**SIZE CONTROL AND SURFACE
FUNCTIONALIZATION OF
MULTIVALENT NON-COVALENT AND
POROUS NANOMATERIALS**

Raquel Mejia-Ariza

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MESA+
INSTITUTE FOR NANOTECHNOLOGY

UNIVERSITY OF TWENTE.

Size Control and Surface Functionalization of Multivalent Noncovalent and Porous Nanomaterials

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SIZE CONTROL AND SURFACE FUNCTIONALIZATION OF MULTIVALENT NON-COVALENT AND POROUS NANOMATERIALS

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 17, 2015 at 16.45 h

by

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Born on June 27, 1982
in Pereira, Colombia

This dissertation has been approved by:

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"Laughter is timeless. Imagination has no age. And dreams are forever."

Walt Disney

This thesis is dedicated to my family.

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Chapter 1

General introduction

Nanomaterials are objects or structures at a scale on the order of a few hundred nm or less. Nanomaterials are one of the main products of nanotechnology, and they can take the shape of nano-scale particles, tubes, rods, or fibers, to mention a few. They are being used successfully in different applications such as electronics,¹ catalysis,² textiles,³ renewable energy^{4,5} and health.^{6,7}

In particular for biomedical applications, nanomaterials have many advantages over traditional approaches including the enhanced bioavailability of drugs and the opportunity to target cells and to create multifunctional diagnostic and therapeutic agents.⁸⁻¹¹ Designing nanoparticles with desired physico-chemical properties such as size, charge, surface hydrophilicity, and the nature and density of surface ligands is important to achieve optimal performance.

The fabrication of nanomaterials through non-covalent interactions, instead of covalent bonds, permits a toolbox approach, in which small molecular and/or nanomaterial components can be designed and assembled into larger functional entities to tune the overall nanomaterials properties.¹² Two examples of such materials classes are supramolecular nanoparticles and metal-organic frameworks.

Supramolecular nanoparticles (SNPs) are particles in which multiple copies of different building blocks are brought together by specific non-covalent interactions, resulting in assemblies that are typically larger than the building blocks themselves.¹³ These SNPs can show good activities in biomedical applications due to their size, surface chemistry (functionalization at the periphery), charge and shape, which, in turn, determine biological properties such as biodistribution, blood circulation, cell uptake and drug release.¹⁴ Molecular recognition has been used to create an easy and flexible toolbox to control the properties of such nanoparticles. The designable and non-covalent nature of the

interactions between the building blocks can be used to control the assembly and disassembly properties, and thereby the uptake and release of cargo.¹⁵

Metal-organic frameworks (MOFs), or porous coordination polymers (PCPs), constitute a heavily investigated class of materials consisting of inorganic (metal ions, metal-organic or inorganic clusters) and organic building blocks (organic ligands, polymers or biomolecules).¹⁶ MOFs have been used in biomedical applications owing to their versatile and controllable properties such as: biodegradability,¹⁷ low toxicity,¹⁸ tunability of pore size to control the encapsulation of molecules such as drugs,¹⁹ gases,²⁰ metal nanoparticles^{21,22} and nucleic acids,²³ water dispersibility,¹⁸ sizes that allow intravenous application,¹⁸ and functionalization of the surface, for example to achieve targeting.^{24, 25}

The research described in this thesis aims to understand the controlled formation of nanosized SNPs and MOFs. The main hypothesis investigated in this thesis is whether these, otherwise very different, materials can be controlled in size and outer functionalization according to the same concept, which is the ratiometric variation of multivalent crosslinkers and monovalent stoppers under stoichiometric control. For SNPs, we have employed multivalent/monovalent host-guest interactions using cyclodextrin host and adamantyl or ferrocenyl guest moieties (Chapters 3 and 4). For MOFs, we used the MIL-88A system, which is based on the Fe_3O secondary binding unit and fumarate as the crosslinker, and incorporated different monovalent carboxylates as capping groups (Chapters 5-7). Control over the assembly and the understanding of the role of different capping groups in size control, surface functionalization and loading of molecules are major objectives of the work presented here.

Chapter 2 provides a literature overview of SNPs and MOFs. Emphasis is placed on the control of formation and size of these materials, the functionalization of their surface, and the loading of molecules for biomedical applications.

In Chapter 3, a turbulent reactor, a so-called multi-inlet vortex mixer, was used to form supramolecular hybrid gold nanoparticle network aggregates using specific host-guest interactions between cyclodextrin and adamantyl groups. The sizes of these aggregates were studied as a function of the host-guest ratio. Finally, the formation of SNPs under turbulent flow was compared with laminar flow and the by-hand technique.

In Chapter 4, SNPs are described that are formed by a redox-switchable assembly/disassembly mechanism, employing ferrocene (Fc) as a guest for binding to cyclodextrin for its loss of affinity upon oxidation. Different parameters, such as ionic strength, the host-guest stoichiometry, and the affinity of the guest moiety and PEG length of the monovalent stabilizer, were varied to determine their influence on SNP formation, size, growth rate, and stability. The reversibility of the SNPs was assessed by studying the influence of oxidation of the Fc moieties.

Chapter 5 describes the synthesis and functionalization of nanoMOFs as multivalent materials. Here, the concept of size control and surface functionalization in one step is applied by controlling the ratio between mono- and multivalent building blocks while maintaining a 1:1 stoichiometry between the binding groups. Different monovalent carboxylates were used as capping ligands to achieve surface functionalization, size control, and biomolecular functionalization.

Based on those findings, in Chapter 6, nanoMOF particles were formed by varying the PEG length and concentration. The PEG coverage was assessed and compared to models to investigate the PEG conformations on the MOF surface. The effect of PEG length on the loading and unloading of a dye, which functions as a model for drug encapsulation, was studied as well.

Finally, Chapter 7 deals with the simultaneous formation of MOF particles and their functionalization to create peptide nucleic acid (PNA)-functionalized MIL-88A for sensing DNA. Both non-covalent and covalent functionalization strategies were investigated. Different complementary and non-complementary DNA strands were used to assess the selectivity of the nanomaterials.

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Chapter 2

Multivalent non-covalent and porous nanomaterials for biomedical applications

Nanomaterials for molecular imaging and therapeutics have become a main focus of research over the last decade. One of the main requirements is the controlled reversibility of assembly and disassembly of the nanoparticles, in order to tune their loading and unloading properties at will. Such properties can potentially be achieved using multivalent non-covalent and porous nanomaterials because of the inherently reversible nature of non-covalent interactions. In this review, two such materials classes are discussed: supramolecular nanoparticles (SNPs) and metal-organic frameworks (MOFs). The choice for these materials is governed by the fact that both contain both multivalent and monovalent building blocks, which provides handles for size control and surface functionalization. For both materials classes, their size, surface functionalization and loading/unloading properties are discussed here.

SNPs are formed using electrostatic and/or host-guest interactions. Particle formation and size are controlled by different parameters, such as the concentration and composition of the building blocks, competition between mono- and multivalent guests and ionic strength. Surface functionalization of SNPs with PEG and tumor-targeting ligands provides improved SNP circulation in the blood stream as well as delivery efficacy. Drug/imaging agents have been encapsulated inside SNPs using electrostatic interactions, covalent bonds and inclusion complexes.

MOFs are composed of metal ions or clusters coordinated with organic building blocks. The following methods are discussed to form and control the sizes of MOFs: hydro/solvothermal, microwave-assisted hydro/solvothermal, ultrasonic, coordination modulation, microfluidic, microemulsions, spray-drying techniques and incorporation of capping ligands. Surface functionalization with PEG is used to prevent opsonic interaction, macrophage uptake and to prolong blood circulation. The MOF surface can further be functionalized with different targeting ligands. Three different methods have been used to encapsulate drug/imaging agents into MOFs. One method consists of using drug/imaging agents as linkers. The second method is the encapsulation of drugs inside the MOF matrix. The third is the encapsulation of drugs by their adsorption into the MOF's pore structure. Finally, an application is discussed showing the encapsulation of nitric oxide in MOFs.

Generally, the stable and reversible nature of SNPs and MOFs make these systems promising candidates for developing drug delivery vehicles, in which control over their size, targeting properties, and drug encapsulation and release can be achieved.

2.1 Introduction

The use of nanoparticles (NPs) for biomedical applications has attracted a lot of interest, in particular nanoparticle-based diagnostics that focuses on *in vitro* biomarker detection and *in vivo* imaging. Nanoparticles have been extensively used to enhance the contrast in magnetic resonance imaging (MRI),¹ positron emission tomography (PET),² and computed tomography (CT).³ Nanoparticles are also considered for therapeutics, such as drug delivery,⁴ tissue engineering,^{5, 6} and the separation and purification of biological molecules and cells.⁷ All these applications are possible owing to the chemical and physical properties originating from the nanoparticles' small dimensions, such as size, surface chemistry, charge, morphology, reactivity, optical and magnetic properties. General requirements for designing nanoparticles for diagnostic and therapeutic applications include: control of particle formation and size, colloidal stability, biocompatibility of the materials by surface functionalization, binding of particles to proteins and cell membranes, biodistribution (clearance by liver and kidney; entry into tumors (Enhanced Permeability and Retention, (EPR)), cell uptake (phagocytosis, endocytosis, and pinocytosis),⁸ and circulation in the blood stream.⁹ Researchers have used different nanomaterials to achieve these properties such as liposomes,¹⁰ polymeric nanoparticles,¹¹ micelles,¹² and inorganic particles.¹³⁻¹⁵

For drug encapsulation and delivery, controlled reversibility of the assembly and disassembly of the nanoparticles is needed to control the release of the drugs after encapsulation. To achieve an efficient therapy with nanocarriers, certain requirements should be met:¹⁶ 1) efficient encapsulation of drugs with high payloads, 2) controlled drug release to avoid the 'burst effect', 3) control over the matrix degradation, 4) the possibility to easily engineer the nanoparticles' surface for *in-vivo* applications, and 5) the ability to detect the nanocarriers using imaging techniques. These challenges can be overcome by designing nanoparticles with certain physico-chemical properties such as size, charge, surface hydrophilicity, and the nature and density of the surface ligands.

Using non-covalent interactions to create nanomaterials permits a toolbox approach, in which small components are chosen to tune the overall materials properties.¹⁷ The inherently reversible nature of non-covalent interactions further allows a reversible

assembly process which can be used to provide (triggered) release. Therefore, multivalent non-covalent and porous nanomaterials, like supramolecular nanoparticles (SNPs) and metal-organic frameworks (MOFs), are used to construct reversible systems which meet the requirements discussed above. These materials provide a high functional affinity during formation, and thus can produce nanoparticles adapted to specific applications. Therefore, SNPs and MOFs will be discussed here as design platforms to form multifunctional nanoparticles for medical diagnostic and therapeutic applications.

To build three-dimensional structures of non-covalently interacting building blocks, multivalency is important. It describes the interaction between multivalent receptors and multivalent ligands.¹⁸ Reasons for the prevalence of supramolecular multivalent bonds in nature are the stability, reversibility and specificity that can be achieved with this type of interaction.¹⁹ An example of multivalency in biological processes is the ligand-receptor interactions that occur at cell surfaces.^{20, 21} It plays a fundamental role in several intra- and intercellular processes as well.²² Multivalency is also important to enhance the overall affinity of non-covalent protein-protein and carbohydrate-protein interactions.²³ These interactions facilitate recognition processes, immune response and cell differentiation. Signal transduction mechanisms also use multivalency.²⁴ For example, some membrane receptors (such as protein kinases) can be held in close proximity to other transmembrane proteins by a multivalent ligand. This promotes the formation of clusters and the phosphorylation of cytosolic fragments of substrate proteins. This type of interaction provides a way to propagate the signal through the cell membrane.

SNPs and MOFs constitute advantageous 3D reversible porous materials. On the one hand, SNPs have the advantage of a design platform which provides a quick method to generate structural diversity to obtain the formulation with the best biological performance.¹⁷ On the other hand, MOFs are important because they are crystalline and porous materials with low densities ($0.2\text{--}1\text{ g/cm}^3$), high surface areas ($500\text{--}4500\text{ m}^2/\text{g}$), high porosities and reasonable thermal and mechanical stability.^{25, 26} The field of SNPs can be divided into two different classes. We focus here on soft SNPs, which are formed based on electrostatic or host-guest interactions for biomedical applications. Numerous reviews have appeared that summarize the formation of SNPs, using either hydrogen bonding,²⁷⁻²⁹ solely electrostatic interactions³⁰⁻³² or organic-inorganic hard SNPs.³³⁻³⁶ With respect to MOFs,

our sole focus is on MOFs for biomedical applications. Several reviews have appeared that summarize the use of MOFs for other applications such as catalysis,^{37, 38} gas storage³⁹ and gas separation.⁴⁰

2.2 Supramolecular nanoparticles

Supramolecular nanoparticles (SNPs) are particles in which multiple copies of different building blocks are brought together by specific non-covalent interactions, resulting in assemblies that are typically larger than the building blocks themselves.⁴¹ The activity of a nanoparticle in a biological environment depends on its size, surface chemistry (functionalization at the periphery), charge and shape.⁴² Researchers aim to use molecular recognition to create an easy and flexible toolbox to control the properties of these nanoparticles, and to employ the non-covalent nature of the interaction to control the assembly and disassembly properties, and thereby the uptake and release of cargo.⁴³

2.2.1 Formation and size control of SNPs

SNPs (see Figure 2.1) consist of building blocks that are assembled by multiple non-covalent interactions. Each building block has a given number of host and guest moieties. One of the main hosts used in supramolecular nanoparticles is β -cyclodextrin (CD), which interacts with a multitude of small organic molecules by hydrophobic interaction. CDs are used in a wide range of biomedical applications because they enhance the solubility of encapsulated molecules, such as drugs, in aqueous solution.⁴⁴⁻⁴⁶ As a guest moiety, adamantane (Ad), among others, has been extensively used owing to its high binding affinity with CD. A monovalent guest is added during the SNP assembly process to avoid unlimited growth and possible precipitation of the particles. The forces involved in the host-guest complexation are Van der Waals, hydrophobic, and dipolar interactions.⁴⁷

Figure 2.1 Schematic representation of SNP formation.

One of the first approaches (see Figure 2.2) used for a non-viral gene delivery system was introduced by Davis *et al.*⁴⁸ In this approach, the core of the SNPs is formed solely by electrostatic interactions, while CD was introduced to allow additional stabilization and functionalization using monovalent Ad derivatives. Correspondingly, the core of these SNPs is composed of positively charged CD-containing polymers and negatively charged small interfering RNA (siRNA), which form polyplexes. Because these polyplexes are unstable under *in-vitro* conditions, a monovalent stabilizer is added. This monovalent stabilizer is a neutral adamantyl-grafted poly(ethylene glycol) (Ad-PEG) and is incorporated at the surface using host-guest interactions between CD and Ad. The PEG chains are assembled on the outside of the SNPs, providing stability and prohibiting aggregation at physiological ionic strength and increasing cellular uptake of the gene delivery vehicles.^{49, 50}

In a related concept, aiming for gene delivery, imaging and drug encapsulation, SNPs are formed solely by host-guest interactions (see Figure 2.3).⁵¹⁻⁵⁸ A modular synthetic approach was reported⁵¹ with CD/Ad recognition to achieve self-assembly of SNPs using three different molecular building blocks, as shown in Figure 2.3. This SNP system is similar to the one shown in Figure 2.1, having a core composed of multivalent hosts and multivalent guests linked by host-guest interactions. A monovalent stabilizer is pivotal for avoiding aggregation and precipitation. The three building blocks are poly(ethylene imine) grafted with CD (CD-PEI) as a multivalent host, Ad-poly(amidoamine) dendrimer (Ad-PAMAM) using two different generations (with 4 and 8 Ad moieties, respectively) as a multivalent guest, and Ad-PEG as a monovalent guest and stabilizer. In this work,

multivalent/monovalent competition between Ad-PAMAM and Ad-PEG was put forward as the means to control the particle size. The SNP size could be tuned in the range of 30 to 450 nm by increasing the amount of multivalent guests in the core, by keeping the host and stopper concentrations constant, and by having an excess of stopper to avoid precipitation.

Figure 2.2 a) SNP formation by polyplex formation stabilized by addition of Ad-PEG and Ad-PEG functionalized with a cell-targeting ligand. b) Chemical structures involved in the formation.⁴⁸

Figure 2.3 a) SNP formation mediated by host-guest interactions of Ad₈-PAMAM and Ad-PEG with CD-PEI. b) Structures CD-PEI, Ad₈-PAMAM and Ad-PEG.⁵¹

In order to provide better control over the assembly process and the size distribution, Tseng's group developed a digital microfluidic droplet generator under laminar flow.^{59, 60} Using this technique, the creation of a library of 648 different SNPs within 2.5 h was possible with a broad variation in structure, functionality, size, surface chemistry, and DNA loading capacity. The processing parameters were precisely controlled with this approach, and multiple samples were prepared in parallel. This enabled a high batch-to-batch reproducibility and robust production of SNPs with a narrow size distribution. The advantages of this technique are reduced human operational errors, accelerated handling procedures, enhanced experimental fidelity, and economical use of reagents. In addition, the surface chemistry of the SNPs was fine-tuned by incorporating additional building blocks functionalized with specific ligands for targeting cells. The size and surface properties of these SNPs correlated well with the efficiency of the cellular uptake. This study thus showed a feasible method for microfluidics-assisted SNP production and provided a means for preparing size-controlled SNPs with a desired surface ligand coverage.

Larsen *et al.*⁶¹ showed the formation of SNPs composed of two types of polymers with the same dextran backbone, modified with Ad or CD. The size of the SNPs was controlled by substitution of CD and Ad and the concentration and composition of the mixtures. Our group⁶² formed SNPs using a multicomponent system based on a linear negatively charged polymer and a monovalent stabilizer as shown in Figure 2.4. The SNPs dispersed in water and phosphate-buffered saline (PBS) did not need any stabilizer and particles were stable over time. However, upon increasing the ionic strength above 1 M KCl, a stopper was needed to prevent aggregation. The main finding in this work is that SNP size is controlled by a balance of forces between attractive supramolecular and repulsive electrostatic interactions.

Figure 2.4 a) Schematic representation of SNPs using poly(isobutyl-alt-maleic acid) (PiBMA) grafted with CD and *p*-tert-butylphenyl groups (TBP). b) Building blocks: molecular structures of the host polymer PiBMA grafted with CD (PiBMA-CD), and the guest polymer PiBMA grafted with TBP (PiBMA-TBP).⁶²

Our group⁴¹ showed the formation of size-tunable SNPs using cucurbit[8]uril (CB[8]) as the host, instead of CD that was used in previous examples. Four different building blocks were used in this case, namely methyl viologen (MV)-functionalized PEI (MV-PEI), naphthol-PAMAM dendrimer (Np-PAMAM), Np-PEG, and CB[8], which led to SNPs held together by multiple ternary complexes as shown in Figure 2.5. Here, size control of these SNPs was achieved by varying the ratio of monovalent Np-PEG to multivalent Np-PAMAM, while keeping the overall Np concentration constant and maintaining an equimolar CB[8]–MV–Np stoichiometry. Particle formation was shown to depend on the temperature. At higher temperatures the required rearrangement of building blocks to form SNPs was enhanced which led to faster SNP formation. This time-dependent particle formation has not been observed so far for SNPs based on CD–Ad host–guest interactions. In all previously reported cases, SNP formation was observed directly after mixing the supramolecular components.

The concept of forming ternary complexes with CB[8] was also applied to create supramolecular nanoparticle clusters (SNPCs) with controlled sizes, mediated by multiple hetero-ternary host–guest interactions, using a microfluidic device.⁶³ Here the diffusive mixing was determined by the diffusion profiles of the molecular components CB[8], Np₈-

PAMAM, and Np-PEG into the SiO₂-MV nanoparticle stream. The size of the SNPCs strongly depended on the concentration of multivalent Np dendrimers, the residence time of the interacting building blocks within the microchannel, and the stoichiometry of the ternary host-guest binding partners. Kinetic control appeared to be mandatory to obtain stable SNPCs. This was proven by comparing the residence time variation as well as the discrepancy between clustering experiments carried out in bulk and in the microfluidic reactor. The molecular weight of the PEG stopper offered an additional kinetic control parameter to the assembly process. Apparently, a longer stopper led to slower diffusion into the NP stream and concomitantly a later termination of the clusters.

The CB[8] system was further used to form dual stimuli-responsive SNPs.⁶⁴ Azobenzene (Azo) groups were used instead of Np to allow phototriggered disassembly of the SNPs. The SNPs were based on the ternary host–guest interaction generated from CB[8], MV polymer, and mono- and multivalent Azo, and the particle size was controlled by the stoichiometry of the multi/monovalent Azo derivatives. Disassembly of the SNPs was observed after photochemical conversion from trans- to cis-Azo upon irradiation with UV light. Isomerization back to trans-Azo was induced by visible light, which led to full restoration of the SNPs. Furthermore, the SNPs were disassembled by chemical reduction of the MV moieties.

Figure 2.5 a) Supramolecular nanoparticle formation by ternary complex formation between CB[8], MV and Np moieties. b) Supramolecular building blocks involved in particle formation: MV-PEI, Np-PEG, CB[8], Np-PAMAM.⁴¹

2.2.2 Functionalization of the SNP surface

SNPs have been functionalized for drug targeting and improving SNP circulation in the blood stream (see Figure 2.6).^{52, 56, 60, 65, 66} PEG has been used as a capping ligand to suppress protein adsorption and to increase retention time.^{52, 53, 66} PEG minimizes the surface charges of the nanoparticle because of its neutral and well-hydrated polymer backbone, which reduces aggregation by particle-particle interactions. It also limits potential electrostatic interactions with other materials during circulation which are mainly negatively charged, including the plasma membrane of cells.⁸

Targeting ligands have been incorporated to modify the surface chemistry of SNPs. These ligands can dock onto overexpressed receptors observed at the surface of tumor cells to improve the delivery efficacy.^{17, 67} Examples of targeting ligands are RGD peptides which have been covalently attached to Ad-PEG.^{52, 60} These ligands have been assembled on the surface of SNPs together with unmodified Ad-PEG and free CD to provide combined PEGylation and presentation of targeting ligands. The RGD sequence improved the cellular uptake efficacy in tumor tissue because RGD-binding $\alpha_v\beta_3$ integrins are overexpressed on the surface of tumor cells. Davis *et al.*⁴⁸ used Ad-PEG-transferrin (Ad-PEG-Tf) to target cancer cells. SNPs as shown in Figure 2.2 were used, where Tf was used to bind transferrin receptors that are commonly upregulated in cancer cells.

Figure 2.6 Two-step assembly for the preparation of a small library of DNA-encapsulated supramolecular nanoparticles with controllable sizes and tunable RGD ligand coverage (© RSC⁵²).

2.2.3 Loading of SNPs

Many potential drug candidates fail in preclinical studies due to low solubility, low stability and high toxicity. The advantage of using SNPs for drug encapsulation is that they provide the option of incorporating different components including drugs, as well as different pathways to release these drugs in a controlled manner.¹⁷

Negatively charged drugs have been encapsulated by binding to positively charged building blocks, in which case the electrostatic interaction is at the same time the main driving force for the SNP assembly process.^{48, 66} Using this concept, SNPs (Figure 2.2) were formed to encapsulate and deliver siRNA in humans, as shown in Figure 2.7. These SNPs were sufficiently large (~70 nm) to avoid clearance via the kidney and were found to localize in tumors. The Tf on the nanoparticle is able to bind to transferrin receptors (TfR) on cancer cells, and the SNPs were internalized via receptor-mediated endocytosis. The CD-PEI contained amine groups that were protonated at a pH of about 6. This chemical sensing mechanism provided an escape route from endocytic vesicles and allowed nanoparticle-mediated release of the nucleic acid cargo into the cytoplasm. The components were small enough to be cleared from the body via the kidney upon disassembly of the SNPs.

Charged drugs have been encapsulated into a positively charged core of pre-formed SNPs held together by host-guest interactions. Tseng's group used this concept to incorporate multivalent guests into the core of SNPs to encapsulate different molecules using electrostatic interactions such as DNA^{52, 53, 67} for the targeted delivery of genes (see Figure 2.6) and transcription factor (TF) vectors (see Figure 2.8a).⁵⁶ The DNA loading capacity of SNPs depends on the net cationic charges present in the interior of the Ad-PAMAM/CD-PEI hydrogel network.⁵² As mentioned before, Tseng's group also constructed a rapid development pathway toward highly efficient gene delivery systems using a microreactor.^{59, 60} Using this microreactor, a broad structural/functional diversity can be programmed into a library of DNA-encapsulated SNPs by systematically altering the mixing ratios of the molecular building blocks and a DNA plasmid.⁵³ TF was encapsulated into cationic SNPs by introducing anionic characteristics to the TF. A DNA plasmid with a matching recognition sequence specific to a TF was employed to form an anionic TF/DNA complex, which was subsequently encapsulated into SNPs.⁵⁶ Moreover,

Zhao *et al.*⁶⁸ fabricated polyacrylate SNPs for loading of the anticancer drug doxorubicin (DOX) for targeted delivery both *in vitro* and *in vivo*. Here, the SNPs were formed through the self-assembly of CD-modified polyacrylic acid (PAA), Ad-modified polyacrylic acid and Ad-PEG in the presence of DOX and Ad-conjugated fluorescein for fluorescence-tracing purposes. The DOX encapsulation relied on the formation of electrostatic interactions between the amino group of DOX and the free carboxylate groups on the PAA backbone within the SNPs.

Figure 2.7 Schematic representation of how SNPs target cancer cells: a) Assembly of the SNPs. b) SNPs in aqueous solution infused into patients. c) SNPs circulate in the blood stream and escape via the “leaky” blood vessels in tumors. d) SNPs penetrate through the tumor and enter into cells by receptor-mediated endocytosis (transmission electron micrograph of 50 nm nanoparticles entering a cancer cell). e) Targeted SNPs can have numerous interactions (e.g., Tf with its receptor) with the surface of the cancer cell that stimulate entrance into the cell (© ACS⁴⁸).

Imaging agents/drugs have been covalently attached to one of the SNP building blocks. For example, a gadolinium(III) (Gd^{3+}) MRI contrast agent (see Figure 2.8b) was incorporated using this concept to improve relaxivity and sensitivity.⁵⁵ Here, the Gd^{3+} complex was covalently conjugated onto a CD-PEI building block. The distinct chemical environment with a high Gd^{3+} loading capacity inside the pseudo-porous polymer-dendrimer hydrogel network enhanced the relaxivity and provided good water accessibility. By altering the mixing ratios of Gd^{3+} -DOTA-CD-PEI and the other two molecular building blocks (Ad-PAMAM and Ad-PEG), a small library of SNPs of different sizes containing Gd^{3+} was produced. In another example, the anticancer drug camptothecine (CPT) was covalently attached (see Figure 2.8c).⁵⁷ CPT was covalently attached to the negatively charged poly(L-glutamic acid) (PGA) (CPT-PGA). By the same electrostatic concept, the negatively charged CPT-PGA interacted with the positively charged CD-PEI. The encapsulation of CPT-PGA into SNPs was achieved by simply mixing CPT-PGA with CD-PEI, Ad-PAMAM and Ad-PEG.

Figure 2.8 Different examples of how drugs/imaging agents have been encapsulated inside SNPs: a) delivery agent of TF (using electrostatic interactions) (© Wiley⁵⁶). b) Gadolinium(III) MRI contrast agent (using covalent bonds) (© Elsevier⁵⁵) c) Therapeutic efficacy anticancer drug: camptothecine (using covalent bonds) (© Elsevier⁵⁷) d) Magnetothermally responsive DOX-encapsulated SNPs (using π - π stacking) ((© Wiley⁵⁸).

Other methods were used to encapsulate drugs inside SNPs. For example, DOX was encapsulated in the core of SNPs using intermolecular π - π stacking as shown in Figure 2.8d.⁵⁸ These SNPs contained superparamagnetic nanoparticles to give a responsive system by which the disassembly of the SNPs was triggered using an alternating magnetic field. Couvreur *et al.*⁶⁹ encapsulated benzophenone in SNPs using two methods, either using an inclusion complex with CD before mixing or by direct loading into preformed nanoassemblies using CD-PEI as a host and dextran-bearing hydrophobic lauryl side chains as a guest building block. Moreover, Ma *et al.*⁶⁵ encapsulated and released dexamethasone, a highly hydrophobic steroidal anti-inflammatory, by inclusion complexation with CD-PEI as host and poly(β -benzyl-L-aspartate) as guest.

2.3 Metal-organic frameworks

The field of biomedicine needs well-defined carriers of, for example, drugs or imaging agents, often in the form of nanoparticles. To use nano-sized MOFs for intravenous drug delivery,⁷⁰ different properties are required such as: biodegradability,⁷¹ low toxicity,¹⁶ tuned pore size to control the encapsulation of molecules such as drugs,⁷² gases,³⁹ metal nanoparticles,^{73, 74} and nucleic acids,⁷⁵ water dispersibility,¹⁶ monodisperse particle sizes tunable to less than 200 nm,¹⁶ and controllable composition and functionalization of the surface.^{76, 77}

2.3.1 Formation and size control of MOFs

Metal-organic frameworks (MOFs), or porous coordination polymers (PCPs), constitute a heavily investigated class of materials consisting of inorganic (metal ions, metal-organic or inorganic clusters) and organic building blocks (organic ligands, polymers or biomolecules) as shown in Figure 2.9.⁷⁸ MOFs have strong bonds which provide robustness and a geometrically well-defined structure.²⁶ MOF compositions are tuned by varying the metal or the organic linker and by functionalizing the organic linker during or after synthesis.⁷⁹

Figure 2.9 Schematic representation of MOFs.

In particular the particle size is a limiting factor for intravenous administration. For that reason, the preparation of monodisperse, well-defined, reproducible, and stable nanoparticles has been investigated using different methods as shown in Figure 2.10.⁸⁰ The desired shape and size of MOFs are achieved by choosing suitable synthetic strategies and reaction conditions, such as the type of solvent,⁸¹ the nature, concentration and stoichiometry of the reagents,⁸² reaction conditions (temperature, time and radiation intensity),⁸³ and emulsion parameters such as the ratio between the surfactant, the oil and the aqueous phase.⁸⁴

Figure 2.10 Overview of synthesis methods, possible reaction temperatures, and final reaction products in MOF synthesis (© ACS⁸⁰).

Different hydro/solvothermal methods have been used to synthesize and control the sizes of MOFs. For example, MIL-88A (MIL stands for Materials from Institut Lavoisier) was synthesized in water, methanol and dimethylformamide (DMF).⁸³ Here the solvent proved crucial to tune the size of the nanoparticles. Nanoparticles formed in water and methanol resulted in particle diameters of 1050 nm and 960 nm, respectively, while formation in DMF gave smaller particle diameters of 430 nm. This was attributed to the higher solubility of fumaric acid in DMF than in alcohols and water as well as the higher dipole moment of DMF compared to other solvents. A higher dipole moment of the solvent typically changes both the solubility of the linker and the solvent–nanoparticle interface, changing the interfacial tension and thus strongly affecting the nanoparticle size. Increasing the pH or the reaction time led to the formation of inorganic by-products. Temperature and reaction time had also an effect on nanoparticle formation. For example, nanoparticles formed in water at 65 °C led to a diameter of 275 nm. By increasing the temperature to 150 °C, the diameter increased to >1200 nm. At short reaction times (0.5 h at 65 °C), nanoparticles were formed with particle diameters around 110 ± 25 nm, while after 24 h nanoparticles were obtained with diameters around 1030 ± 90 nm.

The microwave (MW)-assisted hydro/solvothermal method is one of the most common procedures to synthesize nanoMOFs. This technique allows for faster crystallization times, phase selectivity, narrower particle size distributions and often controls the morphology.⁸³ It also speeds up nucleation rather than crystal growth. A high dielectric absorptivity of polar solvents results in thermal conversion of the energy and thus in more efficient heating of the solution, which promotes the dissolution of the reactants. This rapid and local superheating is used to overcome the nucleation energy barrier at high local concentrations (hot spots) above the supersaturation threshold, which leads to fast and homogeneous nucleation. MOFs were synthesized using this methodology to obtain environmentally favorable aqueous solutions of non-toxic iron(III) carboxylate nanoMOFs (MIL-53, MIL-88A, MIL-88B, MIL-89, MIL-100 and MIL-101_NH₂) for the delivery of anticancer or antiviral drugs.¹⁶ In this work, particle diameters were obtained ranging from 50–350 nm. The same group synthesized MIL-88A in aqueous solution at different temperatures, reaction times, concentrations and pressures.⁸³ As discussed above for hydrothermal conditions, the particle diameter increased at increased reaction temperature and time.

Moreover, if the concentration and pressure were increased, the particle diameters increased as well. It was demonstrated that this method was the fastest and easiest route when compared with other hydrothermal and ultrasonic methods. The same group also studied the size of MIL-100(Fe) in different simulated physiological media at 37°C.⁸⁵ The purpose of this investigation was to study the colloidal and chemical stability of nanoMIL-100(Fe) for intravenous and oral administration. Nanoparticles in water were formed with a diameter of ~141 nm. Using different simulated physiological media, the nanoparticle diameter increased up to 254 nm. Here, the presence of phosphates replaced the carboxylates from the nanostructure. This provoked a progressive degradation of MIL-100(Fe). Finally, the same group synthesized MIL-100(Fe) functionalized with CD with a particle size of ~200 nm in diameter for drug encapsulation and release for HIV treatment.⁸⁶

Ultrasound is a rapid, facile and environmentally friendly technique, widely used for organic synthesis.⁸³ This method provides a rapid synthesis and allows tuning of the reaction kinetics. It, however, leads sometimes to metastable or new phases. The first example of a MOF synthesized using ultrasound was $\text{Zn}_3(\text{BTC})_2 \cdot 12\text{H}_2\text{O}$, (where BTC is 1,3,5-benzenetricarboxylic acid) used for the selective sensing of organoamines.⁸⁷ This MOF was synthesized at ambient temperature and atmospheric pressure for different reaction times. Here, nanoparticles were obtained with diameters of 50–100 nm for short reaction times (5 and 10 min). After 30 min, the diameters increased to 100–200 nm and after 90 min, the diameters increased to 700–900 nm. Moreover, MIL-88A was synthesized using this procedure.⁸³ Small nanoparticles with diameters of 380 ± 35 nm were obtained by adding acetic acid (using low concentrations at 15 min) while the addition of NaOH led to the formation of larger and highly polydisperse nanoparticles with diameters > 1200 nm. Finally, by further decreasing the temperature down to 0 °C using dilute conditions (0.01 and 0.008 M of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), smaller monodisperse nanoparticles with particle diameter < 330 nm were obtained with or without additives.

The coordination modulation method was introduced by Kitagawa *et al.*⁸⁸ to synthesize nanoMOFs. This method employs capping ligands (modulators) which affect the framework extension of MOFs and determine the crystal features. The modulators have the same chemical functionality as the organic linkers: to impede the coordination interaction

between the metal ions and the organic linkers. This generates a competition that regulates the rate of framework extension and crystal growth. In this study, the MOF was synthesized by adding a solution of 1,4-naphthalenedicarboxylic acid and 1,4-diazabicyclo[2.2.2]octane in DMF to a solution of copper acetate in the presence of an excess of acetic acid (modulator). This resulted in nanorods with average diameters of the major and minor axes of 392 ± 210 nm and 82 ± 23 nm, respectively. Zhang *et al.*⁸⁹ used this method to synthesize nanoMOFs with a combination of a proper acid-base environment. Here the Dy(BTC)(H₂O) MOF with a diameter of 50 nm was fabricated using sodium acetate as the modulator. The proper acid–base environment governs deprotonation of the organic linker and hence the nucleation process. The diameter of Dy(BTC)H₂O was controlled from the micrometer to the nanometer range altering the amount of sodium acetate added to the synthesis solution. However, an excess of sodium acetate led to amorphous materials. Sodium acetate significantly accelerated the rate of crystal growth. This acceleration was attributed to the basicity of the sodium acetate which promoted the deprotonation of the organic linkers and hence the nucleation rate.

A microfluidic approach was used to synthesize HKUST-1 (HKUST stands for Hong Kong University of Science and Technology), UiO-66 (UiO stands for Universitetet i Oslo) MOF-5, and IRMOF-3 (IR stands for isorecticular = based on the same net, having the same topology) under various solvothermal conditions.⁹⁰ This technique provides an alternative platform with continuous and ultrafast production of distinctive morphologies. These MOFs were confined in droplets within a few minutes, with higher reaction kinetics than in conventional batch processes. First HKUST-1 was synthesized with 5–15 μ m in diameter. The production rate of HKUST-1 in the microfluidic system was ~ 5.8 kg/(m³ day), whereas in a small-scale laboratory, the production rate was between 0.1 and 1 kg/(m³ day). MOF-5 and IRMOF-3 were produced with cubic crystals in the diameter range of 10–15 μ m, which was smaller than the diameter obtained from conventional solvothermal products but close to the size of sonochemical products.

The microemulsion method was also used to synthesize MOFs. The first MOF example synthesized using this method was the formation of nanorods of Gd(BDC)_{1.5}(H₂O)₂ (where BDC is 1,4-benzenedicarboxylate).⁸⁴ The morphology and size of the nanorods were influenced by the *w* value (defined as the water/surfactant molar ratio)

of the microemulsion systems. For example, nanorods of 100-125 nm in length and 40 nm in diameter were obtained at $w = 5$ and with a Gd^{3+} concentration of 50 mM. As w was increased to 10, nanorods of 1-2 μm in length and 100 nm in diameter were obtained. The particle size was also affected by the reagent concentration and their ratio. A decrease in the concentration of the reagents or a deviation of the metal-to-ligand molar ratio resulted in a decrease of the particle size. Although such a synthetic procedure produced nanoMOFs of several metal/ligand combinations, it led to gel-like amorphous materials due to a rapid and irreversible metal–ligand coordination bond formation at room temperature. Therefore, the same group used this concept at elevated temperatures and demonstrated the potential use of nanoMOFs as imaging and optical contrast agents.⁹¹ For this synthesis, $w = 10$ was used to form nanoparticles of $\text{Gd}_2(\text{bhc})(\text{H}_2\text{O})_6$. The resulting MOFs were block-like nanoparticles of approximately 25 by 50 by 100 nm. These particles were found to have a modest longitudinal relaxivity and a high transverse relaxivity. The same group used Mn^{2+} instead of Gd^{3+} because Mn^{2+} is less toxic.⁹² This concept was used to obtain MOFs with a diameter of 58 ± 11 nm for the delivery of Pt-based drugs to cancer cells.⁹³

Maspoch *et al.*⁹⁴ used for the first time the spray-drying method to synthesize nanoMOFs. This technique drastically reduced the production time and costs of nanoMOF formation, and enabled a continuous and scalable synthesis as well as solvent recovery. The concept of this strategy mimics the emulsion methodology. However, spray drying does not require secondary immiscible solvents, surfactants, emulsifiers or agitation (i.e. stirring or sonication). NanoHKUST-1 crystals were first formed using this technique. To obtain this, superstructures of HKUST-1 with diameter 2.5 ± 0.4 μm were synthesized at first. Then, nanoHKUST-1 particles with diameters of 75 ± 28 nm were obtained by sonicating these superstructures. The same concept was applied to form other types of nanoMOFs: Cu-bdc (105 ± 18 nm), NOTT-100 (60 ± 16 nm), MIL-88A (300 ± 60 nm), MIL-88B (130 ± 20 nm), MOF-14 (98 ± 26 nm), Zn-MOF-74 (130 ± 25 nm) and UiO-66 (30 ± 6 nm). The same group designed MOFs containing polymer composites with enhanced hydrolytic stability to protect the MOFs from hydrolytic degradation.⁹⁵ In a proof-of-concept study, HKUST-1 was chosen because it is a water-sensitive MOF, very porous, and with a high surface BET area of ~ 1400 m^2/g , and is considered among the best MOFs for CH_4 storage

at room temperature. Polystyrene was chosen as the organic polymer because it is strongly hydrophobic and water resistant.

Incorporation of capping ligands has been used to control the MOF size. This method offers size control and surface functionalization at the same time. One example is by Gref *et al.*,¹⁶ where PEG capping ligands were added to synthesise nanoMOFs, MIL-88A (150 nm), MIL-89 (50-100 nm), and MIL-100 (200 nm). Moreover, Lin *et al.*⁹⁶ synthesized nanoUiO-66 with silica and poly(ethylene glycol) (PEG) with hydrodynamic diameters of 246 nm. These particles contain high contents of Zr and Hf metals, making them good contrast agents.

2.3.2 Functionalization of the MOF surface

Surface functionalization of nanoMOFs is essential for their use in biomedical applications. Specifically for intravenous applications, adjuvants and active ligands are needed. Adjuvants are needed to prevent aggregation between particles and to enhance biocompatibility in physiological media. Active ligands provide specific binding to guide the particles to the desired place in the body.

PEG is one of the most common adjuvants to shield the surface and to prevent opsonic interaction, macrophage uptake, and to guarantee prolonged blood circulation. One example has been reported by Gref *et al.*¹⁶ where they incorporated PEG on the surface of MIL-88A and MIL-89. Moreover, as mentioned in the previous section, nanoUiO-66 was synthesized and functionalized with silica and PEG to enhance biocompatibility and stability.⁹⁶ Another approach was used to introduce PEG on the surface of the nanoMOFs.⁸⁶ MIL-100(Fe) was first synthesized and functionalized with CDs. Then, Ad-PEG was incorporated on the surface using inclusion complexes between Ad and CD. These complexes bound strongly to the nanoMOFs, and were firmly anchored. In contrast, PEG chains without inclusion complexes poorly interacted with the nanoMOFs.

MOF surfaces have been functionalized with targeting ligands in many studies. Boyes *et al.*⁷⁶ developed Gd MOF nanoparticles, the surface of which was modified by covalent attachment of polymers containing both a targeting ligand and an antineoplastic agent to produce a novel theranostic nanodevice (see Figure 2.11). GRGDS was used here as the targeting ligand. Fluorescent microscopy demonstrated that there was no specific binding

for the nanoparticles. Moreover, nanorods composed of $\text{Mn}(\text{BDC})(\text{H}_2\text{O})_2$ were synthesized by coating with silica shells to stabilize and provide biomolecular functionalization using RGD and a fluorophore for the use in MRI and in targeting cancer cells.⁹² A post-synthetic modification by click chemistry was used to covalently functionalize the surface of MOFs with oligonucleotides.⁷⁵ This was possible by using the strain-promoted click reaction between DNA appended with dibenzylcyclooctyne (DBCO) and azide-functionalized UiO-66- N_3 to create a nanoparticle-nucleic acid MOF as shown in Figure 2.12. Finally, a mannose-bearing CD derivative was functionalized to the surface of the nano MIL-100(Fe). Human retinoblastoma cell line Y79, known to overexpress the mannose receptor, was incubated with the nanoMOF and the nanoparticle uptake was evaluated by intracellular iron quantification.⁸⁶

Figure 2.11 Polymer-modified Gd nanoMOFs as a nanomedicine system for targeted imaging and treatment of cancer (© ACS⁷⁶)

Figure 2.12 a) DNA functionalization of UiO-66- N_3 nanoparticles using DNA functionalized with DBCO. b) Click reaction between a MOF strut and DNA (© ACS⁷⁵).

2.3.3 Loading of drugs into MOFs

NanoMOFs are developed to deliver cancer therapeutics and as bioimaging agents, owing to their high porosity, good biocompatibility, low toxicity, and controlled drug encapsulation and release.⁹⁷ MOFs can be composed of nontoxic metals (Fe, Zn, Ca, Mg, etc.) and low toxicity carboxylic or phosphonic acids. Most MOFs are to some degree biodegradable upon exposure to an aqueous medium. The structure is composed of hydrophilic–hydrophobic matter that can host a large variety of active molecules with different chemistries. Drug delivery is possible by tuning the host–guest interactions through the incorporation of polar or apolar functional groups. Also the diffusion of the drugs through the porous structure is controlled by changing the structure of the solid (interconnectivity, pore size, flexibility).⁹⁸ A general scheme (see Figure 2.13) shows how drugs are encapsulated and released under *in vivo* conditions.⁹⁹

Figure 2.13 a) Generalized scheme for the use of MOFs as drug delivery vehicles. b) *In vivo* conditions involved in the slow release of drugs (© ACS⁹⁹).

Few groups encapsulated imaging agents/drugs as linkers inside the MOFs. Lin *et al.*⁹³ synthesized nanoMOFs with a diameter of 58 ± 11 nm composed of Pt(IV) based anticancer drugs as linkers and Tb^{3+} metal ions, as shown in Figure 2.14. These particles were stabilized by shells of amorphous silica to control the release of the Pt species. The anticancer efficacy of the nanoMOFs was demonstrated on multiple cancer cell lines *in vitro*. The same group synthesized nanoMIL-101(Fe) with an average diameter of ~ 200 nm.¹⁰⁰ The presence of amino groups on the nanoMIL-101(Fe) allowed a covalent attachment of an imaging contrast agent and an anticancer drug (prodrug of cisplatin) using post-synthetic modifications. Because of the instability of the nanosized MIL-101(Fe) particles in PBS buffer, particles were coated with a thin silica layer to slow down the cargo release. The nanosized MIL-101(Fe) particles thus provide an efficient platform for delivering an optical contrast agent *in vitro*.

Figure 2.14 NanoMOFs composed of a Pt(IV) based anticancer drug precipitated from an aqueous solution of the components via the addition of a poor solvent (© ACS⁹³).

The following examples show the encapsulation of drugs inside the MOF matrix. Maspoch *et al.*¹⁰¹ used this method to encapsulate anticancer drugs (DOX, SN-38, camptothecine and daunomycin) as shown in Figure 2.15. The MOF used here was $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$. The drug encapsulation was possible by the addition of an aqueous solution of $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ to an ethanolic solution of 1,4-bis(imidazol-1-ylmethyl)benzene (bix) containing the drug. Encapsulation efficiencies of up to 21% of the

initial drug concentration were obtained. The drugs were quickly released due to desorption of drug adsorbed on the sphere surface and by the degradation of the MOFs. In another study, bio-MOF-1 (with an anionic framework) was used to encapsulate a cationic drug (procainamide with 22 wt%) and was released within three days under simulated biological conditions as shown in Figure 2.17.¹⁰² To verify that procainamide release was mediated by the buffer cations, a control experiment was performed in which the MOF was placed in water. In this case, only 20% of the procainamide was released after 3 days, which likely corresponds to the molecules associated to the exterior surfaces of bio-MOF-1. Amino-functionalized MIL-101(Fe) nanoparticles were used to encapsulate and deliver ethoxysuccinato-cisplatin with an overall payload of 12.8 wt % using post-surface functionalization of the linkers.¹⁰⁰ In another study, caffeine was encapsulated with a very high drug loading of 35 wt% using NH₂-MIL-88B(Fe), where the caffeine played the role of a structure-directing agent or template.¹⁰³ The drug was released at a constant rate for 3 days in water at 37 °C. Complete release occurred when the MOF was heated to 60 and 80 °C. In water the temperature acted as an activator. However, in PBS the drug was released in about 2 days at 37 °C due to the degradation of the MOF. Tsung *et al.*¹⁰⁴ developed a synthetic route to encapsulate fluorescein and the anticancer drug camptothecin inside the ZIF-8 (ZIF stands for Zeolitic Imidazolate Framework) as shown in Figure 2.17. The authors demonstrated cell internalization and minimal cytotoxicity of fluorescein-encapsulated ZIF-8 nanospheres in the MCF-7 breast cancer cell line. The small size of the particles (70 nm) facilitated cellular uptake, and the pH-responsive dissociation of the ZIF-8 framework resulted in endosomal release of the small-molecule cargo. Also, iron oxide nanoparticles were encapsulated into the ZIF-8 nanospheres. Finally, Wuttke *et al.*¹⁰⁵ showed the efficiency of peptide coupling reagents using post-synthetic modification of MIL-101(Al)-NH₂. After this, covalent attachment of drugs and biomolecules inside the pores of MOFs with moderate chemical stability was possible using mild reaction conditions at room temperature.

Figure 2.15 Encapsulation of drugs into nanoMOFs by the connection of Zn^{2+} ions through multitopic organic ligands (© RSC¹⁰¹).

Figure 2.16 a) Cation-triggered procainamide release from bio-MOF-1. b) Procainamide release profiles from bio-MOF-1 (blue, PBS buffer; red, DI water) (© ACS¹⁰²).

Figure 2.17 Iron oxide particles and drug molecules were encapsulated inside the ZIF-8 framework. The nanoMOF facilitated cellular uptake, and the pH-responsive dissociation of the ZIF-8 framework resulted in endosomal release of the small-molecule cargo (© ACS¹⁰⁴).

The following examples show the encapsulation of drugs by adsorption into the MOF's porous interior. Gref *et al.*¹⁶ encapsulated anticancer and antiviral drugs (busulfan (Bu), cidofovir (CDV), and azidothymidine-triphosphate (AZT-TP)) and cosmetic agents by adsorbing these drugs into the porous structure of nanoMOFs in saturated drug solutions as shown in Figure 2.18. The nanoMOFs used here are MIL-100, MIL-88A, MIL-53 and MIL-89. The loading of Bu in the rigid mesoporous MIL-100 was very high (25 wt%). This result was five times higher than for the best polymer nanoparticle system (5-6 wt%) and 60 times higher than with liposomes (0.4 wt%). This high encapsulation was possible due

to the large pore volume. The Bu entrapment in microporous flexible structures (MIL- 88A, MIL-53, MIL-89) was lower than for MIL-100, but significantly larger than for the existing materials. Moreover, MIL-100 nanoparticles could be loaded with up to 21, 16 and 29 wt% of AZT-TP, CDV and DOX, respectively. A high loading of 42 wt% was achieved for AZT-TP and CDV with MIL-101_NH₂ nanoparticles. Release of AZT-TP, CDV and DOX was studied using MIL-100 nanoparticles and showed no 'burst effect'. The comparison between kinetics of drug delivery and the degradation profiles suggested that the delivery process was governed mainly by diffusion from the pores and/or drug-matrix interactions and not by MOF degradation. Furthermore, experiments carried out with nanoparticles of smaller pore size than the drug dimensions showed very low drug encapsulation and 'burst' release kinetics suggesting that the drug was adsorbed only on the external surface and not within the pores.

Figure 2.18. Drug molecules with different structures and sizes encapsulated into MOFs and then released slowly over several days. The plot shows the release profile for cidofovir (top), azidothymidine triphosphate (middle), and DOX (bottom) (© Wiley⁹⁸).

In another study, the same group used MIL-100(Fe) functionalized with CD to encapsulate and release AZT-TP with a loading of 8 wt%.⁸⁶ Drug release in PBS was progressive and practically the same for coated and uncoated nanoMOFs. In contrast, the AZT-TP-loaded nanoMOFs modified with PEG released the drug much faster, with more than 80% released after 5 h of incubation. This 'burst' release could be related to the presence of mobile and hydrated PEG chains inside the interconnected porous structure, which led to drug expulsion from the matrix. Therefore, it was concluded that CD coatings do not interfere with drug entrapment and release, in contrast with the deleterious effect of a direct PEG coating of nanoMOFs.

Gref *et al.*¹⁰⁶ showed that drug loading capacity was affected by the number of phosphate groups per nucleoside using AZT, AZT-MP, and AZT-TP and their interactions with the Lewis acid sites of the nanoMOFs (using nanoMIL-100(Fe)). In the absence of phosphate groups, as in the case of AZT, no significant interaction took place with the nanoparticles with a very poor encapsulation of 1.2 wt% and an efficiency of ~10%. In contrast, phosphorylated drugs were efficiently adsorbed within the nanoMOF cavities with encapsulation efficiencies close to 100%. Loadings as high as 36 wt% and 24 wt% were obtained for AZT-MP and AZT-TP, respectively. The interaction with the nanoMOFs was possible by the formation of strong ionic-covalent links between the drugs' phosphate groups and the iron(III) Lewis acid sites from the nanoparticles. Due to their strong complexation capability, the AZT-TP molecules replaced some residual coordinated trimesic acid bound to the iron metal sites. Finally, as a consequence of the weaker interaction with the nanoMOFs, AZT-MP molecules were released faster in physiological buffer compared to AZT-TP. However, the release of AZT-MP was still progressive, with less than 60% of the drug released after 8 h of incubation. Experimental encapsulation of AZT-TP was in agreement with molecular modelling predictions, indicating maximal loadings of 33 wt% and preferential location of the drug in the large cages.¹⁰⁷ In another study, Serre *et al.*¹⁰⁸ combined experimental and computational methods to elucidate the driving forces that govern encapsulation and release. The encapsulation of active ingredients depended on the solvent, initial material dehydration, drug/material ratio, immersion time, and the amount of consecutive impregnations. The kinetics of drug delivery depended on the different topologies, connectivities, and chemical compositions of

the nanoMOFs. Finally, the confinement of caffeine in a series of UiO-66(Zr)-type MOFs, functionalized with $-H$, $-NH_2$, $-Br$, and $-2OH$ groups was investigated using a multitechnique approach.¹⁰⁹ Here, caffeine was shown to be preferentially located in the smaller cages, with only weak interactions with the functional groups grafted onto the organic linker.

MOFs have been used for storage, separation and delivery of biologically and medically active gases such as nitric acid (NO).¹¹⁰⁻¹¹⁷ These gases are vital in mammalian biology (in the right amounts).⁹⁸ NO is a well-known gas since the discovery of its role in the cardiovascular system was recognized with the Nobel Prize in Medicine. Thereafter, its application in biology and chemistry has been explored.¹¹⁸ NO is an important biological signaling molecule, and its delivery is attractive for many applications such as *in vitro* and *in vivo* antibacterial, antithrombotic, and wound healing.¹¹⁹⁻¹²¹

MOF is a great candidate for NO storage because of its high storage capacity and the strong interaction between the gas and the framework, which can be controlled by changes of the MOF composition.¹¹⁰⁻¹¹³ Morris *et al.*¹¹⁴ showed the exceptional behavior of MOFs over the entire cycle: adsorption, storage, and water-triggered delivery of NO as shown in Figure 2.19. The same group evaluated the adsorption and release of NO over a series of highly flexible iron(III) dicarboxylate MOFs of the MIL-88 structure type, bearing fumaric or terephthalic spacer, and functionalized with or without polar groups (NO_2 , $2OH$).¹¹⁵ Serre *et al.*¹¹⁶ reported a novel microporous MOF incorporating the biocompatible and bioactive calcium. The presence of coordinative unsaturated metal sites allowed the storage and release of NO at biological levels, making this MOF an interesting candidate for biomedical applications. The same group proved the controlled release of NO,¹¹⁷ and indicated that only a partial release of NO took place due to both the coordination of NO over the Lewis acid sites and the stronger binding of NO to the additional iron(II) sites.

Figure 2.19. Cycle of activation, loading, storage, and delivery that determines the success of a gas-storage material (Co- and Ni-MOF). Color key: cyan, Ni/Co; red, oxygen; gray, carbon; pink, oxygen of coordinated water molecules. Hydrogen atoms and noncoordinated guest molecules have been omitted for clarity (© ACS¹¹⁴).

2.4 Conclusions

The first part of this chapter has described the design and application of SNPs. Here, self-assembly and molecular recognition were used to form nanostructures using non-covalent bonding between the multiple building blocks. The size of SNPs was controlled by tuning the composition and concentrations of the building blocks, competition between the mono- and multivalent guest moieties, and by ionic strength. A microfluidic device gave better control over the formation of SNPs as compared to batch conditions and provided insight into the kinetics of SNP formation. The core and shell of the formed SNPs were modified to allow a variety of functional molecules for imaging, drug delivery and biomolecular recognition. Because of the chemical flexibility of the building blocks, different targeting ligands were incorporated into the shell of SNPs to target cell receptors.

Incorporation of stimulus-responsive moieties allowed the formation of responsive SNPs to achieve a controlled assembly/disassembly system.

The second part of this chapter has discussed the use of MOFs for biomedical applications. MOFs can exhibit advantages of both the organic and inorganic constituents to create a multifunctional system. Formation and size of MOFs were tuned by controlling the self-assembly process and by confining the supramolecular assembly to specific locations. Surface functionalization was mainly demonstrated by post-functionalization of the organic linkers or by adding monovalent capping ligands with the same functionality as the multivalent linkers. Finally, because of the high porosity of the MOFs and the large internal surface area, different drug molecules were encapsulated with high efficiency, such as anticancer and antiviral drugs. Additionally, because of the metal clusters, MOFs were used as imaging agents.

Both areas, a better understanding of the thermodynamics, formation, stability and disassembly of SNPs and MOFs in a biological media is essential to eventually use them in for medical applications. Moreover, a better understanding is required of the mechanisms involved in the encapsulation and release of drugs in SNPs and MOFs.

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Chapter 3

Formation of hybrid gold nanoparticle network aggregates by specific host-guest interactions in a turbulent flow reactor

A multi-inlet vortex mixer (MIVM) was used to investigate the formation of hybrid gold nanoparticle network aggregates under highly turbulent flow conditions. To form aggregates, gold nanoparticles were functionalized with β -cyclodextrin (CD) and mixed with adamantyl (Ad)-terminated poly(propylene imine) dendrimers. Ad-terminated poly(ethylene glycol) was added as a stabilizer to cap the supramolecular aggregates and to provide steric repulsion. Aggregates were characterized using dynamic light scattering, UV-vis spectroscopy, and transmission electron microscopy. It was demonstrated that the growth of the aggregates is driven by specific host-guest interactions between CD and Ad moieties. The size of the supramolecular gold aggregates (20-1000 nm) was controlled by varying the ratio of the Ad and CD concentrations. The turbulent flow was found to have a minor effect on the supramolecular aggregate size at relatively low Ad/CD ratios, whereas it had a substantial effect at high Ad/CD ratios, leading to larger agglomerates in solution compared to laminar flow and manual conditions.

3.1 Introduction

Functional materials are important in the areas of materials science, chemistry, physics, and engineering owing to their precisely controlled composition, size, shape, surface properties and their sensitivity to stimuli.¹ Combination of “bottom-up”, such as self-assembly, and “top-down” technologies provides new possibilities for the creation of functional materials and devices.² Self-assembly employs supramolecular interactions which provide functional materials with high specificity, controlled affinity, and reversibility, and with tunable morphology, size and composition.³ Supramolecular functional materials have been formed using micro/nanostructured building blocks using electrostatic interactions,⁴ hydrogen-bonding,⁵ coordination chemistry,⁶ and host-guest interactions.⁷ In particular, the use of host-guest interactions has allowed the preparation of size-controlled multifunctional materials for biomedical applications.^{2, 4, 8-12}

On a supramolecular level, self-assembly is the underlying paradigm of creating supramolecular network aggregates. At the same time, it is known – but underappreciated – that the resulting aggregates are commonly not at thermodynamic equilibrium but represent some kinetically trapped state. That means that different fabrication methods or reaction conditions could lead to different products, in this case regarding size, composition, density, stability, etc.

By far the largest number of studies on supramolecular nanoparticles use common solution-based self-assembly to control particle size and functionality.¹⁰⁻²¹ Many studies²²⁻²⁴ on supramolecular assemblies have employed cyclodextrin (CD) and adamantyl (Ad) as host and guest because they interact specifically, strong, and reversibly. CD is a natural host molecule for organic guest molecules, such as Ad and ferrocene, that forms specific and kinetically labile inclusion complexes in aqueous media via hydrophobic interactions. The binding constant of a single CD-Ad interaction is $\sim 1 \times 10^5 \text{ M}^{-1}$. The noncovalent host-guest complexation of CD has been applied to assist micro- and nanoparticle assembly.²⁵ A special type of supramolecular nanoparticles is that in which the particle size and stability of a core consisting of multivalently interacting molecules are controlled by the addition of a monovalent stopper, e.g. Ad-terminated polyethylene glycol (Ad-PEG) which simultaneously provides enhanced colloidal stability by the PEG chains and terminates the

multivalent network. Davis *et al.*^{8, 26} used such supramolecular nanoparticles to create the first targeted delivery of small interfering RNA in humans. Here, two-vial formulations were used and combined to give ~70 nm nanoparticles. Moreover, Tseng *et al.*¹⁹ developed a supramolecular approach, solely using host-guest interactions, to prepare size-controlled nanoparticles for targeted gene delivery,¹⁷ treatment of cancer cells,¹² delivery of a transcription factor,¹¹ and a therapeutic efficacy application.²¹ Here, a Hamilton syringe was used for mixing to control the nanoparticle formation. In order to have a better control over the specific aggregation process and the size control, this group designed a new approach using a digital microfluidic droplet generator.^{16, 27} However, all the above methods use laminar flow and little is known about the self-assembly of such functional materials under turbulent conditions.

Formation of functional nanomaterials in a turbulent reactor is possible using a multi-inlet vortex mixer (MIVM).²⁸ This technique has been demonstrated to be an efficient and robust process for the formation of multifunctional nanoparticles using the assembly of hydrophobic solutes and amphiphilic block copolymers.²⁸⁻³⁴ Gindy *et al.* used the MIVM for the formation of multi-component copolymer-protected nanoparticles comprised of a model therapeutic agent and gold nanoparticles.³³ Ungun *et al.*³¹ fabricated multi-component, layered nanoparticles for photodynamic therapies using the flash nanoprecipitation method. Also, lipid-polymer hybrid nanoparticles as a controlled drug delivery platform have been constructed using this technique.³⁴ Using the MIVM, parameters such as the concentration of the compounds and the flow rate, which controls the Reynolds number (Re), can be precisely controlled. This technique displays superior characteristics as compared to common methods, such as a very high mixing energy/volume ratio, high loading capacity, facile route toward production, as it is a rapid, continuous, and scalable process. The MIVM has four inlet streams which allows the insertion of different materials (with equal or unequal volumes) prior to mixing, and their concentrations can thus be precisely manipulated for the encapsulated and well-mixed components by varying stream velocities for potential use in biomedical applications. To the best of our knowledge, the MIVM has so far not been used in the assembly of functional materials based on specific host-guest interactions.

Here, we describe the use of the MIVM in the formation of supramolecular hybrid gold nanoparticle network aggregates using specific host-guest interactions. As a proof of concept, we used gold nanoparticles functionalized with β -cyclodextrin (Au-CD) as a host, multivalent Ad-terminated poly(propylene imine) dendrimers (Ad-PPI) as a guest with multivalent functionalities, and Ad-terminated poly(ethylene glycol) (Ad-PEG) as a stabilizer. Combining these three building blocks, gold nanoparticle aggregates were formed in which the core is composed of Ad-PPI and the surface is covered with Ad-PEG chains. The aggregates were studied using dynamic light scattering (DLS), UV-vis spectroscopy, and transmission electron microscopy (TEM). Formation of these aggregates was investigated using specific host-guest interactions in a turbulent regime. The sizes of the aggregates were studied as a function of the host-guest ratio. Finally, the effect of the turbulent flow was compared with laminar flow and the by-hand technique.

3.2 Results and discussion

3.2.1 Characterization of Au-CD particles

The physical properties of the Au-CD nanoparticles (NPs) are important for understanding the hybrid gold nanoparticle network aggregates. Au-CD NPs were characterized using TEM, DLS, and UV-vis spectroscopy to compare these values with the resulting aggregates. A TEM image of individual gold nanoparticles is shown in Figure 3.1a. Image analysis was used to obtain the particle core size distribution showing an average diameter of 3.1 ± 0.9 nm (Figure 3.1b). The hydrodynamic number diameter obtained for the Au-CD NPs in solution was 12 ± 3 nm by DLS. The discrepancy between the hydrodynamic number diameter and the core diameter by TEM is attributed partially to the CD shell, which is not visible in the TEM images, and partially to the well hydrated nature of the shell. Figure 3.1c shows the surface plasmon absorption band with a maximum absorbance wavelength (λ_{max}) of 522 ± 2 nm which is expected for well-dispersed Au NPs of this size.³⁷ Thermogravimetric analysis showed the particles consist of 11% w/w organic fraction (thiol-CD) and 89% w/w Au (see Figure 3.1d). Using these data, we calculated that there are on average 16 CD units on the surface of each gold nanoparticle. This value is important for tuning the host-guest ratio using multivalent

interactions. In summary, the Au-CD nanoparticles are well dispersed in the absence of host-guest interactions.

Figure 3.1 Characterization of Au-CD NPs: a) TEM image and b) corresponding size distribution of the core of the gold nanoparticles; c) UV-vis spectrum of an aqueous solution of Au-CD NPs, $[\text{Au-CD}] = 9 \mu\text{M}$; d) thermogravimetric analysis.

3.2.2 Host-guest complexation in solution using turbulent flow

Next, the hybrid gold nanoparticle network aggregates were formed by specific host-guest interactions in a turbulent flow reactor using a multi-inlet vortex mixer (MIVM) as illustrated in Figure 3.2. Throughout this study, the concentration of Au-CD is expressed as the molar concentration of the monovalent, functional group, i.e. CD. In one stream, Au-CD was injected, and in a second stream, Ad-PPI dendrimers (with native CD for solubility reasons) were injected, while the third and fourth streams contained Ad-PEG to function as a stabilizer. These four streams allow precise control of the building blocks. Each building block can be inserted separately avoiding any previously non-specific interactions. Also,

the concentration and volume of each building block is precisely controlled. This is a clear advantage of the MIVM as compared with manual methods. The [CD]:[Ad-PPI]:[Ad-PEG] ratio was kept at 1:4:8 using $[\text{Au-CD}]_{\text{final}} = 2.25 \mu\text{M}$ after mixing. This ratio was chosen because there is a clear formation of aggregates at this ratio. In all cases, the ratio of Ad-PEG to Ad-PPI was 2:1 as preliminary experiments showed that lower amounts of Ad-PEG were insufficient for obtaining stable particles. Upon mixing in the reaction chamber, the different components self-assemble into hybrid gold nanoparticle network aggregates as schematically shown in Figure 3.3.

Figure 3.2 Schematic illustration of the multi-inlet vortex mixer (MIVM) used for the formation of the hybrid gold nanoparticle network aggregates.

Figure 3.3 a) Formation of the hybrid gold nanoparticle network aggregates and b) chemical structures of the building blocks.

Evidence of formation of gold nanoparticle network aggregates is shown in Figure 3.4a which shows an increase in hydrodynamic diameter from 12 ± 3 nm for the individual particles to 35 ± 14 nm when aggregates were formed. The λ_{max} increased from 522 nm for the starting Au-CD nanoparticles to 529 nm for the aggregates as shown in Figure 3.4b. Figure 3.4c shows a representative TEM micrograph of the network aggregates. Contrast in the TEM image is provided only by the Au cores as the PEG and dendrimer are not visible. The individual Au nanoparticles are clearly visible, and the interparticle distance indicates close contact between the CD shells. The measured aggregate size by TEM is 25 ± 10 nm. Assuming that the aggregates have flattened during drying, this corresponds to a 3D aggregate diameter of 16 ± 5 nm. These values are somewhat lower than the hydrodynamic diameters, probably due to the presence of the PEG chains on the outside of the aggregates. These experiments thus qualitatively prove, for the first time, the successful use of the turbulent mixer for host-guest systems.

To test whether these interactions are specific, two different control experiments were performed. For the first control, tetra(ethylene glycol)-coated Au NPs (Au-TEG), instead of Au-CD were mixed with Ad-PPI, in the absence of Ad-PEG, in the MIVM. The Au-TEG and Ad-PPI should not form aggregates because the former lacks the host groups to specifically interact with the Ad moieties of Ad-PPI. The addition of Au-TEG to a solution of Ad-PPI led neither to precipitation nor to changes of the surface plasmon absorption band, showing a maximum absorption wavelength of 515 ± 3 nm before and of 515 ± 1 nm after mixing. Also, the TEM images before and after mixing did not change (see Figure 3.5). The second control was achieved by mixing Au-CD and free CD in the absence of Ad-PPI and Ad-PEG in order to prove the need for multivalent interactions to form the nanoparticles. Again, neither precipitation nor aggregation were observed using DLS, TEM, and UV-vis (see Figure 3.6). These two controls clearly showed that specific host-guest interactions between Ad and CD are needed to form the gold nanoparticle network aggregates.

Figure 3.4 Characterization of hybrid gold nanoparticle network aggregates using [CD]:[Ad-PPI]:[Ad-PEG] = 1:4:8: a) comparison of the size distribution and b) UV-vis spectra and compared with Au-CD nanoparticles (solid line) and host-guest aggregates (dashed line) and c) TEM image.

Figure 3.5 TEM images of a) as-prepared Au-TEG NPs and b) after attempted assembly of Ad dendrimers and Au-TEG NPs in the MIVM. For the MIVM, the initial concentration of Au-TEG in streams 1 and 3 is 0.1 mg/mL, and that of Ad-PPI in streams 2 and 4 is 18 μ M, using $Re = 15,000$.

Figure 3.6 a) DLS, b) TEM image and c) UV absorbance of Au-CD NPs and free CD upon mixing in the MIVM. For the MIVM, the initial concentration of Au-CD in streams 1 and 3 is 0.1 mg/mL, and that of CD in streams 2 and 4 is 19.8 mM, using $Re = 15,000$.

In order for the network aggregates to remain stable in solution, a stabilizer, Ad-PEG, was added, as has been done in the work of Davis and Tseng.^{8, 11, 12, 17, 21} To prove the role of Ad-PEG, a control was performed by forming the network aggregates in the absence of Ad-PEG. After 10 min upon collection, the sample precipitated leading to phase separation. This observation was confirmed by UV-vis spectroscopy: The λ_{max} before complexation was 522 nm, and it increased to 534 nm after complexation, using a [Ad-PPI]:[CD] ratio of 5. However, when Ad-PEG was incorporated under similar conditions, the aggregates were stable for several days. Uncontrolled aggregation of Au NPs and Ad-modified dendrimers was also shown before by us, as witnessed by visible precipitation.²³ In summary, this control showed the need of Ad-PEG as a stabilizer to control and cap the network aggregates.

3.2.3 Tuning of aggregate size by varying the host-guest ratio

Size control of hybrid gold nanoparticle network aggregates was achieved using a turbulent flow reactor by varying the [Ad-PPI]/[CD] ratio while keeping the [Ad-PEG]/[Ad-PPI] ratio constant. These experiments are important to demonstrate that, under turbulent flow, a large range of sizes can be obtained in a short time under different conditions. For all the experiments, the Au-CD concentration was kept constant using [CD] = 2.25 μ M in the mixer, Ad-PPI was injected with varying [Ad]/[CD] from 1-7.

By simply using varying ratios of [Ad-PPI]/[CD] and keeping the [Ad-PEG]/[Ad-PPI] ratio constant, we were able to obtain a collection of supramolecular gold nanoparticle network aggregates of varying sizes as shown in Figure 3.7 by TEM. The hydrodynamic diameters and the λ_{max} of the aggregates as a function of the [Ad-PPI]/[CD] ratio are presented in Figure 3.8.

The size of the hybrid gold nanoparticle network aggregates depends strongly on the ratio between multivalent interactions in the core (between Ad-PPI and Au-CD) and the monovalent interactions (of Ad-PEG and Au-CD) at the surface of the aggregates. For example, Figures 3.7 and 3.8 show an increase in size as the [Ad-PPI]/[CD] ratio increases. Figure 3.8 shows there was no aggregate formation at a ratio below 2. At ratios of 2 and 3, the aggregates started to form, although the aggregate size increased with small variation, and the aggregates remained stable for several days. At ratios of 5 and higher, larger

agglomerates were formed, which flocculated after several hours. In all cases, there was a concomitant red-shift of λ_{max} which increased with increasing $[\text{Ad-PPI}]/[\text{CD}]$. Thus, we have shown that the aggregate size can be controlled by changing the $[\text{Ad-PPI}]/[\text{CD}]$ ratio under turbulent conditions. This is comparable with the results of Tseng *et al.*¹² who have shown the formation of aggregates composed of Au-Ad nanoparticles using host-guest interactions by CD-poly(ethylene imine) (CD-PEI) as host, and Ad-PEG as a stabilizer, under laminar flow conditions. Overall, our results show that network aggregates of multivalent Au-CD and Ad-PPI building blocks are specific, their size can be tuned by the host-guest ratio, and the stability is to a large extent governed by the presence of Ad-PEG, all shown here, for the first time, under turbulent flow conditions.

Figure 3.7 TEM micrographs of network aggregates obtained at varying $[\text{CD}]:[\text{Ad-PPI}]:[\text{Ad-PEG}]$ ratios: a) 1:2:4, b) 1:5:10 and c) 1:6:12.

Figure 3.8 Variation of the network aggregate size measured by DLS (left, circles) and of λ_{max} by UV-vis spectra (right, diamonds) as a function of $[\text{Ad-PPI}]/[\text{CD}]$ using $Re = 15,000$ and $[\text{Ad-PEG}]/[\text{Ad-PPI}] = 2$. See section 3.5.4 for the calculations of the number average diameter and the standard deviations.

3.2.4 Effect of turbulence on network aggregate size

The MIVM has been previously used by others for nanoparticle formation using a flash-nanoprecipitation concept. This technique uses rapid micromixing, which leads to high supersaturation and kinetically controlled aggregation of hydrophobic compounds.²⁸⁻³⁴ In contrast, in this work in the absence of supersaturation effects, specific host-guest interactions govern the aggregate formation and size. Therefore, this study opens new directions to study new interactions under turbulent conditions. To investigate the effect of turbulence on the aggregate size, we varied Re using a few different host-guest ratios in the ratio-sensitive range ($[Ad-PPI]/[CD] = 4-6$, see Figure 3.9). Therefore, supramolecular network aggregates were formed by three different methods: manually (by hand), under laminar flow ($Re = 150$) and under turbulent flow ($Re = 15,000$). Figure 3.9 clearly shows the advantages of using the MIVM. First, we can precisely control the residence time (τ_{res}), during which the aggregates mix in the chamber, as compared with the manual techniques. Second, τ_{res} is shorter for $Re = 15000$ vs $Re = 150$; for $Re = 15000$, $\tau_{res} = 18$ ms, while for the $Re = 150$, the τ_{res} is 100 times larger. This is also a control parameter to scale up this process. The short mixing time ensures the solvent replacement starts homogeneously, so that the properties of the nanoparticles depend on the competitive kinetics of the building blocks. This brief mixing time ensures that all the building blocks are mixed homogeneously in the MIVM upon which the gold nanoparticles are kinetically frozen, entrapped between specifically bound Ad-PPI molecules, while Ad-PEG serves as a stabilizer.

The hydrodynamic diameters for all cases were measured by DLS and plotted in Figure 3.9. Figure 3.9 clearly shows the effect of turbulence on aggregate size. In general, the aggregate diameter was always found to be larger when using the MIVM. At relatively low $[Ad-PPI]/[CD]$ ratios, however, the diameter was less dependent on Re compared to higher $[Ad-PPI]/[CD]$ ratios. To prove that the differences in particle diameter formed under turbulent flow vs laminar flow and by hand are significant, a statistical analysis was performed using a t-test on the data shown in Figure 3.9, as shown in Figures 3.12-3.14 (histograms; see section 3.5.4 below) and Tables 3.2-3.4 (p values). For the case of the $[CD]:[Ad-PPI]:[Ad-PEG]$ ratio of 1:4:8, the p values between turbulent vs laminar and laminar vs by hand are 0.11 and 0.07, respectively, while the p value for turbulent vs by hand is 3.0×10^{-6} (see Table 3.2). When taking a cut-off of $p = 0.05$, these values confirm

that at low $[\text{Ad-PPI}]/[\text{CD}]$ the diameter is higher using the turbulent flow as compared to by hand experiments. However, the difference in diameter between turbulent and laminar flow may not be significant. However, for aggregates formed at ratios of 1:5:10 and 1:6:12, all p values are below 0.05 for all cases, indicating that the differences in particle diameter are significant for all comparisons. At high ratios, the turbulence has apparently a stronger effect on the supramolecular assembly process leading to larger network aggregates. The size enhancement effect of turbulence may arise from an extra force and concomitant accelerated collision leading to enhanced aggregate formation. This work clearly shows that simply changing the flow conditions can lead to differently sized and shaped products. Thus, this technique - using strongly turbulent conditions - provides a novel approach to control the size of network aggregates held together by multivalent host-guest interactions.

Figure 3.9 Variation of aggregate size as a function of turbulence, as measured by DLS at varying $[\text{Ad-PPI}]/[\text{CD}]$ ratios (while keeping $[\text{Ad-PEG}]/[\text{Ad-PPI}] = 2$) using three different techniques: manually (by hand), laminar flow ($Re = 150$), and turbulent flow ($Re = 15,000$). See section 3.5.4 for the calculations of the number average diameter and the standard deviations.

3.3 Conclusions

This chapter demonstrates the benefits of using a turbulent-flow reactor - such as the multi-inlet vortex mixer (MIVM) - for the formation of hybrid gold nanoparticle network aggregates formed by specific host-guest interactions. It was demonstrated that the sizes of aggregates are tuned using a turbulent flow. Leaving out either the multivalent host or the guest did not provide network aggregates, confirming that specific host-guest interactions are needed for aggregation to occur. Moreover a stabilizer in the form of monovalent Ad-PEG is needed to avoid flocculation. Most importantly, the size of the supramolecular network aggregates was higher under turbulent flow conditions as compared to laminar flow and the manual technique. This effect increased as the [Ad-PPI]/[CD] ratio was increased. Thus this study contributes as well to the rapidly growing interest and body of out-of-equilibrium self-assembled systems.

To our knowledge, this work is the first example of nanoparticle network aggregation by specific host-guest interactions using turbulent flow. It offers clear advantages, first of all by having a new way to control aggregate size, but also to have a much larger-scale and more rapid aggregate formation than can be achieved under laminar flow in microfluidic devices. As such it may offer a new and complementary technique to create supramolecular materials.

3.4 Acknowledgments

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3.5 Experimental

3.5.1 Materials

Reagents and solvents were purchased from Sigma-Aldrich and used as received without further purification otherwise noted. Chemicals were obtained from commercial sources and used as such. β -Cyclodextrin (CD) was dried in vacuum at 80 °C in the presence of P_2O_5 for at least 5 h before use. Perthiolated CD was synthesized according to

a literature procedure.³⁵ Gold(III) chloride, sodium borohydride, and (11-mercaptoundecyl)tetra(ethylene glycol) were obtained from Sigma-Aldrich. Millipore water with a resistivity of 18.2 M Ω •cm at 25 °C was used in all the experiments. First-generation poly(propylene imine) dendrimer (PPI) with amino terminal groups was purchased from Symo-Chem.

3.5.2 Synthetic procedures

Synthesis of gold nanoparticles (NPs) coated with β -cyclodextrin

Gold nanoparticles functionalized by CD (Au-CD) were synthesized by adapting a procedure described in the literature³⁷ by the reduction of HAuCl₄ (184.3 mg, 0.54 mmol) with NaBH₄ (247.5 mg, 6.54 mmol) in dimethylsulfoxide (DMSO) solution (20 mL) containing perthiolated CD³⁵ (26.8 mg, 0.024 mmol) (CD/Au = 0.044/1). The reaction mixture colored deep brown immediately after the addition of the reducing agent and the reaction was allowed to continue overnight. Next, CH₃CN (40 mL) was added to precipitate the nanoparticles and collect them by centrifugation, followed by washing in CH₃CN:DMSO (1:1 v/v, 60 mL, 3x), and ethanol (60 mL, 3x), dissolution in water, and freeze drying.

Synthesis of gold nanoparticles (NPs) coated with tetra(ethylene glycol)

Tetra(ethylene glycol) (TEG)-coated Au NPs (Au-TEG) were prepared following a procedure described in the literature.²³ HAuCl₄ (47.3 mg, 0.14 mmol) was dissolved in methanol (15 mL) and acetic acid (2.5 mL). After stirring for 5 min, (11-mercaptoundecyl)tetra(ethylene glycol) (HS(CH₂)₁₁(OCH₂CH₂)₄OH) (8.1 mg, 0.02 mmol) was added to the above mixture and dissolved by stirring for 5 min. NaBH₄ (42.2 mg, 1.11 mmol) was dissolved in Millipore water and added drop-wise to the solution during rapid stirring. The reaction mixture turned deep brown immediately. After 2 h, methanol was evaporated, and the NPs were purified and collected using several washings with methanol (60 mL, 4x), followed by dissolution in water and storage in an aqueous solution.

Synthesis of Ad-PPI

This compound was prepared as reported before.³⁶ To a solution of PPI-(NH₂)₄ (0.2526 g) in dry chloroform (5 mL) was added 1-adamantyl isocyanate (0.6138g). After stirring for 2 h, the homogeneous solution was precipitated in diethyl ether to remove the excess of isocyanate. Then, the precipitated was filtered off and dried in vacuo. Ad-PPI was analyzed by ¹H-NMR on a Bruker 400 MHz system. ¹H-NMR (CDCl₃): δ = 1.41 (s, 4H, NCH₂CH₂CH₂CH₂N), 1.62-1.65 (m, 32H, NCH₂CH₂CH₂NHCONH and H-2), 1.93-2.03 (m, 39H, H-4 and H-3), 2.35-2.40 (m, 12H, NCH₂CH₂CH₂CH₂N and NCH₂CH₂CH₂NHCONH), 3.17 (q, 8H, NCH₂CH₂CH₂NHCONH), 5.07 (s, 4H, CH₂NHCONH), 5.76 (t, 4H, CH₂NHCONH). The mass analysis was performed using the electrospray ionization with a Voyager DE-RP and a micromass LCT from Waters. Mass: Calcd. For C₆₀H₁₀₀N₁₀O₄: 1025.5. Found 1025.3.

Synthesis of Ad-PEG₅₀₀₀

The mono Ad-functionalized poly(ethylene glycol) (Ad-PEG₅₀₀₀) was synthesized as described before.¹⁹

3.5.3 Methods

Preparation of gold clusters in the turbulent reactor

The multi-inlet vortex mixer (MIVM) has four inlets tangential to the boundary of the mixing chamber. The final product exits the mixing chamber at the center, as shown in Figure 3.2. A schematic picture and dimensions of the mixer are shown in Figure 3.10 and Table 3.1, respectively. All the compounds were dissolved in Millipore water. Syringe pumps were used to accurately control the flow rate of each phase into the reaction chamber. All the solutions were fed at a fixed flow rate into the MIVM. The four water streams were controlled by a Harvard Apparatus Remote Infuse/Withdraw PHD 2000 Hpsi Programmable syringe pump. The Ad-terminated PPI dendrimers were first dissolved in water at pH 2 using 1.1 equiv of CD relative to the Ad units. Then, after the compounds were fully dissolved (by sonication), the pH was increased up to ~ 5.4. In all experiments, the Au-CD concentration was kept constant and injected into one stream with [Au-CD] = 9 μ M (in CD concentration), in a second stream a variable amount of adamantyl-terminated

poly(propyleneimine) PPI (Ad-PPI) dendrimers were injected with concentrations of $[Ad] = 9, 18, 27, 36, 45, 54, 63 \mu\text{M}$. In the third and fourth streams, Ad-PEG was injected with $[Ad-PEG] = 9, 18, 27, 36, 45, 54, 63 \mu\text{M}$ for each stream. The concentration of Au-CD was always checked prior to the injection using a UV-vis calibration curve as shown in Figure 3.11. The hydrodynamic diameters and UV-vis spectra of the aggregates were measured after 30 min. The Reynolds number (Re) was calculated using the formula:

$$Re = D_{mixer} \cdot \sum_i (V_i / \nu_i),$$

where D_{mixer} is the diameter of the mixer, V_i is the velocity of each inlet stream, and ν_i is the kinematic viscosity of each solvent (here water).

Figure 3.10 Schematic of the MIVM.

Table 3.1 Dimensions of the MIVM.

diameter _{mixer} (m)	0.0059
diameter _{inlet stream} (m)	0.0011
diameter _{outlet stream} (m)	0.0013

Figure 3.11 UV-vis calibration curve of Au-CD for different concentrations in DI water.

Turbulent flow with $Re = 15,000$ was produced with a flow rate for each stream of 33.7 mL/min and a residence time of 18 ms in the mixing chamber. Laminar flow with $Re = 150$ was produced with a flow rate for each stream of 0.337 mL/min and a residence time of 1777 ms. The procedure for by hand complexation was as follows to a solution of 0.5 mL of Au-CD particles ($[Au-CD] = 9 \mu M$ (in CD concentration)) in DI-water solution (pH = 5.4) under vigorous stirring, 0.5 mL of Ad-PPI (of $[Ad] = 9, 18, 27, 36, 45, 54, 63 \mu M$) and 1 mL of Ad-PEG ($[Ad-PEG] = 9, 18, 27, 36, 45, 54, 63 \mu M$) were added using a micropipette. The sample was mixed for 30 min and then the hydrodynamic diameters and UV-vis spectra of the aggregates were measured.

3.5.4 Calculations of the number average diameter and the standard deviations

For the aggregate size analysis of Figures 3.8 and 3.9, the log values of the number average diameter (d_n) were calculated using the arithmetic mean from all the measurements, and the min and max errors bars were calculated using the 0.159 and 0.841 percentiles (corresponding to ± 1 standard deviation for a Gaussian distribution), respectively, from all the measurements.

Statistical analysis for Figure 3.9

First, the $^{10}\log$ of each d_n for each measurement was calculated. Then, the histograms of all these values were plotted as shown in Figures 3.12-3.14. This histograms were assumed to follow a log-normal distribution. Then, the arithmetic mean of the $^{10}\log$ number diameter was calculated for each run. Finally, the t-test was performed using the previous values for all the cases in Figure 3.9 as shown in Tables 3.2-3.4. We used the two-tailed, two-sample t-test with unequal variance (heteroscedastic).

Figure 3.12 Histograms of network aggregates obtained at a [CD]:[Ad-PPI]:[Ad-PEG] ratio of 1:4:8 from DLS a) by hand, b) under laminar flow and c) under turbulent flow.

Table 3.2 t-test for network aggregates obtained at a [CD]:[Ad-PPI]:[Ad-PEG] ratio of 1:4:8

Cases	<i>p</i>
Turbulent vs Laminar	0.11
Turbulent vs By hand	2.97×10^{-6}
Laminar vs By hand	0.07

Figure 3.13 Histograms of network aggregates obtained at a [CD]:[Ad-PPI]:[Ad-PEG] ratio of 1:5:10 from DLS a) by hand, b) under laminar flow and c) under turbulent flow.

Table 3.3 t-test for network aggregates obtained at a [CD]:[Ad-PPI]:[Ad-PEG] ratio of 1:5:10.

Cases	<i>p</i>
Turbulent vs Laminar	0.012
Turbulent vs By hand	2.21×10^{-7}
Laminar vs By hand	5.83×10^{-3}

Figure 3.14 Histograms of network aggregates obtained at a [CD]:[Ad-PPI]:[Ad-PEG] ratio of 1:6:12 from DLS a) by hand, b) under laminar flow and c) under turbulent flow.**Table 3.4** t-test for network aggregates obtained at a [CD]:[Ad-PPI]:[Ad-PEG] ratio of 1:6:12.

Cases	<i>p</i>
Turbulent vs Laminar	9.95×10^{-4}
Turbulent vs By hand	2.63×10^{-7}
Laminar vs By hand	2.37×10^{-2}

3.5.5 Equipment

Dynamic light scattering (DLS)

The hydrodynamic sizes of individual gold nanoparticles and aggregates were characterized by dynamic light scattering at 25 °C using the Microtrac Zetratrac. The average translational diffusion coefficient and the hydrodynamic diameters (number

average diameters) were determined by using the Stokes-Einstein equation. For the aggregates, the samples were measured as originally obtained (without any dilution). The number average diameters and the standard deviations are shown in the supporting information.

Transmission electron microscopy (TEM)

All TEM images were performed using a Philips CM300 microscope operating at 300 kV. Samples for imaging were deposited onto a 200 mesh copper grid and the liquid was allowed to dry in air at room temperature.

Thermogravimetric analysis (TGA)

TGA was performed using a Netzsch STA449 F3 on dried Au-CD NPs under air flow (50 mL/min) at a heating rate of 10 °C/min from 25-600 °C.

UV-vis spectroscopy

UV-vis spectra were recorded at room temperature on a Perkin Elmer Lambda 850 UV-vis spectrophotometer in the range of 300-800 nm.

NMR spectroscopy

¹H NMR spectra was recorded on Bruker 400 MHz spectrometer.

Mass spectroscopy

The mass analysis was performed using the electrospray ionization with a Voyager DE-RP and a micromass LCT from Waters.

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Chapter 4

Size-controlled and redox-responsive supramolecular nanoparticles

Control over the assembly and disassembly of nanoparticles is pivotal for their use as drug delivery vehicles. Here, we aim to form supramolecular nanoparticles (SNPs) by combining advantages of the reversible assembly properties of SNPs using host-guest interactions and of a stimulus-responsive moiety. The SNPs are composed of a core of positively charged poly(ethylene imine) grafted with β -cyclodextrin (CD) and a positively charged ferrocene (Fc)-terminated poly(amidoamine) dendrimer, with a monovalent stabilizer at the surface. Fc was chosen for its loss of CD-binding properties when oxidizing it to the ferrocenium cation. The ionic strength was shown to play an important role in controlling the aggregate growth. The attractive supramolecular and repulsive electrostatic interactions constitute a balance of forces in this system at low ionic strengths. At higher ionic strengths, the increased charge screening led to a loss of electrostatic repulsion and therefore to faster aggregate growth. A Job plot showed that a 1:1 stoichiometry of host and guest moieties gave the most efficient aggregate growth. Different stabilizers were used to find the optimal stopper to limit the growth. A weaker guest moiety was shown to be less efficient in stabilizing the SNPs. Also steric repulsion is important for achieving SNP stability. SNPs of controlled particle size and good stability (up to seven days) were prepared by fine-tuning the ratio of multivalent and monovalent interactions. Finally, reversibility of the SNPs was confirmed by oxidizing the Fc guest moieties in the core of the SNPs.

4.1 Introduction

Supramolecular chemistry involves two main concepts: self-assembly and molecular recognition. It offers a convenient and versatile approach for the formation of nanostructured materials using molecular building blocks.¹ Of particular interest are supramolecular nanoparticles (SNPs), which are particles composed of multiple copies of different building blocks forming assemblies by specific noncovalent interactions that are typically larger than the building blocks.² They have the potential to be used in biomedical applications owing to control over their size, their assembly/disassembly, and the modular character for the versatile incorporation of agents aiming for imaging,³ photothermal therapy,⁴ drug delivery⁵⁻⁷ and gene delivery⁸⁻¹⁰ applications.

Different approaches have been used to form SNPs. Davis *et al.*⁸ showed the formation of SNPs using attractive electrostatic interactions between positively charged β -cyclodextrin (CD)-containing polymers and negatively charged siRNA at the core. Neutral adamantyl-grafted poly(ethylene glycol) (Ad-PEG) was incorporated at the surface to stabilize these SNPs using host-guest interactions between CD and Ad. Tseng *et al.*^{11, 12} studied the formation of SNPs that are assembled solely by host-guest interactions. Here, the core is composed of multivalent interactions between positively charged CD-grafted polymers and positively charged PAMAM dendrimers, and a monovalent neutral Ad-PEG stopper is introduced at the surface for stabilization. The SNP size was increased by increasing the amount of multivalent guest molecules in the core, while keeping the host and stopper concentration constant and having an excess of stopper to avoid precipitation. Wintgens *et al.*¹³ showed the formation of SNPs by controlling the host-guest ratio and the total concentration of components with a neutral polymer backbone. Recently, our group^{2,14,15} formed SNPs by varying the ratio of neutral monovalent stoppers and multivalent, positively charged guest dendrimer. Here, the overall concentration of the building blocks was kept constant while maintaining an equimolar stoichiometry of host and guest moieties. Moreover, our group¹⁶ showed that SNP formation is controlled by a balance of forces between attractive supramolecular and repulsive electrostatic interactions using a multicomponent system based on a linear, negatively charged polymer. The force balance used in the latter approach was only observed with negatively charged polymers at

low ionic strengths, and it is not known whether this balance occurs also for positively charged polymers and dendrimers.

In order to use these SNPs for biomedical applications, in particular for drug delivery, a stimulus-responsive self-assembled system is desired for controlled cargo release. Fc is one of the most common redox-responsive compounds and undergoes a reversible one-electron oxidation. Fc has the unique property that it can be used as a guest for CD in its reduced state.¹⁷ The formed CD-Fc inclusion complex disassembles when the Fc moiety is converted to the ferrocenium cation by electrochemistry¹⁸ or by adding an oxidizing agent.¹⁹ Different studies have employed this concept to form redox-responsive systems applied, for example, in self-healing materials,¹⁹ polymeric hydrogels,^{20, 21} voltage-responsive vesicles,²² ultrasensitive enzyme sensors,²³ and as a plasma membrane protein isolation method.²⁴ So far, this concept has not been applied to SNPs.

Here, we aim to make SNPs with a redox-switchable assembly/disassembly mechanism. As a proof of concept, we used positively charged CD-grafted poly(ethylene imine) (CD-PEI) as a host, positively charged ferrocene-terminated poly(amidoamine) (Fc₈-PAMAM) dendrimer as the multivalent guest and a monovalent stabilizer. Different stabilizers were used such as: adamantyl-terminated poly(ethylene glycol) (Ad-PEG), Fc-terminated poly(ethylene glycol) (Fc-PEG), methoxypoly(ethylene glycol) (mPEG), and Ad-tetraethylene glycol (Ad-TEG). The effect of the following parameters on the formation of these SNPs is investigated: the role of ionic strength on SNP formation, the role of host-guest stoichiometry on the growth rate of the SNPs, and the influence of the affinity of the guest moiety and the PEG length of the stabilizer on the SNP stability. The size of the SNPs is controlled by the stoichiometry of the multivalent guest and the monovalent stabilizer. Finally, the reversibility of the SNPs is assessed by studying the influence of oxidation of the Fc moieties.

4.2 Results and discussion

4.2.1 Characterization of building blocks

The positively charged host CD-PEI was synthesized according to earlier reports with slight modifications.^{25, 26} A reaction between 6-mono-tosyl- β -cyclodextrin (TsCD) and PEI

in DMSO was performed using excess of triethylamine as a base, followed by purification by dialysis. In order to control the stoichiometry of the host and guest moieties, the number of CDs per PEI was determined using microcalorimetry and NMR. According to the ^1H -NMR spectrum, there are, on average, 8 CD units grafted on a branched PEI backbone in the polymer building block CD-PEI. To assess the CD concentration in a CD-PEI stock solution, a calorimetric titration was performed using CD-PEI as the host and Ad-TEG as a guest, as shown in Figure 4.1a. Fitting the results by optimizing ΔH° , K and the CD host concentration gave a concentration of 0.39 mg/mL of CD-PEI, which is equivalent to a concentration of 0.088 mM of CD moieties participating in host-guest interactions. The results gave a binding constant (K_a) of $3 \times 10^4 \text{ l mol}^{-1}$. This is slightly lower than the interaction between native CD and Ad-TEG, for which a K_a value of $5 \times 10^4 \text{ l mol}^{-1}$ has been determined (see Figure 4.1b). It can therefore be concluded that the grafting of CD to PEI has a minor effect on the host-guest binding affinity.

Figure 4.1 Microcalorimetric titrations of a) CD-PEI (CD concentration of 0.088 mM, cell) with Ad-TEG (1.1 mM, burette) and b) Ad-TEG (1.1 mM, cell) with CD (10 mM; burette). H = host (CD from CD-PEI or native CD) and G = guest (Ad from Ad-TEG). Experimental binding curve (markers) and best fit to a 1:1 model (line).

The positively charged Fc_8 -PAMAM multivalent guest was prepared according to a procedure developed in our group.²⁷ The positively charged Ad-terminated poly(amidoamine) dendrimer (Ad_8 -PAMAM), used as a control, was prepared according to a literature procedure.¹¹ The neutral Fc-PEG stabilizer was synthesized by a reaction of 1-(chlorocarbonyl)-ferrocene with the terminal amino group of methoxypoly(ethylene glycol)

amine (MW 5000 g/mol) in dichloromethane, using an excess of triethylamine as a base, followed by precipitation from diethyl ether. To evaluate the association constant of the Fc moiety with free CD, and to confirm the degree of functionalization, a calorimetric titration was performed with native CD, as shown in Figure 4.2. This titration confirmed that nearly 100% of Fc-PEG was formed. The K_a of Fc-PEG with native CD is $1.2 \times 10^3 \text{ l mol}^{-1}$, which is comparable to the binding constant of Fc dendrimers with CD.²⁸

Figure 4.2 ITC titration of Fc-PEG (1.03 mM; cell) with native CD (10 mM; burette). H = host and G = guest. Experimental binding curve (markers) and best fit to a 1:1 model (line).

The neutral stabilizer Ad-PEG was synthesized according to a literature procedure,¹¹ by the reaction of 1-adamantylamine with the succinimidyl ester of carboxymethyl-PEG (MW 5000 g/mol) in dichloromethane with an excess of triethylamine.

4.2.2 Formation and size control of SNPs

Figure 4.3a shows the concept of forming SNPs based on host-guest interactions, and the possible or impossible redox-induced disassembly when using Fc or Ad as the guest moiety, respectively. Throughout this study, concentrations of all the building blocks were expressed as the molar concentrations of the monovalent groups, i.e. CD, Ad and Fc.

Figure 4.3 a) Schematic representation of the supramolecular nanoparticle (SNP) self-assembly and redox-triggered disassembly of the host-guest complex. b) Chemical structures of the building blocks used here. c) Binding of Fc by CD and subsequent dissociation upon oxidation of Fc.

Influence of the ionic strength

The SNPs used here are formed using host-guest interactions between Fc₈-PAMAM and CD-PEI. These molecules have positive charges that can influence the growth by repulsive interactions, which is an additional parameter that can influence the formation of

SNPs. Moreover, Fc is used as the guest moiety as its stimulus-responsive properties lead to a triggered assembly/disassembly system. In order to study the influence of ionic strength on SNP formation, we used 0 to 0.2 M NaCl solutions and different guest-host ratios when assembling SNPs from CD-PEI and Fc₈-PAMAM in the absence of stabilizer. SNPs were formed by adding Fc₈-PAMAM to an aqueous solution of CD-PEI ([CD] = 100 μ M) in aqueous NaCl. To confirm particle formation, SNPs were characterized using DLS and SEM (Figure 4.4). DLS measurements of the particles in water without salt showed nanoparticles of comparable hydrodynamic diameters (d) for the different Fc/CD ratios. The size for 0% Fc (only CD-PEI) was approx. 70 nm, which is attributed to the fact that the concentration of CD-PEI is above its critical aggregation concentration. These results show that the particle size remains similar for the range of Fc/CD ratios shown here (in the absence of salt).

Similar experiments were performed at three different salt concentrations, 0.1, 0.15 and 0.2 M NaCl, while keeping [CD] = 100 μ M. Particle formation and growth was observed by DLS after 20 min and 3 h. Figure 4.5 shows an increase of particle size with increasing salt concentration at ionic strengths above 0.1 M, and the effect is stronger after 3 h, indicating a slow growth process. Up to an ionic strength of 0.1 M, however, no change of particle size was apparent. At this concentration of 0.1 M, the Debye screening length is approx. 1 nm. Zeta potential (ζ) measurements were performed using [CD] = 100 μ M (CD is the number of moieties from CD-PEI) and [Fc] = 50 μ M (Fc is the number of moieties from Fc₈-PAMAM) at different salt concentrations after 20 min, as shown in Table 4.1. ζ decreased at increased ionic strengths, and values below 20 mV were observed at ionic strengths of 0.1 M and higher, indicating an absence of stabilization by charge repulsion. These results demonstrate, as shown before for negatively charged polymers,¹⁶ that a balance between repulsive electrostatic forces and attractive host-guest interactions exists at low ionic strengths, leading to stable SNPs even in the absence of a stabilizer. At higher ionic strengths, however, the increased charge screening leads to a loss of electrostatic repulsion and therefore to aggregates that grow over time.

Figure 4.4 Size determination of SNPs prepared from CD-PEI and Fc_8 -PAMAM: SEM images (a-c) of the resulting SNPs as a function of the $[\text{Fc}]/[\text{CD}]$ ratio (in Fc and CD moieties from Fc_8 -PAMAM and CD-PEI, respectively) in aqueous solution (without salt) (a: 0, b: 0.5 and c: 1) used during supramolecular assembly keeping constant the total concentration using $[\text{CD}] = 100 \mu\text{M}$ and d) d by DLS and size by HRSEM.

Figure 4.5 DLS size determination of SNPs prepared from CD-PEI and Fc_8 -PAMAM by increasing the $[\text{Fc}]/[\text{CD}]$ ratio (in Fc and CD moieties from Fc_8 -PAMAM and CD-PEI, respectively) at different salt concentrations (0-0.2 M NaCl) keeping constant the total concentration using $[\text{CD}] = 100 \mu\text{M}$ after: a) 20 min and b) 3 h.

Table 4.1 Hydrodynamic diameters, d , and zeta potentials, ζ , measured by DLS of SNPs prepared at increasing salt concentrations (0–0.2 M NaCl) using CD-PEI and Fc₈-PAMAM keeping the total concentration constant at [CD] = 100 μ M and [Fc] = 50 μ M (in Fc and CD moieties from Fc₈-PAMAM and CD-PEI, respectively).

Salt concentration (M)	d (nm)	ζ (mV)
0	51	33
0.1	58	17
0.15	247	15
0.20	441	15

Influence of the host-guest stoichiometry on SNP formation

To assess whether all host and guest moieties of CD-PEI and Fc₈-PAMAM are engaged in interactions, a Job plot was performed varying the host-guest ratio while keeping the sum of the concentrations constant. The SNP growth at high ionic strength was used as a sign of interactions between the multivalent host and guest molecules. When increasing the Fc content to 0.5 (i.e., a host-guest ratio of 1:1), an increase in particle size was observed, but the particle size remained constant as the Fc concentration was increased further (data not shown). Therefore, 2 mM of native CD was added in an attempt to suppress non-specific, hydrophobically driven aggregation at excess Fc moieties. Figure 4.6a shows, however, a very similar picture, with particle sizes increasing as the Fc fraction was raised from 0 to 0.5, and a plateau of constant size at higher Fc fractions. Apparently, the addition of native CD was insufficient to cap excess free Fc groups, due to lack of affinity. To verify that this low affinity is the main reason for the continued particle growth observed at Fc contents above 0.5, the Ad dendrimer analog was used as a control. Similar to the Fc case, increase of the Ad fraction up to 0.5 (see Figure 4.6b) led to an increase of the SNP size. Higher Ad contents in the presence of 2 mM native CD, however, led to a decrease in particle size, indicating that an excess of Ad is efficiently blocked by CD, which is in line with the approx. 30 times higher binding affinity of Ad (see above). Most importantly, this graph (Figure 4.6b) confirms a 1:1 binding stoichiometry of the system. These results

demonstrate that SNPs form optimally at a 1:1 stoichiometry at which all available host and guest moieties are simultaneously engaged in host-guest interactions.

Figure 4.6 Hydrodynamic diameter, d , of SNPs prepared from CD-PEI and Fc_8 -PAMAM or Ad_8 -PAMAM measured by DLS as a function of the $[guest]/([guest]+[CD])$ ratio for: a) CD-PEI and Fc_8 -PAMAM $[CD+Fc] = 50 \mu M$ (in CD and Fc moieties), $I = 0.4 M$ NaCl, with 2 mM native CD measured after 10 min, and b) CD-PEI and Ad_8 -PAMAM $[CD+Ad] = 200 \mu M$ (in CD and Ad moieties), $I = 0.2 M$ NaCl, with 2.0 mM native CD measured after 6 min.

Effect of a monovalent stopper

In order to limit particle growth and achieve stabilization, a proper stabilizing agent should be found. The strategy on stoichiometry remains the same as previously described, keeping the host-guest ratio at 1:1 and a high ionic strength of 0.2 M NaCl. Two different parameters were considered to study the effect of a stopper: length and binding affinity. The formation of SNPs was studied using constant concentrations of $[CD] = 100 \mu M$ (CD is the number of moieties from CD-PEI) and $[Fc + \text{guest-stabilizer}] = 100 \mu M$ (Fc is the number of moieties from Fc_8 -PAMAM), thus keeping the molecular recognition moieties in a 1:1 stoichiometry ratio. Therefore, Fc_8 -PAMAM was dissolved in DMSO and aqueous solutions of CD-PEI, and stabilizers (Ad-PEG, mPEG, Fc-PEG, Ad-TEG and without stabilizer (see Figure 4.3b)) were prepared prior to mixing. Size tuning of the SNPs was assessed by using two different concentrations of Fc_8 -PAMAM dendrimers and stabilizer, while keeping the overall concentration of the guest moieties constant. The formation of SNPs was evaluated by DLS after 20 min and 4 h. Figure 4.7 shows the strong effect of the

use of a stabilizer on the SNP size and, as shown before, that the SNP size is further increased by increasing the fraction of multivalent Fc moieties at the core of the particles. These results show that the smallest sizes and most stable particles were formed when using Ad-PEG as the stabilizer. Larger particles were observed for Fc-PEG than for Ad-PEG, but these also appeared stable (sizes after 20 min and 4 h are similar). The shorter Ad-TEG was less efficient in capping and stabilizing the SNPs compared to polymeric Ad-PEG. Leaving out the guest moiety, by using mPEG as a stabilizer, led to uncontrolled growth as was also observed in the absence of PEG. In summary, these results demonstrate that a guest moiety is important, and that a weaker guest is less efficient in stabilizing the particle. Moreover, steric repulsion by having a long polymer chain present on the stopper is important for achieving SNP stability.

Figure 4.7 DLS size determinations of SNPs prepared from CD-PEI, Fc₈-PAMAM, in the absence or presence of a monovalent stopper, for two [Fc]/[CD] ratios (in Fc and CD moieties from Fc₈-PAMAM and CD-PEI, respectively) keeping constant both [CD] = [Fc] + [stopper] = 100 μ M (where [stopper] is the concentration of the monovalent stopper), using 0.2 M NaCl and different stoppers: Ad-PEG, mPEG (no guest moiety), Fc-PEG, Ad-TEG and without stabilizer after: a) 20 min and b) 4 h.

Size control by changing the stoichiometric composition

SNP size control was achieved by changing the stoichiometry of the multivalent guest and the monovalent stabilizer while keeping the overall host-guest ratio constant and equimolar. SNPs were observed by SEM and DLS for all samples as shown in Figure 4.8.

The particle sizes determined by SEM (see Figure 4.8a-c) with sizes of 49 ± 13 nm (Fc fraction of 0.375), 61 ± 17 nm (Fc fraction of 0.5) and 67 ± 21 nm (Fc fraction of 0.625) were much smaller than those measured by DLS. This can be possibly due to drying effects. Figure 4.8d-f shows an increasing size with increasing fraction of the multivalent Fc_8 -PAMAM and they are stable up to 7 days. In summary, we have demonstrated the formation of stable and size-tunable SNPs by varying the multivalent vs monovalent stoichiometry.

Figure 4.8 Size determinations of SNPs prepared from CD-PEI, Fc_8 -PAMAM and Ad-PEG: SEM images (a-c) of the resulting SNPs by increasing $[\text{Fc}]/[\text{CD}]$ ratios (in Fc and CD moieties from Fc_8 -PAMAM and CD-PEI, respectively) using 0.2 M NaCl (a: 0.375, b: 0.50 and c: 0.625) used during supramolecular assembly using $[\text{CD}] = 100 \mu\text{M}$ and CD : Ad + Fc stoichiometry, and DLS data (d-f) after: d) 20 min, e) 4 h and f) 7 days

4.2.3 Stimulus-responsive disassembly by oxidation

The redox-triggered disassembly of the Fc-containing SNPs (see Figure 4.3a) makes use of the redox-responsive properties of Fc and the resulting loss of binding affinity for CD upon oxidation of Fc to the ferrocenium cation. We chose Ce^{4+} as the oxidizing agent to perform the disassembly experiments because of its proven effectiveness in a Fc/CD system similar to ours.²⁹ SNPs composed of CD-PEI, Fc_8 -PAMAM and Ad-PEG were

formed using a ratio of CD : (Ad + Fc) = 1 : 1. The hydrodynamic diameter d by DLS was found to be 210 nm. Directly thereafter, a small volume of a Ce^{4+} stock solution was injected into the sample (Ce:Fc = 10). The SNP size was then monitored by DLS over time as shown in Figure 4.9a before (red) and after addition of Ce^{4+} (green) at 10 min. These results show a quick breakdown of the aggregates in the first 20 min. Sizes measured thereafter resemble the size measured for CD-PEI only. To prove that particle disassembly requires the redox-active Fc group, a similar experiment was performed using the redox-silent Ad_8 -PAMAM dendrimer (see Figure 4.3a) as a control. SNPs composed of CD-PEI, Ad_8 -PAMAM and Ad-PEG were formed using $[\text{CD}] = 100 \mu\text{M}$ (CD is the number of moieties from CD-PEI), $[\text{Ad}] = 37.5 \mu\text{M}$ (Ad is the number of moieties coming from Ad_8 -PAMAM) and $[\text{Ad}] = 62.5 \mu\text{M}$ (from Ad-PEG) in 0.2 M NaCl. A size of $d \sim 150$ nm was measured by DLS. Figure 4.9b shows the hydrodynamic diameter of these aggregates over time before (red) and after addition of Ce^{4+} (green) at 10 min. These results shows that the Ad-based SNPs do not disassemble in the presence of oxidant. Therefore we conclude that Fc groups are needed to equip the SNPs with a triggered disassembly mechanism, attributed to the oxidation of the Fc groups of Fc_8 -PAMAM to the ferrocenium cation resulting in decomplexation of the guest groups and concomitant loss of multivalent links between the CD-PEI units in the SNPs.

Figure 4.9 DLS size determination before (red) and after the addition of the oxidant agent Ce^{4+} (green) for as-prepared SNPs: a) $[\text{CD}] = 100 \mu\text{M}$ (in CD moieties from CD-PEI) $[\text{Fc}] = 50 \mu\text{M}$ (in Fc moieties from Fc_8 -PAMAM) and $[\text{Ad}] = 50 \mu\text{M}$ (from Ad-PEG) and b) $[\text{CD}] = 100 \mu\text{M}$ and $[\text{Ad}] = 37.5 \mu\text{M}$ (in Ad moieties from Ad_8 -PAMAM) and $[\text{Ad}] = 62.5 \mu\text{M}$ (from Ad-PEG) (control) in 0.2 M NaCl. 10 eq. of Ce^{4+} relative to Fc was added to the SNPs. Experimental d measurements (markers) and trendlines (line, guide to the eye).

4.3 Conclusions

In conclusion, we have displayed a supramolecular strategy for the self-assembly of size-controlled supramolecular nanoparticles based on host-guest interactions and using a stimulus-responsive moiety. The SNP size and stability were found to be controlled owing to a balance of forces between attractive supramolecular host-guest interactions and repulsive electrostatic interactions between the host and the guest-functionalized positively charged polymers at low ionic strengths. At higher ionic strengths, the increased charge screening led to a loss of electrostatic repulsion and therefore to larger aggregates. Optimal self-assembly of the multivalent components was observed at a 1:1 stoichiometry of the host:guest moieties. A stabilizer with high binding affinity and sufficient steric repulsion is needed to obtain stable and small particles, thus Ad-PEG was observed to be the optimal stopper. Variation of the mono- to multivalent guest ratio provided a range of SNP sizes, and the SNPs were stable up to 7 days. The particles were successfully disassembled using a chemical oxidant. The understanding of the forces involved in SNP formation, and control over their stability and responsive character makes these SNPs a promising candidate for developing a drug delivery vehicle where control over the drug encapsulation and release can be achieved.

4.4 Acknowledgments

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4.5 Experimental

4.5.1 Materials

Reagents and solvents were purchased from Sigma-Aldrich and used as received without further purification, unless noted otherwise. Millipore water with a resistivity of $18.2 \text{ M}\Omega\cdot\text{cm}$ at 25°C was used in all the experiments. The amine-terminated poly (amido amine) dendrimer was purchased from Symo-Chem and received as a solution in methanol (20 w/w%).

4.5.2 Synthetic procedures

The 6-monotosyl- β -cyclodextrin was synthesized according to a literature procedure.³⁰ The multivalent Fc_8 -PAMAM was prepared according to a procedure developed in our group.²⁸ Synthesis of Ad_8 -PAMAM and the Ad-PEG (MW 5000 g/mol) were synthesized according to a literature procedure,¹¹ as well as Ad-TEG.³¹

Synthesis of CD-PEI

The procedure for preparing β -CD-functionalized PEI polymer was based on a literature procedure.¹¹ DMSO was freshly distilled under argon. Then, to a solution of branched poly(ethylene imine) [MW $\sim 10,000 \text{ g/mol}$] (250 mg, 0.025 mmol) dissolved in 45 mL DMSO under argon at 60°C , a solution of 6-monotosyl- β -cyclodextrin (1.4 g, 1.1 mmol) and 0.5 mL triethylamine in 35 mL of DMSO was added slowly under argon by using a syringe while stirring. The resulting solution was stirred at 60°C for three days under argon. The solution was cooled to room temperature and diluted with 40 mL deionized water with a resulting pH of 10.9. The solution was transferred to a Spectra/Por MWCO 6-8 kD membrane and dialyzed against water for 4 days. The dialyzed solutions were filtered over paper and lyophilized to afford 189 mg of a fluffy, near-white solid. $^1\text{H-NMR}$ (400 MHz, D_2O): δ (ppm) 5.05-5.39 (br, 7H, C_1H of CD), 3.56-3.87 (m, 42.1, C_{2-6}H of CD), 2.2-3.5 (br, 115.6, OCH_2 of PEI).

Synthesis of Fc-PEG

Methoxypoly(ethylene glycol) amine (MW 5000 g/mol; 250 mg, 0.050mmol) and 0.2 mL triethylamine was dissolved in 15 mL CH_2Cl_2 under argon in a 100 mL one-necked round-bottom flask. While stirring, a solution of ferrocenoyl chloride (500 mg, 2.0 mmol) in 15 mL dichloromethane was added dropwise by using a syringe. The mixture was allowed to stir overnight at room temperature under argon. The solvent was removed leaving an orange residue. The residue was dissolved in 20 mL chloroform. The chloroform mixture was washed with 10 mL aqueous saturated NaHCO_3 solution after which the organic layer was dried using MgSO_4 . After filtration over paper, the solvent was removed by evaporation and the remaining precipitate was redissolved in 2 mL chloroform. The chloroform solution was added dropwise to 40 mL of diethyl ether, giving immediate precipitation of a yellow solid, which was filtrated and dried in a vacuum oven at 40 °C overnight. This yielded 136 mg of a slightly yellow solid. $^1\text{H-NMR}$ (400 MHz, D_2O): δ (ppm) 4.78 (m, 2H, Fc), 4.53 (t, 2H, Fc), 4.28 (s, 5H, Fc), 3.85 (t, 2H, $\text{CH}_2\text{CH}_2\text{NHCO}$), 3.50 (t, 2H, CH_2NHCO), 3.36 (s, 3H, OCH_3).

4.5.3 Methods

Supramolecular nanoparticle assembly as a function of ionic strength

For the preparation of SNPs as a function of ionic strength (0-0.2 M NaCl), aqueous solutions of CD-PEI and NaCl and Fc_8 -PAMAM in DMSO were prepared before mixing. The concentration of CD-PEI was kept constant. As an example, for preparing a solution of 50% Fc entities derived from the Fc dendrimer in 0.1 M NaCl, first 100 μL of aqueous CD-PEI solution (500 μM in CD moieties), 60 μL of aqueous NaCl solution (833.3 mM) and 340 μL of water were mixed for 30 s. After mixing, 7.5 μL of Fc_8 -PAMAM solution in DMSO (3336 μM in Fc moieties) was injected to the previous solution while sonicating.

Job plot using Fc_8 -PAMAM

For the preparation of SNPs using 0.4 M NaCl, an aqueous solution of CD-PEI, free CD, NaCl and a solution of Fc_8 -PAMAM in DMSO were prepared before mixing. The concentration of total moieties was kept constant at 50 μM . As an example, for preparing a solution of 50% Fc entities (25 μM) derived from the Fc dendrimer, first 125 μL of

aqueous CD-PEI/free CD solution (100 μM in CD moieties; 2 mM free CD), 240 μL of aqueous NaCl/free CD solution (833.3 mM NaCl; 2 mM free CD) and 135 μL of aqueous free CD solution (2 mM), were mixed for 30 s. After mixing, 7.5 μL of Fc₈-PAMAM solution in DMSO (1664 μM in Fc moieties) was injected to the previous solution while sonicating.

Job plot using Ad₈-PAMAM

For the preparation of SNPs using 0.2 M NaCl, aqueous solution of CD-PEI, free CD, NaCl and solution of Ad₈-PAMAM in DMSO were prepared before mixing. The concentration of total moieties was kept constant at 200 μM . As an example, for preparing a solution of 50% Ad entities (100 μM) derived from the Ad dendrimer, first 125 μL of aqueous CD-PEI/free CD solution (400 μM in CD moieties; 2 mM free CD), 120 μL of aqueous NaCl/free CD solution (833.3 mM NaCl; 2 mM free CD) and 255 μL of aqueous free CD solution (2 mM), were mixed for 30 s. After mixing, 7.5 μL of Ad₈-PAMAM solution in DMSO (6664 μM in Ad moieties) was injected to the previous solution while sonicating.

Supramolecular nanoparticle assembly as a function of different stoppers

For the preparation of SNPs using 0.2 M NaCl as a function of stoppers at two different Fc fractions, various aqueous solutions of CD-PEI, PEG modified (Ad-TEG, Ad-PEG (MW=5000), Fc-PEG (MW=5000), mPEG (MW=5000) and using two different concentrations of Fc₈-PAMAM in DMSO were prepared. The concentration of CD-PEI was kept the same. As an example, for preparing a solution of 50% Fc entities derived from the Fc dendrimer using Ad-PEG, first 100 μL of aqueous CD-PEI solution (500 μM in CD moieties), 100 μL of aqueous Ad-PEG solution (250 μM), 120 μL of aqueous NaCl solution (833.3 mM) and 180 μL of DI-water were mixed for 30 s. After mixing, 7.5 μL of Fc₈-PAMAM solution in DMSO (3336 μM in Fc moieties) was injected to the previous solution while sonicating.

Supramolecular nanoparticle assembly as a function increasing multivalent guest

For the preparation of SNPs in 0.2 M NaCl various aqueous solution of CD-PEI and Ad-PEG and Fc₈-PAMAM in DMSO were prepared before mixing. The concentration of CD-PEI was kept the same. As an example, preparing a solution of 50% Fc entities derived from the Fc dendrimer, first 100 μL of aqueous CD-PEI solution (500 μM in CD moieties), 100 μL of aqueous Ad-PEG solution (250 μM), 120 μL of aqueous NaCl solution (833.3 mM) and 180 μL of DI-water were mixed for 30 s. After mixing, 7.5 μL of Fc₈-PAMAM solution in DMSO (3336 μM in Fc moieties) was injected to the previous solution while sonicating.

Triggered disassembly of SNPs

To evaluate the redox responsiveness of the particles, SNPs containing [CD] = 100 μM (in CD moieties from CD-PEI), [Fc] = 50 μM (in Fc from Fc₈-PAMAM) and [Ad] = 50 μM (from Ad-PEG) were prepared in 0.4 M NaCl. The d was measured over time after the injection of Ce⁴⁺ (10 eq. of Ce⁴⁺ relative to Fc was added to the SNPs). To evaluate whether the SNP disassembly was due to the oxidation of the ferrocene groups, SNPs containing [CD] = 100 μM (in CD moieties from CD-PEI), [Ad] = 37.5 μM (in Ad from Ad₈-PAMAM) and [Ad] = 62.5 μM (from Ad-PEG) were prepared in 0.2 M NaCl. The d was measured over time after the injection of Ce⁴⁺ (10 eq. of Ce⁴⁺ relative to Ad was added to the SNPs).

4.5.4 Equipment

Dynamic light scattering (DLS)

Hydrodynamic diameters and zeta potentials were measured on a Zetasizer NanoZS (Malvern Instrument Ltd, Malvern, United Kingdom) at 20 °C, with a laser wavelength of 633 nm and a scattering angle of 173°.

High resolution scanning electron microscopy (HR-SEM)

All SEM images were taken with a Carl Zeiss Merlin scanning electron microscope. The samples were prepared by drop-casting 10 μL of a SNP solution onto a silicon wafer. After 60 s, excess of water was removed by filter paper. The particle dimensions are

obtained from SEM images with ImageJ software. For each sample at least 100 particles were measured.

Calorimetric analysis

Calorimetric titrations were performed at 25 °C using a Microcal VP-ITC titration microcalorimeter. Sample solutions were prepared in Millipore water.

NMR spectroscopy

¹H NMR spectra was recorded on a Bruker 400 MHz NMR spectrometer. ¹H chemical shift value, 400 MHz is reported as δ using the residual solvent signal as internal standard at ~ 22 °C.

Mass spectroscopy

Mass analysis was done using matrix-assisted laser desorption ionization (MALDI) on a Waters Synapt G1 using 2,5-dihydroxybenzoic acid as the matrix.

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Chapter 5

MOFs as multivalent materials: size control and surface functionalization by monovalent capping ligands

Control over particle size and composition are pivotal to tune the properties of metal organic frameworks (MOFs), for example for biomedical applications. Particle size control and functionalization of MIL-88A were achieved by using stoichiometric replacement of a small fraction of the divalent fumarate by monovalent capping ligands. A fluorine-capping ligand was used to quantify the surface coverage of capping ligand at the surface of MIL-88A. Size control at the nano-scale was achieved using monovalent carboxylic acid-functionalized poly(ethylene glycol) (PEG-COOH) ligands at different concentrations. Finally, a biotin-carboxylic acid capping ligand was used to functionalize MIL-88A to bind fluorescently labeled streptavidin as an example towards bioapplications.

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5.1 Introduction

Metal-Organic Frameworks (MOFs), or Porous Coordination Polymers, constitute a heavily investigated class of materials consisting of metal ions or clusters thereof, and multivalent organic linkers.¹ MOFs are crystalline materials with high porosities (with reports of up to 90% free volume) and large internal surface areas (over 4000 m²/g).² MOF pore sizes range from several Å up to tens of nanometers. MOF compositions can be readily tuned by varying the metal or the organic linker and by the ability to functionalize by grafting the organic linker during or after synthesis.³ The resulting properties make these MOFs suitable for various applications such as catalysis,⁴ gas sorption⁵ and drug delivery.^{3,4,6}

In the field of biomedicine, there is the need for well-defined carriers of, for example, drugs or imaging agents, often in the form of nanoparticles.^{3,7,8} To use nano-sized MOFs for intravenous drug delivery, different properties are required: biodegradability,⁹ low toxicity,⁸ tuning the pore size to control the encapsulation of molecules such as drugs¹⁰⁻¹² gases,¹³⁻¹⁵ metal nanoparticles,¹⁶⁻¹⁷ and nucleic acids,¹⁸ water dispersibility,⁸ monodisperse particle size tunable to less than 200 nm,^{8,19-21} and controllable composition and functionalization of the surface.^{22,23} A general and versatile fabrication methodology is crucial to control the MOF particle size and the chemical and physical properties of the particles for introducing ligands e.g. for dispersibility or targeting, and to achieve colloidal and in vivo (bio)chemical stability.

In particular the particle size is a limiting factor for intravenous administration, but also for other applications, such as for embedding MOFs in other material matrices like polymers, nanosize control is important. For that reason, the preparation of monodisperse, well-defined, reproducible, and stable nanoparticles has been investigated using different methods such as hydrosolvothermal,^{8,13,24-27} reverse-phase microemulsions,²⁸⁻³¹ interfacial,³² sonochemical,^{24,33-35} and microwave assisted syntheses,^{8,21,24,36} and spray-drying.³⁷ Of particular interest is the coordination modulation method,^{19,26,38-40} in which monovalent capping ligands are added, in large excess, to control the nucleation phase by serving as a stopper of the crystal growth to form nanoMOFs. However, still a molecular and

physicochemical concept to quantitatively control the MOF particle size together with the outer functionalization is lacking.

In the realm of supramolecular chemistry, on the other hand, the formation of supramolecular nanoparticles (SNPs)^{41,42} is controlled by multivalent/monovalent competition between the building blocks. Variation of the stoichiometry of these building blocks is a simple and effective means to tune the size of SNPs in a controllable manner as well as their surface chemistry. In this strategy, the monovalent building block by default ends up on the outside of the SNP, and therefore the increase of its fraction at the expense of the multivalent, crosslinking building block present in the core leads to an increase of the total particle surface area and thus to a decrease of the average particle size. Important to note is that the success of this control by stoichiometry is in part due to the strong enthalpic driving forces for binding and the concomitant drive for saturation of sites. Even stronger enthalpies are commonly observed in MOF formation. To the best of our knowledge, multivalent/monovalent competition by variation of the stoichiometry of the building blocks without using an excess of material has so far not been used as a fabrication strategy in the synthesis of nanoMOFs.

In this chapter, the synthesis of functionalized nanoMOFs as multivalent materials is described. We apply the concept of size control and surface functionalization in one-pot by controlling the ratio between mono- and multivalent building blocks while maintaining a 1:1 stoichiometry between the binding groups. In the context of MOFs, the metal ions or secondary building units and the organic linkers are the multivalent building blocks, while a capping ligand that contains a single binding moiety of the same nature as the organic linker as the monovalent building block. Maintaining the stoichiometry enables the saturation of sites by the system. In contrast to the other coordination modulation methods, we do not employ a large excess of capping molecules, but rather replace only a small fraction of the organic linker by a monovalent ligand. To demonstrate this concept, we adapted the MIL-88A system using ferric chloride and fumaric acid as the multivalent building blocks and different monovalent carboxylates as the capping ligands to achieve surface functionalization, size control, and biomolecular functionalization.

5.2 Results and discussion

Figure 5.1 shows the concept of multivalent/monovalent competition in MOF synthesis. By substituting a small part of the multivalent bridging ligand (fumaric acid) for a monovalent carboxylate capping ligand while keeping the number of moles of carboxylate groups constant in the reaction mixture, we aim to control particle size and surface functionality. If saturation of metal sites is an important driving force, it is expected that the MOF particle size will decrease when minor quantities (up to a few percent) of capping ligand are introduced. The monovalent capping ligand effectively terminates the surface which inhibits particle growth. The capping ligands used in this study are: (i) a fluorine-containing capping ligand ($\text{CF}_3\text{-cy-COOH}$) to introduce an element (F) that can be detected both by bulk elemental analysis and the surface-sensitive X-ray photoelectron spectroscopy (XPS), (ii) a poly(ethylene glycol) (PEG) capping ligand with a molecular weight of 2000 g/mol (PEG-COOH), which provides water solubility, to study the effect of molecular size and steric interactions between the capping ligands on MOF particle size, and (iii) a biotin capping ligand (biotin-COOH) which allows biofunctionalization via the coupling with streptavidin.

5.2.1 Synthesis and characterization of unmodified MIL-88A

The iron(III) fumarate MOF MIL-88A has been synthesized according to an adapted microwave synthesis of Chalati et al.²⁴ The synthesis yielded elongated particles (aspect ratio of 4.7) with a length of $1.70 \pm 0.55 \mu\text{m}$ and a width of $0.36 \pm 0.11 \mu\text{m}$ (see Figure 5.2a-b for SEM and TEM images). Analysis using X-ray diffraction (XRD) as shown in Figure 5.3 confirms that MIL-88A is crystalline. Moreover, total reflection X-ray fluorescence (TXRF) and elemental analysis (see Table 5.1 and Table 5.2) show similar Fe weight percentages, 23.89 wt% and 25.33 wt% for TXRF and elemental analysis, respectively. These values are similar to the theoretically expected value of 25.32 wt% using the chemical structure of MIL-88A as shown below. Calculations of area and volume for MIL-88A (non-functionalized and functionalized particles) are given in section 5.5.4. Sorption experiments (see Figure 5.4) of MIL-88A gave a Brunauer–Emmett–Teller (BET) surface area of $348 \text{ m}^2/\text{g}$, which is similar to previous results.¹⁶

Figure 5.2 Characterization of MIL-88A: a) SEM and b) TEM images.

Figure 5.3 XRD spectrum of MIL-88A.

Table 5.1 TXRF analysis for MIL-88A and MIL-88A functionalized with 1% PEG.

Sample	Mass Fe (mg)	Mass sample (mg)	%w _{Fe}
MIL-88A (control)	0.43	1.80	23.89
MIL-88A with 1% PEG	0.51	2.40	21.25

Table 5.2 Elemental analyses (w%) of MIL-88A without and with substitution of 1% PEG-COOH capping ligand.

% Ligand	0	1
C	25.02	24.93
Cl	5.36	4.99
Fe	25.33	24.09

Figure 5.4 BET surface areas determined by N₂ sorption experiments.

5.2.2 Surface functionalization of modified MIL-88A

Synthesis of MIL-88A functionalized with CF₃-cy-COOH was performed by substituting 1, 3 or 5% of fumaric acid for CF₃-cy-COOH (see Figure 5.5 for SEM images). XRD experiments confirmed that all samples were crystalline MIL-88A (see Figure 5.6). Note that the powder XRD spectra patterns of the different MIL-88A differ from reported values due to differences in pore opening as a consequence of their flexible structure, their smaller size and the effect of water absorption inside the pores as described before.^{43,44} Additionally, as is shown below, a fraction of the F capping ligand can be

incorporated at an internal coordination site, which may cause some crystal damage leading to smaller crystallite sizes. Sorption experiments of MIL-88A functionalized with 1% F (see Figure 5.4) shows that upon addition of the F capping ligand, the BET surface area decreased by 40%. This decrease in the presence of the F capping ligand is explained in three ways: (i) presence of the ligand at the surface can block the pore entrances, (ii) binding of the ligand inside the lattice at a coordination site may cause crystal damage, smaller crystallite sizes and possible pore blockages and (iii) incorporation of the ligand as a counterion, loosely connected to the lattice, could lead to reduced accessibility.

Figure 5.5 SEM images of MIL-88A with increasing substitution of $\text{CF}_3\text{-cy-COOH}$ capping ligand for a) 1%, b) 3%, and c) 5%.

Figure 5.6 XRD spectra of MIL-88A with increasing substitution of $\text{CF}_3\text{-cy-COOH}$ capping ligand for a) 1%, b) 3% and c) 5%.

XPS was used to analyze the surface elemental composition. Figure 5.7 shows the XPS F1s spectrum of the 3% and 5% ligand samples, qualitatively indicating the presence of F at the surface of the MOF particles. In contrast, the native MIL-88A particles did not show a peak in this region (data not shown). An increase in fluorine content was observed with increasing amount of capping ligand (see Table 5.3). Elemental analysis (Table 5.4) was used to analyze the bulk elemental composition. The fluorine content was found to increase significantly whereas the chlorine content decreased slightly. In order to explain these trends, one has to look at the structure: MIL-88A is composed of Fe_3O inorganic building blocks which are linked together by fumarate ligands. In order to obtain charge neutrality, one negative charge per Fe_3O unit is needed which is a chloride or a negatively charged monovalent capping ligand supplied from the synthesis mixture during preparation. In functionalized MIL-88A, the structure is $\text{Fe}_3\text{O}(\text{OOC}-\text{C}_2\text{H}_2-\text{COO})_3\text{Cl}_z\cdot\text{R}-\text{COO}_{1-z}\cdot 3\text{H}_2\text{O}$, where $\text{R}-\text{COO}$ is the monovalent capping ligand. Based on a statistical composition of these anions (chloride and capping ligand) by assuming equal probabilities for incorporation into the MOF crystal according to their presence in the synthesis solution, the value for z is calculated as 0.980 and 0.968 for 3% and 5% substitution, respectively, by the $\text{CF}_3\text{-cy-COOH}$ capping ligand (see equation 5.14).

Figure 5.7 XPS F1s spectra for MIL-88A functionalized with a) 3% and b) 5% $\text{CF}_3\text{-cy-COOH}$ capping ligand.

Table 5.3 Experimental F/Fe ratios as a function of the fraction of CF₃-cy-COOH capping ligand, measured by XPS.

% CF ₃ -cy-COOH	F/Fe ratio
1	0.047
3	0.116
5	0.294

Table 5.4 Elemental analysis (wt%) of MIL-88A without and with CF₃-cy-COOH capping ligand.

% CF ₃ -cy-COOH	0	3	5
C	25.02	27.83	25.44
Cl	5.36	4.56	4.72
Fe	25.33	21.68	22.50
F	0.00	0.98	1.56

The Cl contents are in good agreement with the stoichiometric value of 0.33 for Cl/Fe since one chloride anion should compensate for the charge of one Fe₃O block (see Figure 5.8). The decrease in chloride content in the MOF crystal upon increase of capping ligand is qualitatively observed as expected from the described statistical counterion model, but the experimental Cl/Fe ratio is not decreasing as much as the model predicts. At the same time, the increasing amounts of F cannot be explained solely by surface occupation. This suggests that a small fraction of CF₃-cy-COOH capping ligand is inside the crystal as a counterion and an even smaller fraction possibly at internal coordination sites, possibly causing crystal defects.

Figure 5.8 Cl/Fe ratios using statistical composition and bulk elemental analysis as a function of CF₃-cy-COOH capping ligand.

For comparing the bulk and surface composition of the MOF, the ratio between fluorine (F) and iron (Fe) is used. In Figure 5.9, the F/Fe atom% ratios are shown for increasing quantities of fluorine-capping ligand as predicted by the statistical counterion model, and obtained from experimental data, bulk elemental analysis and surface-specific XPS analysis. These results show that the MOF contains more fluorine compared to statistical replacement of chloride counterion for both the bulk of the material as well as the surface. This increase is tentatively attributed to population of the capping ligand at the MOF particle surface. For MIL-88A particles of the size observed here, approx. 1.4% of the unit cells is located at the surface (see below), which can explain the observed increase in bulk F content well. With 5% of the CF₃-cy-COOH capping ligand, substantially enhanced fluorine contents in XPS has been observed (see Figure 5.7b), indicating that the surface is the prominent position for the capping ligand.

Figure 5.9 F/Fe atom% ratios using statistical composition, bulk elemental analysis and XPS.

Relative surface densities of the capping ligand have been calculated (equations 5.17-18) by comparing the XPS data with a model for depth-dependent X-ray penetration and photoelectron emission. The model assumes full surface site saturation by CF₃-cy-COOH and statistical counterion incorporation of the monovalent capping ligand replacing a small part of the chloride (see Equation 5.16). Parameters for X-ray penetration, such as the probability of an electron to escape which depends on the depth of excitation and the inelastic mean free path of the electron, are given in Section 5.5.6. The F/Fe ratio resulting from this model is plotted as a function of depth as shown in Figure 5.10. In this way we estimated maximum F/Fe ratios (0.31-0.33) for the different ligand percentages and compared these values to the ones obtained by XPS. Relative surface coverages of 15.3%, 36.8% and 90.0% were estimated (equation 5.18) accordingly for the samples with 1%, 3% and 5% CF₃-cy-COOH capping ligand, respectively (see Table 5.5).

Figure 5.10 F/Fe atom% ratio as a function of depth for a model assuming coordination of all surface Fe_3O sites with the monovalent F capping ligand.

Table 5.5 Coverage percentages for different MIL-88A functionalized with various percent $\text{CF}_3\text{-cy-COOH}$ capping ligands. Experimental F/Fe are measured using XPS analysis.

% $\text{CF}_3\text{-cy-COOH}$	Experimental F/Fe	Theoretical F/Fe	Coverage percentage on the surface (%)
1	0.047	0.3064	15.3
2	0.116	0.3155	36.8
5	0.294	0.3266	90.0

5.2.3 Size control towards nanoMIL-88A

Subsequently, the effect of monovalent capping ligands on particle size and aspect ratio was studied by using the fluorinated ligand and a PEG-functionalized capping ligand. XRD experiments confirmed that all samples were crystalline MIL-88A (see Figure 5.11). For the fluorinated ligand, a significant particle size effect was observed (see Figure 5.5 and Figure 5.12). A statistical analysis (t-test) on the data shown in Figure 5.12 (Table 5.6) showed that all p values are below 0.05 for both 0% vs 1% F and 0% vs 3% F for both length and width, indicating that the differences in particle length and width are significant for all comparisons. For the PEG ligand, the decrease in particle size was more pronounced, as witnessed by a decrease of the length by a factor of 2 when using only 0.1% of PEG

ligand (see Figure 5.13a). Higher quantities of PEG-functionalized capping ligand induced an even stronger particle size decrease, reaching lengths down to ~ 200 nm. Beyond 0.5% of PEG capping ligand (see SEM image in Figure 5.13b), the particle size did not decrease significantly anymore. The width of the PEG-capped particles is less affected as witnessed by an aspect ratio of 3.1 compared to the standard MIL-88A particles (aspect ratio 4.8). Yet, the crystals retained their characteristic elongated shape and triangular faceted ends. The particle sizes using only 0.5% of PEG-COOH are similar to those reported earlier⁸ when using 2% of PEG functionalized with amino groups, with similar size distributions.

Figure 5.11 XRD spectra of MIL-88A with increasing substitution of PEG-COOH capping ligand for a) 0%, b) 0.1%, c) 0.5%, d) 1%, e) 3% and f) 5%.

Figure 5.12 Particle length (left, triangles) and width (right, squares) as a function of percentage CF₃-cy-COOH capping ligand.

Table 5.6 Statistical t-test results for comparisons of the length and width of native MIL-88A and functionalized CF₃-cy-COOH capping ligand.

Cases	<i>p</i> (length)	<i>p</i> (width)
0% vs 1%	1.7×10^{-5}	1.5×10^{-4}
0% vs 3%	6.2×10^{-3}	1.7×10^{-6}

Figure 5.13 Size determinations of MIL-88A modified with PEG-COOH capping ligand: SEM images (a-e) of the resulting MIL-88A as a function of percentage PEG-COOH a) 0.1%, b) 0.5%, c) 1%, d) 3%, e) 5% and f) particle length and g) particle width. Experimental measurements (markers) and trendlines (line, guide to the eye).

The surface fraction of unit cells (defined as the number of unit cells at the surface versus the total number of unit cells see equation 5.15) increases if the particles become smaller. For the standard MIL-88A, 1.0% of the unit cells were at the surface. There is a remarkable difference between PEG- and $\text{CF}_3\text{-cy-COOH}$ -capped MIL-88A: the PEG-capped particles with only 0.5% substitution showed a significant increase in surface fraction of unit cells up to about 4.5% (see Figure 5.14). However, for $\text{CF}_3\text{-cy-COOH}$ -capped particles only a minor increase was observed up to 1.4% (see Figure 5.14). PEGs attached to surfaces can occupy a larger area, thus enhancing the capping effect compared to small capping ligands, and thereby apparently reduce the particle size more strongly. Elemental analysis of MIL-88A functionalized with 1% PEG-COOH (Table 5.2) showed that the %Cl decreased only slightly from 5.36 to 4.99 for 0% to 1% PEG-COOH, respectively, indicating no considerable uptake of the PEG-COOH in the bulk of the crystal according to chloride counterion replacement. At the same time, the C/Fe ratio increase slightly from 0.99 to 1.03 for 0% to 1% PEG-COOH, respectively, which is attributed fully to surface occupation of PEG. The BET surface area (Figure 5.4), however, decreased strongly as compared with the F capping ligand to a value of $58.2 \text{ m}^2/\text{g}$. These measurements show that the porosity is hampered to some extent by the outer functionalization; it appears thus to be dependent on the length of the outer ligand and compares well with earlier observed PEG-covered systems.⁴⁵

Figure 5.14 Surface fraction of unit cells (%) as a function of percentage of $\text{CF}_3\text{-cy-COOH}$ (diamond) and PEG-COOH (square) capping ligands.

5.2.4 Biomolecular surface functionalization of MIL-88A

As a proof of concept for possible biomedical application, MIL-88A was functionalized using a biotin-containing capping ligand. This was achieved by substituting 1% of fumaric acid with biotin-COOH followed by binding of Alexa-Fluor488-streptavidin (AF488-SAv). SEM imaging (Figure 5.15a) shows biotin-MIL-88A particles with a length of $1.50 \pm 0.48 \mu\text{m}$ and a width of $0.13 \pm 0.03 \mu\text{m}$. Additionally, analysis using XRD (Figure 5.16) confirmed the successful synthesis of biotin-MIL-88A. Upon incubation with AF488-SAv, fluorescence imaging (Figure 5.15b) showed a strong fluorescence intensity demonstrating that biotin had been successfully bound to the MIL-88A surface and interacted with AF488-SAv. To test whether these interactions were specific, a control experiment was performed by mixing MIL-88A (without biotin) and AF488-SAv (Figure 5.15c). As expected, this led neither to any significant aggregation nor fluorescence due to the absence of biotin groups on the MOF surface.

Figure 5.15 a) SEM image of biotin-MIL-88A, and overlay images of optical and fluorescence confocal microscopy for b) biotin-MIL-88A after adsorption of AF488-SAv, c) MIL-88A after adsorption of AF488-SAv(control) and d) biotin-MIL-88A after adsorption of AF488-SAv that was partially blocked with 0.8 equiv of biotin.

Figure 5.16 XRD spectrum of MIL-88A functionalized with 1% of biotin-COOH.

Apart from the clear fluorescence of SAV, Figure 5.15b also shows strong aggregation of the MIL-88A-biotin particles upon incubation with SAV. This is attributed to the valency of the SAV: the protein has four identical binding pockets which can induce crosslinking upon interaction with biotin moieties of different particles. Also, this aggregation is promoted by the large ratio of biotin to SAV of 71.6 (see section 5.5.7). In order to suppress the aggregation, we pre-blocked a large fraction of the SAV sites with free biotin before incubation with biotin-MIL-88A. Fluorescence imaging (Figure 5.15d) shows lower cluster formation even for 80% blocked SAV. In addition, the fluorescence intensity for the blocked AF488-SAV experiment was still higher than the blank (Figure 5.17). In summary, these results showed that excluding multivalent specific interactions between MIL-88A-biotin and partially blocked SAV decreased the clustering while maintaining the specific interactions.

Figure 5.17 Mean fluorescence intensity as measured from the fluorescence confocal microscopy images, for MIL-88A after incubation with AF488-SAv (diamonds), biotin-MIL-88A after incubation streptavidin with free biotin blocking 80% of the receptors (squares) and biotin-MIL-88A after incubation with AF488-SAv (triangles). Each measurement in the x-axis is coming from each image taken. The mean fluorescence intensity corresponds to AF488 for each corresponding sample.

5.3 Conclusions

In conclusion, this work demonstrates the benefit of monovalent/multivalent competition under stoichiometric control by using a monovalent capping ligand, to simultaneously control the size and the functionalization of MIL-88A. The surface coverage of MIL-88A was assessed using a capping ligand with fluorine which allowed its quantitative analysis. Both small and large capping ligands significantly provide decrease of the MOF particle size. However, a larger capping ligand is more efficient for decreasing the size due to its larger molecular size. Combining these physico-chemical properties, makes this MOF system a promising candidate for developing a targeting platform for biomolecules such as proteins and nucleic acids and thus potentially for drug delivery applications. For example, the here shown biotin-MOFs may be tuned down in size into the nanoregime by straightforward use of the here developed concept, i.e., by using biotin-PEG-COOH as a linker. This well controlled system will also be beneficial for other materials, such as polymer blends, and other types of MOF systems.

5.4 Acknowledgments

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5.5 Experimental

5.5.1 Materials

The following materials have been used as received from the supplier: Ferric chloride hexahydrate (Acros, 99%), fumaric acid (Fluka, purum), trans-4-(trifluoromethyl)cyclohexane carboxylic acid (Aldrich, 96%), O-methyl-O'-succinylpoly(ethylene glycol)-2000 (Sigma, MW~2000), biotin functionalized with poly(ethylene oxide)₄ carboxylic acid (Uptima, MW=491.6), biotin (Sigma, ≥99%), Alexa Fluor® 488 conjugate with streptavidin (Invitrogen), and absolute ethanol (Merck, p.a.). High purity water was freshly obtained from an Aquatron A4000D system (Barloworld Scientific, Nemours Cedex, France) and was used for the X-ray fluorescence analysis. Milli-Q water with a resistivity of 18.2 MΩ•cm at 25° C was used for the synthesis of MOFs and the rest of the experiments. Gallium standard solution (c = 1,000 mg/L) used for X-ray fluorescence analysis was purchased from Fluka Chemie GmbH (Buchs, Switzerland). Nitric acid (70%; Primar Plus®; Trace Analysis Grade) was purchased from Fisher Scientific (Loughborough, UK). All reagents were purchased in the highest quality available.

5.5.2 Synthetic procedures

Synthesis of MIL-88A

In typical MIL-88A synthesis, ferric chloride (270 mg; 1 mmol) and fumaric acid (116 mg; 1 mmol) were mixed in 5 mL of MilliQ water for at least 20 min under vigorous stirring. The samples were mixed in Pyrex glass vials of 10 mL and closed before heating. A CEM Discover microwave was set at 100 °C with a maximum power of 300W, with the stirring settings at its maximum, and reaction time was set at 5 min. After cooling down to room temperature the reaction mixtures were transferred to a centrifuge tube, centrifuged (eppendorf centrifuge 5804, 10k rpm for 10 min) and redispersed in fresh water twice. Finally the sample was freeze-dried and activated in a vacuum oven at 60 °C for at least 3 h.

Synthesis of MIL-88A functionalized with capping ligands

The procedure for linker implementation was similar to the one described for MIL-88A, except for the steps described here. For the fluorine-containing capping ligand (CF_3 -cy-COOH), a percentage (1, 3 and 5%) of linker (fumaric acid) was replaced by trans-4-(trifluoromethyl)cyclohexane carboxylic acid (4, 12 and 20 mg), respectively. For the polyethylene glycol (PEG) capping ligand (PEG-COOH) of MW of 2000, a percentage (0.1, 0.5, 1.0, 3.0 and 5.0%) of linker (fumaric acid) was replaced by PEG-functionalized capping ligand O-methyl-O'-succinylpoly(ethylene glycol)-2000 (4, 20, 40, 120 and 200 mg) respectively. For biotin capping ligand (biotin-COOH), 1 % linker (fumaric acid) was replaced by biotin-tetra(ethylene oxide)-carboxylic acid (10 mg). The number of COOH groups in all these systems was kept constant by substituting fumarate for capping ligand. Samples were centrifuged once more in ethanol to remove the excess of capping ligands. In total three centrifugations were performed (in the sequence water, ethanol, water) before freeze-drying. Samples using CF_3 -cy-COOH and biotin-COOH capping ligands were dialyzed against water using a dialysis membrane with a MWCO of 1000 for 2 days to remove free linkers.

5.5.3 Methods

Biotin-streptavidin interactions

Phosphate buffered saline (PBS) (0.01 M phosphate; NaCl 0.138 M; KCl - 0.0027 M; pH 7.4, at 25 °C) was used for all experiments with streptavidin. The PBS was mixed with 0.05% of Tween-20. 1 mg/mL of MIL-88A-biotin was dispersed in 0.5 mL PBS and mixed with 1 µg/mL of Alexa-Fluor488-streptavidin (AF488-SAv) in 0.5 mL PBS. The suspension was incubated for 1 h followed by centrifugation at 1000 rpm for 10 min. Then, the suspension was washed three times for 1 h using 1.5 mL PBS. Finally, the sample was washed with 1.5 mL PBS to remove the Tween-20. The same experiment was performed also with a control using non-functionalized MIL-88A. Similar experiments were performed by first blocking 80% of the SAv (with higher concentration of 10 µg/mL) with free biotin and the rest of the experiment was kept the same. After that, images were taken using a confocal microscope.

Statistical analysis for Figure S10

The t-test was performed using the length and width values from Figure 5.12 for the cases of 0% vs 1% F and 0% vs 3% F as shown in Table 5.6. We used the two-tailed and two-sample t-test with unequal variance (heteroscedastic).

5.5.4 Calculations of surface fraction of unit cells for MIL-88A (non-functionalized and functionalized particles)

In this calculation, the length (L) and width (W) of the particles are needed. The L is defined as complete length (including both tips) and W is the complete particle width as shown in Figure 5.18. From Figure 5.18, we define the following parameters:

$$\tan \alpha = \frac{I}{0.5W} \quad \text{Equation 5.1}$$

$$I = 0.65W \quad \text{Equation 5.2}$$

$$0.5W = y \cos \alpha \quad \text{Equation 5.3}$$

$$\cos \beta = \frac{\frac{1}{4}W}{y} = 0.30 \quad \text{Equation 5.4}$$

$$\beta = 72^\circ \quad \text{Equation 5.5}$$

$$\tan \beta = \frac{x}{1/4W} = 3.12 \quad \text{Equation 5.6}$$

$$x = 0.78W \quad \text{Equation 5.7}$$

Then, the area (a) of a triangle is:

$$a_{triangle} = \frac{1}{2} \cdot x \cdot \frac{1}{2}W = 0.195W^2 \quad \text{Equation 5.8}$$

A crystal has 6 main plains of $1/2W \cdot (L-2I)$ and 12 triangles of $0.195 W^2$. Then the total area of the MOF is as follows:

$$a_{total} = 3 \cdot W(L - 1.3 \cdot W) + 2.34W^2 \quad \text{Equation 5.9}$$

For the volume (v) of MOF, one can calculate the linear part and the tips separately and then add them up for the total volume. In this case we have the following:

$$v_{linear} = 1.3 W^2 \cdot (L - 2I) \quad \text{Equation 5.10}$$

$$v_{tip} = 0.2815 W^3 \quad \text{Equation 5.11}$$

$$v_{total} = 1.3 W^2 \cdot (L - 2I) + 2 \cdot 0.2815 W^3 \quad \text{Equation 5.12}$$

For the surface fraction of unit cell (SFUC) defined as the number of unit cells at the surface versus the total number of unit cells is calculated as follows:

$$m = \frac{a_{total}}{a_{unit-cell}} \quad \text{Equation 5.13}$$

$$n = \frac{v_{total}}{v_{unit-cell}} \quad \text{Equation 5.14}$$

$$SFUC = \frac{m}{n} \cdot 100 \quad \text{Equation 5.15}$$

Where m is the # of unit cells in particle surface (UCPS), n is the # unit cells in bulk (UCB), $a_{unit-cell}$ and $v_{unit-cell}$ are the area and volume of a unit cell with values of $163.2 \text{ \AA}^2$ and $1580.6 \text{ \AA}^3$, respectively.⁴⁶

Figure 5.18 Crystal structure of MIL-88A.

5.5.5 Calculations of counterion replacement (z values)

For the functionalized MIL-88A, the structure is $\text{Fe}_3\text{O}(\text{OOC}-\text{C}_2\text{H}_2-\text{COO})_3\text{Cl}_z \cdot \text{R}-\text{COO}_{z-1} \cdot 3\text{H}_2\text{O}$, where $\text{R}-\text{COO}$ is the monovalent capping ligand. Based on the reaction compositions the value for z is calculated as follows: for 3% functionalized $\text{CF}_3\text{-cy-COOH}$ capping ligand, 1 mmol of ferric chloride (3 mmol chloride), 0.97 mmol of fumaric acid and 0.06 mmol of $\text{CF}_3\text{-cy-COOH}$ are used. Then z is calculated as follows:

$$z = \frac{\text{mols}_{\text{Chloride}}}{\text{mols}_{\text{Chloride}} + \text{mols}_{\text{CF}_3\text{-cy-COOH}}} \quad \text{Equation 5.16}$$

5.5.6 Calculations of surface coverage

To estimate the percentage of F ligand-capped surface sites, several assumptions were made. Using the unit cells one can determine the amount of free ligand sites. In this case we can see in Figure 5.19, that there are two free sites for binding at the flat surface of the particle and three free sites at the tip of the particle. Using these values we can determine the number of free sites if the length and width of the particle are known. For XPS, it was assumed that fluorine-containing capping ligands are linked to the top Fe_3O -unit followed by subsequent layers of Fe_3O and fumaric acid up to a depth of 150 Å and that the MOF particle surface is flat. Moreover, also the z value is taken into account of incorporated counterions in the pores. So in the case of 5% $\text{CF}_3\text{-cy-COOH}$ capping ligand MIL-88A there is $100 - 98 = 2\%$ of capping ligand in the porous fumaric acid layer.

Figure 5.19 Schematic view of crystal structure from including free sites for binding with ligand (red arrow) and occupied sites (blue cross). For the straight side, there is 2 capping ligands per repeating unit (red box). For the tip/angled side, there is 3 capping ligands per repeating (green box). Figure adapted from Férey *et al.*⁴⁷

Also, the relative lengths of the molecules have been estimated using ChemBioDraw software. The intensity of the XPS signal is depth dependent since the electron has a lower

probability to reach the detector at a greater excitation depth. This relation follows an exponential function (Equation 5.17), where P is the probability of escape, d is the depth of excitation in Å and λ is the inelastic mean free path (IMFP) of the electron in Å. The latter variable λ is the average distance an electron can travel through the material without scattering (and therefore not reaching the detector). This parameter is highly material-specific and dependent on X-ray energy as well.

$$P(d) = e^{\left(-\frac{d}{\lambda}\right)} \quad \text{Equation 5.17}$$

This latter value is dependent on the X-ray source and the nature of the solid. The IMFP was estimated based on the model of Cumoson & Seah from 1997⁴⁸ which takes qualitatively the respective atoms and the total density of the material into account. The IMFP allows one to estimate the probability of escape as a function of excitation which is directly related to the signal intensity in the XPS spectrum. The estimated λ is 89 Å using a density of 0.5 g/mL.⁴⁹ Then, the theoretical F/Fe was calculated and plotted as shown in Figure 5.11. The F/Fe value plotted here is limiting towards 0.33, this value is then used to compare with the F/Fe values obtained for XPS. These F/Fe ratios are 0.047 for 1%, 0.116 for 3% and 0.294 for 5% CF₃-cy-COOH capping ligand MIL-88A, respectively.

$$\text{coverage percent} = \frac{\left(\frac{F}{Fe}\right)_{\text{experimental}}}{\left(\frac{F}{Fe}\right)_{\text{theoretical}}} \cdot 100 \quad \text{Equation 5.18}$$

5.5.7 Calculation of the biotin to streptavidin ratio

First the surface and volume of a unit cell were calculated using the following values: $a = 11.184$ Å (unit cell width along axis a), $c = 14.591$ Å (unit cell width along axis c). The surface of unit cell is $a \times c = 11.184$ Å $\times$ 14.591 Å = 163 Å² and the volume of unit cell is 1580.6 Å³. Then, the number of biotins per unit cell can be calculated using the surface coverage for 1% using F-capping ligands with a value of 15.3%. The number of biotin capping ligands (CP) on the surface is calculated as follows:

$$CP = 0.153 \left(2 \cdot \frac{a_{\text{linear}}}{a_{\text{total}}} + 3 \cdot \frac{a_{\text{tip}}}{a_{\text{total}}} \right) = 0.344 \quad \text{Equation 5.19}$$

For the MOF-biotin particle, m and n are calculated using equations 5.13 and 5.14 as follows:

$$m = \frac{0.778 \times 10^{-12} m^2}{163 \times 10^{-20} m^2}$$

$$m = 4.77 \times 10^5$$

$$n = \frac{0.032 \times 10^{-18} m^3}{1580.6 \times 10^{-30} m^3}$$

$$n = 2.02 \times 10^7$$

The # of MOF particles per volume is calculated as follows:

$$\# \text{ MOF particles/volume} = (c_{exp}/\rho_{MOF})/V_{MOF} \quad \text{Equation 5.20}$$

where $c_{exp-initial}$ is the Experimental concentration of 1 mg/mL of MOF particles in PBS Tween-20, ρ_{MOF} is the density of the MOF of 1.73 g/mL, V_{MOF} is the Volume per unit MOF of 3.2×10^{-14} mL/per unit MOF. Then the # of MOFs per volumes is:

$$\frac{\# \text{ MOF particles}}{\text{volume}} = 1 \times 10^{-3} \frac{g}{mL} \cdot \frac{1 mL}{1.73 g} \cdot \frac{\text{unit of MOF}}{3.2 \times 10^{-14} mL}$$

$$\frac{\# \text{ MOF particles}}{\text{volume}} = 1.81 \times 10^{10} \cdot \frac{\text{unit of MOF}}{mL}$$

The # biotins per volume is calculated using 0.344 biotin moieties per unit cell, with $m=4.77 \times 10^5$ per MOF. Then, the # biotins per particle is UCPS / per MOF $\times$ 0.344 Biotins per unit cell with a total of 1.64×10^5 biotin/unit MOF. The # biotins per volume (n_{Biotin}) can be calculated as follows:

$$n_{Biotin} = (\#_{Biotin}/\text{unit MOF}) \cdot (\text{unit MOF}/mL) \quad \text{Equation 5.21}$$

$$\begin{aligned} n_{Biotin} &= 1.64 \times \frac{10^5 \text{ Biotin}}{\text{unit}} \text{ MOF} \cdot 1.81 \times 10^{10} \frac{\text{unit of MOF}}{mL} \\ &= 2.97 \times 10^{15} \frac{\text{number of Biotin}}{mL} \end{aligned}$$

MOFs as multivalent materials: size control and surface functionalization

The number of streptavidin (SAv) per volume (n_{SAv}) is calculated as follows:

$$n_{SAv-cavities} = (C_{SAv-initial} \cdot 4 \cdot N_A) / MW_{SAv} \quad \text{Equation 5.22}$$

Where $C_{SAv-initial}$ is the initial concentration of 1 $\mu\text{g/mL}$ in PBS-0.05% Tween 20, MW_{SAv} is 58000 g/mol and N_A is the Avogadro's number. Then, n_{SAv} is calculated as follows:

$$n_{SAv-cavities} = \frac{1 \times 10^{-6} \text{ g}}{\text{mL}} \cdot \frac{1 \text{ mol SAv}}{58000 \text{ g}} \cdot \frac{4 \text{ cavities}}{1 \text{ SAv}} \cdot \frac{6.022 \times 10^{23} \text{ unit SAv}}{\text{mol}}$$

$$n_{SAv-cavities} = 4.15 \times 10^{13} \frac{\text{units SAv}}{\text{mL}}$$

Finally, the ratio between biotin and SAv (r) is calculated as follows:

$$r = n_{Biotin} / n_{SAv-cavities} \quad \text{Equation 5.23}$$

$$r = 2.97 \times 10^{15} / 4.15 \times 10^{13} = 71.6$$

5.5.8 Equipment

X-ray powder diffraction (XRD)

For X-ray diffraction experiments a D2 Phaser (Bruker) using a Cu X-ray source was used.

Scanning electron microscopy (SEM)

For SEM characterization, a FEI ESEM XL30 has been used. Before introducing the samples in the SEM a layer of gold was sputtered to enhance the signal. For high resolution (HR) SEM characterization a Zeiss 1550 FE-SEM was used. The particle dimensions are obtained from SEM images with ImageJ software, for each sample at least 50 particles were measured.

Transmission electron microscopy (TEM)

Transmission electron microscopy (TEM) was performed using a Philips CM300 microscope operating at 300 kV. Samples for imaging were deposited onto a 200 mesh copper grid and the liquid was allowed to dry in air at room temperature.

X-ray fluorescence analysis (TXRF)

X-ray fluorescence analysis (TXRF) was carried out on a S2-Picofox instrument (Bruker AXS, Berlin, Germany) with an air-cooled molybdenum anode for X-ray generation. The excitation settings were 50 kV and 750 mA and quartz glass disks were used as sample carriers. As internal standard, gallium with a concentration of 5 mg/L was applied. The samples were weighed into a vial, mixed with the same volume of the 5 mg/L gallium standard solution and nitric acid, to dissolve them, 1:1 (v/v) up to 2 mL. 5 μ L aliquots of the samples were placed on the sample carriers and evaporated to dryness. The analysis was performed by signal integration over 500 s. For the determination, the signal of iron ($K\alpha_1 = 6.405$ keV) and gallium ($K\alpha_1 = 9.251$ keV), as internal standard, were used. Quantification was performed by the Bruker Spectra software (version 6.1.5.0) and based on the known concentration of the internal gallium standard. Nitrogen sorption experiments are performed with a Autosorb by Quantachrome instruments, prior to analysis samples are outgassed at 155°C for several hours until no significant weight loss was observed.

X-ray photoelectron spectroscopy (XPS)

X-ray photoelectron spectroscopy (XPS) was performed using a PHI Quantera ESCA Microprobe with Al-K α source for all the CF₃-cy-COOH functionalized MIL-88A samples.

Elemental analysis

The MIL-88A functionalized with 0, 3 and 5 % CF₃-cy-COOH capping ligand was analyzed by elemental analysis in the Mikroanalytisches Laboratorium Kolbe as follows: C,H was measured with a CHNOS-Analyzer from Elementar, Fe has been measured after an acidic destruction with an Analyst 200 AAS from Perkin Elmer and Cl and F have been measured with a IC 883 from Metrohm.

Confocal laser scanning microscope

To visualize the biotin-streptavidin interactions, a drop of PBS containing particles was flattened between two glass slides and placed on a Nikon Eclipse Ti confocal laser scanning microscope fitted with a 100x oil objective (NA 1.49, WD 0.12). Differential interference contrast images were taken to examine particle size and morphology.

Streptavidin coupled to Alexa-488 was visualized using a 488 laser to quantify the amount of fluorescence at the particles

Gas sorption

The BET measurements were performed on an Autosorb-1 from Quantachrome. The samples were degassed at 155°C for 3 hours.

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Chapter 6

The effect of PEG length on the size and guest uptake of PEG-capped MIL-88A particles

PEG surface functionalization of MOFs is important for their use in biomedical applications. Here, the effect of the molecular weight of a monovalent PEG-carboxylate capping ligand was investigated in a newly developed stoichiometric procedure that aims to functionalize MIL-88A particles and achieve size control at the same time. The size of the PEG-functionalized MIL-88A decreased with increasing MW_{PEG} , and nanoMOFs were obtained for long (≥ 2 kDa) PEG chains. For lower MW_{PEG} , higher concentrations of PEG were needed to obtain the maximum size effect, but the resulting sizes were still larger than for long PEGs. BET surface area measurements, elemental analyses and zeta potential data showed that the PEG chains were attached to the surface of the MOF particles and not in their interior. Moreover, it was demonstrated that longer chains occupy a larger surface area, and that the PEG chains adopt the low-density brush conformation. Uptake and release experiments with sulforhodamine B dye (as a model drug) showed a higher and faster uptake and release for MIL-88A functionalized with PEG (20 kDa) than the native MIL-88A, which is attributed to a larger surface-to-volume ratio (smaller particles) for the PEG-covered particles, and to the well-hydrated and accessible nature of the PEG layer in aqueous medium. Complete release of the dye was achieved in PBS, the majority by counter ion exchange, and a smaller fraction in the salt form.

6.1 Introduction

Metal-organic frameworks (MOFs), or porous coordination polymers (PCPs), constitute a heavily investigated class of materials consisting of inorganic (metal ions, metal-organic or inorganic clusters) and organic building blocks (organic ligands, polymers or biomolecules).¹ MOFs have strong bonds which provide robustness and a geometrically well-defined structure.² MOF compositions are tuned by varying the valencies of the building blocks and by functionalizing the organic linker during or after synthesis.³

Recently, MOFs have been used in the field of biomedicine.⁴⁻¹⁶ In particular for intravenous drug delivery, nanoMOFs are needed with sizes smaller than 200 nm to freely circulate through the smallest capillaries. NanoMOFs are potential nanovectors for delivering therapeutic agents to targeted areas of the body,¹⁷ as they have large surface-to-volume ratios and high porosities, and can be functionalized easily, thus providing opportunities for drug loading and controlled drug release. A prerequisite for the successful targeted delivery is the ability of nanoparticles to circulate in the bloodstream for a prolonged period of time.³ To accomplish this, hydrophilic “stealth” polymers such as poly(ethylene glycol) (PEG) have been used as coating materials.³⁻⁵

PEG is one of the most common coating materials to shield the surface of nanomaterials (including nanoMOFs), to prevent opsonic interaction and macrophage uptake, and to guarantee prolonged blood circulation.^{18,19} As reported by Gref et al.,⁴ PEG has been incorporated on the surface of MIL-88A, MIL-89 and MIL-100 for the encapsulation and release of anticancer and antiviral drugs. Moreover, nanoUiO-66 has been used as a contrast agent, functionalized with silica and PEG to enhance its biocompatibility and stability.²⁰ Agostoni used another approach, introducing PEG on the surface of nanoMOFs to encapsulate and release a drug for HIV treatment.⁵ In this case, MIL-100(Fe) was functionalized with cyclodextrins (CD), after which the surface was decorated with adamantyl-PEG (Ad-PEG) using inclusion complexes between Ad and CD.

In particular the group of Férey has carried out pioneering work on the exploitation of biomedical applications of surface-modified nanoMOFs.⁴ One of these MOFs is MIL-88A, which has been used for drug encapsulation and as a contrast agent. MIL-88A is very promising because it has a flexible framework, low toxicity, endogenous degradations

products (iron and fumaric acid), is synthesized in an aqueous medium, has good biocompatibility, degradability and imaging properties and high loading capacities compared with other systems. However, the mechanism of drug encapsulation and release using MIL-88A, and the influence that a PEG coating exerts on these properties, has not been determined so far.

Here, we aim to control the size of MIL-88A nanoMOF particles by varying the PEG length and concentration, and to study the effect of PEG length on the loading and unloading of a dye, which functions as a model for drug encapsulation. We adapted the MIL-88A system using Fe_3O and fumaric acid as the multivalent building blocks and used different MWs of monovalent PEG carboxylates (PEG-COOH) as capping ligands, a concept developed in Chapter 5. In this earlier study, we showed that replacement of only a small fraction of fumarate with a stoichiometric amount of monovalent PEG-COOH ($\text{MW}_{\text{PEG}} = 2 \text{ kDa}$) had a strong influence on the particle size. Here, the size of MIL-88A is controlled by varying the PEG length (MW_{PEG}) and its concentration. In order to determine the packing density of PEGs on the surface of MIL-88A, the ratio of organic to inorganic components of MIL-88A as a function of PEG length was measured and compared with theoretical calculations (assuming mushroom and brush conformations). Finally, a model drug was encapsulated using MIL-88A with and without surface functionalization with PEG-COOH to determine the exchange mechanism.

6.2 Results and discussion

6.2.1 Formation and size control of MIL-88A as a function of PEG length

Figure 6.1a shows the concept of forming nanoMIL-88A using different PEG lengths. As shown in Chapter 5, MIL-88A is formed by the coordination of metal clusters, Fe_3O , and organic ligands, fumaric acid, at the core and the surface is functionalized with PEG-COOH. Using monovalent PEG carboxylates with different lengths, the effect of polymer length and concentration on the formation of MIL-88A is evaluated.

Figure 6.1 a) MIL-88A synthesis: conceptual image of the introduction of different lengths of monovalent capping ligands (red) which terminate the particle surface, thereby controlling the particle size and b) Chemical structures of the building blocks used here, with the different PEG lengths corresponding to MW_{PEG} of 178, 588 Da, 2, 5 and 20 kDa. Picture adapted from previous literature.²¹

The formation of MIL-88A using different PEG lengths and concentrations was studied using the procedure described in Chapter 5 for ferric chloride, fumaric acid, and a monovalent PEG-COOH capping ligand (MW 2 kDa), keeping the number of Fe_3O_4 coordination sites and carboxylate moieties (of the bivalent fumaric acid and monovalent PEG-COOH together) constant at a 1:1 stoichiometry. Previous results (Chapter 5) have shown that 1% of this PEG capping ligand was sufficient to achieve most or all of the size decrease. However, in particular for the smaller PEGs, higher fractions (3 or 5%) are needed to obtain the maximum effect, as was shown in Chapter 5 for the fluor-containing small-molecule capping ligand.

Here, MIL-88A was synthesized with 1% of PEG-COOH as a function of MW_{PEG} . X-ray powder diffraction (XRD) measurements were taken for all the samples to confirm that they were crystalline, as shown in Figure 6.2. Note that the powder XRD spectra vary from sample to sample due to differences in capping ligand size and because of their flexible structure.^{22,23} SEM images of all samples are shown in Figure 6.3a-f, and the length and the

width were averaged and plotted as a function of PEG length, as shown in Figure 6.3g. The size of the MIL-88A particles rapidly decreased with increasing MW_{PEG} . For $MW_{\text{PEG}} \geq 5$ kDa, the size reached a plateau of ~ 290 nm in length and ~ 119 nm in width, which is the desired size for intravenous applications. Moreover, for all MW_{PEG} , the MOFs were synthesized as a function of % PEG as shown in Figure 6.4. At low MW_{PEG} (178 or 588 Da), higher fractions (3 or 5%) were needed to reach the maximum size decrease, and even then the lengths remained too large (700-1000 nm). In contrast, for $MW_{\text{PEG}} \geq 2$ kDa, only 0.5% of PEG carboxylate was needed to obtain nanoMOFs. These results are in line with the findings for small and large molecular capping ligands described in Chapter 5, and demonstrate the importance of having steric effects induced by the polymer, next to a termination of coordination sites, to reduce the size of the particles.

Figure 6.2 XRD spectra of a) MIL-88A, and MIL-88A functionalized with 1% of PEG-COOH capping ligand as a function of MW_{PEG} for b) 178, c) 588 Da, d) 2, e) 5, and f) 20 kDa.

Figure 6.3 SEM images of a) MIL-88A and MIL-88A functionalized with 1% PEG-COOH capping ligand as a function of MW_{PEG} for b) 178, c) 588 Da, d) 2, e) 5, and f) 20 kDa. Average values from SEM images (a-f) for: g) length and h) width, as a function of MW_{PEG}. Experimental measurements (markers) and trendlines (lines, guide to the eye).

Figure 6.4 Particle a) length and b) width as a function of the percentage of PEG-COOH capping ligand from SEM analysis using different MW: 0 (■), 178 (●), 588 Da (◆), 2 (○), 5 (—) and 20 kDa (×).

Brunauer–Emmett–Teller (BET) surface areas were calculated from N_2 sorption isotherm measurements for four samples: non-functionalized MIL-88A, and MIL-88A functionalized with 1% of PEG-COOH with a MW_{PEG} of 178 Da, 2 and 20 kDa to study the effect of PEG length (see Figure 6.5). These results show that addition of capping ligands led to a decrease of the BET surface area, from $347 \text{ m}^2/\text{g}$ for unfunctionalized MIL-88A to $165 \text{ m}^2/\text{g}$, $58.2 \text{ m}^2/\text{g}$ and $5.8 \text{ m}^2/\text{g}$ for the functionalized MOFs. This strong decrease is consistent with the observations made in Chapter 5 and other studies.⁵ These results suggest that PEG is blocking the access to the pores (which is more drastic for longer PEGs) and impedes the access of gases to the MOF's interior.

Figure 6.5 BET surface areas determined from N₂ sorption experiments for MIL-88A made in the absence or presence of 1% of PEG-COOH capping ligand (MW_{PEG} of 0.178, 2 or 20 kDa).

Elemental analysis was measured for all samples, as shown in Table 6.1. In functionalized MIL-88A, the structure is $\text{Fe}_3\text{O}(\text{OOC-C}_2\text{H}_2\text{-COO})_3 \cdot (\text{fumaric acid})_x \cdot \text{Cl}_z \cdot \text{R-COO}_{1-z} \cdot 3\text{H}_2\text{O}$, where R-COO is the monovalent capping ligand and fumaric acid is occasional, freely adsorbed fumaric acid in the MOF. In order to obtain charge neutrality, one negative charge per Fe_3O unit is needed, which is supplied by a chloride or negative monovalent capping ligand (or divalent fumarate) from the synthesis mixture during preparation. The high atomic Cl/Fe ratio shown in Table 6.1 indicates that Cl is practically the sole counterion. More importantly, this ratio is not changing for longer PEGs. This is in agreement with the results reported in Chapter 5, where no fumaric acid (mono or bis-charged) or little monovalent capping ligand (only for the small molecular weight ligands) was observed as a counterion. Moreover, little fumaric acid is bound inside the lattice, indicated by low fumaric acid fractions. At the same time, the C/Fe ratio is increasing, which is attributed to surface-attached PEG. In summary, we can conclude that PEG-COOH is not incorporated as a counterion and is only attached at the surface of MIL-88A.

Table 6.1 Elemental analyses (w%) of MIL-88A made in the absence or presence of 1% of PEG-COOH capping ligand using different MW_{PEG}.

MW _{PEG} (kDa)	C (w%)	H (w%)	Fe (w%)	Cl (w%)	Cl/Fe*	Fe ₃ O(OOC-C ₂ H ₂ - COO) ₃ ·(fumaric-acid) _x ·Cl _z ·R- COO _{1-z} ·3H ₂ O **
0	25.02	2.47	25.33	5.36	1.00	x = 0.4 and z = 1
0.178	24.96	2.57	24.37	5.17	1.00	x = 0.3 and z = 1
0.588	23.97	2.86	27.52	5.53	0.95	x = 0.04 and z = 1
2	24.93	2.74	24.09	4.99	0.98	x = 0.1 and z = 1
20	25.30	3.59	21.81	5.48	1.19	x = 0.03 and z = 1

* atomic ratio = (w%Cl/AW_{Cl})/(w%Fe/AW_{Fe}) in which AW are the atomic weights (35.45 and 167.55 Da, respectively) ** Fit parameters of the elemental analysis data to the empirical formula.

In order to confirm that PEG is present on the MOF surface, zeta potential (ζ) measurements were conducted, as shown in Figure 6.6. These results show first a sharp decrease, then a gradual increase of ζ . The zeta potential of non-functionalized MIL-88A is positive because of an excess of free Fe sites at the surface. Probably the native MIL-88A has not only free Fe sites, but also sites capped by fumaric acid, ending in a carboxylate. The Fe sites are in slight excess, causing the overall positive charge. After incorporation of small capping ligands (MW_{PEG}=178 or 588 Da), these ligands block some of the surface Fe sites which leads to a decrease of about 50% of ζ . PEG itself can cause a negative zeta potential, attributed to the attraction of OH⁻.²⁴ At higher MW_{PEG}, PEG occupies a larger surface area, resulting in the occupation of fewer Fe sites (see also the PEG packing densities discussed below). This explains the upward trend in ζ for higher MW_{PEG} as observed in Figure 6.6. In summary, these results show that longer PEGs occupy a larger surface area leading to fewer chains (in comparison with shorter chains) per unit surface area of MOF.

Figure 6.6 Zeta potential, ζ , measurements for MIL-88A in the absence or presence of 1% of PEG-COOH capping ligand using different MW_{PEG}.

In summary, we have shown here that the formed nanoMOFs have sizes appropriate for biomedical applications, in particular for MW_{PEG} $\geq$ 2 kDa. As observed in Chapter 5, we see a dependence on the PEG capping ligand concentration for all MW_{PEG}. At low MW_{PEG} ($\leq$ 588 Da), high PEG concentrations are needed to observe a large effect on the size, while at high MW_{PEG} ($\geq$ 2 kDa), 0.5% PEG is enough to obtain nanoMOFs. At MW_{PEG} = 2, 5 and 20 kDa, higher concentrations of capping ligand do not cause a further reduction in size. This shows the importance of steric shielding from the PEG chains during the MOF synthesis and suggests that PEG is present only on the surface of MIL-88A.

After showing that the MOF size is controlled by the PEG length and concentration, understanding the packing of the PEG chains on the MOF surface as a function of PEG length is important to understand the kinetics of drug release. Thermogravimetric analysis (TGA) was used to measure the organic (m_{org}) to inorganic mass (m_{inorg}) ratio of functionalized MIL-88A as a function of PEG chain length. Two examples, for unfunctionalized MIL-88A and with 20 kDa PEG, are shown in Figure 6.7a,b. A first weight loss occurred below 100 °C, which is attributed to moisture and gas molecules adsorbed to the MIL-88A surface and inside the pores. The weight of MIL-88A remained nearly stable up to $\sim$ 200°C, when fumaric acid and PEG started to decompose, until reaching 400 °C. Subsequently, the weight of MIL-88A remained unchanged until 600 °C.

The remaining mass at 600 °C is m_{inorg} . For m_{org} , the loss of water (m_{water}) was calculated first and then m_{org} was calculated by subtracting m_{water} and m_{inorg} from the initial total mass. The m_{org} to m_{inorg} ratio was plotted as shown in Figure 6.7c. The m_{org}/m_{inorg} value of approx. 1.5 is in agreement with a weight ratio of fumaric acid and Fe_3O_4 in correspondence with the composition of fumaric acid and Fe_3O in the bulk of MIL-88A. The results for the functionalized MOFs (Figure 6.7c) exhibit high standard deviations and the ratios are similar for functionalized and unfunctionalized MOFs, indicating that the PEG outer layer has little or no influence on the TGA results. T-tests for different sets (for selected $MW_{PEG}=0, 2$ and 20 kDa) showed no statistically significant differences between these cases, with p values higher than 0.05 (Figure 6.7d). In summary, we conclude that TGA is not sensitive enough to differentiate between different PEG lengths on the MOF surface. This indicates that the expected increase of the amount of PEG (in weight) that is attached to the MOF surface is too little to observe by TGA.

Figure 6.7 TGA plots for a) unmodified MIL-88A and b) MIL-88A functionalized with 1% PEG-COOH (MW=20 kDa). c) The m_{org}/m_{inorg} ratios of the TGA results for different MW_{PEG} . d) t-test for unmodified MIL-88A vs MIL-88A functionalized with 1% PEG-COOH (MW = 2 kDa), unmodified MIL-88A vs MIL-88A functionalized with 1% PEG-COOH (MW = 20 kDa) and MIL-88A functionalized with 1% PEG-COOH (MW = 2 kDa) vs MIL-88A functionalized with 1% PEG-COOH (MW = 20 kDa).

There are several ways in which PEG may cover the surface of a material. At low surface coverage, the PEG chains exist as isolated free coils in solution, also called the “mushroom” regime. If the PEG density is increased, excluded volume interactions between chains will force extension of the PEG chains more and more, and the layer will increase in thickness. Such extended polymer conformations are called “brushes”. The “brush” regime usually denotes the equilibrated state where the free energy of the crowded polymer chains in the steric layer balances the energy of creation of interface between the solution phase and the core phase.²⁵

To study surface packing, Prud’homme²⁶ formed core-shell nanoparticles where the core was composed of polystyrene (PS) and the surface was covered with PEG chains. They measured experimentally the PEG coverage per particle and compared these values with theoretical values using mushroom and brush regimes. Because of the large PS particle size, the surface was assumed to be flat. Here, we use the analytical approach of Prud’homme, using the same assumptions, except for the core which we take to be composed of magnetite (Fe₃O₄) only and the surface is covered with PEG. We also applied other parameters for the surface tensions using MOFs²⁷ and zeolites²⁸ instead of magnetite at the core. These calculations (not shown here) led to brush density predictions that are of about the same order of magnitude as when using the parameters of magnetite. All gave (much) higher densities than for the mushroom conformation, and the calculations for magnetite lie in between those of the others. For both regimes, the projected diameter, ξ , i.e., the diameter of the area that a PEG chain occupies at the surface, is calculated. For the mushroom regime, the blob size is calculated using the Flory radius in a good solvent (water) as follows:

$$\xi_{\text{mushroom}} = 2 \cdot R_f = 2 \cdot a \cdot N^{3/5} \text{ (m)} \quad \text{Equation 6.1}$$

where ξ_{mushroom} is the diameter of a free polymer coil assuming no interactions between the neighboring chains, R_f denoting the Flory radius of gyration of each blob, a is the size of the individual ethylene glycol monomer (0.35 nm)²⁹ and N is the number of monomer repeat units. For surface packing density calculations (below), we assume a densest hexagonal packing of such PEG chains to obtain the mushroom values.

For the brush regime, the PEG spacing and brush thickness are calculated assuming an energy equilibrium of the polymer layer at the surface. Since the radius of the nanoMOF particle is much larger than the brush length, a flat plate approximation is appropriate to estimate the projected size of a PEG brush. The approximation is based on the minimization of free energy of PEG at the interface.^{26,30} The free energy (F) is composed of (1) a surface energy term and (2) an entropic contribution term due to the stretching of the polymer chain and is given by:^{30,31}

$$F = \gamma_{\text{eff}} \pi \frac{\xi_{\text{brush}}^2}{4} + \frac{3}{2} kT \frac{d^2}{Nb^2} \quad \text{Equation 6.2}$$

where γ_{eff} is the effective surface energy, ξ_{brush} is the projected diameter of a PEG molecule in a brush regime, k is the Boltzmann constant, T is the temperature, d is the end-to end distance of a single PEG brush, N is the number of PEG monomers in a chain, and b is the Kuhn monomer length of PEG. If g is the number of monomers in an extended PEG molecule, then $\xi_{\text{brush}} = bg^v$, where $v = 3/5$ for a real chain,³¹ and d , is described as:

$$d = \xi_{\text{brush}} \frac{N}{g} = \xi_{\text{brush}}^{(v-1)/v} Nb^{1/v} \quad \text{Equation 6.3}$$

Substituting d as described in Equation 6.3 into the free energy expression in Equation 6.2, the following expression is obtained:

$$F = \gamma_{\text{eff}} \pi \frac{\xi_{\text{brush}}^2}{4} + \frac{3}{2} kT \frac{(\xi_{\text{brush}}^{(v-1)/v} Nb^{1/v})^2}{Nb^2} \quad \text{Equation 6.4}$$

Minimizing the free energy expression in Equation 6.4 with respect to ξ_{brush} yields the following expression:

$$\xi_{\text{brush}} = \left(\frac{4kTNb^{4/3}}{\gamma_{\text{eff}} \pi} \right)^{3/10} \quad (\text{m}) \quad \text{Equation 6.5}$$

The surface tension of a material, γ , consists of two terms: a dispersive term (γ^d) and a polar term (γ^p) and is calculated as follows:³⁰

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$$\gamma = \gamma^p + \gamma^d \quad \text{Equation 6.6}$$

The interfacial tension between the material (a) and water (b), γ_{ab} can be approximated using harmonic mean expression as follows:³¹

$$\gamma_{ab} = \gamma_a + \gamma_b - 4 \frac{\gamma_a^d \cdot \gamma_b^d}{\gamma_a^d + \gamma_b^d} - 4 \frac{\gamma_a^p \cdot \gamma_b^p}{\gamma_a^p + \gamma_b^p} \quad \text{Equation 6.7}$$

Then, the interfacial tension of the MOF functionalized with PEG suspended in water can be represented by:

$$\gamma_{eff} = \frac{b^2}{\xi_{brush}^2} \gamma_{magnetite,PEG} + \left(1 - \frac{b^2}{\xi_{brush}^2}\right) \gamma_{magnetite,water} \quad \text{Equation 6.8}$$

From here on, the following equations show how to calculate the theoretical m_{org} to m_{inorg} ratios for the mushroom and brush regimes. After $\xi_{mushroom}$ and ξ_{brush} are calculated, the number of chains present on a particle in each regime, $\#PEG_{mushroom}$ and $\#PEG_{brush}$ ($\#PEG_i$), are first calculated using the following equation, with a_{MOF} the particle surface area covered by PEG, as calculated from the experimental lengths and widths (see Figure 6.4) using the crystal facet description of Chapter 5.

$$\#PEG_i = \frac{a_{MOF}}{\pi \left(\frac{\xi}{2}\right)^2} \quad \text{Equation 6.9}$$

Next, the ratio m_{org} to m_{inorg} ratios for both regimes is calculated using the following equation.

$$\frac{m_{org}}{m_{inorg_i}} = \frac{\#PEG_i \cdot MW_{PEG} + \#fumaric\text{-}acid \cdot MW_{fumaric\text{-}acid}}{\#Fe_3O_4 \cdot MW_{Fe_3O_4}} \quad \text{Equation 6.10}$$

where $\#fumaric\text{-}acid$ and $\#Fe_3O_4$ are calculated using the unit cells in bulk as shown already in Chapter 5. $MW_{fumaric\text{-}acid}$ and $MW_{Fe_3O_4}$ are the molecular weights of fumaric acid and Fe_3O_4 , respectively.

Values for PEG, magnetite, and water as shown in Table 6.2 were used to calculate the m_{org}/m_{inorg} ratios for the brush and mushroom regimes using equation 6.10. All theoretical values together with the experimental values are plotted in Figure 6.8. These results show

that the brush values go significantly higher than the experimental values, also when taking the large standard deviations (Figure 6.7c) into account. The experimental values are more dense than the maximum mushroom packing density, but the difference is statistically insignificant. Therefore we conclude the PEG chains to be in a low-density brush conformation, and assembled in a packing in which the chains have steric interactions with each other. In a study of MIL-88A functionalized with PEG of 5 kDa,⁴ the authors concluded that the PEG chains adopted a superficial PEG 'brush' sterically protecting the nanoparticles from aggregation. In summary, together with the enhanced size control effect of PEGs for the higher MWs, we assume that the PEG coverages are in the low-density brush conformation.

Table 6.2 Values to calculate ζ_{brush} and surface tension values for PEG,³⁰ magnetite³² and water.²⁷

k	$1.38 \times 10^{-23} \text{ J/K}$
T	298 K
N	44 g/mol
b	1.1 nm^{26}
γ_{PEG}^p	12 mN/m
γ_{PEG}^d	30.9 mN/m
$\gamma_{magnetite}^p$	3.5 mN/m
$\gamma_{magnetite}^d$	48.9 mN/m
γ_{water}^p	50.7 mN/m
γ_{water}^d	22.1 mN/m

Figure 6.8 m_{org}/m_{inorg} ratios of MIL-88A functionalized with 1% PEG-COOH capping ligand as a function of MW_{PEG} (kDa). Experimental (♦, black) and theoretical ratios for no PEG (●, gray), mushroom conformation (■, gray), and brush conformation (▲, gray). Trendlines are guide to the eye. For mushroom and brush regimes, it is assumed that the PEG chains are attached on a flat surface. For all the calculations, dimensions given in Figure 6.3 are used.

A comparison of the packing densities of the different models is performed and compared to the density of surface coordination sites and to literature values of packing densities. Hereto, the densities of PEG (σ , mol/cm²) were calculated from the ξ values as a function of the molecular weight, as shown in Figure 6.9. When comparing the packing densities of the mushroom and brush conformations to the density of available coordination sites, we observe that the brush and mushroom packings occur always at lower densities than that of the available coordination sites. This implies that PEG functionalization leads to partial use of the coordination sites and to steric interactions between the PEG chains regardless of the length of the PEG. This is in agreement with the zeta potential results discussed above. Only at low molecular weights, on the order of about 100 Da, full occupation of coordination sites can be expected without strong steric repulsion, in line with the practically complete functionalization using the small-MW fluor-containing capping ligand described in Chapter 5.

In order to compare our model values to literature data, we first calculated the experimental number of polymer chains (#PEGs) per surface area using the ratio of m_{org} and m_{inorg} from TGA and equation 6.10, and compared these values with our calculations and with values from the literature. Two series of literature values were plotted together

with the mushroom, brush calculations and experimental values as shown in Figure 6.9. The experimental values with $MW_{\text{PEG}} \geq 2\text{kDa}$ fit well between the brush and mushroom calculations and match well with the observations above that PEG chains are in a low-density brush conformation. The first literature series is a “cloud” with densities around 10^{10} mol/cm^2 at MWs of about 10^4 Da , obtained for surface-initiated atom-transfer radical-polymerization of methyl methacrylate at different types of surfaces (flat, convex, and concave/convex) and different kinds of materials (silicate and gold).³³ These chain brushes fit between the model lines for the coordination sites and the brush regime. These indicates higher, more compressed, brush densities than expected from the thermodynamics model described above because these chains are growing from the surface starting from a high initiator density. The second data set has been obtained for PEG chains end-grafted to a gold substrate surface³⁴ and for PEG grafted at a PS core.²⁶ Here, the authors calculated the densities of these PEG chains assuming they are in the mushroom regime. However, these values are higher than our calculated mushroom values and very similar to our experimental values. All-in-all, we conclude that the PEG chains in our MOF system are in a low-density brush regime and use only part of the available coordination sites.

Figure 6.9 Packing densities, σ (mol/cm^2), for different models: experimental ($\circ$), mushroom ($\blacklozenge$), brush ($\blacksquare$), coordination sites for the linear and tip sections of a MIL-88A crystal ($\blacktriangle, \times$), and values in the literature for different brushes on flat surfaces³³($\bullet$) and PEG chains assuming that they are in mushroom regime (+).^{26,34} For the mushroom and brush regimes, equation 6.9 was used: $\sigma = \# \text{PEG} / (a_{\text{MOF}} \cdot N_A)$, where N_A is Avogadro's number. The coordination site values are calculated from Chapter 5. Trendlines (lines, guide to the eye).

6.2.2 Encapsulation and release of sulforhodamine B

The encapsulation and release properties of the PEG-functionalized nanoMOFs was explored to study the effect of the PEG shell. We used the anionic sulforhodamine B as a model drug and studied its encapsulation and release using PEG-functionalized MIL-88A with different MW_{PEG} . Because of the presence of the chloride anion in the lattice, a possible uptake and release mechanism is by counterion exchange, as shown in Figure 6.10. Sulforhodamine B dye was chosen as a model drug because it is overall negatively charged and it is easy to quantify its encapsulation and release properties using fluorescence and UV/vis spectroscopy.

Figure 6.10 Encapsulation and release of sulforhodamine B by counterion exchange.

In order to study the effect of PEG on the MOF surface, sulforhodamine B was encapsulated into two different MOFs, non-functionalized MIL-88A and MIL-88A functionalized with 1% PEG-COOH ($MW_{\text{PEG}}=20$ kDa; PEG20k-MIL-88A). First, the dye, in the mono Na^+ form, was encapsulated by adsorption into the porous interior of the MOF using a saturated aqueous solution, in the absence of additional salts. The loaded particles were rinsed multiple times with water, until no leaching was observed anymore. As shown in Figure 6.11, 8 washings were sufficient. Subsequently, all the dye-loaded particles were washed eight times, then freeze-dried. The amount of encapsulated dye was determined by degrading the particles with HCl for 16 h, followed by raising the pH to 7 and quantifying the dye in solution using a fluorescence calibration line (see Figure 6.14), yielding dye encapsulation results of 0.052 ± 0.007 wt% (for MIL-88A) and 0.095 ± 0.008 wt% (for

PEG20k-MIL-88A). This shows that PEG20k-MIL-88A can encapsulate more dye than non-functionalized MIL-88A. A possible reason for this enhanced uptake could be entrapment of dye by the PEG layer. However, this layer is very hydrophilic and well hydrated, so it is unlikely that the PEG layer can hold the dye upon prolonged washing with water. In case the dye diffuses into the MOF interior, this diffusion is probably difficult and slow because a relatively large molecule has to move into a pore of approximately the same size. This effect has been observed for zeolites to result in preferential location of the dye in the pores that are closer to the surface.³⁵ In this case, the dye loading will depend on the total particle surface area, which is corresponding to the surface fraction of unit cells (SFUC) (see Equation 5.15, Chapter 5). Thus, the dye loading of MIL-88A and PEG20k-MIL-88A was plotted vs. SFUC, as shown in Figure 6.12. The linear trend observed here supports the idea that the loading of the dye depends on the surface to volume ratio of the particles.

Figure 6.11 Monitoring the cumulative leaching of sulforhodamine B from freshly loaded MIL-88A particles (without PEG) as a function of the number of washing steps (with water) measured using UV/vis; see calibration lines in Figure 6.15. All experiments were carried out in triplicate.

Figure 6.12 Dye-loading as a function of the surface fraction of unit cells (SFUC, equation 5.15 from Chapter 5) for MIL-88A and PEG20k-MIL-88A.

The release of sulforhodamine B from the loaded particles was studied for both unmodified MIL-88A and PEG20k-MIL-88A, in the absence and presence of salt in the washing solution. To determine the release over time, we placed both samples in water and in phosphate buffer saline (PBS) (pH 7.4, 0.137 M NaCl). For each data point, half of the supernatant was removed from the dye-loaded MOF suspension and replaced by fresh water or PBS, and the amounts of sulforhodamine B in the supernatant aliquots were analyzed. The dye release was measured for up to 5 days as shown in Figure 6.13a. The results show a very quick release on the first day, but the sampling was done here more frequently. When looking at the released dye per sampling point, the change in released dye is practically monotonous during the first 3 days. This indicates that equilibrium between bound and free dye is reached before a new aliquot is taken. The release is substantially higher for PBS than for water in both of samples. Moreover, PEG20k-MIL-88A releases the dye slightly faster than MIL-88A, again in agreement with the higher surface area of the former sample. After 5 days, the samples in PBS had completely released the dye, while the samples rinsed with water still contained approx. 60-75% wt% of the initially loaded dye. These results indicate that counterion exchange is the most important uptake and release mechanism. At the same time, there is a notable fraction of dye released from water, which indicates that part of the dye has also been taken up as a salt.

Figure 6.13 Sulforhodamine B released (measured using UV/vis; see calibration lines (in Figure 6.15) in water (diamonds) and PBS (circles) using MIL-88A (black) and PEG20k-MIL-88A (gray), refreshing the solvent at least daily for 5 d (a), and hourly during 6.5 h (b). All experiments were carried out in triplicate (a) or duplicate (b).

To study better the early release stage, possibly before reaching equilibration, we refreshed the solution more often. Thus, the experiments were repeated for a period of 6 h while refreshing the solvent every hour, as shown in Figure 6.13b. We observed qualitatively the same behavior: complete release was achieved for PEG20k-MIL-88A and MIL-88A in PBS after 6 h showing that exchange of anions is needed to achieve complete dye release as compared to when only water is used. The overall release rates were somewhat faster than when the solutions were refreshed less frequently. In both solvents, the dye was released slightly faster when PEG was present on the surface, as explained by the higher surface fraction for this MOF as discussed above. Still, it is surprising to note that the PEG layer has no retarding effect on the release kinetics, this in stark contrast to the much lower BET values as discussed above. As a possible explanation, the PEG layer is well hydrated in water, providing practically unhindered access of the dye to the MOF pores from water, whereas gas adsorption in the dry state encounters a collapsed and dense PEG polymer layer that does affect its uptake.

6.3 Conclusions

In conclusion, we have studied the dependence of the size and properties of MIL-88A by varying the length of the capping ligand, PEG-COOH. At low MW_{PEG} , the size of MIL-88A was found to be more sensitive to changes of the PEG length. A capping ligand with $MW_{\text{PEG}} \geq 2$ kDa is needed to obtain nanoMIL-88A suitable for biomedical applications. At low MW_{PEG} (178 and 588 Da), a higher PEG concentration is needed to obtain the smallest possible particle size, but these sizes are still larger than for longer PEGs. At high MW_{PEG} (2, 5 and 20 kDa), a small PEG fraction (0.5% PEG) is sufficient to obtain nanoMOFs.

It was demonstrated that PEG chains are not incorporated in the bulk of the crystal and only attach to the surface of the MOF. Experimental organic and inorganic fractions were compared with theoretical compositions and suggest that PEG chains are present in a low-density brush conformation, slightly more densely packed than mushroom conformation. A capping ligand needs to have a small MW (about 100 Da) to be able to occupy all coordination sites on the MOF surface; larger capping ligands lead to fractional occupation of surface sites and steric interactions between the ligands.

Encapsulation and release were demonstrated using MIL-88A and MIL-88A functionalized with PEG-COOH (20 kDa) and it was demonstrated that negatively charged molecules can be encapsulated by counterion exchange and, to a smaller extent, by diffusion into the MOF interior as a salt. Complete dye release was possible by counterion exchange using PBS. Notably, encapsulation and release was higher and faster in the presence of PEG. The higher capacity is attributed to slow diffusion within the MOF core and thus to dependence on the total surface area, which is higher for the PEG-covered MOF because of the smaller observed particle sizes. The faster release indicates that the PEG layer has no strong influence on the accessibility of the pores for guests in water, which is in stark contrast to the much lower BET values observed for gas sorption measurements in the dry state.

The improved understanding of the formation of ligand-capped MIL-88A using different PEG lengths and of the uptake and release mechanisms underlying guest encapsulation, may lead to further support for MIL-88A as a promising candidate for theranostic multifunctional nanocarriers.

6.4 Acknowledgments

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6.5 Experimental

6.5.1 Materials

The following materials have been used as received from the supplier: Ferric chloride hexahydrate (Acros, 99%), fumaric acid (Fluka, purum), 2-[2-(2-Methoxyethoxy)ethoxy]acetic (Sigma, MW 178.18), O-(2-carboxyethyl)-O'-methyl-undeca(ethylene glycol) (Sigma, MW 588.68), O-methyl-O'-succinyl-poly(ethylene glycol) (Sigma, MW~2000 and ~5000), O-[2-(3-succinylamino)ethyl]-O'-methyl-polyethylene glycol (Sigma, MW~20,000), sulforhodamine B (Sigma, dye content 75 %), ethylenediaminetetraacetic acid dipotassium salt dihydrate (EDTA) (Sigma, ≥99.0%), ethanol absolute (Merck, p.a.) and PBS (Sigma). Milli-Q water with a resistivity of 18.2 MΩ•cm at 25°C was used for the synthesis of the MOFs and for the drug encapsulation and release of sulforhodamine B.

6.5.2 Synthetic procedures

Synthesis of MIL-88A and PEG-covered MIL-88A

The native and PEG-covered MOFs were synthesized using the procedures described in Chapter 5 for the native MOF and the MOF using the PEG capping ligand with MW_{PEG} = 2 kDa.

6.5.3 Methods

Sulforhodamine B encapsulation in MIL-88A and in PEG20k-MIL-88A ($MW_{\text{PEG}} = 20$ kDa)

Sulforhodamine was adsorbed into the pores of MIL-88A or PEG20k-MIL-88A using a suspension of 61 or 63.8 mg of dehydrated solid and 536 or 443.4 mg of sulforhodamine B in 24.4 or 25.52 mL of water, respectively at room temperature by mixing for 16 h. The sulforhodamine-loaded particles were collected by centrifugation at 8000 rpm for 10 min and washed 8 times with water followed by freeze-drying.

In order to measure the amount of encapsulated dye, ~ 1.5 mg of the sulforhodamine-loaded particles was degraded under acidic conditions (~ 1.5 mL of 5 M aqueous HCl at 50°C overnight). Experiments were done in triplicate. Then, EDTA was added to the solution, the pH of the solution was raised to 7 and the solution was measured using fluorescence spectroscopy using a calibration curve as shown in Figure 6.14.

Figure 6.14 Fluorescence calibration curve of sulforhodamine B in water.

Sulforhodamine B release in water and PBS

The release of sulforhodamine B was studied by suspending ~ 2 mg of non-functionalized and functionalized MIL-88A nanoparticles containing sulforhodamine B in 1 mL water or PBS (pH 7.4). These suspensions were kept by mixing for different incubation times (from 30 min to 5 d, or from 30 min to 6.5 h). At each time point, an

aliquot of 0.5 mL of supernatant was recovered by centrifugation (1000 rpm/10 min) and replaced with the same volume of fresh solvent. The dye release was measured using the UV calibration curves shown in Figure 6.15. Experiments were done in triplicate (series from 30 min to 5 d) or duplicate (series from 30 min to 6.5 h).

Figure 6.15 UV-calibration curves of sulforhodamine B in a) PBS and b) water.

Statistical analysis

The t-test was performed using two-tailed and two-sample t-test with unequal variance (heteroscedastic).

6.5.4 Equipment

X-ray powder diffraction (XRD)

For XRD experiments a D2 Phaser (Bruker) using a Cu X-ray source was used.

High resolution scanning electron microscopy (HRSEM)

For HRSEM a Zeiss 1550 FE-SEM was used. The particle dimensions are obtained from SEM images with ImageJ software, for each sample at least 50 particles were measured.

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Elemental analysis

The MIL-88A samples functionalized with 1% PEG-COOH capping ligand with different MW_{PEG} were analyzed by elemental analysis at the Mikroanalytisches Laboratorium Kolbe (Mülheim an der Ruhr, Germany), as follows: C, H were measured with a CHNOS-Analyzer from Elementar, Fe was measured after an acidic destruction with an Analyst 200 AAS from Perkin Elmer, and Cl and F were measured with a IC 883 from Metrohm.

Gas sorption

The BET measurements were performed on an Autosorb-1 from Quantachrome. The samples were degassed at 155°C for 3 h.

Thermogravimetric analysis (TGA)

TGA was performed using a Pyris 1 TGA Perkin Elmer on dried modified and unmodified MIL-88A under oxygen flow (20 mL/min) at a heating rate of 10 °C/min from 40-600 °C.

Zeta potential

Zeta potentials were measured on a Zetasizer NanoZS (Malvern Instrument Ltd, Malvern, United Kingdom) at 20 °C, with a laser wavelength of 633 nm and a scattering angle of 173°.

Fluorescence spectroscopy

Fluorescence spectra were recorded using a Perkin Elmer LS 55 fluorescence spectrophotometer equipped with a high energy pulsed Xenon source for excitation. Emission and excitation slits were kept constant at 10 nm and scan speed of 100 nm/min using an excitation wavelength of 560 nm sulforhodamine B.

UV/vis spectroscopy

UV/Vis absorption spectra were recorded using a Perkin Elmer Lambda 850 UV-Vis spectrometer. Two calibration curves of sulforhodamine in water and PBS were performed.

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The effect of PEG length on the size and guest uptake of PEG-capped MIL-88A particles

Chapter 7

Engineering the surface of MIL-88A for DNA detection

Surface ligands determine, to a great extent, the biologically relevant functions of various kinds of nanosized particles. This is particularly important for drug delivery, where active ligands provide specific interactions to guide the particles to the desired place in the body, and for biosensing, where moieties that recognize the analyte of choice provide the necessary selectivity in binding the analyte to the sensor surface. Here, we demonstrate a versatile technique to modify nanocarriers such that their surface can subsequently be functionalized with a desired recognition moiety for sensing DNA. Two strategies were employed to functionalize the surface, using covalent or non-covalent interactions. To demonstrate this concept, we synthesized (i) alkyne-MIL-88A and (ii) biotin-MIL-88A. Covalent surface coupling between an azide-dye and the alkyne-MIL-88A was achieved. Non-covalent surface interactions were achieved between streptavidin and biotin-MIL-88A. For DNA sensing, biotin-PNA was linked to the streptavidin-biotin-MIL-88A particles. Confocal imaging and flow cytometry results demonstrated that DNA can be bound selectively to the MOF surface. Flow cytometry results showed that the MOF particles should be washed at 40 °C to remove non-specific interactions. Flow cytometry provided better statistics compared to confocal microscopy due to the larger numbers of particles that could be analyzed. In summary, PNA was needed for the specific binding of DNA, and this platform was able to discriminate between complementary, single-base mismatched, and mismatched DNA targets.

7.1 Introduction

Metal-organic frameworks (MOFs), also known as coordination polymers, are a relatively new class of crystalline porous materials that are formed by the assembly of metal ions with rigid rod-like organic ligands in suitable solvents.¹⁻³ The preparation procedure of MOFs is simple and highly efficient. They offer ultrahigh porosity, large internal surface-to-volume ratios, tunable structures, and tailorable chemistry.^{4,5} MOFs have attracted considerable interest in many potential applications.⁶⁻¹² Particularly the use of MOFs in biomedicine is growing rapidly because of their advantages in drug delivery and imaging applications over traditional carriers such as organic polymers¹³ and inorganic materials.^{14,15} Therefore, surface modification strategies are important for tuning the properties of MOFs toward specific applications, such as drug delivery and biosensing.

The ideal nanocarriers for biomedicine are multifunctional. They possess (i) a particle size appropriate for intravenous applications,¹⁶ (ii) a specific trigger for assembly and disassembly that allows controlled drug encapsulation and release,^{11,17} (iii) the ability to serve as a contrast agent^{18,19} (e.g., to differentiate between normal and cancerous cells), (iv) a surface functionalized with stabilizers^{16,20} (such as PEG) to permit circulation in the bloodstream for a prolonged period of time, and (v) cell-targeting surface moieties to allow targeted drug delivery.^{19,21} Recently, DNA moieties have been incorporated on MOF surfaces to allow particle stabilization, DNA detection, and cellular entry.²²⁻²⁶ Moreover, nanosized MOFs (nanoMOFs) were non-covalently linked to fluorescent PNA for miRNA detection.²⁷

Mirkin *et al.* created the first nucleic acid-MOF nanoparticle hybrids, in which the oligonucleotides were used to create a steric and electrostatic barrier that stabilizes the particles in highly dielectric media and at the same time allows them to enter cells.²² This was achieved by covalently functionalizing the organic linkers in the MOF structure with oligonucleotides, utilizing a strain-promoted click reaction between DNA appended with dibenzylcyclooctyne and azide-functionalized UiO-66-N₃.

Many researchers used MOFs as sensing platforms for DNA detection using electrostatic interactions and π - π stacking. Chen *et al.*²³ developed a selective fluorescence sensor for sequence-specific recognition of HIV ds-DNA in vitro, using MOF as the

sensing platform. Here, the MOF was functionalized with triplex-forming oligonucleotide (TFO) probes by π - π stacking, which quenched the fluorescence. Upon the addition of the target ds-DNA, the TFO probe interacted with the bases in the major groove of the target ds-DNA to form a rigid triplex structure via Hoogsteen hydrogen bonds. After hybridization, this structure was released from the MOF platform and fluorescence was recovered accordingly. Using a similar approach, Li *et al.*²⁴ introduced MIL-101 as a fluorescence anisotropy (FA) amplifier for DNA detection. This MOF provided a strong affinity for negatively charged ss-DNA through π - π stacking and electrostatic interactions. First, the ss-DNA probe was adsorbed onto the MOF surface. In this way, the fluorophore label on the ss-DNA was rotated, resulting in enhanced FA values. When the ss-DNA hybridized with the target DNA, the resulting structure pointed away from the MOF surface, resulting in lower FA values. Jiang *et al.*²⁵ used a similar approach but using UiO66-NH₂ instead of MIL-101. Here, DNA was detected by electrostatic interactions and π - π stacking between ss-DNA and the benzyl-amino group in UiO-66-NH₂. The authors developed a platform which is capable of distinguishing complementary and mismatched target sequences with high sensitivity and selectivity. Kang *et al.*²⁷ used this concept for sensing multiplexed miRNAs in living cancer cells by a peptide nucleic acid labeled with a fluorophore adsorbed on a nanoMOF.

In Chapter 5, a strategy was presented to control the size and surface functionalization of MOF particles simultaneously. MIL-88A was synthesized and its size was tuned by varying the stoichiometric ratio between multivalent and monovalent ligands. At the same time, surface functionalization was successfully accomplished and the surface coverage was quantified using a fluorine-containing capping ligand. Moreover, surface functionalization with PEG and biotin groups was shown, and the latter were bound to fluorescently labeled streptavidin.

Here, we have used the concept of simultaneous particle formation and surface functionalization of MOFs for the development of more general surface functionalization strategies, using both covalent and non-covalent approaches. The click reaction and the biotin-streptavidin interaction have been used for these approaches, respectively. We here apply the non-covalent strategy to the formation of peptide nucleic acid (PNA)-

functionalized MIL-88A, which is used subsequently to test the selectivity in DNA binding at the MOF surface.

7.2 Results and discussion

7.2.1 Synthesis and characterization of functionalized MIL-88A

MIL-88A was functionalized with alkyne (alkyne-MIL-88A) or biotin moieties (biotin-MIL-88A) using the methodology from Chapter 5 by replacing a small fraction of the organic linker fumaric acid with 10-undecynoic acid (5%) or biotin-COOH (1%), respectively, as shown in Figure 7.1a. Powder X-ray diffraction (XRD) (see Figure 7.2) showed that all MOFs were semi-crystalline. The changes observed in the spectra of the functionalized MOFs compared to the native MIL-88A are attributed to the flexibility of the framework and the effect of water absorption into the pores.^{28,29} Figure 7.3 shows scanning electron microscopy (SEM) images and the average length and width of MIL-88A, alkyne-MIL-88A and biotin-MIL-88A particles, which confirm the successful synthesis of the MOFs. As observed before (Chapter 5), adding small-MW ligands to the MOF surface has only limited effect on the MOF particle size. Here, we only observe a significant decrease of the width but not of the length of the particles. Since functionalization was our primary target here, we did not investigate the size effects further.

N₂ sorption isotherms were measured for two samples, non-functionalized MIL-88A and biotin-MIL-88A. Addition of biotin led to a strong decrease of the Brunauer–Emmett–Teller (BET) surface area, from 347 m²/g for unfunctionalized MIL-88A to 13.4 m²/g for biotin-MIL-88A. This strong decrease is consistent with the observations made in Chapters 5 and 6, and in other studies.⁵ Given the similar molecular weight of undecynoic acid compared to the fluorine-containing capping ligand described in Chapter 5, we presume the BET areas to be similar. The zeta potential values of uncoated MIL-88A (19.4 ± 3.4 mV) were shifted to more neutral values of 12.4 ± 0.3 mV and 12.4 ± 0.4 mV in the case of alkyne-MIL-88A and biotin-MIL-88A, respectively, in line with a partial capping of free Fe coordination sites at the surface, as also discussed in Chapter 6. In summary, we

successfully functionalized the surface of MIL-88A with alkyne and biotin groups, which can be used for further covalent and non-covalent functionalization, respectively.

Figure 7.1 Schemes of a) synthesis of MIL-88A functionalized with a capping ligand, b) covalent surface functionalization by the click reaction between alkyne-MIL-88A and azide-coumarin, c) non-covalent surface interactions between biotin-MIL-88A, AF488-SAv, and biotin-PNA, and d) chemical structures used here.

Figure 7.2 XRD spectra of a) MIL-88A and MIL-88A functionalized with b) 5% 10-undecynoic acid, and c) 1% biotin-COOH capping ligand.

Figure 7.3 SEM images of a) MIL-88A and MIL-88A functionalized with b) 5% 10-undecynoic acid and c) 1% biotin-COOH capping ligand; d) average particle dimensions from SEM images (a-c): length (dark gray) and width (light gray).

7.2.2 Covalent surface functionalization of MIL-88A using click chemistry

Covalent surface functionalization was achieved by employing the Cu(I)-catalyzed click reaction at the surface of alkyne-MIL-88A (see Figure 7.1b). Coumarin azide was used as the reagent, resulting in coumarin-MIL-88A. The reaction was carried out by adding an excess of coumarin azide in the presence of $[\text{Cu}^{\text{I}}(\text{CH}_3\text{CN})_4]\text{PF}_6$ (catalyst) and tris-(benzyltriazolylmethyl)amine (TBTA; ligand for stabilization of the Cu^{I} catalyst) using two different sets of concentrations: 0.49 mM of alkyne and 3.3 mM of coumarin azide, or 0.87 mM of alkyne and 5.6 mM of coumarin azide, followed by continuous stirring overnight at room temperature.

After the click reaction, optical and fluorescence microscopy images (Figure 7.4a-b) were taken. These show MOF particles with a high fluorescence intensity, demonstrating the successful functionalization of the surface of MIL-88A at both low and high (data not shown here) concentrations. The fluorescence mean intensities were quantified for these two cases giving values of 48 ± 21 a.u. and 65 ± 25 a.u. at low and high concentrations, respectively. The higher concentration apparently resulted in an intensity increase of approx. 35%. To test whether the coupling was specific, two control experiments were performed. Control 1 was performed by reacting MIL-88A (without alkyne) with coumarin azide, while control 2 was performed by mixing alkyne-MIL-88A with coumarin azide in the absence of the Cu^{I} catalyst, at both concentrations. The optical and fluorescence images shown in Figure 7.4c-f (for the low concentrations; for high concentrations: data not shown) indicate the absence of fluorescence in all cases, which confirms the need of alkyne groups on the MOF surface and the presence of the catalyst to achieve the successful click synthesis. In summary, these results demonstrate the specific covalent surface functionalization of MIL-88A using the Cu(I)-catalyzed click reaction.

Figure 7.4 Optical (a, c, e) and fluorescence (b, d, f) microscopy images of MIL-88A after reaction of alkyne-MIL-88A with coumarin azide (a, b), control 1 (c, d; using native MIL-88A instead of alkyne-MIL-88A), and control 2 (e, f; without catalyst), using 0.49 mM of alkyne and 3.3 mM of coumarin azide.

7.2.3 Non-covalent surface functionalization of MIL-88A with PNA, and selective DNA binding

In Chapter 5, we demonstrated the specific interaction between biotin-MIL-88A and Alexa-Fluor488-labeled streptavidin (AF488-SAv). Here, we applied this concept to bind biotinylated peptide nucleic acid (PNA) (as shown in Figure 7.1c) to create a MOF platform for the selective binding of DNA, as shown in Figure 7.5. Three different DNA targets were chosen to evaluate the binding selectivity of the MOF-PNA system: T_1 is the complementary strand, T_2 is a single-base mismatched target, and T_3 is a multiple-mismatched target.

Figure 7.5 a) Scheme of non-covalent surface interactions between biotin-MIL-88A, AF488-SAv, and biotin-PNA and further hybridization with DNA and b) structures of the oligonucleotides used here.

To demonstrate the feasibility of employing MIL-88A as a platform for PNA/DNA functionalization using DNA as the ultimately selective binder, the functionalization of MIL-88A with PNA with a particular sequence, already tested in other sensing applications,^{30,31} was employed as a model system to test its ability to recognize DNA sequences when immobilized at the MOF surface. First, AF488-SAv was attached to biotin-MIL-88A using a SAv/biotin ratio of 15 in order to suppress the particle aggregation observed in Chapter 5. The fluorescence intensities of AF488-SAv and Cy5 (which was used to label the DNA targets) were measured using flow cytometry as shown in Figure 7.6. The biotin-MIL-88A sample (Figure 7.6a) shows no intensity of AF488-SAv above a threshold of 100. Figure 7.6b shows a positive (i.e., above threshold) fluorescence intensity of AF488-SAv confirming the conjugation between biotin-MIL-88A and AF488-SAv. In order to analyze only the particles conjugated with AF488-SAv, we selected in all cases the

particles that passed the minimum threshold of 100 for the fluorescence intensity of AF488-SAv (Figure 7.6c). Thus, only Cy5-labelled oligonucleotides are captured that are adsorbed on the fraction of MOF covered with AF488-SAv. This enables easier visualization of differences in selectivity for the different Cy5-labelled DNA target probes used here. The few data points above the Cy5 intensity threshold observed in Figure 7.6c (pink section) are attributed to background noise since Cy5 is not present in this sample. However, for the PNA-DNA hybridization we used a fluorescence intensity threshold of 32 for Cy5 and used the same threshold settings of both dyes for all experiments described below.

Figure 7.6 Flow cytometry results for a) biotin-MIL-88A, and for the conjugate of biotin-MIL-88A and AF488-SAv, b) without applying a threshold, and c) selecting only those particles that pass a threshold of 100. The violet regions indicate intensities below the threshold for both dyes, green indicates above threshold for the AF488 dye only (indicative of conjugation between biotin-MIL-88A and AF488-SAv), and pink represents above-threshold intensities for both AF488-SAv and Cy5. The intensities of Cy5 and AF488 are given in arbitrary units.

Subsequently, biotin-functionalized PNA (biotin-PNA) was incubated at room temperature with the conjugate of biotin-MIL-88A and AF488-SAv using a PNA/SAv ratio of 3. Thereafter, hybridization between PNA and complementary T_1 , labeled with Cy5, was performed at 30 °C for 1 h using a PNA/DNA ratio of 1, followed by washing at room temperature and at 40 °C. The same procedure was followed for the hybridization with targets T_2 and T_3 .

The samples were characterized using flow cytometry and confocal microscopy. Figure 7.7a,b shows the flow cytometry results for T_1 , indicating a high intensity of Cy5 (signal from T_1) and AF488 (from SAV-modified MIL-88A), both upon washing at room temperature and at 40 °C. Fluorescence imaging (Figure 7.7c) confirmed the local co-occurrence of high fluorescence intensities of AF488-SAV as well as Cy5. These results indicate that PNA and T_1 have successfully hybridized at the MOF surface. To test whether these interactions were specific, a control experiment was performed by incubating AF488-SAV-modified biotin-MIL-88A (MOF) with T_1 in the absence of biotin-PNA. Flow cytometry showed non-specific interactions between T_1 and MOF when rinsing was performed at room temperature (see Figure 7.7d). These interactions are attributed to weak electrostatic interactions between positively charged MIL-88A and negatively charged DNA. When rinsed at 40 °C, these non-specific interactions were drastically reduced as shown in Figure 7.7e. Fluorescence imaging (see Figure 7.7f) confirmed the success of removing nonspecific interactions between DNA and non-PNA-modified MIL-88A, showing a strong intensity from AF488-SAV only. In summary, these results showed that biotin-SAV interactions at the MIL-88A surface can provide a platform for PNA-DNA hybridization.

In addition to T_1 , the mismatched targets T_2 and T_3 were used to investigate the selectivity of the sensing platform. All samples were washed at 40 °C to avoid non-specific interactions followed by characterization using flow cytometry (Figure 7.8a,c) and confocal microscopy (Figure 7.8b,d). Flow cytometry showed high intensities for the adduct of MOF+PNA and T_2 (Figure 7.8a) but low intensities for the adduct of MOF+PNA and T_3 (Figure 7.8c). In the latter case, the fluorescence intensity distribution was similar to the control without biotin-PNA (Figure 7.7e). Fluorescence imaging (Figure 7.8c,d) confirmed these findings by the local co-occurrence of fluorescence of AF488-SAV and Cy5 (Figure 7.8c) for the interaction between MOF+PNA and T_2 , and a strong intensity from AF488-SAV only (Figure 7.8d) for MOF+PNA with T_3 .

Figure 7.7 Flow cytometry (a, b, d, e) and confocal microscopy (c, f) results for biotin-MIL-88A particles after incubation with AF488-SAv and biotin-PNA (a, b, c) or with AF488-SAv only (d, e, f), followed by hybridization with T_1 after washing at RT (a, d) and at 40 °C (b, c, e, f). In the flow cytometry graphs, only the fractions of particles are shown for which the AF488 intensity passed the threshold intensity of 100. The green regions indicate a high intensity of AF488 but below the Cy5 threshold, and pink represents a high intensity for both AF488-SAv and Cy5. In the confocal images (c, f), the top left panel corresponds to AF488-SAv, top right to Cy5 fluorescence bound to T_1 , bottom left is the optical image, and bottom right is the overlay. Scale bars are 10 μm .

Fluorescence intensity profiles were taken from several confocal images as shown in Figure 7.9a-d. The ratio (r) of the differences of ΔI_{Cy5} and ΔI_{AF488} between the peak and background intensities of Cy5 and AF488-SAv, respectively, was measured and plotted in Figure 7.9e for all cases. This ratio r was calculated as follows,

$$r = \frac{\Delta I_{\text{Cy5}}}{\Delta I_{\text{AF488}}} = \frac{q_{90}(\text{Cy5}) - q_{20}(\text{Cy5})}{q_{90}(\text{AF488}) - q_{20}(\text{AF488})} \quad \text{Equation 7.1}$$

where $q_{x(\text{dye})}$ represents the x -th percentile, i.e., the intensity value below which x (90 or 20) percent of the intensity values in each profile may be found, for both dyes Cy5 and AF488-SAv. Figure 7.9e shows that the hybridization of MOF+PNA with complementary T_1

exhibited the largest r value, followed by MOF+PNA with single-base mismatched T_2 . The adducts of MOF (without PNA) with T_1 and of MOF+PNA with T_3 show very small r values, due to very low Cy5 intensities in these cases. Statistical analyses (t-tests) as shown in Table 7.1 were performed for the adduct of MOF+PNA with T_1 in comparison with MOF (no PNA) with T_1 , MOF-PNA with T_2 , and MOF-PNA with T_3 . These tests showed p values below 0.05 only for the comparisons with MOF (no PNA) with T_1 and MOF-PNA with T_3 . In the case of MOF-PNA with T_2 , the confocal intensity results were not significantly different. The main reason for this is most likely the lack of a sufficiently large number of profiles.

Figure 7.8 Flow cytometry (a, c) and confocal microscopy (b, d) results for biotin-MIL-88A particles after incubation with AF488-SAv and biotin-PNA followed by hybridization with (a, b) T_2 and (c, d) T_3 , after washing at 40 °C. In the flow cytometry graphs, only the fractions of particles are shown for which the AF488 intensity passed the threshold intensity of 100. The green regions indicate a high intensity of AF488 only, and pink represents a high intensity for both AF488-SAv and Cy5. In the confocal images (b, d), the top left panel corresponds to AF488-SAv, top right to DNA-Cy5 fluorescence, bottom left is the optical image, and bottom right is the overlay. Scale bars are 10 μm.

Figure 7.9 Typical fluorescence intensity profiles from confocal images for a) MOF-PNA + T₁, b) MOF (no PNA) + T₁, c) MOF-PNA + T₂, d) MOF-PNA + T₃, after washing at 40 °C. Green = AF488 and red = Cy5 fluorescence channels. e) average intensity ratio (*r*) for different confocal images.

Table 7.1 Statistical t-test results for MOF-PNA + T₁ vs different cases using the average *r* values from Figure 7.9e (confocal imaging) and the log intensity values from Figure 7.10 (FACS).

MOF-PNA + T ₁ vs	<i>p</i>	
	confocal imaging	FACS
MOF	-	< 0.0001
MOF + T ₁	0.026	< 0.0001
MOF-PNA + T ₂	0.340	< 0.0001
MOF-PNA + T ₃	0.023	< 0.0001

In order to analyze the flow cytometry results more deeply, we divided the counts for the different particles in fractions. We only analyzed those particles that showed an AF488 intensity above 100 and a Cy5 intensity above 32. The latter threshold was used to eliminate the fraction of particles that appeared fully non-fluorescent for Cy5. Table 7.2 reports the fractions of particles for which the Cy5 intensity was above or below 100, thus analyzing the data shown in Figure 7.7b,e and Figure 7.8a,c. Clearly, the case of MOF-PNA + T₁ shows much more particles with a Cy5 intensity above 100 in comparison with all other cases, indicating higher degrees of hybridization. The case of MOF-PNA + T₂ (single-base mismatch) has a high-intensity fraction that is only slightly higher than for MOF, MOF (no PNA) + T₁, and MOF-PNA + T₃. Figure 7.10 shows the Cy5 log fluorescence mean intensities (for all particles with a Cy5 intensity above 32). The values for MOF incubated with T₁ (in the absence of PNA; 1.48) and MOF+PNA with T₃ (1.60) are comparable to the value of MOF (1.60). Successful hybridization between MOF-PNA and T₁ yielded a Cy5 log fluorescence intensity of 1.85. However, when MOF-PNA was incubated with T₂, the intensity was only slightly less, with a log intensity value of 1.81. Yet, a statistical analysis (t-test) of the data shown in Figure 7.10 (Table 7.1) showed that all *p* values for comparisons of the adduct of MOF+PNA with T₁ in comparison with those of MOF (no PNA) with T₁, MOF-PNA with T₂, and MOF-PNA with T₃ were below 0.05. Thus, we conclude that PNA immobilized on MIL-88A is able to distinguish between complementary T₁ and single-base mismatched T₂ when analyzing the data using flow cytometry, in the sense that the distributions for sufficiently large numbers of particles can be distinguished. In summary, these results suggest that (i) PNA is mandatory for the specific binding of DNA, (ii) T₁ and T₂ are hybridized with this PNA but to measurably different extents, and (iii) this sensing platform is able to differentiate between the complementary T₁ and mismatched T₂ and T₃.

Table 7.2 Fractions (%) and total numbers of particles with a Cy5 intensity below or above 100, as measured by flow cytometry, for MOF (= adduct of AF488-SAv and biotin-MIL-88A), MOF-PNA + T₁, MOF (no PNA) + T₁, MOF-PNA + T₂, MOF-PNA + T₃ after washing at 40 °C, for only the particles above intensity thresholds of 100 and 32, for AF488-SAv and Cy5, respectively.

Sample name	fraction I < 100	fraction I > 100	# of particles
MOF	89.9	10.1	200,000
MOF-PNA + T ₁	28.2	71.8	191,927
MOF (no PNA) + T ₁	92.5	7.5	50,000
MOF-PNA + T ₂	71.4	28.6	200,000
MOF-PNA + T ₃	88.2	11.8	45,000

Figure 7.10 Average log fluorescence intensities as measured by flow cytometry for MOF, MOF-PNA + T₁, MOF (no PNA) + T₁, MOF-PNA + T₂, MOF-PNA + T₃ after washing at 40 °C. The vertical bars denote the standard deviation.

7.3 Conclusions

In conclusion, we have developed versatile methods for the surface functionalization of MIL-88A using either covalent or non-covalent interactions. Moreover, we demonstrated that the MOF surface can be equipped with PNA to achieve DNA binding and to assess the DNA binding selectivity. In order to remove non-specific adsorption between DNA and the

particles, the MOFs should be washed at elevated temperature. Overall, the sensing platform was able to distinguish between complementary, single-base mismatched and multiple mismatched sequences. The difference between complementary and single-base mismatched DNA was rather small, but was statistically significant when analyzing large numbers of particles using flow cytometry. The difference between these targets can possibly be improved further by washing at a temperature closer to the melting temperature of the DNA-PNA duplex. In the future we envisage the creation of different DNA binding strategies for recognizing proteins, transcription factors, etc. This method can in principle be used to assay different analytes by introducing probes that selectively bind to the analytes. These features contribute to a more simple, efficient, and more general platform which can be expanded to other MOFs and applications.

7.4 Acknowledgments

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7.5 Experimental

7.5.1 Materials

The following materials have been used as received from the supplier: ferric chloride hexahydrate (Acros, 99%), fumaric acid (Fluka, purum), 10-undecynoic acid (Sigma-Aldrich, 95%), 3-azido-7-hydroxycoumarin (Carbosynth), tris-(benzyltriazolylmethyl)amine (TBTA; Sigma), tetrakis(acetonitrile)copper(I) hexafluorophosphate (Sigma), biotin functionalized with tetra(ethylene oxide) carboxylic

acid (Uptima), Alexa Fluor® 488 conjugate with streptavidin (Invitrogen), m-cresol (Fluka), diisopropylethylamine (DIPEA), trifluoroacetic acid (TFA), *N,N*-dimethylformamide (DMF), acetonitrile, dichloromethane, diethyl ether, Methanol (Sigma), absolute ethanol (Merck, p.a.), O-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HBTU), $N\alpha$ -Fmoc-N ϵ -[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl]-D-lysine, hydroxybenzotriazole (HOBT), biotin, acetic anhydride, and 1-methyl-2-pyrrolidinone (NMP). Oligonucleotides were purchased from Eurofins and used as received without further purification. Milli-Q water with a resistivity of 18.2 M Ω •cm at 25°C was used for the synthesis of MOFs and all assembly experiments.

7.5.2 Synthetic procedures

Synthesis of MIL-88A

See Chapter 5.

Synthesis of MIL-88A functionalized with capping ligands

Alkyne-MIL-88A and biotin-MIL-88A were synthesized in a similar manner as described in Chapter 5 for biotin-MIL-88A.

Synthesis of biotin-PNA: biotin-AEEA-AEEA-AAA CCC TTA ATC CCA-Gly-NH₂

The synthesis of biotin-PNA was performed by automated Fmoc-SPPS on an ABI 433A synthesizer on a Rinkamide ChemMatrix resin (loading 0.23 mmol/g, scale 5 μ mol). The monomers were introduced by using the HBTU/DIPEA coupling protocol, the Fmoc group was deprotected by piperidine/DMF (20%). Biotin was introduced by coupling the *N*-terminus of the last monomer of 2-(2-aminoethoxy)ethoxyacetic acid (AEEA) and the carboxylic group of biotin, using HBTU/DIPEA coupling. Free biotin-PNA was cleaved from the resin by TFA/m-cresol (9:1) and precipitated in ether. The purification was carried out by RP-HPLC on a XTerraPrepRP18 column (flow rate: 4 mL/min and gradient elution from 100% water (0.1% TFA, eluent A) to 100% acetonitrile (0.1% TFA, eluent B) in 31 min. The purified product was characterized by UPLC-ESI-MS (Acquity UPLC BEH C₁₈). The gradient conditions for UPLC and the retention time of the PNA are 0.9 minutes in H₂O (0.2% formic acid, FA), then linear gradient to 50% MeCN (with 0.2% FA) in 5.7

minutes at a flow rate of 0.25 mL/min, retention time of biotinylated PNA: 2.99 min. This yields the following distribution of signals: calcd. m/z : 907.7 $[MH5]^{5+}$, 756.6 $[MH6]^{6+}$, 648.7 $[MH7]^{7+}$, 567.7 $[MH8]^{8+}$; found m/z : 908.9, 759.3, 650.9, 569.7. After MS analysis, the biotin-PNA was stored in the freezer at -20°C in MilliQ water.

7.5.3 Methods

Click synthesis at low concentration

Under nitrogen, alkyne-MIL-88A (3.7 mg, 0.08 μmol alkyne) was dissolved in 162 μL methanol, and coumarin azide (0.54 μmol , 6.75 equiv.), TBTA (0.04 μmol , 0.50 equiv.) and $\text{Cu}^{\text{I}}(\text{CH}_3\text{CN})_4\text{PF}_6$ (0.41 μmol , 5.13 equiv.) were added to the reaction mixture, which was allowed to stir overnight at room temperature. For purification, the samples were washed six times using 10 mL of methanol followed by three times washing with water using centrifugation for 10 min at 1000 rpm followed by freeze drying.

Control 1: without alkyne groups at low concentration

Under nitrogen, MIL-88A (5 mg) was dissolved in 219 μL methanol, and coumarin azide (0.73 μmol), TBTA (0.05 μmol) and $\text{Cu}^{\text{I}}(\text{CH}_3\text{CN})_4\text{PF}_6$ (0.55 μmol) were added to the reaction mixture, which was allowed to stir overnight at room temperature. For purification, the samples were washed six times using 10 mL of methanol followed by three times washing with water using centrifugation for 10 min at 1000 rpm followed by freeze drying.

Control 2: without catalyst at low concentration

Under nitrogen, alkyne-MIL-88A (5 mg, 0.11 μmol alkyne) was dissolved in 214 μL methanol, and coumarin azide (0.73 μmol , 6.64 equiv.) and TBTA (0.05 μmol , 0.45 equiv.) were added to the reaction mixture, which was allowed to stir overnight at room temperature. For purification, the samples were washed six times using 10 mL of methanol followed by three times washing with water using centrifugation for 10 min at 1000 rpm followed by freeze drying.

Click synthesis at high concentration

Under nitrogen, alkyne-MIL-88A (5 mg, 0.11 μmol alkyne) was dissolved in 126 μL methanol, and coumarin azide (0.73 μmol , 6.64 equiv.), TBTA (0.05 μmol , 0.45 equiv.) and $\text{Cu}^{\text{I}}(\text{CH}_3\text{CN})_4\text{PF}_6$ (0.55 μmol , 5 equiv.) were added to the reaction mixture, which was allowed to stir overnight at room temperature. For purification, the samples were washed three times using 10 mL of methanol followed by one time washing with water using centrifugation for 10 min at 1000 rpm followed by freeze drying.

Control 1: without alkyne groups at high concentration

This procedure was similar to the one described for click synthesis, except for replacing alkyne-MIL-88A by MIL-88A.

Control 2: without catalyst at high concentration

This procedure was similar to the one described for click synthesis, except for the absence of the catalyst $\text{Cu}^{\text{I}}(\text{CH}_3\text{CN})_4\text{PF}_6$.

Fabrication of MOF (adsorption of AF488-SAv-modified biotin-MIL-88A)

Phosphate-buffered saline (PBS) (0.01 M phosphate; 0.138 M NaCl; 0.0027 M KCl; pH 7.4, at 25 $^{\circ}\text{C}$) was used for all experiments with streptavidin. PBS was mixed with 0.05% of Tween-20 (PBS-tween). 1 mg of biotin-MIL-88A (0.45 nmol of biotin) was dispersed in 50 mL PBS-tween and mixed with 500 μg of Alexa-Fluor488-streptavidin (AF488-SAv, 1.72 nmol). The suspension was incubated for 1 h followed by centrifugation at 1000 rpm for 30 min. Thereafter, the suspension was washed two times for 1 h using 7.5 mL of PBS-tween followed by centrifugation at 1000 rpm for 30 min each time. The sample was resuspended in 7.5 mL PBS (without tween) to obtain MOF.

Fabrication of MOF-PNA

For this experiment, PBS was used without tween. Here to a 4.5 mL suspension of MOF, 31.2 μL of biotin-PNA (492.53 μM , ~ 15 nmol) was added to give a PNA/streptavidin molar ratio of 3. The suspension was incubated for 1 h followed by centrifugation at 1000 rpm for 30 min. Then, the suspension was washed two times for 1 h

using 4.5 mL of PBS followed by centrifugation at 1000 rpm for 30 min each time. The sample was resuspended in 4.5 mL PBS.

DNA hybridization

For each DNA hybridization, 1.5 mL of MOF-PNA is incubated with three different DNAs: 29.4 μL of T_1 (170 μM , ~ 5 nmol), 30.1 μL of T_2 (165 μM , ~ 5 nmol), and 89.1 μL of T_3 (55.9 μM , ~ 5 nmol) for 1 h at 30 $^\circ\text{C}$. For all these samples the PNA/DNA molar ratio was 1. For the control experiment without biotin-PNA, 1.5 mL of MOF was added to 29.4 μL of T_1 (170 μM , ~ 5 nmol). All the suspensions were washed four times for 1 h at RT using 1.5 mL of PBS followed by centrifugation at 1000 rpm for 30 min each time. These samples were analyzed using flow cytometry and confocal microscope. Then, all the suspensions were washed one time for 40 min at 40 $^\circ\text{C}$ using 1.5 mL of PBS followed by centrifugation at 1000 rpm for 46 min each time and samples were analyzed using flow cytometry and confocal microscopy.

Statistical analysis for Figure 7.9e and Figure 7.10

Two t-tests were performed for the results for MOF-PNA + T_1 vs different cases using the average r values from Figure 7.9e (confocal imaging) using the two-tailed and two-sample t-test with unequal variance (heteroscedastic) and the log intensity values from Figure 7.10 (FACS) using the unpaired t-test³² using the mean, the standard deviation, and the number of particles.

7.5.4 Equipment

X-ray powder diffraction (XRD)

For X-ray diffraction experiments a D2 Phaser (Bruker) using a Cu X-ray source was used.

High resolution scanning electron microscopy (HRSEM)

For HRSEM characterization a Zeiss using ImageJ software. For each sample at least 50 particles were measured.

Confocal fluorescence microscopy

A drop of MOF particles suspended in PBS was put between two glass slides and placed on a Nikon Eclipse Ti confocal laser scanning microscope fitted with a 100x oil objective (NA 1.49, WD 0.12). Differential interference contrast images were taken to examine particle size and morphology. Streptavidin coupled to Alexa-488 was visualized using a 488 nm laser and DNA labeled with Cy5 was visualized using a 633 nm laser.

Fluorescence microscopy

Fluorescence microscopy images were taken using an Olympus inverted research microscope IX71 equipped with a mercury burner U-RFL-T as the light source and a digital Olympus DR70 camera for image acquisition. The filter used for all covalent surface experiments had an excitation of 350 nm and an emission of 460 nm using an Olympus filter cube. All fluorescence images were acquired in air.

Gas sorption

The BET measurements were performed on an Autosorb-1 from Quantachrome. The samples were degassed at 155°C for 3 hours.

Zeta potential (ZP)

ZP 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°.

Flow cytometry

For the flow cytometry analysis, a BD (Becton Dickinson) FACS Aria II flow cytometer has been used. A 488 nm laser excited the AlexaFluor-488 dye, while the emission was measured using a 530 nm emission filter with a 20 nm bandwidth. The Cy5 dye was excited by a 633 nm laser and the emission was measured using a 660 nm emission filter with a bandwidth of 20 nm. For all samples, 45,000-200,000 events were measured at the lowest flow rate of 1 (A.U.) and using a Neutral Density filter of 1.5.

7.6 References

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Summary

The use of nanomaterials for molecular imaging and therapeutics has flourished over the past decade. Controlled assembly and disassembly of nanoparticles is important for applications such as drug delivery systems in order to tune their loading and unloading behavior. Such properties can be accomplished using multivalent non-covalent and porous nanomaterials, such as supramolecular nanoparticles (SNPs) and metal-organic frameworks (MOFs), because of the inherently reversible nature of non-covalent interactions. The main hypothesis investigated in this thesis is whether these, otherwise very different, materials can be controlled in size and outer functionalization according to the same concept, which is the ratiometric variation of multivalent crosslinkers and monovalent stoppers under stoichiometric control.

Chapter 2 presents a literature review about how size control, surface functionalization and tuning of loading/unloading properties of SNPs and MOFs are achieved. SNPs were formed using electrostatic and/or host-guest interactions. The SNP size has been controlled by tuning the concentration and composition of the building blocks in solution, the stoichiometric ratio between mono- and multivalent guests, and the ionic strength of the solution. Surface functionalization with PEG and tumor-targeting ligands led to improved SNP circulation in the blood stream as well as delivery efficacy. Furthermore, drugs and imaging agents have been encapsulated inside SNPs using electrostatic interactions, covalent bonds and inclusion complexes. For MOFs, different methods have been used to control their formation and size, such as hydro/solvothermal, microwave-assisted hydro/solvothermal, ultrasonic, coordination modulation, microfluidic, microemulsions, spray-drying techniques and incorporation of capping ligands. Surface functionalization with PEG has been used to prevent opsonic interaction, macrophage uptake and to prolong blood circulation. The MOF surface can further be functionalized with different targeting ligands. Three different methods have been used to encapsulate drug or imaging agents into MOFs: as linkers, inside the MOF matrix and by adsorption into the pores of the MOFs.

Chapter 3 has described the formation of hybrid gold nanoparticle network aggregates under highly turbulent flow conditions using a multi-inlet vortex mixer (MIVM). These

aggregates were formed by mixing gold nanoparticles functionalized with β -cyclodextrin and adamantyl (Ad)-dendrimers. Ad-terminated poly(ethylene glycol) was added as a stabilizer at the surface of the supramolecular aggregates to provide steric repulsion. The ratio of the Ad and CD concentrations was used to control the size of these aggregates. The turbulent flow had a minor effect on the supramolecular aggregate size at relatively low Ad/CD ratios, whereas it had a substantial effect at high Ad/CD ratios, leading to larger agglomerates in solution compared to laminar flow and manual conditions.

Chapter 4 shows the formation of SNPs composed of positively charged poly(ethylene imine) grafted with β -cyclodextrin (CD) and positively charged ferrocene (Fc)-terminated poly(amidoamine) dendrimers, with a monovalent stabilizer at the surface. It was shown that there is a balance of forces at low ionic strength, composed of attractive host-guest and repulsive electrostatic interactions, but this balance can be disrupted at high ionic strength. The stabilizer plays an important role to obtain stable SNPs in solution at high ionic strength, where the optimal stabilizer is a long chain polymer with high binding affinity. The particle size was controlled by fine-tuning the ratio of multivalent and monovalent interactions with good stability up to seven days. Finally, it has been shown that the disassembly of SNPs was triggered by oxidation of the Fc groups.

Chapter 5 shows that control of particle size and functionalization of MIL-88A are possible using a stoichiometric replacement of a small fraction of the divalent fumarate by a monovalent capping ligand. The surface coverage was quantified using a fluorine-containing capping ligand. Size control at the nano-scale was achieved using monovalent carboxylic acid-functionalized poly(ethylene glycol) (PEG) ligands at different concentrations. Finally, to demonstrate the bioapplicability of this system, a biotin-carboxylic acid capping ligand was used to functionalize MIL-88A which allowed to bind fluorescently labeled streptavidin.

In Chapter 6, we used the methodology developed in Chapter 5 to study the effect of the molecular weight (MW) of a monovalent PEG-carboxylate capping ligand on particle size and PEG conformation. The size of the PEG-functionalized MIL-88A particles decreased with increasing MW, and nanoMOFs were obtained for long PEG chains. It was demonstrated that longer chains occupied a larger surface area, and these chains adopt a low-density brush conformation. A larger surface-to-volume ratio of PEG-covered MOF

particles increased the uptake and release of a dye, while the shielding effect of the PEG layer appeared to be minimal. Finally, the dye was completely released in salt solution, whereby the majority of the dye was released by counter ion exchange, and a smaller fraction as a salt form.

In Chapter 7 the MOF surface was functionalized using covalent and non-covalent interactions. Covalent surface interactions were achieved using click chemistry between azide-dye and alkyne-modified MIL-88A. Non-covalent surface interactions were performed between biotin-MIL-88A and streptavidin. PNA-biotin was linked subsequently to these particles for sensing DNA, and it was shown that this platform was able to distinguish between complementary and random DNA sequences.

In summary, the work described in this thesis provides a unified strategy for the formation of porous and self-assembled nanomaterials. The concept relies on ratiometric control over the multivalent and monovalent building blocks while maintaining stoichiometry between the interacting moieties. In this way, size control and surface functionalization are achieved simultaneously. We expect that the concept can lead to the development of multifunctional nanoparticles for biomedical applications.

Summary

Samenvatting

Het gebruik van nanomaterialen voor moleculaire beeldvorming en geneeskunde is sterk toegenomen in het afgelopen decennium. Gecontroleerde assemblage en desintegratie van nanodeeltjes is belangrijk voor toepassingen zoals medicijnafgiftesystemen, zodat het opname- en afgiftegedrag geregeld kan worden. Zulke eigenschappen kunnen worden gerealiseerd door het gebruik van multivalente, niet-covalente en poreuze nanomaterialen, zoals supramoleculaire nanodeeltjes en metaal-organische netwerken, vanwege de inherent reversibele aard van niet-covalente interacties. De voornaamste hypothese die in dit proefschrift wordt onderzocht is of deze, op zich erg verschillende, materialen kunnen worden gecontroleerd in grootte en uitwendige functionalisatie volgens hetzelfde concept, namelijk de ratiometrische verandering van multivalente crosslinkers en monovalente stoppers in stoichiometrische verhouding.

Samengevat geeft het werk dat in dit proefschrift beschreven wordt een consistente strategie om poreuze en zelf-geassembleerde nanomaterialen te maken. Het concept is gebaseerd op ratiometrische controle van de multivalente en monovalente bouwstenen terwijl de stoichiometrie tussen de interacterende groepen behouden blijft. Op deze manier kunnen controle over de grootte en oppervlaktefunctionalisatie tegelijk gerealiseerd worden. We verwachten dat het concept kan leiden tot de ontwikkeling van multifunctionele nanodeeltjes voor biomedische toepassingen. Meer details zijn te vinden in de Engelse versie van de samenvatting.

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About the author

Raquel Mejia-Ariza was born in Pereira, Colombia on June 27, 1982. She studied Chemical Engineering at the University of Puerto Rico at Mayagüez. After graduating in 2006, she went to Virginia Polytechnic Institute and State University where she got a master's degree in Macromolecular Science and Engineering. For her master's project she designed, synthesized, and characterized magnetite clusters using a multi-inlet vortex mixer under the direction of Dr. Richey M. Davis.

On January 2011 she started her PhD project in the MnF group of Prof. Dr. ir. Jurriaan Huskens at the University of Twente. The aim of her research is size tuning and surface functionalization of supramolecular nanoparticles and metal-organic frameworks for biomedical applications. The results of this research are described in this thesis.

Since May 2015 she works as a postdoc in the MnF group on the Nanopill 2.0 project on the development of a bladder cancer detection kit in collaboration with other groups at the University of Twente and on the fabrication of an electronic nose using functionalized cantilevers for disease detection via breath diagnostics.

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