relatively short reaction times (2 hours). The influence of the ligands and the fluoride source on the particle size and

luminescence properties was systematically evaluated. The method is highly versatile, because apart of allowing the use of a variety of lanthanides, it is also possible to employ various fluoride sources and ligands to obtain particles with different properties (blue and green DCNPs, UCNPs) and sizes. Also, since the synthetic conditions are very mild, it allows the introduction of reactive groups or molecules on the surface of the NPs. The DCNPs have a small crystallite size varying from 9 nm (AA) to 24 nm (PEI) and the ones prepared with PAA as capping ligand present the most intense emission bands. In the case of the UCNPs, NPs prepared with CD-PiBMA as capping ligand exhibit the best characteristics: smaller particle size (11.7 nm) and strong up-conversion bands.

In comparison with the normal two-step procedures in the literature for the water solubilisation of this type of particles,^{14,15,39,40} this one-step method saves time (2 hours versus several days), is relatively easy, and it offers mild conditions, which allows the incorporation of functional molecules on the surface of the NPs during particle preparation. Consequently, it grants the easy incorporation of CDs on the surface of the NPs by simply grafting them into the polymer capping ligand PiBMA prior to particle synthesis, obtaining a CD surface density of 0.28 CD nm⁻². This will allow the formation of nanoassemblies with appropriate guests, hence, the easy encapsulation of drugs or genetic material, and furthermore the bonding to the surface of targeting moieties also via host-guest interactions, which has potential for biomedical applications.

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6.5 Experimental

6.5.1 Materials

Chemicals and solvents were obtained from Sigma-Aldrich and used as received. CD-PiBMA and CD-PiBMA-DOD were prepared as described in Chapters 3 and 4.^{41,42} MilliQ water with a resistivity of 18.2 MΩ cm at 25 °C was used when indicated.

6.5.2 Synthetic procedures

Green DCNPs

PEI, AA or PAA functionalized Ce³⁺/Tb³⁺-doped KY₃F₁₀ nanoparticles were prepared as follows. To a heated mixture (70 °C) of Y(NO₃)₃·6H₂O (306.40 mg, 0.80 mmol, 383 g mol⁻¹), Ce(NO₃)₃·6H₂O (65.13 mg, 0.15 mmol, 434 g mol⁻¹), and Tb(NO₃)₃·6H₂O (21.75 mg, 0.05 mmol, 435 g mol⁻¹) in distilled water (10 mL) was added dropwise a clear hot solution (70 °C) of KF (580 mg, 10 mmol, 58 g mol⁻¹) and the corresponding ligand (PEI, 2 mL (0.08 mmol, 25000 g mol⁻¹), AA (1169 mg, 8 mmol, 146.14 g mol⁻¹), or PAA (0.4 mL, 0.06 mmol, 50000 g mol⁻¹) in 10 mL of water/ethanol (1 : 1). The solution was heated for 2 h at 70 °C. After the solution was cooled down to room temperature, a white precipitate was collected by centrifugation (7500 rpm, 10 min). The particles were purified by washing with ethanol twice, followed by a last wash with milliQ water.

Blue DCNPs

PAA, CD-PiBMA-DOD, L-Cys functionalized Dy³⁺ doped Sr₂GdF₇ nanoparticles were produced in this manner. To a heated mixture (80 °C) of Sr(NO₃)₃ (423.26 mg, 2 mmol, 211.63 g mol⁻¹), Gd(NO₃)₃·H₂O (424.60 mg, 0.98 mmol, 433.34 g mol⁻¹), and Dy(NO₃)₃·5H₂O (8.77 mg, 0.02 mmol, 438.59 g mol⁻¹) in distilled water (10 mL) was added dropwise a clear hot solution (80 °C) of NaF (294 mg, 7 mmol, 42 g mol⁻¹) and the corresponding ligand (Na-PAA (0.4 mL, 0.02 mmol, 8000 g mol⁻¹), CD-PiBMA-DOD (50 mg, 0.002 mmol, 22000 g mol⁻¹), or L-Cys (484.64 mg, 4 mmol, 121.16 g mol⁻¹) in 10 mL of water/ethanol (1 : 1). The solution was heated for 2 h at 80 °C. After the solution was cooled down to room temperature, a white precipitate was collected by centrifugation (7500 rpm, 10 min). The particles were purified by washing with ethanol twice, followed by a last wash with milliQ water.

UCNPs

PAA, CD-PiBMA-DOD, CD-PiBMA functionalized $\text{Yb}^{3+}/\text{Tm}^{3+}$ doped Sr_2GdF_7 nanoparticles were prepared as follows. To a heated mixture (80 °C) of $\text{Sr}(\text{NO}_3)_3$ (423.26 mg, 2 mmol, 211.63 g mol⁻¹), $\text{Gd}(\text{NO}_3)_3 \cdot 5\text{H}_2\text{O}$ (366.17 mg, 0.84 mmol, 433.34 g mol⁻¹), $\text{Yb}(\text{NO}_3)_3 \cdot 5\text{H}_2\text{O}$ (67.36 mg, 0.15 mmol, 449.13 g mol⁻¹), and $\text{Tm}(\text{NO}_3)_3 \cdot 5\text{H}_2\text{O}$ (2.22 mg, 0.005 mmol, 445.03 g mol⁻¹) in distilled water (10 mL) was added dropwise a clear hot solution (80 °C) of NaBF_4 (192.13 mg, 1.75 mmol, 109.79 g mol⁻¹) and PAA (0.4 mL, 0.06 mmol, 50000 g mol⁻¹), or NaF (294 mg, 7 mmol, 42 g mol⁻¹) and CD-PiBMA-DOD (50 mg, 0.002 mmol, 22000 g mol⁻¹), or CD-PiBMA (50 mg, 0.003 mmol, 18300 g mol⁻¹) in 10 mL of water/ethanol (1 : 1). The solution was heated for 2 h at 80 °C. After the solution was cooled down to room temperature, a white precipitate was collected by centrifugation (10000 rpm, 15 min). The particles were purified by washing with ethanol twice, followed by a last wash with milliQ water.

6.5.3 Methods**XRD measurements**

Powder X-ray diffraction measurements were performed on a D2Phaser (Bruker) using a Cu-K α X-ray source at a scanning rate of 1° min⁻¹ in the 2 θ range from 20° to 90°. The powdered samples were spread evenly on a quartz slide.

TEM measurements

TEM samples were prepared by placing a drop of the particles onto a carbon coated Cu grid (200 meshes) and dried in air at room temperature. The measurements were carried out using a high-resolution Philips CM300ST-FEG transmission electron microscope (300 KeV) equipped with a GATAN Tridiem energy filter (2k x 2k CCD camera), and extra GATAN Ultrascan 1000 CCD camera (2k x 2k).

 ζ Potential measurements

See Chapters 4 and 5.

Photoluminescence measurements

The photoluminescence measurements were performed of liquid samples (DCNPs) at a concentration of 0.5 mg mL⁻¹ or of solid samples (UCNPs) using a special holder with a Horiba Jobin Yvon Fluorolog.

6.5.4 Calculation of the particle size from the powder XRD patterns

The average crystallite size was calculated by the Debye-Scherrer equation, where λ is the wavelength of X-ray (0.15418 nm), θ is the full width at half-maximum (FWHM) of a diffraction peak, ϑ is the diffraction Bragg's angle, and k is a constant (0.9).

$$d = \frac{k\lambda}{\beta \cos \theta}$$

6.5.5 Calculation of the number of CDs per particle

The number of CDs per particle in the NPs functionalized with CD-PiBMA-DOD or CD-PiBMA was calculated from the TGA graphs (Fig. 6.10) as described in the next paragraphs and considering the density of the UCNPs.³⁶

In the case of CD-PiBMA-DOD as ligand (Fig. 6.10a), the loss of water accounts for 3% of the total weight of the particles. The loss of organic material happens between the two plateau regions: at 97% and at 89.5%. Therefore, the amount of organic material ($w\%_{org}$) in the UCNPs is 7.5%, and the remaining is supposed to be the inorganic part, $w\%_{inorg}$, attributing to 89.5%.

This polymer is grafted with a number of CDs, $N_{CD,pol}$, which is 12.0 (MW_{CD} 1135 g mol⁻¹), and the overall molecular weight of the polymer, MW_{pol} is 22054 g mol⁻¹. Therefore, the weight percentage of CD ($w\%_{CD}$) in the particles is given by:

$$w\%_{CD} = w\%_{org} \times (N_{CD,pol} \times MW_{CD} / MW_{pol}) \quad (1)$$

With the values given above, this amounts to $w\%_{CD} = 4.6\%$ in the UCNPs functionalized with CD-PiBMA-DOD.

The crystallite size of these UCNPs is 14.1 nm. Therefore, the V_c of one crystallite, assuming a sphere, is 1468 nm³, and the surface area A_c is 625 nm².

Considering the density (4.21 g cm⁻³ for NaYF₄) of the bulk material of the UCNPs, the mass of one crystal (m_c) is 6.18×10^{-18} g.

Given the ratio of the weight fractions of CD vs. the inorganic part, 4.6% vs. 89.5%, the mass of CDs per particle, m_{CD} , is 3.18×10^{-19} g, which leads to a number of CDs per particle, $N_{CD,NP}$, of 168. Taking into account the surface area per particle ($A_C = 625 \text{ nm}^2$), a surface density, Γ_{CD} , of 0.27 CDs per nm^2 .

Similarly, in the case of CD-PiBMA, the water loss is 4%, the organic weight is 10%, and the inorganic fraction is 86% of the total weight of the particles. Considering that MW_{pol} is in this case 18300 g mol^{-1} , and that the polymer has 9.0 CDs per polymer backbone, the $w\%_{CD}$ in this case is 5.6% wt.

Taking into account that the crystallite size is in this case 11.7 nm, $V_C = 839 \text{ nm}^3$, $A_C = 430 \text{ nm}^2$, and $m_C = 3.53 \times 10^{-18}$ g. Therefore, the mass of CD, m_{CD} , is 2.30×10^{-19} g, which leads to a number of CDs per particle, $N_{CD,NP}$, of 122, and a surface density, Γ_{CD} , of 0.28 CD per nm^2 .

6.5.6 ICSD PDF card of the standard tetragonal phase

Fig. 6.11 shows the powder XRD pattern of the standard KY_3F_{10} and Sr_2GdF_7 crystals of tetragonal phase.

Fig. 6.11. ICSD PDF card of the pure standard KY_3F_{10} and Sr_2GdF_7 crystals of tetragonal phase.

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Chapter 7

Host-Guest and Electrostatic Interactions in Supramolecular Nanoparticle Clusters

In this Chapter, the influence of the charge, structure, and host-guest interactions of different soft polymeric components in the assembly with negatively charged inorganic gold and up-converting nanoparticles functionalized with cyclodextrins (CD-AuNPs and CD-UCNPs) was studied. The inter-intramolecular interplay was evaluated by changing the morphology and the charge of the polymeric component. Using a linear, anionic guest-functionalized polymer resulted in the formation of a thin coating layer around the CD-AuNPs and CD-UCNPs without further NP aggregation. When the branched cationic polymer PEI was used instead, small clusters were obtained before the electrostatic neutralization point, while large NP aggregates were obtained above the neutralization point. When using branched cationic guest-functionalized dendrimers, clusters were obtained at a 1 : 1 host-guest stoichiometry. In this case, self-assembly occurred when still far away from the neutrality point, indicating that this cluster formation is driven by supramolecular host-guest interactions. The luminescence of the CD-UCNPs with the linear polymer was quenched due to C-H vibrational modes of the guest polymer, while in the case of electrostatic cluster formation by PEI, the luminescence of CD-UCNPs was enhanced after the neutralization point due to the formation of densely packed NP assemblies. These findings will be useful for the design of hybrid polymeric-inorganic assemblies for sensing and biomedical applications.

7.1 Introduction

Inorganic nanoparticles (NPs) have a core in the range of 1-100 nm, with new properties compared to the bulk material that arise from their nm size.^{1,2} The core of these nanoparticles can be made from a large variety of materials, such as iron oxide, gold (AuNPs), silver, semiconductors (quantum dots), and lanthanide oxides or fluorides (up-converting and down-converting NPs, UCNPs and DCNPs). From these types of inorganic nanoparticles, AuNPs and UCNPs are particularly interesting for nanomedicine, in particular for photothermal therapy (AuNPs) and for bioimaging applications (UCNPs).^{3,4}

Inorganic nanoparticles are generally synthesized with a stabilizing coating layer, which varies from different charged ligands⁵⁻⁷ to host or guest supramolecular components.⁸⁻¹⁰ The presence of this coating allows the interaction of the inorganic nanoparticles with soft materials, and provides water solubility at the same time which is essential for biomedical applications. Non-covalent interactions with soft materials, such as polymers and dendrimers, results in the organization, by controlled aggregation, of the inorganic nanoparticles into hybrid supramolecular assemblies creating network aggregates.¹¹⁻¹⁶ Depending on the chosen soft component, it is possible to control these assemblies in size, shape, and morphology, and thereby to tune the distance between the inorganic nanoparticles within the assembly.¹⁴ This is important for the targeted applications, especially for sensing and imaging.^{14,17-19} When using supramolecular host-guest interactions as the way to direct the self-assembly process, studies exist on the influence of the valency, the morphology and flexibility of the used guest on the formation of intra- or intermolecular assemblies.^{9,20-22} However, a systematic study of these parameters in combination with the influence of electrostatic interactions on the formation of hybrid network aggregates, especially of UCNPs, is lacking.

In the previous Chapters 3, 4, and 5,²³⁻²⁵ supramolecular nanoparticles (SNPs) composed of soft anionic polymeric host and guest components were prepared and characterized, and the influence of electrostatic interactions on the particle formation and stability was studied. In Chapter 6, inorganic lanthanide-doped UCNPs, surface-modified with cyclodextrins (CDs), were synthesized and characterized. Here, we aim to study the formation of hybrid inorganic-organic, supramolecular nanoparticle clusters (SNPCs), composed of inorganic NPs

functionalized with CDs (CD-AuNPs and CD-UCNPs) and different types of soft polymers (see Fig. 7.1).

Fig. 7.1. a) Self-assembly of the components to form supramolecular clusters of CD-AuNPs or CD-UCNPs. b) Components of the system: AuNPs grafted with CD (CD-AuNPs), UCNPs grafted with CD (CD-UCNPs), guest polymer grafted with *p*-tert-butylphenyl groups (TBP-PiBMA), poly(ethylene imine) (PEI), and PAMAM dendrimer grafted with adamantyl groups (Ad₈-PAMAM).

In the choice of the polymeric component of the SNPs, we take into account three parameters: host-guest interactions, electrostatic forces (attractive and repulsive), and the structure of the polymer (linear and branched). We focus on the difference between intra- and intermolecular assembly formation between the components when changing the

morphology, charge, and guest of the soft polymeric component. The inorganic nanoparticles and their assemblies are characterized by dynamic light scattering (DLS), ζ potential measurements, transmission electron microscopy (TEM) and, in the case of UCNPs, also photoluminescence (PL).

7.2 Results and discussion

7.2.1 Synthesis and characterization of the components

AuNPs surface-functionalized with β -CD (CD-AuNPs) were synthesized following a procedure reported before,^{8,9} by reducing HAuCl_4 with NaBH_4 in DMSO containing perthiolated β -CD. The CD-AuNPs were purified by precipitation in acetonitrile and ethanol and collected by freeze-drying. The CD-AuNPs were characterized by TEM, DLS, and UV/Vis (Fig. 7.2). The size of the core of the CD-AuNPs was 3 ± 1 nm by TEM (Fig. 7.2a). The hydrodynamic diameter was 10.5 ± 1.8 nm as measured by DLS (Fig. 7.2b). This difference in size stems from the presence of CDs on the surface of the particles, which are not visible by TEM, and from the hydrated nature of the NPs during DLS. UV/Vis spectroscopy (Fig. 7.2c) exhibited the characteristic surface plasmon absorption band,²⁶ with the maximum absorbance wavelength (λ_{max}) at 516 nm. There are on average 14 CDs per particle. This number was calculated taking into account that the particle is fully covered with a CD shell, that the diameter of the core of the particles is 3 nm, and that the distance between CDs is 2 nm.²⁷

Fig. 7.2. Characterization of CD-AuNPs by: a) TEM, b) DLS, and c) UV/Vis absorbance.

The β -CD-grafted poly(isobutyl-*alt*-maleic acid)-capped, Yb^{3+} (15%)/ Tm^{3+} (0.5%)-doped Sr_2GdF_7 nanoparticles (CD-UCNPs) were prepared and characterized as described in Chapter

6. The crystallite size of the NPs was 11.7 nm as determined by powder X-ray diffraction (XRD), and by thermogravimetric analysis (TGA) the amount of CD was estimated to be 5.6 wt%, which means that a solution of CD-UCNPs of 0.5 mg mL^{-1} provides a CD concentration of $15 \text{ }\mu\text{M}$. These particles were characterized further by DLS and ζ potential measurements. To study the cluster formation, the concentration of CD-UCNPs used was $3 \text{ }\mu\text{M}$ of CD, because at higher concentration the CD-UCNPs were not fully stable in water and started to flocculate. Since this CD concentration is too low to detect cluster formation by DLS (see Chapters 3, 4, and 5), free CD-PiBMA ($9 \text{ }\mu\text{M}$ CD) was also added to the solution to reach a sufficiently high CD concentration ($12 \text{ }\mu\text{M}$ CD). This addition of free CD-PiBMA assisted also in the water solubilization of the UCNPs. By DLS (Fig. 7.3), a hydrodynamic diameter, d_h , of $60 \pm 9 \text{ nm}$ was obtained. The d_h differs significantly from the crystallite size by powder XRD and the particle core size by TEM. We attribute this difference to: a) the presence of the polymer coating around the NPs that is not detected by XRD and TEM, b) the hydration of the NPs during DLS measurements, and c) some degree of particle aggregation. The ζ potential ($-39 \pm 2.5 \text{ mV}$) indicates the presence of negatively charged moieties due to the negatively charged carboxylate groups of the capping ligand.

Fig. 7.3. DLS of CD-UCNPs ($3 \text{ }\mu\text{M}$ CD) + CD-PiBMA ($9 \text{ }\mu\text{M}$ CD) in water.

7.2.2 Cluster formation with the linear polymer TBP-PiBMA by host-guest interactions

The negatively charged linear polymer TBP-PiBMA was added to the negatively charged CD-AuNPs at 1 : 1 and 1 : 4 CD-TBP ratios, at a CD concentration of $13.1 \text{ }\mu\text{M}$ in water. The hydrodynamic diameter was followed by DLS over time (Fig. 7.4a). The results show that,

although initially there was a slight increase in particle size from 10.5 nm of the individual particles to 15 nm (at the 1 : 4 CD-TBP ratio), d_h rapidly dropped to the starting value. Therefore, it is concluded that no cluster formation occurred, irrespective of the host-guest ratio and of the time upon addition. These results suggest instead that the guest polymer forms a thin coating around the CD-AuNPs. The formation of a thin coating in the absence of particle aggregation indicates efficient binding of all or most guest moieties to the same particle. This is attributed to the linear structure of the guest polymer and the effective molarity (EM) of the guest TBP in that polymer.²⁹⁻³¹ EM can be defined as the probability that two different binding sites (for example, a host and a guest group) meet forming an intramolecular complex, and its value depends on the length and flexibility of the components. It thus provides a measure of the local concentration of the guest binding sites. Here, the EM of TBP in TBP-PiBMA is likely to be higher (mM range) than the concentration of TBP in solution (μ M range) and therefore, intramolecular binding forming a capping layer around the CD-AuNP, instead of intermolecular binding between different particles, is favored. Consequently, cluster formation is suppressed. The linear nature of the guest polymer is contributing strongly to this effect and a similar thin layer formation has been observed on CD monolayers before.³² In contrast, steric hindrance occurring in branched molecules, such as dendrimers, prevents full use of all their guest moieties thus exposing free guest sites for intermolecular, crosslinking interactions.³³

In the case of CD-UCNPs, the negatively charged linear polymer TBP-PiBMA was added to the CD-UCNPs at a 1 : 1 CD-TBP ratio and 12 μ M total CD concentration. The cluster formation was followed by DLS over time (Fig. 7.4b). Upon addition of the linear polymer, the particle size increased from 60 to 225 nm. However, with time, the clusters started to break down, and after two days the particle size was the same as before the addition of the guest polymer. Therefore, we conclude that the same behavior occurs as with the CD-AuNPs: intramolecular bonding is favored over intermolecular interactions, and the guest polymer TBP-PiBMA arranges itself as a thin coating layer around the CD-UCNPs while directing all or most of its guest moieties to the CDs on the particle surface. The initial formation of clusters and their slower breakdown compared to the CD-AuNPs may be attributed to the larger size of the CD-UCNPs.

Fig. 7.4. DLS measurements of the self-assembly of TBP-PiBMA and a) CD-AuNPs or b) CD-UCNPs (3 μ M CD) + CD-PiBMA (9 μ M CD) in water.

The up-converting luminescence properties of the CD-UCNPs after excitation at 980 nm were measured before and after (3 days) addition of the linear guest polymer TBP-PiBMA (Fig. 7.5). After the addition (Fig. 7.5b), the luminescence was a lot lower, which confirms the difference in the particle environment before and after adding the linear polymer. This quenching of the luminescence is likely to be caused by C-H vibrational modes of the guest polymer.³⁴ Moreover, the blue luminescence band at 450 nm is quenched considerably stronger in comparison to the 800 nm band, indicating a lower efficiency of the up-conversion process compared to the uncomplexes UCNPs. This strong quenching might be caused by the formation of a densely packed coating layer around the CD-UCNPs due to multivalent host-guest interactions between CD (CD-PiBMA) and TBP (TBP-PiBMA).

Fig. 7.5. Photoluminescence spectra upon excitation at 980 nm of CD-UCNPs (3 μ M CD) + CD-PiBMA (9 μ M) a) before and b) after (3 days) addition of TBP-PiBMA.

7.2.3 Cluster formation with the cationic polymer PEI by electrostatic interactions

In order to study the effect of attractive electrostatic interactions to induce the aggregation of CD-UCNPs, the positively charged hyperbranched polymer PEI (MW 10 kDa) was added to the CD-UCNPs by stepwise addition in phosphate buffer (1 mM, pH 7.4). In this case in the absence of complementary guest moieties, cluster formation can be induced only by attractive electrostatic forces between the negatively charged CD-UCNPs (see Chapter 6) and the protonated polymer backbone of PEI. The size of the clusters as a function of the concentration of PEI was monitored by DLS (Fig. 7.6a). Before reaching a concentration of PEI of 0.7 μM , relatively small clusters of well-defined size and small standard deviation were obtained. Above 0.7 μM , large clusters started to appear. By ζ potential measurements (Fig. 7.6b), a similar trend was observed. Up to a concentration of 0.7 μM of PEI, the clusters were negatively charged, while at that concentration, the neutralization point was reached. Above 0.7 μM PEI the clusters became positively charged, but the ζ potential remained below 10 mV, indicating that no more PEI is adsorbing onto the, then positively charged, clusters. The theoretical neutralization point of the clusters was calculated to be at 0.65 μM PEI by comparing the amounts of positive charges of PEI and negative charges of the ligands on the UCNPs. This value agrees very well with the experimental point of neutralization obtained by DLS and ζ potential measurements. The process of electrostatically controlled self-assembly corresponds to that observed for anionic SNPs to which PEI was added (Chapter 4):²³ upon reaching the neutralization point the SNPs lost colloidal stability, and only large aggregates remained in solution.

Fig. 7.6. characterization of the clusters between PEI and CD-UCNPs by a) DLS and b) ζ potential.

The clusters were visualized by TEM. Fig. 7.7 shows images of the clusters after addition of PEI a) before (0.34 μM PEI) and b) after (0.85 μM PEI) the neutralization point. In Fig. 7.7a, irregular clusters with a size of approx. 150 nm are visible, while in Fig. 7.7b large aggregates of indefinite size are displayed. These results agree with those of DLS (Fig. 7.6a). In conclusion, all data confirm the formation of well-defined clusters between CD-UCNPs and PEI due to electrostatic interactions as long as the clusters remain negatively charged, while crossing the point of neutrality leads to uncontrolled aggregation.

Fig. 7.7. TEM images of the clusters between PEI and CD-UCNPs a) before (0.34 μM PEI) and b) after (0.85 μM PEI) the neutralization point.

The up-converting luminescence properties of the CD-UCNPs/PEI clusters were studied by measuring their photoluminescence (PL). Fig. 7.8 shows the PL spectra a) of the CD-UCNPs

alone, b) after adding PEI before the neutralization point and c) after the neutralization point. The luminescence characteristics did not change significantly after adding PEI before the neutralization point, which suggests that the environment around the particles in the clusters formed by electrostatic interactions did not change. On the other hand, the luminescence increased significantly once the neutralization point was reached. Interestingly, the intensity of the blue band at 450 nm increased proportionally more than the one at 800 nm (1.5- versus 1.2-fold), which is most probably caused by the increase in size of the clusters once neutralization is reached.³⁵ The increase in luminescence is caused most probably by a combination of two factors: first, a smaller amount of water is present in the clusters because they are more densely packed and solvent is removed upon clustering, and second, the CD-UCNP inter-particle distance is reduced upon cluster formation.³⁶ One of the main current problems with UCNPs is the loss of luminescence intensity after transferring them into water due to the OH vibrations of the solvent. Therefore, the strategy followed here, forming dense structures via electrostatic interactions, is an interesting and straightforward way to increase the luminescence of these particles in aqueous solvents.

Fig. 7.8. Photoluminescence spectra upon excitation at 980 nm of CD-UCNPs (3 μ M CD) + CD-PiBMA (9 μ M) a) before adding PEI, after adding PEI b) before (0.34 μ M PEI) and c) after (0.85 μ M PEI) the neutralization point.

7.2.4 Cluster formation with the branched dendrimer Ad₈-PAMAM by host-guest and electrostatic interactions

The positively charged, branched, guest-functionalized dendrimer Ad₈-PAMAM was added to the CD-UCNPs, at 1 : 1 host-guest (CD-Ad) ratio (12 μ M CD, with 3 μ M from CD-UCNPs and 9 μ M from CD-PiBMA), in phosphate buffer (1 mM, pH 7.4). The branched guest dendrimer Ad₈-PAMAM was used instead of the linear TBP polymer in order to promote

inter-particle binding. Within minutes after addition, the d_h increased from 60 to 225 nm with a PDI of 0.2, which indicates the formation of clusters (Fig. 7.9a). Clusters of similar size were still present after 24 hours, indicating that the cluster formation was not reversible as in the case of the linear polymer. However, the PDI increased almost three-fold (to 0.6) at the same time, which indicates that the clusters were not homogeneous in size. The addition of Ad₈-PAMAM caused the formation of clusters due to attractive host-guest and electrostatic interactions. The clusters increased almost four times in size although the (calculated) neutralization point is still far away: the concentration of positive charges (coming from the dendrimer) is approx. 23 μ M, while the concentration of negatively charges (“free” carboxylate groups in solution) is approx. 75 μ M, thus there are approx. three times more negative charges present in solution. Therefore, we conclude that the SNPC formation, as witnessed from the increase of d_h , is primarily caused by the attractive host-guest interactions maintaining the 1 : 1 host-guest ratio. These results correspond to those obtained with CD-AuNPs, in which large aggregates of CD-AuNPs with an Ad-functionalized poly(propylene imine) dendrimer were obtained in the absence of a monovalent capping agent.⁸ The formation of clusters was also visualized by TEM (Fig. 7.9b), which shows large and compacted structures after the addition of Ad₈-PAMAM.

Fig. 7.9 a) DLS results and b) TEM image of SNPCs between CD-UCNPs and Ad₈-PAMAM in water at a 1 : 1 host-guest ratio between CD-UCNP (3 μ M CD) + CD-PiBMA (9 μ M CD) and Ad₈-PAMAM (12 μ M). In a) case 1 corresponds to free CD-UCNPs before addition of Ad₈-PAMAM (PDI 0.3), case 2 to clusters 15 min after adding Ad₈-PAMAM (PDI 0.2), and case 3 to clusters 1 day after addition of Ad₈-PAMAM (PDI 0.6).

7.3 Conclusions and outlook

In this Chapter, the influence of the charge, structure, and host-guest interactions of different soft polymeric components on the supramolecular assembly with hard, negatively charged inorganic nanoparticles (CD-AuNPs and CD-UCNPs) was studied. While linear and flexible anionic guest polymers favored intramolecular assemblies forming a soft thin coating around the particles, branched cationic guest dendrimers favored inter-particle interactions and therefore cluster formation, obtaining clusters at a 1 : 1 host-guest ratio, while still far off from the electrostatic point of neutrality. In the case of using only electrostatic forces, when using the branched, densely positively charged PEI, cluster formation was induced by attractive electrostatic interactions. The size of the aggregates increased upon increasing the number of positive charges. However, above the neutralization point, large aggregates were obtained. The luminescence properties of the CD-UCNPs were also evaluated: with the linear polymer, the luminescence was quenched considerably due to C-H vibrational modes of the guest polymer. In the case of cluster formation with PEI, the luminescence after the neutrality point increased considerably, due to the formation of dense structures which bring the particles closer together and also reduces the amount of water around them.

In conclusion, this study presents and clarifies how to control and tune the network aggregate formation of inorganic nanoparticles by changing the nature and structure of the guest component inducing intra- or intermolecular assemblies via host-guest and/or electrostatic interactions. Moreover, an easy and straightforward way to increase the luminescence of UCNPs in water is presented, by forming dense assemblies by attractive electrostatic interactions. These results could be useful for developing new inorganic nanoparticle-based systems for bioimaging applications.

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7.5 Experimental

7.5.1 Materials and equipment

Chemicals and solvents were obtained from Sigma-Aldrich and used as received. TBP-PiBMA and CD-PiBMA were prepared as described in Chapters 3 and 4.^{23,24} Ad₈-PAMAM was prepared as described by Tseng and coworkers.³⁷ MilliQ water with a resistivity of 18.2 MΩ cm at 25 °C was used when indicated. For the equipment details, see Chapter 6.

7.5.2 Methods

Preparation of the CD-AuNPs assemblies with TBP-PiBMA for DLS experiments

The CD-AuNPs assemblies with TBP-PiBMA were prepared in MilliQ water at 1 : 1 and 1 : 4 host-guest ratios at 13.1 μM CD, from stock solutions of CD-AuNPs, and TBP-PiBMA (3.87 μM TBP) by mixing the components with a vortex. The final composition of these SNPs was CD-AuNPs (13.1 μM CD) and TBP-PiBMA (13.1 and 52.4 μM TBP). The particle size was measured at pre-established size intervals.

Preparation of the CD-UCNPs assemblies with TBP-PiBMA for DLS, ζ potential, TEM, and PL experiments

The CD-UCNPs assemblies with TBP-PiBMA were prepared in phosphate buffer (1 mM, pH 7.4) at a 1 : 1 host-guest ratio at 12 μM CD (3 μM CD from UCNPs and 9 μM CD from TBP-PiBMA) from stock solutions of CD-UCNPs (16 μM CD from CD-UCNPs and 48 μM CD from CD-PiBMA), and TBP-PiBMA (3.87 μM TBP) by mixing the components with a vortex. The final composition of these SNPs was CD-UCNPs (12 μM CD) and TBP-PiBMA (12 μM TBP). The particle size was measured at pre-established size intervals.

Preparation of the CD-UCNPs assemblies with Ad₈-PAMAM for DLS and TEM experiments

The CD-UCNPs assemblies with Ad₈-PAMAM were prepared in phosphate buffer (1 mM, pH 7.4) at a 1 : 1 host-guest ratio at 12 μM CD (3 μM CD from UCNPs and 9 μM CD from TBP-PiBMA) from stock solutions of CD-UCNPs (16 μM CD from CD-UCNPs and 48 μM CD from CD-PiBMA) in MilliQ water and Ad₈-PAMAM (750 μM Ad) in DMSO by mixing the components with a vortex. The final composition of these SNPs was CD-UCNPs (12 μM CD) and Ad₈-PAMAM (12 μM TBP). The particle size was measured at pre-established size intervals.

Preparation of the CD-UCNPs assemblies with PEI for DLS, ζ potential, TEM, and PL experiments

The CD-UCNPs assemblies with PEI were prepared in phosphate buffer (1 mM, pH 7.4) at different cationic : anionic ratios at 12 μM CD (3 μM CD from UCNPs and 9 μM CD from TBP-PIBMA) from stock solutions of CD-UCNPs (16 μM CD from CD-UCNPs and 48 μM CD from CD-PIBMA), and PEI (25 μM) by mixing the components with a vortex. The final composition of these SNPs was CD-UCNPs (12 μM CD) and PEI (0-1.2 μM). The particle size and ζ potential were measured at pre-established size intervals. TEM and PL were measured before (0.34 μM PEI) and after (0.85 μM PEI) the neutralization point.

7.5.3 Calculation of the number of charges in the system

The number of charges in the assemblies was calculated based on the concentration of the different species, in the same way as in Chapters 4 and 5. The concentration of negative charges, coming from the carboxylic groups is 75.3 μM .

The number of positive charges is coming from the PEI and is calculated in the same way as in Chapter 4. The results are summarized in Table 7.1.

Table 7.1. Changes in the amount of charges of the clusters upon addition of PEI

[PEI], μM	[amine], μM
0.0	0
0.34	39
0.67	78
0.85	99
1.17	136

7.6 References

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Summary

Supramolecular nanoparticles (SNPs) have emerged recently as a novel and powerful tool for new developments in nanomedicine. The high versatility of SNPs together with their modular character make them ideal for various biomedical applications. In particular the modular character brings the possibility of easily incorporating targeting moieties, imaging agents, and drug molecules. In recent years, several biomedical systems based on SNPs underwent clinical studies, and some of them have been approved to be used in humans. Most SNPs rely on the assembly of multiple (multivalent) host and guest moieties which have been anchored to building blocks such as molecules, biomolecules, polymers, dendrimers, and inorganic nanoparticles. However, other non-covalent, most notably electrostatic, interactions are usually also involved in their formation. The interplay between the electrostatic and the multivalent host-guest interactions has often been ignored. The work described in this thesis aims to clarify this important aspect of SNP formation, which is essential to understand SNP characteristics such as SNP assembly mechanism, kinetics, disassembly, and stability.

Chapter 2 reviews the most important aspects related to the formation of cyclodextrin (CD)-based SNPs and their biomedical applications. The particle formation is evaluated in terms of the different forces involved. Also, the formation of hybrid organic-inorganic SNPs is reviewed. In the second part of this chapter, the use of these SNPs in biomedicine, for therapeutic and imaging applications, is described.

In the first part of the thesis (Chapters 3, 4, and 5), a new CD-based SNP system based on the negatively charged linear polymer poly(isobutyl-*alt*-maleic acid) (PiBMA) has been developed. Chapter 3 has focused on the mechanism underlying the formation of these particles. The particles were formed by multivalent supramolecular interactions between the host (CD) and the guest (*tert*-butylphenyl group, TBP), while their size and stability were controlled by a balance between attractive host-guest and repulsive electrostatic (between the polymer backbones) interactions. Disturbing this balance, for example, by increasing the

ionic strength of the medium, resulted in particle destabilization and aggregation. In Chapter 4, the effect of loading a fluorescent, heavily charged cargo (polyethylene imine, PEI, grafted with fluorescein, FL) into these SNPs has been investigated. The environment inside the SNPs changed from negatively to positively charge upon addition of the cargo, which also affected the particle size and stability. The SNPs were made fluorescent by grafting the dye rhodamine B (RhB) onto the host polymer. Since FL and RhB form a FRET couple, it was possible to observe a FRET effect upon loading the SNPs with the FL-labeled cargo. In Chapter 5, a pH-sensitive, peptide uptake and release system was developed based on these negatively charged fluorescent SNPs. Here, instead of the heavily charged cationic cargo PEI, oligoarginines containing a cyanine dye (Cy5) were used. RhB and Cy5 also form a FRET couple, and thus the FRET effect was used as a sensor to study the encapsulation and release of the peptide cargo. Lowering the pH resulted in release of the encapsulated peptides because of protonation of the SNP components. Most importantly, this release occurred at pH values which mimic lysosome conditions. The encapsulation of the peptides into SNPs was shown to have a clear positive effect on the uptake of the peptides by cells, as witnessed by *in vitro* studies in prostate cancer cells.

The second part of this thesis (Chapters 6 and 7) has described the formation of hybrid organic-inorganic SNPs based on lanthanide-doped nanoparticles. In Chapter 6, hydrophilic lanthanide-doped nanoparticles (both down- and up-converting, DCNPs and UCNPs) doped with different lanthanides and bearing different capping ligands were synthesized by a versatile and fast colloidal method at a relatively low temperature. By changing the dopant lanthanides, the luminescence of the NPs could be changed from blue or green emitting DC to UC. The influence of the ligand and the fluoride source on the particle size and luminescence properties, while using the same core composition of the particles, was evaluated. Since PiBMA can be easily grafted with different functional groups, such as CD, it was possible to obtain UCNPs bearing CDs on the surface (CD-UCNPs) using this one-step method. In Chapter 7, assemblies of negatively charged gold NPs functionalized with CDs (CD-AuNPs) or the CD-UCNPs developed in Chapter 6 with different types of soft polymeric components were studied. By changing the morphology and the size of the polymeric components, different network aggregates were prepared. Using PiBMA grafted with TBP

(PiBMA-TBP), a thin coating layer around the CD-AuNPs and CD-UCNPs was obtained by mainly intra-particle host-guest interactions between CD and TBP. However, using branched polymers resulted in NP clustering and thus hybrid SNP formation. The different assembly properties also resulted in different effects on the luminescence properties of the CD-UCNPs.

In summary, the work described in this thesis provides a new strategy for the formation of SNPs using negatively charged and linear polymers. The importance of the fragile balance between the forces which govern the formation and stability of these SNPs is highlighted. Also, the encapsulation and stimulated release of peptide cargo in these SNPs has been reported. Finally, the importance of the morphology and charge of the guest component on the formation of hybrid clusters was evaluated. These findings are essential for understanding the mechanisms underlying SNP formation, as well as of the encapsulation and release of charged cargo into/from charged SNPs, both of which are of utmost importance for therapeutic applications such as drug and gene delivery. In the area of lanthanide-based NPs, exploring new ways of synthesizing them and studying the characteristics of hybrid organic-inorganic SNPs based on these particles, may lead to a better and more versatile use of this interesting class of nanomaterials, which are becoming more and more important in the bioimaging world. Thus, we hope that the knowledge provided in this thesis will further the development of SNPs as drug carriers and bioimaging agents in nanomedicine.

Samenvatting

Supramoleculaire nanodeeltjes (SNPs) zijn recent ontdekt als een nieuw en krachtig systeem voor nieuwe ontwikkelingen in de nano-geneeskunde. De grote veelzijdigheid van SNPs samen met hun modulaire karakter maakt ze ideaal voor verschillende biomedische toepassingen. Met name het modulaire karakter zorgt ervoor dat sturende ('targeting') liganden, contrastreagentia en medicijnen gemakkelijk ingebouwd kunnen worden. Recentelijk zijn verschillende biomedische systemen gebaseerd op SNPs aan klinische studies onderworpen, waarvan sommige goedgekeurd zijn om in mensen gebruikt te worden. Veel SNPs zijn gebaseerd op de assemblage van meervoudige (multivalente) gastheer- en gastgroepen die zijn verankerd aan bouwstenen zoals moleculen, biomoleculen, polymeren, dendrimeren en anorganische nanodeeltjes. Andere niet-covalente interacties, veelal elektrostatische, zijn echter ook vaak betrokken bij de vorming. De wisselwerking tussen de elektrostatische en de multivalente gastheer-gast-interacties wordt vaak buiten beschouwing gelaten. Het werk beschreven in dit proefschrift heeft als doel om dit belangrijke aspect van SNP-vorming te verhelderen, het geen essentieel is om kenmerken van SNPs te begrijpen zoals hun assemblagemechanisme, kinetiek, uiteenvallen en stabiliteit. Meer details zijn te vinden in de Engelse versie van de samenvatting.

Samenvattend biedt het werk beschreven in dit proefschrift een nieuwe strategie voor de vorming van SNPs door het gebruik van negatief geladen, lineaire polymeren. Het belang van de fragiele balans tussen de krachten die de vorming en stabiliteit van deze SNPs reguleren wordt hierbij benadrukt. Daarnaast is ook de inclusie en de stimulus-gestuurde vrijzetting van een peptidelading in deze SNPs beschreven. Ten slotte is ook het belang van de morfologie en de lading van de gast op de vorming van hybride clusters bepaald. Deze bevindingen zijn essentieel voor het begrijpen van de mechanismen die ten grondslag liggen aan de SNP-vorming alsmede van de inclusie en vrijzetting van electrostatisch geladen lading in/vanuit geladen SNPs, welke allebei van uiterst belang zijn voor therapeutische toepassingen zoals medicijn- en genafgifte. Op het gebied van lanthanide-gebaseerde nanodeeltjes, kan het ontdekken van nieuwe synthesesemanieren en het bestuderen van de

eigenschappen van hybride organisch-anorganische SNPs gebaseerd op deze deeltjes, leiden tot een beter en veelzijdiger gebruik van dit interessante type materialen, die steeds belangrijker worden op het gebied van beeldvorming in biologische systemen. We hopen daarmee dat de kennis verkregen in dit proefschrift de ontwikkeling van SNPs als medicijndragers en contrastreagentia in de nano-geneeskunde zal bevorderen.

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About the Author

Laura Graña-Suárez was born 1980 in A Coruña (Spain). She studied chemistry at the University of A Coruña. During her study, she spent one year at the NTNU (Trondheim, Norway) in the Organic Chemistry department as an exchange student. At the NTNU, she investigated the nucleophilic aromatic substitution of nitropyridines. After graduating in Spain in 2006, she worked in Germany for three years at Rilit Lackfabrick GmbH, where she was in charge of the quality testing of several chemical products, and at Elastogran GmbH, where she worked on the development and quality testing of several polyurethane products. In September 2009, she started the master program of chemical engineering at the University of Twente, with specialization in the track “Molecules & Materials”. She did her masters project at the Biomedical Chemistry group, under the supervision of Prof. dr. Piet Dijkstra, where she investigated PEG-PTMC-based hydrogels for drug and protein delivery. Parallel to her study at the University of Twente, she worked at Apollo Vredestein B.V. in the R&D department on the development of a new high-tech product.

Since September 2011, she works as a PhD student at the Molecular Nanofabrication group under the supervision of Prof. dr. ir. Jurriaan Huskens and Dr. Willem Verboom. The aim of her project is to design and study supramolecular systems for biomedical imaging. This project is in collaboration with Dr. Fijs W. B. van Leeuwen from the Leiden University Medical Center.

List of Publications

1. L. Graña-Suárez, W. Verboom, S. Sarkar, V. Mahalingam and J. Huskens, "Host-guest and electrostatic interactions in supramolecular nanoparticles clusters", submitted.
2. L. Graña-Suárez, W. Verboom, S. Sarkar, V. Mahalingam and J. Huskens, "Versatile, fast, and easy one-step method for the synthesis of hydrophilic lanthanide-doped nanoparticles", submitted.
3. S. Sarkar, L. Graña-Suárez, W. Verboom, V. Mahalingam and J. Huskens, "A luminescent nanocrystal marker for the selective and ultrasensitive detection of explosives", *ChemNanoMat*, 2016, DOI: 10.1002/cnma.201600184.
4. L. Graña-Suárez, W. Verboom, T. Buckle, M. Rood, F. W. B. van Leeuwen and J. Huskens, "Loading and release of fluorescent oligoarginine peptides into/from pH-responsive anionic supramolecular nanoparticles", *J. Mater. Chem. B*, 2016, **4**, 4025.
5. L. Graña-Suárez, W. Verboom and J. Huskens, "Fluorescent supramolecular nanoparticles signal the loading of electrostatically charged cargo", *Chem. Commun.*, 2016, **52**, 2597.
6. L. Graña-Suárez, W. Verboom and J. Huskens, "Cyclodextrin-based supramolecular nanoparticles stabilized by balancing attractive host-guest and repulsive electrostatic interactions", *Chem. Commun.*, 2014, **50**, 7280.
7. R. Mejía-Ariza*, L. Graña-Suárez*, W. Verboom and J. Huskens, "Cyclodextrin-based supramolecular nanoparticles for biomedical applications", in preparation (* equal authorship).