Metal Complex-Based Templates and Nanostructures for Magnetic Resonance/Optical Multimodal Imaging Agents

Jealemy Galindo Millán

METAL COMPLEX-BASED TEMPLATES
AND NANOSTRUCTURES FOR
MAGNETIC RESONANCE/OPTICAL
MULTIMODAL IMAGING AGENTS

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RESONANCE/OPTICAL MULTIMODAL IMAGING AGENTS**

PROEFSCHRIFT

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*Nan-in, a Japanese master during the Meiji era (1868-1912),
received a university professor who came to inquire about Zen.
Nan-in served tea. He poured his visitor's cup full, and then kept on pouring.
The professor watched the overflow until he no longer could restrain himself.
"It is overfull. No more will go in!"
"Like this cup," Nan-in said, "you are full of your own opinions and speculations. How can I
show you Zen unless you first empty your cup?"*

Zen Koan #1

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CHAPTER 1

INTRODUCTION

1.1 General Introduction

Molecular imaging has rapidly emerged as a discipline dedicated to address the need for accurate and rapid visualization of the human body in a, desirably, non-invasive way.¹⁻³ Today, the various imaging modalities used in the clinic include those based on nuclear techniques (positron emission tomography -PET- and single photon emission computed tomography -SPECT-), proton relaxivity (magnetic resonance imaging -MRI-) and optical phenomena (e.g., fluorescence, absorption and bioluminescence).³⁻⁵ Despite the valuable information these imaging modalities provide, they fail to discriminate between physiologically/pathologically different tissues. To overcome this issue imaging agents (IAs), designed to aid image contrast without perturbing metabolic processes, are used.⁶

As more metabolic paths of the human body are unveiled by novel IAs and state-of-the-art techniques,^{3,5,7} a door has opened up for chemists, biologists and physicians to explore new approaches towards molecular imaging. Yet, a more conservative approach, where priority is given to well-established imaging modalities, is still preferred. However, the well-known limited spatial resolution and/or low sensitivity these techniques suffer from have led to efforts being focused on the optimization of their synergy rather than on individual advances. Whilst several examples in the literature nicely illustrate the value of this multimodal approach,⁸⁻¹⁰ challenges and issues still remain.

Today, there is a wide variety of high-performance IAs, as well as a large number of well-studied, commercially available dyes, contrast and targeting agents. Many of these IAs have successfully been combined with nanomaterials and self-assembling systems, thereby leading to a large

“molecular imaging library”, where endless combinations are possible. Despite the innumerable successful examples described in the literature,^{8,10-17} the question remains: how can we engineer simple, fast, cheap and safe multimodal IAs? Inspired by the need for novel, versatile multimodal probes, the aim of this thesis is to postulate new approaches directed towards simple and functional agents for MR and fluorescence imaging. The multimodal IAs described in this thesis derive from combinations of (novel) metal complex-based ligands with nanomaterials. The versatility of this approach is reflected in the fact that the ligands presented here can be used as templates/scaffolds for the growth of nanostructures, for other imaging techniques (by complexation with various metals) and can also be combined with an endless number of nanomaterials.

In **Chapter 2**, a literature survey providing a general overview of multimodal IAs, with emphasis in general contrast agent and surface chemistry combined with nanomaterials, is presented. While several strategies are briefly highlighted, this survey mostly deals with multimodal probes where Gadolinium (Gd^{3+}) complexes are used for magnetic resonance, combined with fluorescence as the optical imaging technique.

The aqueous-based, one-step synthesis of hybrid silver nanostructures (*hAgNSs*) grown using the polyamino carboxylic acid scaffold AB-DO3A is presented in **Chapter 3**. A feasible structure for these *hAgNSs* is depicted using UV-Vis absorbance spectroscopy, transmission electron microscopy (TEM), dynamic light scattering (DLS), NMR spectroscopy, mass spectrometry (MS) and fluorescence spectroscopy (lifetime and steady-state).

Pure hierarchical self-assembly of protein cages of the cowpea chlorotic mottle virus (CCMV) around amphiphilic (Gd^{3+})-DOTAC10 micelles is described in **Chapter 4**. Encapsulation of the micelles by the viral nanoparticles was optically confirmed using an amphiphilic Zn^{2+} phthalocyanine (ZnPc) dye and by visualization using MRI. The size and paramagnetic properties of these self-assembled protein cages were studied using TEM and MRI, respectively.

In **Chapter 5**, the synthesis and characterization of multimodal gold nanorods (MMAuNRs) for magnetic resonance and optical imaging are described. The gold nanostructures were modified by thiol-based ligand exchange using various PEG-SH, Gd^{3+} -SUDDO3A and Biotin-SH ratios. The biocompatibility of the MMAuNRs was studied by means of cell viability studies and the gold nanostructures were visualized by MRI and fluorescence microscopy.

In **Chapter 6**, the versatility of the metal complex-based template described in Chapter 3 (and similar to the one designed for Chapter 5) is demonstrated by its incorporation in carbon-based scaffolds such as small organic molecules and carbon nanotubes (CNTs). The synthetic strategies followed are described and the properties of these novel materials are investigated. Preliminary results illustrate the potential of the approach proposed, thereby introducing a short discussion on the challenges and future perspectives of the IAs presented in this thesis.

1.2 References

- (1) Galbán, C. J.; Galbán, S.; Van Dort, M. E.; Luker, G. D.; Bhojani, M. S.; Rehemtulla, A.; Ross, B. D. In *Progress in Molecular Biology and Translational Science*; Raymond, W. R., Ed.; Academic Press: **2010**; Vol. Volume 95, p 237.
- (2) Hausner, S. H. In *Nanoplatfrom-Based Molecular Imaging*; Chen, X., Ed.; John Wiley & Sons, Inc.: **2011**, p 1.
- (3) Weissleder, R.; Mahmood, U. *Radiology* **2001**, *219*, 316.
- (4) Brindle, K. *Nat. Rev. Cancer* **2008**, *8*, 94.
- (5) Cassidy, P. J.; Radda, G. K. *J. Royal Soc. Interface* **2005**, *2*, 133.
- (6) Seaman, M. E.; Contino, G.; Bardeesy, N.; Kelly, K. A. *Expert Rev. Mol. Med.* **2010**, *12*, 1.
- (7) Weissleder, R.; Pittet, M. J. *Nature* **2008**, *452*, 580.
- (8) Frullano, L.; Meade, T. *J. Biol. Inorg. Chem.* **2007**, *12*, 939.
- (9) Jennings, L. E.; Long, N. J. *Chem. Commun.* **2009**, 3511.
- (10) Louie, A. *Chem. Rev.* **2010**, *110*, 3146.
- (11) Cherry, S. R.; Louie, A. Y.; Jacobs, R. E. *P. IEEE* **2008**, *96*, 416.
- (12) Culver, J.; Akers, W.; Achilefu, S. *J. Nucl. Med.* **2008**, *49*, 169.
- (13) Hou, Y.; Hao, R. In *Nanoplatfrom-Based Molecular Imaging*; Chen, X., Ed.; John Wiley & Sons, Inc.: **2011**, p 529.
- (14) Huang, W.-Y.; Davis, J. J. *Dalton Trans.* **2011**, *40*, 6087.
- (15) Kim, J.; Piao, Y.; Hyeon, T. *Chem. Soc. Rev.* **2009**, *38*, 372.
- (16) Mulder, W. J. M.; Strijkers, G. J.; van Tilborg, G. A. F.; Cormode, D. P.; Fayad, Z. A.; Nicolay, K. *Acc. Chem. Res.* **2009**, *42*, 904.
- (17) Pinho, S. L. C.; Faneca, H.; Geraldès, C. F. G. C.; Rocha, J.; Carlos, L. D.; Delville, M.-H. *Eur. J. Inorg. Chem.* **2012**, 2828.

*"The slave begins by demanding justice and
ends by wanting to wear a crown"*
Albert Camus

CHAPTER 2

MULTIMODAL AGENTS FOR MAGNETIC RESONANCE AND FLUORESCENCE IMAGING

Abstract

In medical diagnosis, combinations of different imaging modalities are often used in order to address the shortcomings of each individual technique while, at the same time, taking advantage of their individual strengths. In this chapter, a concise literature overview of various multimodal agents is presented, with particular interest in those combining magnetic resonance with optical imaging techniques. A selection of different multimodal imaging strategies explored until this day, based on advances in surface chemistry, contrast agent synthesis and nanotechnology, are discussed together with some theoretical background.

2.1 Introduction: Molecular Imaging

The need for tools to facilitate the visualization of the different molecular processes occurring in the human body in a non-invasive, accurate way has led to the development of molecular imaging.¹⁻³ Originally started in the field of radiopharmacology, this diagnostic discipline combines knowledge from biology, physics, chemistry and medicine.

2.1.1 General Aspects

In the clinic, established (non-invasive) molecular imaging techniques used nowadays include positron emission tomography (PET), single photon emission computed tomography (SPECT), magnetic resonance imaging (MRI) and several optical imaging techniques based on fluorescence, absorption and bioluminescence.³⁻⁵ For all of these modalities, an imaging agent (IA) is used in order to aid the visualization of certain regions of interest (ROI) without perturbing the molecular pathways to be visualized.⁶ IAs used for the previously mentioned imaging modalities usually consist of radioactive, paramagnetic or fluorescent moieties, alone or in combination with e.g., antibodies or peptides to target specific cells in the body.

General requirements for these IAs, especially when it comes to *in vivo* applications, include high kinetic⁷ and thermodynamic stability, water solubility, chemical stability at physiological pH, long-term stability at kidney environment (pH~ 4.5), and low toxicity. Other features, such as target specificity and excretion time after administration are crucial in a more advanced stage of clinical testing and once the previously mentioned criteria are met.

2.1.2 The Need for Different Modalities: Complementarity

In spite of the various imaging techniques available nowadays, there is not a single technique that provides all the information needed in a single scan. Typical examples are techniques derived from nuclear medicine, such as PET and SPECT, which offer physiological information and are highly sensitive, as they allow detection of IAs in the pico-molar range (Figure 2.1). However, these modalities suffer from limited spatial resolution and, therefore, require a second technique (e.g. computed tomography -CT-) to provide anatomical detail that can be correlated to the information gathered.

Figure 2.1. Sensitivity vs. spatial resolution of selected imaging modalities, including the position of the ideal imaging modality (red) and its real/achievable positioning (green). Adapted from.⁴

Alternative techniques such as MRI offer anatomic (detailed) information, soft tissue contrast and excellent spatial resolution in the sub millimeter range. Still, MRI suffers from a rather low sensitivity. On a cellular level, optical techniques based on absorption and (self)emission of light using near-infrared dyes and bioluminescent compounds (enzymatic assays)

have become very popular due to the high sensitivity they offer. For most of these techniques, with the exception of green fluorescent protein (GFP) imaging, the limited spatial resolution remains an issue. Other drawbacks include tissue penetration depth, altered photon transport as a result of scattering and absorption phenomena, photobleaching, autofluorescence and (sometimes) the need for preparates for *ex vivo* analysis.^{3,4}

Ideally, the ultimate imaging technique will offer the best spatial resolution, excellent soft tissue contrast and a very high sensitivity (Figure 2.1, red square). While with the present technical knowledge and imaging modalities available this seems unlikely, the merge of two (or more) imaging techniques appears to be the most intuitive way to reach a compromise between high spatial resolution and sensitivity (Figure 2.1, green square). In order to obtain information from different imaging techniques about a certain ROI, the so-called multimodal agents are used: these are IAs where active moieties for different imaging modalities are combined.

2.2 Multimodal Imaging

Multimodal probes have become increasingly popular due to their several advantages, which include the possible visualization of biological material using different, complementary techniques. Additionally, multimodal IAs can help see “hot spots” in the operation room after localization with non-invasive routine techniques⁸ and are excellent labels for *in vivo* validation of (new) agents. There are innumerable approaches ranging from pure synthetic chemistry (in order to make probes with the different imaging techniques combined in one molecule), to the modification of fairly established IAs that can be combined, with the help of surface chemistry,

to nanostructures/nanomaterials. These advances have led to a complete library of combinations of various imaging techniques, which will be highlighted in the next sections.

2.2.1 The Concept of Multimodality

In literature, terms such as multimodality probes,⁸ multimodal agents⁹ and and/or dual agents¹⁰ are used when referring to systems where various IAs are combined into one whole. In this way, detection using different imaging modalities can be achieved. Nevertheless, there is some ambiguity regarding the concept of duality, since the terms dual probe or dual agent are also used when referring to probes where little variations (e.g., by changing the lanthanide in a molecule)^{11,12} lead to separate IAs that can be used for different modalities.

2.2.2 Combinations of Imaging Modalities

Throughout the years, multimodal agents with functionalities for optical, magnetic and/or nuclear imaging techniques have been developed, studied and, in some cases, pre-clinically tested.^{8,10} An excellent example from the past few years regards the development of the PET/MRI combination.¹³⁻¹⁵ Synergistically, the spatial resolution issues of PET are addressed by MRI while, at the same time, the low sensitivity of MRI is overcome by the pico-molar range concentrations that can be detected using PET (Figure 2.2).¹⁶

Figure 2.2. Spatial resolution of a) MRI vs. b) PET, illustrated using a Derenzo phantom containing Mn-doped magnetism-engineered iron oxide nanoparticles (MnMEIO). Yellow circle: water (control). c) Sensitivity-related information illustrated by MR (left) and PET (right) images of free ^{124}I , ^{124}I -SA-MnMEIO and SA-MnMEIO contrast agents at different concentrations (SA: serum albumin). Adapted with permission from.¹⁶ Copyright 2008 John Wiley & Sons.

Another example is the combination optical/nuclear imaging, where the unlimited depth penetration of nuclear imaging is combined with the better resolution and (sometimes) higher sensitivity that optical imaging provides for superficially localized lesions.^{10,17,18} In addition to this, nuclear imaging can also be used as a validation technique for (new) optical IAs.^{19,20} Despite the innumerable technical/chemical/biological developments related to the previously mentioned combinations, magnetic resonance/optical imaging remains by far the most studied and developed blend.⁹

2.3 Magnetic Resonance and Optical Imaging

When it comes to multimodal IAs, one of the prevalent combinations is optical/MR imaging, with the latter technique remaining one of the most popular and investigated imaging modalities today. Interest in MRI arises not only from the excellent spatial resolution and soft tissue contrast it offers, but also because of its non-ionizing character that distinguishes it from nuclear imaging techniques. Additionally, MRI has been used for a broader range of applications, including blood flow, metabolic and cell density studies.²¹ Optical imaging, on the other hand, helps balance out the sensitivity issues related to MRI while still offering spatial resolutions in the sub-micron range and a non-ionizing character.^{22,23}

2.3.1 Magnetic Resonance Imaging (MRI)

Magnetic resonance imaging (MRI) is a non-invasive technique mostly associated with medical diagnostics due to its ability to discriminate between different kinds of tissue, in 2 and 3-dimensional views, of a certain part of the human body. Unlike other commonly used imaging techniques such as PET, MRI has a non-ionizing character, making it suitable for cell research since no cell damage occurs. Typical magnetic field strengths used for medical/diagnostic imaging include 0.5, 1.5 and 3 Tesla, the latter being the most used in modern hospitals.

In the presence of a strong external magnetic field B_0 , the nuclear magnetic moments of protons (^1H , quantum spin number $I = \frac{1}{2}$) stop being randomly oriented and adopt parallel or anti-parallel orientations to the magnetic field due to energetic preferences. The population difference that arises from the adopted spin states ($\pm \frac{1}{2}$) in the presence of B_0 is known as the macroscopic (or bulk) magnetization M_0 (average magnetic moment,

Figure 2.3a,b). If an applied radiofrequency (rf) pulse generates an electromagnetic field of a resonance frequency that can be absorbed by the spins (known as the resonance frequency), the position of the magnetization vector M_0 changes in space as it inclines away from the reference axis (Figure 2.3c). The phenomenon of realignment of the M_0 with the external magnetic field B_0 is known as relaxation (Figure 2.3d).

Figure 2.3. a) Population difference, that gives rise to the magnetization vector M_0 , due to the possible spin states. b) Macroscopic magnetization in thermal equilibrium. c) The magnetization vector moves away from the z-axis as an rf pulse is applied. d) Precession of the magnetization vector as it realigns with the magnetic field.

The use of magnetic resonance for medical purposes was first explored in 1971, when Damadian²⁴ reported differences in the (water) proton relaxation of both normal and cancer tissues and proposed scanning of living people. Further studies focused on the development of imaging techniques and by 1973, Paul Lauterbur²⁵ had proven that, in the presence of a magnetic field, a relationship between location and frequency could be established. This is achieved using a spatial gradient of the static field B_0 , which causes linear variations of the spatial location of nuclei with their resonance frequency (Figure 2.4a).²⁶ In this way, spatial resolution is achieved as the position of a given nucleus can be identified. Inversed Fourier Transformation of the signals, expressed as several points in a coordinate system (the so-called reciprocal space), is used to generate

and image which shows the proton distribution in the body (real space) (Figure 2.4b).

Figure 2.4. a) In the presence of a linearly space-dependent magnetic field in the z-direction (gradient), the phase of the magnetization in the y-direction (M_y) changes in time. Adapted from.²⁷ b) MR image is produced by inverse Fourier Transformation (iFT) of the reciprocal space to the real space.

2.3.1.1 Proton Relaxation and Image Contrast

After an rf pulse is applied to a sample in the presence of a magnetic field B_0 , the magnetization vector will tend to return to the thermal equilibrium state; a phenomenon known as relaxation. During this process, two kinds of relaxation occur:

- i) spin-lattice or longitudinal relaxation (T_1 , Figure 2.5a): described as the time it takes for the magnetization vector to return to its thermal equilibrium state (relaxation in the z direction). This enthalpic process, where spins simply loose energy, can take seconds, minutes, hours or even days.
- ii) spin-spin or transverse relaxation (T_2 , Figure 2.5b): described as the time it takes for the magnetization vector to dephase (relaxation in the xy plane). This entropic process, where energy is swapped between spins (flip-flop process), takes usually milliseconds.

Figure 2.5. a) Spin-lattice or longitudinal relaxation (T_1). b) Spin-spin or transverse relaxation (T_2).

Once the magnetization vector returns to thermal equilibrium, no transverse relaxation in the xy plane can occur. For this reason, longitudinal relaxation times T_1 are always larger than transverse relaxation times T_2 . The differences between these two relaxation processes form the basis for MR contrast imaging, generated by optimizing rf pulses and delay times in order to obtain, for instance, T_1 - and T_2 -weighted images.

While water protons of different tissues in the body show differences in their relaxation times, visualization of possible ROIs can be challenging, as tissues with similar relaxation behavior might be histologically different. In such cases a contrast agent (CA) aids the image contrast by shortening T_1 and/or T_2 . Shortening of the longitudinal relaxation time T_1 leads to brighter images due to the increased signal (positive contrast), while shorter transverse relaxation times T_2 lead to negative contrast (darker images) due to signal loss. Typical T_1 CAs are based on metal complexes of paramagnetic ions,²⁸⁻³² being Gd^{3+} the most widely used, while T_2 CAs are mostly based on super-paramagnetic iron oxides (SPIOs).³³⁻³⁵ Other alternatives to Gd^{3+} -based metal chelates are described elsewhere.^{32,36} In the next sections, T_1 relaxivity and Gd^{3+} -based T_1 CAs will be discussed in more detail.

2.3.1.2 *First Generation T_1 Contrast Agents*

The Gd^{3+} ion, with its seven unpaired f -electrons, has a totally symmetric electronic state ($^8S_{7/2}$ ground state) and a high magnetic moment ($7.9 \mu\text{B}$). For these reasons, Gd^{3+} has much longer relaxation time than other lanthanide ions.^{30,37} The 8 water molecules in the first coordination sphere of the Gd^{3+} -aquo ion are responsible for the high relaxivity it offers. However, Gd^{3+} ions are often “clustered” using organic ligands, as this lanthanide is very toxic in its “free” form. For decades, metal complexes have been widely used as IAs/diagnostic tools,³¹ especially for oncologic applications. Since their introduction in the late 80s,³⁸ extracellular CAs based on Gd^{3+} complexes of polyamino polycarboxylate ligands³⁹ are used in approximately one third of all the MRI exams in the world. These CA have an extracellular character since the most stable complexes, formed using negatively charged ligands, are repelled by the negatively charged head groups of the phospholipids in the membrane.

Complexes used nowadays for clinical diagnostics include Gd^{3+} -diethylenetriaminepentaacetic acid (Gd^{3+} -DTPA, Magnevist®) and Gd^{3+} -1,4,7,10-tetraazacyclododecanetetraacetic acid (Gd^{3+} -DOTA, Dotarem®) (Scheme 2.1, left and right, respectively). These CAs enhance the contrast of images in the regions where they are located due to a lowered longitudinal relaxation time T_1 .

Scheme 2.1. Chemical structure of Gd^{3+} -DTPA (left) and Gd^{3+} -DOTA (right).

2.3.1.3 Relaxivity

The relaxation effect observed in the presence of metal complexes is due to the interaction of the electron spins of the paramagnetic ion with the hydrogen nuclei of water. In the presence of paramagnetic species, the observed relaxation rate ($R_i = 1/T_i$)_{obs} can be expressed as the contribution from the relaxation rate in: a) the absence of the paramagnetic solute, known as the diamagnetic term $(1/T_i)_d$ and b) the presence of the paramagnetic substance $(1/T_i)_p$, which is known to be linearly proportional to the concentration of the paramagnetic solute (Equation 2.1).⁴⁰

$$\left(\frac{1}{T_i}\right)_{obs} = \left(\frac{1}{T_i}\right)_d + \left(\frac{1}{T_i}\right)_p = \left(\frac{1}{T_i}\right)_d + r_i[Gd] \quad (2.1)$$

The T_i -shortening efficiency shown by a CA is known as relaxivity r_i ($i=1,2$), defined as the efficacy of a 1 mM solution of CA to increase the relaxation rate R_i of water protons. In Table 2.1, the r_1 relaxivities rates of some commercially available CA for MRI are shown.

Table 2.1. Relaxivity (r_1) values of some clinically used Gd^{3+} -based contrast agents for MRI, measured at 37°C and 1.5 T.³⁰

Trademark	Abbreviation of the active component	$r_1/\text{mM}^{-1} \text{ s}^{-1}$
Dotarem®	Gd^{3+} -DOTA	4.2
ProHance®	Gd^{3+} -HP-DO3A	4.4
Gadovist®	Gd^{3+} -BT-DO3A	5.3
Magnevist®	Gd^{3+} -DTPA	4.3
Omniscan®	Gd^{3+} -DTPA-BMA	4.6
OptiMARK®	Gd^{3+} -DTPA-BMEA	5.2
MultiHance®	Gd^{3+} -BOPTA	6.7
Primovist®	Gd^{3+} -EOB-DTPA	7.3
Vasovist®	MS-325	19

As shown in Equation 2.2, two mechanisms contribute to the paramagnetic relaxation enhancement effect: inner-sphere relaxation, resulting from the exchange of coordinated water molecules (first coordination sphere) with the bulk environment, and outer-sphere relaxation, related to water molecules in the surroundings:

$$\left(\frac{1}{T_i}\right)_p = \left(\frac{1}{T_i}\right)_{\text{inner-sphere}} + \left(\frac{1}{T_i}\right)_{\text{outer-sphere}} \quad (2.2)$$

Inner-Sphere Relaxation. The inner-sphere contribution to the relaxation rate is usually modeled using the Solomon-Bloembergen-Morgan (SBM) theory.^{29,40} According to this theory, two relaxation mechanisms contribute to both the longitudinal (R_1) and transverse (R_2) relaxation rates, namely the dipole-dipole (DD) and scalar (SC) mechanisms, generally expressed as follows:

$$\frac{1}{T_{iM}} = \frac{1}{T_i^{DD}} + \frac{1}{T_i^{SC}} \quad i = 1, 2 \quad (2.3)$$

$$\frac{1}{T_1^{DD}} = \frac{2}{15} \frac{\gamma_I^2 g^2 \mu_B^2 S(S+1)}{r^6} \left[\frac{3\tau_{c1}}{(1 + \omega_I^2 \tau_{c1}^2)} + \frac{7\tau_{c2}}{(1 + \omega_s^2 \tau_{c2}^2)} \right] \quad (2.4)$$

$$\frac{1}{T_1^{SC}} = \frac{2}{3} S(S+1) \left(\frac{A}{\hbar} \right)^2 \left[\frac{\tau_{e2}}{(1 + \omega_s^2 \tau_{e2}^2)} \right] \quad (2.5)$$

$$\frac{1}{T_2^{DD}} = \frac{1}{15} \frac{\gamma_I^2 g^2 \mu_B^2 S(S+1)}{r^6} \left[\frac{3\tau_{c1}}{(1 + \omega_I^2 \tau_{c1}^2)} + \frac{13\tau_{c2}}{(1 + \omega_s^2 \tau_{c2}^2)} + 4\tau_{c1} \right] \quad (2.6)$$

$$\frac{1}{T_2^{SC}} = \frac{1}{3} S(S+1) \left(\frac{A}{\hbar} \right)^2 \left[\frac{\tau_{e2}}{(1 + \omega_s^2 \tau_{e2}^2)} + \tau_{e1} \right] \quad (2.7)$$

$$\frac{1}{\tau_{ci}} = \frac{1}{T_{ie}} + \frac{1}{\tau_M} + \frac{1}{\tau_R} \quad i = 1, 2 \quad (2.8)$$

$$\frac{1}{\tau_{ei}} = \frac{1}{T_{ie}} + \frac{1}{\tau_M} \quad i = 1, 2 \quad (2.9)$$

Where: $\tau_{ci} - \tau_{ei}$: correlation times dipole-dipole and scalar relaxation
 γ : nuclear gyromagnetic ratio
 g : electron g factor
 S : spin quantum number
 μ_B : Bohr magneton
 ω_s and ω_i : electron and nuclear Larmor precession frequencies, respectively ($\omega = \gamma B$, where B is the magnetic field)
 r : electron spin-solvent nuclear spin distance
 $A/\hbar$: electron-nuclear hyperfine coupling constant
 τ_R : molecular rotational coordination time or tumbling rate
 τ_m : water residence time
 T_{ie} : electronic longitudinal ($i = 1$) and transverse ($i = 2$) relaxation times for the metal ion

The SBM equations above (Equation 2.3-2.9) show that relaxation rates (interpreted in terms of zero-field splitting interactions -ZFS-) are field dependent, since the gyromagnetic ratio γ depends on the magnitude of

this field ($\omega = \gamma B$). Therefore, the maximum achievable relaxivity decreases as the strength of the magnetic field increases. In such cases, the outer-sphere relaxation mechanisms become more important (see next section).

Extra details regarding the SBM theory can be found elsewhere.^{28,38}

Since relaxed water protons exchange rapidly with molecules from the surroundings, relaxation is dictated by the number of water molecules coordinated to the paramagnetic center (q , known as the hydration number), their residence time (τ_m), the rotational correlation time or tumbling rate (τ_R) and the diffusion correlation time (τ_D , Figure 2.6).

Figure 2.6. Inner- and outer-sphere parameters that dictate the relaxivity of Gd^{3+} -based CAs. Adapted from.^{28,30}

Optimization of τ_m is necessary as the time a coordination site on the metal ion is occupied by a water molecule dictates the relaxation efficiency of a CA. The tumbling rate τ_R (which becomes slower when the size/volume of CAs increases) also plays a key role as lowered global rotational motion of CAs leads to higher relaxivity values. Intuitively, the relaxivity is strongly affected by the number of bound water molecules per metal ion. However, the ability to increase q is limited by safety issues because, typically, the

more coordination sites available for water ligation, the lower the thermodynamic stability of the complex. Second generation CAs based on hydroxypyridinone ligands,⁴¹⁻⁴⁷ which will not be discussed in this literature review, address this issue with rather respectable results.

Outer-Sphere Relaxation. The paramagnetic relaxation effect is not only limited to solvent molecules in the first coordination sphere, as some degree of relaxivity has been observed in complexes with $q = 0$.⁴⁸ When the solvent molecules diffuse to the (magnetized) “external” solute sphere, their protons experience variations in the local magnetic field.⁴⁹ While the exact outer-sphere contributions to the relaxivity are not well understood yet, it is known that the diffusion correlation time τ_D of water molecules,²⁹ as well as relaxation by dipolar mechanisms of hydrogen bonded water molecules in the second-sphere,^{50,51} play an important role.

Relaxivity at High Magnetic Fields. Typical magnetic fields used for clinical imaging include 0.5, 1.5 and 3 T. At these field strengths, an optimal balance between fast water exchange and enhanced tumbling rate of the complex is desired, together with a higher contribution from the second coordination sphere water molecules.⁵² As higher magnetic fields are implemented, the maximum relaxivity achievable decreases and tends to depend on careful “fine-tuning” of τ_m and τ_R .²⁹ Yet, this decrease in the relaxivity achievable is compensated by the higher sensitivity (due to greater signal-to-noise ratio) and increased spatial resolution that can be attained using high-field MRI instrumentation.

2.3.1.4 The Sensitivity Gap: Increasing the MRI Signal

One of the major challenges when developing new CAs for MRI is dealing with the inherent low sensitivity and the need for high concentrations of CAs: *“in order to induce observable contrast in a robust clinical exam, a relaxation rate change of at least about 0.5 s^{-1} is required. For extracellular commercial contrast agents with a relaxivity of $\sim 4\text{ mM}^{-1}\text{ s}^{-1}$, this means a concentration of $\sim 125\text{ }\mu\text{M}$. For targeted imaging and assuming a 1:1 binding stoichiometry (Gd^{3+} :target molecule), this would require a biological target present with a concentration at least $125\text{ }\mu\text{M}$ ”*⁵³

Through the years, efforts have focused on the design of new ligands to form metal complexes with optimized relaxivity-related parameters such as the hydration number q ,⁴¹⁻⁴⁴ the water residence time τ_m ⁵⁴⁻⁶⁰ and the molecular tumbling rate τ_R . Slower global rotational motion, and therefore higher relaxivity, has successfully been achieved by increasing the molecular weight of the metal complexes. This has been done by means of covalent and non-covalent attachment to e.g., proteins,^{61,62} peptides,⁶³ human serum albumin⁶⁴ and ATP.⁶⁵ An alternative strategy followed involves the attachment of Gd^{3+} complexes to macromolecules⁶⁶ in order to: i) decrease the tumbling rate and ii) increase the local paramagnetic moiety concentration.^{67,68} There are numerous examples in the literature illustrating the improved relaxivity properties that can be achieved by incorporating Gd^{3+} complexes to, e.g., dendrimers,⁶⁹⁻⁷⁵ proteins (polypeptides),^{61,76-79} polymers,^{80,81} proteins cages such as wild-type cowpea mosaic virus (CPMV),⁸² HSP capsids,⁸³ cowpea chlorotic mottle virus (CCMV) capsids⁸⁴ and MS2 viral capsids,⁸⁵⁻⁸⁷ cyclic oligosaccharides,⁸⁸ carbohydrates,⁸⁹ peptides,⁹⁰⁻⁹² metallostars^{93,94} and supramolecular aggregates such as micelles and liposomes.⁹⁵⁻¹⁰⁰

A more recent strategy uses advances in the field of nanotechnology in order to decrease the molecular motion of complexes (by increasing their volume) and boost the local Gd^{3+} concentration. Most of these approaches, based on surface modification of diverse nanomaterials, not only show improved relaxivities but also require less synthetic input. In addition to this, surface modification opens up the door to the facile incorporation of other moieties for, e.g., biocompatibility and targeting. Examples of such “paramagnetic nanosystems” include quantum dots,¹⁰¹⁻¹⁰⁴ nanodiamonds,¹⁰⁵ (silica) nanoparticles,¹⁰⁶⁻¹⁰⁸ mesoporous silica nanospheres,¹⁰⁹ nanoassembled capsules of polymer aggregates inside silica shells,¹¹⁰ carbon nanotubes,¹¹¹⁻¹¹³ gold nanoparticles¹¹⁴⁻¹¹⁸ and gold nanorods.^{119,120}

2.3.2 Optical Imaging

Whereas standard optical techniques are based on interactions of photons with matter, these elementary particles can also interact with tissue. Optical imaging of biological tissues relies on the fact that photons can interact with certain tissue components thereby affecting the number of transmitted photons that pass through irradiated ROIs. This imaging modality is usually associated with traditional microscopy techniques which are known to be limited by the mean free path (MFP) of a photon, which is around 100 μm in tissue.¹²¹ Contrary to nuclear or magnetic resonance imaging techniques, where detection is mainly performed *in vivo*, some optical imaging techniques involve *in vitro* as well as *ex vivo* experimentation, where thin tissue sections are usually required. More recently, new optical techniques based on image fusion, multichannel imaging, fiber-optic approaches and tomographic reconstruction are used to generate images *in vivo*.²²

2.3.2.1 Optical Imaging Techniques

Typical optical imaging techniques currently used in the clinic are based on: i) absorption (absorption of photon energy by tissue) and ii) scattering phenomena (light deviated from its original path) that can occur in three spectral regions, ultraviolet (UV, < 450 nm), visible (Vis, 450-650 nm) and near-infrared (NIR, 650-1000 nm). In the next sections, selected examples of optical absorption-based techniques, such as bioluminescence and fluorescence imaging, will be discussed. State-of-the-art optical imaging modalities, e.g. based on protein-protein interactions, will not be discussed here.^{22,121}

Bioluminescence Imaging (BLI). Biological activity can be tracked *in vivo* using enzymes that form light emitting molecules (bioluminescence imaging). Enzymes such as luciferases are known for using energy and oxygen in order to oxidize luciferin, thereby leading to the self-emission of visible light (yellow to green). Emission without the need for external light sources for excitation is particularly interesting, as biological tissues are known to absorb and emit light in the visible range. Using firefly luciferase (FL), non-invasive imaging of gene expression in mice has successfully been achieved (Figure 2.7).¹²² However, the need for detecting devices with sufficient sensitivity remains one of the major issues of this imaging modality. Other examples illustrating the successful implementation of BLI and new developments are described in reviews on optical imaging.^{3,5,123-}

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Figure 2.7. Cooled charge-coupled device (CCD) images on a Swiss Webster mouse showing the magnitude and duration of firefly luciferase (FL) gene expression injected. Mice were injected with E1-deleted adenovirus expressing FL driven by a cytomegalovirus promoter (Ad-CMV-FL). Control virus yielded only background signals (55 ± 10 RLU/min). Adapted with permission from.¹²² Copyright 2001 Nature Publishing group.

Fluorescence Imaging. Optical microscopy, using fluorescent molecules such as porphyrins and fluorescein for staining, has been widely used since the 1920s for *in vitro* applications.²² These “fluorescent agents” are used in order to alter the absorption, emission or scattering properties of the tissue where it accumulates. In this way, contrast enhancement is achieved. Taking advantage of the technological progress in microscopy and the development of fiber-optic approaches and new imaging probes, the use of fluorescent dyes has been extended to *ex vivo* and *in vivo* applications. Current techniques used include, amongst others, optical projection tomography (OPT), diffuse optical tomography (DOT), NIR fluorescence and green fluorescent protein (GFP) imaging.^{4,22,121} In particular, organic dyes and inorganic nanocrystals as IAs for imaging in the NIR region have been extensively studied since scattering is known to be weak in this spectral region as NIR photons travel through tissue much more efficiently.¹²⁶ In the next sections, a selection of NIR IAs will be briefly discussed.

2.3.2.2 *Fluorescence Imaging Agents*

Organic Dyes. Typically, organic dyes with absorption and emission properties in the NIR region can be classified in cyanine dyes, tetrapyrroles or lanthanide chelates. Water-soluble cyanine dyes (Scheme 2.2a) are very popular due to their high molar extinction coefficients ($> 150,000 \text{ M}^{-1} \text{ cm}^{-1}$), good fluorescence quantum yields (up to 50%),^{127,128} commercial availability and their ability to be further functionalized. In this way, these IAs can be linked to virtually any probe, e.g., peptides for targeting purposes (Scheme 2.2b). Other compounds used for NIR detection include tetrapyrrole-based dyes such as porphyrins, chlorins and phthalocyanines (Scheme 2.2c). In addition to the above mentioned spectroscopic properties, the latter dyes are also known for their use as photosensitizing agents in photodynamic therapy (PDT).¹²⁷

Scheme 2.2. Chemical structures of selected: a) commercially available carbocyanine dyes. b) Peptide-linked (octreotate) Cypate 9 dye. c) Chlorins (**1,2,3**), phthalocyanines (**4**) and porphyrins (**5**). Adapted from.¹²⁷

Lanthanide-based complexes are not limited to biomedical applications involving paramagnetic properties (for MRI), but can also be used for

alternative detection modalities such as fluorescence.^{11,129} While europium (Eu^{3+}) and terbium (Tb^{3+})^{12,130} are poor MRI CAs compared to Gd^{3+} , they are highly attractive as fluorescent probes due to their long fluorescence lifetimes and large Stoke shifts, which allows detection with high selectivity/sensitivity and limited background interference. For lanthanide ions, electronic transitions in the $4f^n$ configuration are Laporte forbidden, whereby electronic $f \rightarrow f$ transitions are forbidden for molecules exhibiting a center of symmetry. Within the lanthanide series, Eu^{3+} and Tb^{3+} are high emitters and have excited state lifetimes in the 100-3000 μs range. However, parity-forbidden $4f$ - $4f$ absorptions (with very low molar absorption coefficients) are observed as the $4f$ orbitals are shielded by the filled $5p^6 6s^2$ sub-shells.¹³¹ Therefore, the luminescence of lanthanide ions alone is weak and requires sensitization. Usually, carefully chosen “antennae or sensitizers” are integrated in the chelating ligand in order to absorb light and promote transitions into the excited states of the coordinated metal ion (Figure 2.8a).¹³¹ However, sensitization using antennae is a complex process where the energy gap between the lowest ligand triplet state and the lanthanide emitting level needs to be adjusted (Figure 2.8b). Typical examples of sensitizers used for energy transfer include aromatic rings,^{130,132} pyrene,¹³³ azathioxanthone¹³⁴ and triazolophthalazine¹³⁵ or transition metals such as rhenium,¹³⁶ chromium¹³⁷ and ruthenium.¹³⁸

Figure 2.8. a) Sensitization using antennae. b) Diagram showing the main transition states during sensitization of lanthanide luminescence using ligands. Adapted from.¹³¹

Eu^{3+} and Tb^{3+} complexes with various sensitizers have been studied for years¹³⁹ and their potential for imaging applications has been extensively described.¹³⁹⁻¹⁴¹ More recently, Nd^{3+} , Er^{3+} and Yb^{3+} have become highly attractive since their emission occurs in the NIR region.¹⁴²⁻¹⁴⁴ Nevertheless, these metal ions also exhibit some drawbacks, including their short lifetimes (ns to μs , that limit time-resolved detection) and the low sensitization due to the small energy gap between the excited and the ground-state levels.

Inorganic Fluorescent (Semiconductor) Nanocrystals.

Semiconductor nanocrystals or quantum dots (QDs) consist of an inorganic core and a metal shell (with an organic outer layer) and are known for their high quantum yield, resistance to photobleaching and tunable emission wavelengths.¹⁴⁵ Furthermore, ligand exchange of the organic outer layer or (possible) hydrophobic interactions with other molecules provide versatility to these nanoparticles. While their potential use as NIR fluorophores for *in vivo* optical imaging has been explored,¹⁴⁶ the toxicity of these nanocrystals remains an issue.

Other nanocrystals with potential for optical imaging are silver nanoclusters. Consisting of a limited number of silver atoms ($n \sim 2-10$),

nanoclusters are known to be fluorescent¹⁴⁷⁻¹⁵⁰ and show differences in emission spectra when differing in number by one single atom.^{151,152} The potential of nanoclusters as IAs for optical imaging is evidenced by their successful application as single-molecule fluorophores¹⁵³ and cell staining labels.¹⁵⁴⁻¹⁵⁶

2.4 Strategies for Magnetic Resonance/Optical Multimodal Imaging Agents

The potential of multimodal agents for applications in magnetic resonance and optical imaging is evidenced by the dramatic increase in literature during the past decades.^{8,10} Generally speaking, multimodal IAs can be divided in three major groups: small molecule IAS, macromolecular IAs and nanostructures. In the next sections, some representative examples of IAs for these two imaging modalities will be presented and shortly discussed.

2.4.1 Small Molecule Imaging Agents

Mononuclear Dual Imaging Agents. Owing to the small differences in radii between lanthanides, small molecular templates offer great versatility as they can complex either Gd^{3+} (for MRI) or $\text{Eu}^{3+}/\text{Tb}^{3+}/\text{Yb}^{3+}$ (for optical imaging). Additionally, these complexes are expected to show the same biological behavior. This concept, often referred to as duality, has been proved using various chelators.¹⁵⁷ For instance, Picard *et al.*¹² used ligands derived from an N,C-pyrazolylpyridine moiety and iminodiacetate units (**1**, Scheme 2.3) to make lanthanide complexes with high thermodynamic stability. The luminescence and relaxometric properties of the Tb^{3+} and Gd^{3+} chelates, respectively, were investigated in aqueous solutions. The

Tb³⁺ chelate is strongly luminescent, with a remarkable lifetime and quantum yield ($\tau = 1.82$ ms and $\Phi = 0.42$, respectively). Moreover, the longitudinal proton relaxivity of the Gd³⁺ chelate was found to be comparable to that of the clinically used Gd³⁺-DTPA. Cyclic polyaminocarboxylates, such as 10-(2-hydroxypropyl)-1,4,7-tetraazacyclododecane-1,4,7-triacetic acid (HP-DO3A **2**, Scheme 2.3), have also been studied as dual MR/optical imaging IAs. In order to do so, Geninatti Crich *et al.*¹¹ incubated endothelial progenitor cells (EPCs) with Gd³⁺ and Eu³⁺-HP-DO3A complexes. The Gd³⁺ chelates were used as T_1 -CAs for MRI visualization while the Eu³⁺ chelates acted as reporters/“validation” agents for fluorescence microscopy.

Scheme 2.3. Chemical structures of: polyaminocarboxylate ligand derived from N,C-pyrazolylpyridine (**1**),¹² HP-DO3A ligand (**2**),¹¹ methyl pyridine DOTA derivative with bipyridine rhenium complex (**3**)¹³⁶ and DOTA/5A-PADDTA ligand (**4**).¹⁵⁸

Heteronuclear Dual Imaging Agents. Another example of dual IAs is heteronuclear complexes, where two metal ions are integrated in the same molecule. For this purpose, Koullourou *et al.*¹³⁶ used a methyl pyridine DOTA derivative for lanthanide complexation and a bipyridine rhenium chromophore for sensitization (**3**, Scheme 2.3). While the Yb³⁺ complex showed sensitized emission with long luminescence lifetimes, interesting relaxivity properties at high magnetic fields were associated to the Gd³⁺ complex (ionic $r_1 = 8.6$ mM⁻¹ s⁻¹ at 500 MHz).

Heteronuclear Multimodal Imaging Agents. Heteronuclear complexes, where two chelating moieties are combined into one molecule, were proposed by Mamedov *et al.*¹⁵⁸ Consisting of a cyclic DOTA and the acyclic 5-aminoisophthalamide diethylenediamine tetraacid ligand (5A-PADDTA, **4**, Scheme 2.3), this bis-chelate was found to form stable complexes with Eu^{3+} and Gd^{3+} , exhibiting luminescence and relaxometric properties, respectively. Complexation of the acyclic chelator was confirmed by ^1H NMR spectroscopy, where the spectrum of the ligand, its Eu^{3+} complex, and that containing both Eu^{3+} and Tb^{3+} confirmed the existence of the monometallic and bimetallic species. While this ligand is an impressive example of the potential use of heteronuclear complexes as multimodal agents, controlled integration of two different lanthanides in one molecule remains a difficult task.

Targeting Dual and Multimodal Imaging Agents. A more “targeted” approach to new, small molecule imaging probes for multimodality purposes has been proposed by Mindt *et al.*¹⁵⁹ Using the $\text{Cu}(\text{I})$ -catalyzed cycloaddition of terminal alkynes and azides, a “clickable” derivative of folic acid (FA) was prepared. Starting from the γ -azido-FA precursor (**5**, Scheme 2.4), imaging probes for NIR fluorescence (using a Cy5.5 NIR dye) and MR imaging (using a Gd^{3+} -DOTA derivative) were successfully synthesized. Detailed spectroscopic characterization and *in vitro* studies showed the expected properties and the affinity of the FA-derivative CAs towards the FA receptor. Moreover, these probes are suitable for other imaging techniques since the DOTA-based ligand can be used for complexation with ^{67}Ga , ^{111}In , ^{177}Lu (tracer for SPECT) and the γ -azido-FA precursor can be clicked to ^{18}F (tracer for PET) or ^{19}F (for ^{19}F MRI) derivatives. Other targeted CAs include trifunctional macrocyclic ligands

with two phosphonic acid pendant arms for chelation of $\text{Gd}^{3+}/\text{Eu}^{3+}$ and a quinoline chromophore that acts as an antenna (**6**, Scheme 2.4). Manning *et al.*¹⁶⁰ coupled this CA to 1-(2-chlorophenyl)isoquinoline-3-carboxylic acid, known to target peripheral benzodiazepine receptors (PBR). The Eu^{3+} complex showed visibly bright fluorescence and the Gd^{3+} complex had a relaxivity comparable to that of Gd^{3+} -DTPA.

Scheme 2.4. Chemical structures of: folate- γ -(4-azido)-butane amide¹⁵⁹ (**5**), trifunctional macrocyclic ligand¹⁶⁰ (**6**) and carbocyanine dye with integrated DTPA (**7**).¹⁶¹

Li *et al.*¹⁶¹ have used a carbocyanine dye and a Gd^{3+} -DTPA chelate with integrated C16 alkyl tails for labeling of low-density lipoprotein receptors (LDLs) via intercalation into their phospholipid monolayer (**7**, Scheme 2.4). This multimodal IA not only showed a 7-fold increase in ionic r_1 relaxivity than Gd^{3+} -DTPA (which allowed *in vivo* MRI detection of LDL receptors in liver and tumor), but it was successfully observed *in vitro* by confocal fluorescence microscopy.

2.4.2 Macromolecular Imaging Agents

The synthetic approaches proposed for small molecule IAs have certainly led to versatile probes with improved properties. Still, these IAs are limited by their lack of selectivity and usually require the integration of targeting moieties and, therefore, longer synthetic routes. In contrast, macromolecules are an interesting alternative to achieve local high concentrations whilst still being able to integrate different imaging/targeting moieties.¹⁶² In the next sections, representative examples of the various macromolecules currently used for multimodal purposes, as well as the results achieved so far, will be highlighted.

Dendrimers. One of the first macromolecules explored for multimodal purposes were dendrimers.¹⁶³ These repeated branch molecules have been used extensively to conjugate, complex or encapsulate IAs, targeting moieties and therapeutic drugs.¹⁶⁴ Although synthetically demanding, dendrimers are chemically interesting since their surface is an excellent platform for chemical (covalent) modification and their interior is suitable for host-guest chemistry.

Considering the higher sensitivity of optical imaging when compared to MRI, dendrimers are a fascinating platform to reach a high MRI CA ratio per optical imaging unit. For this purpose, Talanov *et al.*¹⁶⁵ developed a generation 6 polyamidoamine (PAMAM) dendrimer functionalized with 2-(4-isothiocyanatobenzyl)-6-methyl-diethylenetriaminepentaacetic acid (1B4M-DTPA) ligands and a Cy5.5 dye. With this nanoprobe, sentinel lymph nodes in mice were visualized *in vivo* using both imaging modalities (Figure 2.9). While an increase in the NIR dye content resulted in partial quenching, its quantum yield was not affected by the presence of Gd^{3+} .

Figure 2.9. a) Optical image obtained under simultaneous white light and filtered (615-665 nm) excitation light. Fluorescence emission was detected in the axillary lymph node of the mouse with the emission filter set to 720 nm. b) Image from a) shown in false color. c) Maximum intensity projection calculated from a 3D spoiled gradient echo MRI, illustrating the draining lymphatics and axillary lymph node depicted in b). Adapted with permission from.¹⁶³ Copyright 1999 American Chemical Society.

Macromolecules offer a distinct advantage since they can be functionalized with targeting units in order to induce selective uptake. One example of such probes was reported by Xu *et al.*,¹⁶⁶ in which dendrimer-based templates were functionalized with avidin-biotin and used as targeted dual-reporters. By reducing the disulfide bond of the core of Gd^{3+} -1B4M-DTPA chelated generation 2 PAMAM dendrimers, followed by subsequent attachment to a maleimide-functionalized biotin, biotinylated dendrimer-based MRI CAs were obtained. In this work, up to 4 copies of these dendron-like structures were successfully immobilized to rhodamine green labeled avidin. In this way, mice bearing ovarian cancer tumors were efficiently targeted and visualized by both MR and optical imaging. Another targeting-integrated approach was proposed by Boswell *et al.*,¹⁶⁷ whereby generation 3 PAMAM dendrimers were covalently adorned with RGD-cyclopeptides, Alexa Fluor 594 dyes and Gd^{3+} -1B4M-DTPA complexes (Figure 2.10). *In vitro* studies demonstrated the capability of these multimodal dendrimers to act as IA for both MR and optical imaging. However, while fluorescence microscopy of the RGD peptide-bearing

dendrimer with empty 1B4M-DTPA ligands showed that selective binding of $\alpha_v\beta_3$ -expressing cells occurred, a decrease in selectivity was observed after complexation with Gd^{3+} . The same effect was observed in *in vivo* studies, as optical fluorescence imaging failed to show appreciable tumor uptake.

Figure 2.10. Structural representation (half-section) of the modified PAMAM dendrimer. The dendrimer core is shown in black, the $\alpha_v\beta_3$ -targeting peptide (c(RGDfK)) in orange, the 1B4M-DTPA ligand in green and the Alexa Fluor 594 dye in pink. Reprinted with permission from.¹⁶⁷ Copyright 2008 American Chemical Society.

Despite several examples of multimodal dendrimers in literature, the use of these macromolecules for biomedical applications is limited by their large size, which often leads to rapid renal excretion even if targeting agents are used. Nevertheless, these macromolecules have successfully been used as IAs for imaging of the lymphatic system.¹⁶⁸

Supramolecular Aggregates. Another interesting type of macromolecules proposed for multimodal purposes are supramolecular aggregates such as micelles and liposomes. These lipid-based “nanoparticles” are known to lead to higher relaxivities due to decreased molecular motion of the monomer units and are excellent scaffolds for higher local Gd^{3+} concentrations.¹⁶⁹ Mulder *et al.*¹⁷⁰ used PEGylated multimodal liposomes for dual detection of the expression levels of molecular markers on human umbilical vein endothelial cells (HUVEC). The paramagnetic PEGylated liposomes consisted of Gd^{3+} -DTPA-bis(stearylamide) (Gd^{3+} -DTPA-BSA), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), cholesterol (Chol), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (rhodamine-PE) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(poly(ethylene glycol))-2000] (PEG-DSPE), with antibodies coupled to the distal end of the PEG-chains (Figure 2.11a). While *in vitro* targeting of HUVEC was shown by fluorescence microscopy (Figure 2.11b), relaxometric studies revealed a higher ionic r_1 relaxivity for the Gd^{3+} -DTPA-BSA when compared to Gd^{3+} -DTPA, especially at higher temperatures (Figure 2.11c).

Figure 2.11. A) Schematic representation of a PEGylated paramagnetic liposome consisting of Gd $^{3+}$ -DTPA-BSA, DSPC, Chol and PEG-DSPE. Note that the antibodies are coupled to the distal end of the PEG-chains. B) TNF α -treated HUVEC showing the immunoliposomes. Inset: fluorescence partly originates from the cell interior (magnification 630x). C) Ionic r_1 relaxivities of free Gd $^{3+}$ -DTPA (○) and Gd $^{3+}$ -DTPA-BSA containing liposomes (■) as a function of the temperature at 300 MHz. Adapted with permission from.¹⁷⁰ Copyright 2004 American Chemical Society.

Kamaly *et al.*¹⁷¹ used Gd $^{3+}$ -2-{4,7-bis-carboxymethyl-10-[(N,N-distearylamidomethyl-N'-amidomethyl)-1,4,7,10-tetraazacyclododec-1-yl]} acetic acid (DOTA-DSA) and rhodamine-labeled 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) in order to obtain Gd $^{3+}$ -liposomes suitable for cell labeling and tumor imaging (Figure 2.12a). After optimization to achieve maximum cellular entry, HeLa cells were successfully labeled *in vitro*. Whereas internalization by the cell was confirmed by optical imaging, (Figure 2.12b), uptake of the Gd $^{3+}$ -liposomes by the incubated cells resulted in lowered T_1 relaxation times (Figure 2.12c).

Figure 2.12. a) Chemical structure of the ligand DOTA-DSA. b) Fluorescence image of HeLa cells labeled with Gd^{3+} -liposome formulation (40x magnification). c) T_1 -weighted MR images of the Gd^{3+} -liposome formulation solution (incubated HeLa cells and controls). Adapted with permission from.¹⁷¹ Copyright 2008 American Chemical Society.

Paramagnetic micelles of PEG-DSPE and Gd^{3+} -DTPA-BSA with either rhodamine-PE or QDs have also been used for multimodality purposes. These micelles were conjugated with macrophage scavenger receptor (MSR)-specific antibodies by Mulder *et al.*¹⁷² The QD-containing micelles were used for identification of abdominal aortas of atherosclerotic apoE-KO mice using UV light, while the rhodamine-PE containing micelles were used as trackers for cellular details by means of fluorescence microscopy (Figure 2.13a). T_1 -weighted high-resolution MR images (before and 24 h after intravenous administration) showed successful imaging of the abdominal aortas (Figure 2.13b).

Metallostars. Moriggi *et al.*,¹⁷³ inspired by the paramagnetic metallostars published by Livramento *et al.*,⁹³ used the same “ Gd^{3+} -complex self-assembly around a transition metal center (iron)” concept in order to engineer a new class of dual IAs. Using two diethylenetriamine- $\text{N},\text{N},\text{N}',\text{N}'$ -tetraacetate (DTTA) chelators integrated in a bipyridyl (bpy) ligand, six paramagnetic centers per molecule were obtained after complexation with ruthenium (Figure 2.14, left). The multimodal properties of the $\{\text{Ru}[\text{Ln}_2\text{bpy-DTTA}_2(\text{H}_2\text{O})_{4,3}]\}^{4-}$ metallostar were illustrated by the enhanced ionic r_1 relaxivity of the Gd^{3+} complex and the luminescence properties, due to

sensitization of Eu^{3+} upon excitation of the $\text{tris}(2,2'\text{-bpy})\text{Ru}^{2+}$ unit, shown by the Eu^{3+} complex.

Figure 2.13. Left: a) Left: UV illumination image of an excised aorta (animal injected with QD-micelles) showing green fluorescence-emitting regions (inset). Right: Fluorescence microscopy image showing uptake of the rhodamine-labeled micelles. b) High-resolution T_1 -weighted MR images before (1, 3, and 5) and 24 h after administration of rhodamine-labeled micelles (2) and QD-micelles (4 and 6). Adapted with permission from.¹⁷² Copyright 2007 John Wiley & Sons.

Figure 2.14. Left: structure of the self-assembling $\{\text{Ru}[\text{Gd}_2\text{bpy-DTTA}_2(\text{H}_2\text{O})]_3\}^{4-}$ metallostar.¹⁷³ Right: structure of the self-assembling $[(\text{Gd}^{3+}\text{-DTPA})_3\text{Ti}(\text{H}_2\text{O})_3]^{5-}$ metallostar.¹⁷⁴

More recently, Dehaen *et al.*¹⁷⁴ engineered metallostars used a chelating ligand bearing a catechol and a DTPA moiety. In this way, lanthanide ions were complexed by the DTPA ligand while the metallostar forms as the with Gd^{3+} complexed ligands self-assemble around a titanium ion (Figure 2.14, right). The $[(\text{Gd}^{3+}\text{-DTPA})_3\text{Ti}(\text{H}_2\text{O})_3]^{5-}$ metallostar showed multimodal properties demonstrated by enhanced ionic r_1 relaxivity upon self-assembly and broadband green emission (400-750 nm) upon UV irradiation.

2.4.3 Inorganic Nanoparticles

The attractiveness of inorganic nanoparticles (NPs) for multimodal imaging purposes lies not only in their intrinsic properties (optical detectability, therapeutical properties, etc.), but also in the large surface area-to-volume ratio they offer. This surface can be (relatively) easily modified by ligand exchange, making them excellent platforms for the integration of various IAs. Many inorganic NPs, such as quantum dots (QDs), gold and silica NPs have been successfully implemented for multimodal imaging purposes, as it will be revealed in the next sections. While the standard ionic r_1 relaxivity (value per Gd^{3+} ion) is still the mostly used way of expressing the relaxivity properties of such systems, the term “nanoparticle r_1 ” is frequently referred to as a measure of the relaxivity per NP. There are no clear guidelines for the calculation of this value and it is usually done either by: i) calculating the relaxivity per NP concentration (using a standard inversion recovery experiment where instead of plotting $1/T_1$ values as a function of the Gd^{3+} concentration, the NP concentration is used) or ii) by multiplying the calculated ionic r_1 relaxivity by the number of Gd^{3+} ions present. Throughout this review, the calculated nanoparticle r_1 value refers to the former procedure.

Quantum Dots. Given the tunable optical properties of QDs, these inorganic NPs have been used as platforms to increase the local Gd^{3+} concentration and tumbling rate of paramagnetic complexes while, at the same time, making use of their optical detectability.⁸ Furthermore, QDs are excellent alternatives to fluorescent organic dyes, as these NPs can be used for imaging over long periods of time since they are resistant to photobleaching.¹⁷⁵ A popular approach followed to develop multimodal IAs is the entrapment of QDs in paramagnetic supramolecular aggregates and some relevant examples will be discussed in the next sections. A review dealing with multimodal assemblies of amphiphiles and inorganic NPs for molecular imaging can be found elsewhere.¹⁷⁶

Mulder *et al.*¹⁷⁷ coated CdSe/ZnS core/shell, tri-octylphosphineoxide/hexadecylamine (TOPO/HAD)-capped QDs with a micellar, paramagnetic coating consisting of PEG-DSPE and Gd^{3+} -DTPA-BSA (Figure 2.15a). By conjugating the fluorescent QDs to cyclic, $\alpha_v\beta_3$ -specific RGD peptides, their specificity for angiogenic blood vessels was tested (Figure 2.15b). *In vitro* studies on cultured HUVEC showed an ionic relaxivity r_1 for these QDs of $12 \text{ mM}^{-1} \text{ s}^{-1}$ ($3 \times \text{Gd}^{3+}$ -DTPA) and a particle relaxivity of $2000 \text{ mM}^{-1} \text{ s}^{-1}$, corresponding to $\sim 150 \text{ Gd}^{3+}$ -DTPA-BSA lipids (Figure 2.15c).

Figure 2.15. a) Schematic representation of the preparation of paramagnetic, micelle-coated QDs. b) UV-Vis absorption (red) and PL (blue) spectra of micelle-coated QDs. c) T_1 -weighted image (at 60 MHz) of cells incubated with RGD-QDs, QDs, or QDs (no CA). Adapted with permission from.¹⁷⁷ Copyright 2006 American Chemical Society.

In another example, Van Tilborg *et.al.*¹⁷⁸ used CdSe/ZnS core/shell, TOPO/HDA-capped QDs coated with a mixture of paramagnetic Gd^{3+} -DTPA-bis(stearylamide) (Gd^{3+} -DTPA-BSA) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(poly(ethylene glycol))-2000] (PEG-DSPE) micelles. Multiple recombinant human annexin A5 protein molecules (AnxA5) were covalently attached to maleimide-labeled PEG-DSPE (Figure 2.16a). These multimodal AnxA5-conjugated NPs (AnxA5-pQD) were fluorescent (Figure 2.16b) and showed enhanced paramagnetic properties, with an ionic r_1 of $12.4 \text{ mM}^{-1} \text{ s}^{-1}$ and a particle relaxivity at least 150 times higher (Figure 2.16c). The potential use of AnxA5-pQDs as *in vivo* probes for apoptotic cell detection was tested and fluorescence microscopy and MRI demonstrated the successful attachment and targeting.

Figure 2.16. a) Schematic representation of AnxA5-pQD. The lipids drawn with the yellow and red head groups represent PEG-DSPE and Gd^{3+} -DTPA-BSA, respectively. b) Cell pellets of the different incubations illuminated with 254 nm UV light. From left to right: Control cells, apoptotic cells incubated with AnxA5-pQDs, vital cells incubated with AnxA5-pQDs, and apoptotic cells incubated with AnxA5-pQDs. c) T_1 -weighted image of the different cell pellets. Adapted with permission from.¹⁷⁸ Copyright 2006 American Chemical Society.

Another way of engineering multimodal IAs using QDs as fluorescent probes is by surface modification. Prinzen *et al.*¹⁷⁹ combined QDs with paramagnetic macromolecules and AnxA5 in order to synthesize multimodal NPs with increased Gd^{3+} payloads. Using the biotin-streptavidin linkage, streptavidin coated QDs (with an estimated 10 streptavidin molecules per QD) were used to obtain two kinds of NPs: a) single-biotinylated AnxA5 QDs prepared using a 1:1 stoichiometry (AnxA5-QD-Gd), with all remaining binding sites filled with biotinylated Gd^{3+} -DTPA and b) Gd^{3+} -lysine wedge biotinylated QDs (AnxA5-QD-Gd-wedge, Figure 2.17), where each wedge contains 8 Gd^{3+} chelates. These QD-based NPs exhibited intense fluorescence and the high Gd^{3+} payloads were evidenced by the nanoparticle r_1 relaxivities measured, which were 420-630 $\text{mM}^{-1} \text{ s}^{-1}$ for AnxA5-QD-Gd and 3000-4500 $\text{mM}^{-1} \text{ s}^{-1}$ for AnxA5-QD-Gd-wedge.

Figure 2.17. a) Chemical structure of biotinylated Gd^{3+} -DTPA (**1**) and biotinylated Gd^{3+} -lysine wedge (**2**). Biotin (red) and Gd^{3+} -DTPA (blue). b) Schematic representation of the two NPs: single biotinylated Gd^{3+} -DTPA QD (**1**, AnxA5-QD-Gd) and Gd^{3+} -lysine-wedge biotinylated QD (**2**, AnxA5-QD-Gd-wedge). Green: QD; yellow: streptavidin; red dot: Gd^{3+} -DTPA; red star: lysine-wedge; blue: AnxA5. Adapted with permission from.¹⁷⁹ Copyright 2006 American Chemical Society.

Yang *et al.*¹⁰³ functionalized water-soluble silica-coated ZnS-passivated CdS:Mn fluorescent QDs with tetraethyl orthosilicate (TEOS), 3-

A synthetically less demanding approach has also been proposed by Jin *et al.*¹⁰¹ To glutathione (GSH, reduced form) coated CdSeTe/CdS QDs, Gd³⁺-DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) was covalently attached by means of an amide bond. These multimodal QDs, which retain 60% of their fluorescence after being coated with GSH, emitted in the NIR region and showed enhanced positive MRI contrast when compared to GSH-QDs alone (Figure 2.19).

Figure 2.19. T_1 -weighted MR images for saline, GSH-QDs and Gd³⁺-DOTA-QDs in PBS. Adapted from.¹⁰¹

Other chelators used for Gd³⁺ complexation include S-2-(4-isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane tetraacetic acid (DOTA-Bn-NCS), used by Bakalova *et al.*¹⁸⁰ and DDTA, used by Park *et al.* (Figure 2.20a and b respectively).¹⁸¹ The latter DTTA-ligated uridine-QD conjugates showed enhanced nanoparticle r_1 relaxivities (655 and 571.2 mM⁻¹ s⁻¹ at 60 and 200 MHz, respectively), payloads of approximately 56 ± 6 Gd³⁺ ions per particle and confirmed uptake by RAW 264.7 cells.

Figure 2.20. a) Schematic representation of multimodal silica-shelled QD probes: the Gd³⁺ complex is conjugated on the surface of the silica sphere. Reprinted with permission from.¹⁸⁰ Copyright 2008 American Chemical Society. b) Schematic representation of DTTA-ligated uridine coated QDs. Adapted from.¹⁸¹

Gold Nanoparticles. Gold NPs (AuNPs) are very attractive for biological applications due to their light absorption and scattering properties derived from localized surface plasmon resonance (LSPR), observed as strong extinction peaks in the visible and near-infrared regions. Song *et al.*¹⁸² used AuNPs in order to create multimodal, Gd³⁺ enriched polyvalent DNA-AuNP conjugates (DNA-Gd³⁺@AuNP). These AuNPs were prepared by reacting citrate-stabilized AuNPs with thiol-labeled 24-mer poly-dT oligonucleotides, the latter containing five conjugation sites for covalent attachment of Gd³⁺-complexes. The multimodal Cy3-DNA-Gd³⁺@AuNPs were obtained by conjugation of Cy3 to the 5' end of the DNA-Gd³⁺ strands oligomers (Figure 2.21a). While cellular uptake and efficient labeling was confirmed by fluorescence microscopy (Figure 2.21b), the calculated ionic r_1 relaxivity of the Gd³⁺ complex after conjugation to DNA (8.7 mM⁻¹ s⁻¹ at 60 MHz) was twice as high as that of the unconjugated Gd³⁺ complex (Figure 2.21c).

Figure 2.21. a) Synthesis of Cy3-DNA-Gd³⁺@AuNP conjugates. b) Confocal fluorescence micrograph of NIH/3T3 cells incubated with 0.2 nM particle concentration of Cy3-DNA-Gd³⁺@AuNPs (red channel) for 4 h and a 24 h leach in fresh media and 1 μm DAPI (4',6-diamidino-2-phenylindole, blue channel) for 10 min. c) T_1 -weighted MR image of NIH/3T3 cells incubated with 5 and 20 μM (Gd³⁺ concentration) of DNA-Gd³⁺@AuNP and Gd³⁺-DOTA for 24 h at 600 MHz. Adapted with permission from.¹⁸² Copyright 2009 John Wiley & Sons.

Other Inorganic Nanoparticles. Bimodal silica NPs (SiO₂ NPs), recently proposed by Pinho *et al.*,¹⁸³ consist of SiO₂ NPs coated with a linker, 3-aminopropyltriethoxysilane (APS), used to covalently attach DTPA ligands for lanthanide complexation (Figure 2.22a). Studies using hybrid SiO₂@APS/DTPA:Eu³⁺Gd³⁺ (1:1) and SiO₂@APS/DTPA:Tb³⁺Gd³⁺ (1:1) NPs showed that the optical properties of Eu³⁺ and Tb³⁺ were not influenced by the presence of Gd³⁺. Also, the moderate ionic r_1 increase observed confirmed the bimodal character of these NPs. Uptake studies on living cells showed increase intensity in the T_1 -weighted MRI images as well as optical detection (Figure 2.22b and c respectively). The same principle has

been used in order to make hybrid NPs with antenna-incorporated ligands, e.g., 2,2',2'',2'''-[(pyridine-2,6-diyl)bis(methylenenitrilo)]tetra-kis(acetic acid) (PMN), showing comparable relaxometric and emission properties.¹⁸⁴

Figure 2.22. a) Schematic representation of a SiO₂ NP functionalized with APS and coupled with DTPA. b) T₁-weighted MR image of cellular pellets corresponding to: I) no NPs internalization (control); II) γ-Fe₂O₃ NPs (T₂) NPs cell internalization and III) SiO₂@APS/DTPA:Eu³⁺Gd³⁺ NPs cell internalization. c) Photograph of cellular pellets, excited at 393 nm, corresponding to: I) no NPs cell internalization (control) and II) SiO₂@APS/DTPA:Eu³⁺Gd³⁺ NPs cell incorporation. Adapted with permission from.¹⁸³ Copyright 2012 Elsevier.

Another type of hybrid silica NPs has been studied by Rieter *et al.*¹⁸⁵ These NPs contain a luminescent [Ru(bpy)₃]Cl₂ core (emission at 595 nm) and a paramagnetic monolayer coating consisting of single or double silylated Gd³⁺-DTTA complexes (**1** and **2** respectively, Figure 2.23, left). While NP **1** contained around 10200 Gd³⁺ ions and showed an enhanced ionic r_1 relaxivity of 19.7 mM⁻¹ s⁻¹, an increase in the number of Gd³⁺ ions did not lead to higher relaxivities, as NP **2** contained 63200 Gd³⁺ ions and showed an ionic $r_1 = 7.8$ mM⁻¹ s⁻¹. This decrease in ionic r_1 is attributed to the non-accessible Gd³⁺ centers in the inner layers of **2**. Also, studies using monocyte cells showed efficient uptake of these NPs, which was confirmed by optical and MR imaging (Figure 2.23, right).

Figure 2.23. Above: schematic representations of hybrid $[\text{Ru}(\text{bpy})_3]\text{Cl}_2$ core + silylated Gd^{3+} DTTA complex NPs. Below: images of 1-labeled monocyte cells: a) optical microscopy; b) laser scanning confocal fluorescence microscopy; c) T_1 -weighted MR image of unlabeled (left) and 1-labeled (right) monocyte cells. Adapted with permission from.¹⁸⁵ Copyright 2007 John Wiley & Sons.

2.4.4 Nanocontainers

Surface modification of NPs by means of ligand exchange is not the only method available for multimodal imaging purposes, as nanostructures can also be used as scaffolds where IAs are “entrapped”. Examples of both organic and inorganic nanocontainers include organic NPs/protein cages and zeolites, respectively.

Organic Nanoparticles. Nam *et al.*¹⁸⁶ recently introduced tumor targeting, self-assembled chitosan NPs (CNPs) for optical and MR imaging. Amphiphilic glycol chitosan 5β -cholanolic acid conjugates were reacted with Cy5.5-NHS (Figure 2.24a) and monoreactive DOTA-NHS in order to obtain Gd^{3+} -encapsulated conjugates. Spontaneous self-assembly of these conjugates in water led to stable, spherical encapsulated chitosan

NPs (Cy5.5-CNP-Gd³⁺) of approx. 350 nm in diameter (Figure 2.24b) with strong NIR fluorescence emission (Figure 2.24c, left). Contrast enhancement in T_1 -weighted MR images showed the paramagnetic properties of these CNPs. Whereas CNPs showed similar enhancement at different pH conditions (Figure 2.24c, right), encapsulation of the Gd³⁺ chelates in the hydrophobic inner part of the CNPs did affect the ionic r_1 relaxivity. Likewise, chitosan has also been used to encapsulate both CdSe/ZnS QDs and Gd³⁺-DTPA, showing optical properties and relatively high relaxivity.¹⁸⁷

Figure 2.24. a) Chemical structure of Cy5.5-CNP-Gd³⁺. b) Schematic representation of the self-assembled chitosan NPs CNPs. Blue: chitosan shell; white: inner part CNP. c) Left: NIR-fluorescence images of the Cy5.5-CNP-Gd³⁺ in PBS. Right: T_1 -weighted spin-echo images of Cy5.5-CNP-Gd³⁺ in acidic (pH 5.2) and physiological conditions (pH 7.4). Adapted with permission from.¹⁸⁶ Copyright 2010 American Chemical Society.

Protein Cages. Owing to their extensive surface and reactive groups available for covalent functionalization, protein cages or virus-like particles (VLP) have been explored as possible carriers of IAs. Anderson *et al.*¹⁸⁸

conjugated Gd^{3+} complexes onto MS2 viral capsids using lysine-reactive 2-(4-isothiocyanatobenzyl)-diethylenetriaminepentaacetic acid (DTPA-ITC). At the same time, VLPs were reacted with fluorescein isothiocyanate (FITC) in order to obtain the multimodal $\text{MS2}(\text{Gd}^{3+}\text{-DTPA-ITC})_m(\text{FITC})_n$ viral capsids (Figure 2.25a). The paramagnetic properties of these labeled VLPs were clearly enhanced (Figure 2.25b), with a calculated ionic r_1 relaxivity for the bimodal $\text{MS2}(\text{Gd}^{3+}\text{-DTPA-ITC})_{360}(\text{FITC})_{55}$ of $16.9 \text{ mM}^{-1} \text{ s}^{-1}$ and a nanoparticle r_1 of $6100 \text{ mM}^{-1} \text{ s}^{-1}$ (at 60 MHz). Using the same approach, Suci *et al.*¹⁸⁹ attached *N*-hydroxysuccinimide derivatives of fluorescein and DOTA to multifunctionalize cowpea chlorotic mottle virus (CCMV) protein cages. This multimodal platform was used to observe penetration into a *Staphylococcus aureus* film, thereby demonstrating their possible application as diagnostic and targeted treatment agents for bacterial infections.

Figure 2.25. a) VLPs used for MRI studies: MS2 capsids, MS2 capsids soaked in Magnevist (control), paramagnetic $\text{MS2}(\text{Gd}^{3+}\text{-DTPA-ITC})_m$ and bimodal $\text{MS2}(\text{Gd}^{3+}\text{-DTPA-ITC})_m(\text{FITC})_n$ ($m + n \approx 540$). b) T_1 -weighted image of the different VLPs. Adapted with permission from.¹⁸⁸ Copyright 2006 American Chemical Society.

Pokorski *et al.*¹⁹⁰ increased the number of functionalities that can be attached to a VLP by using these protein cages as multivalent macroinitiators for atom transfer radical polymerization (ATRP). VPLs functionalized with oligo(ethylene glycol)-methacrylate (OEGMA) and its azido-functionalized analogue (OEGMA-N₃) can be easily modified by means of Cu(I)-catalyzed click chemistry (CuAAC). By allowing the poly(OEGMA-N₃) grafts (**1**) to react with alkyne-substituted Alexa Fluor 488 dyes and DOTAs (under CuAAC conditions), multimodal VPLs were obtained (Figure 2.26a). Successful incorporation of paramagnetic moieties to these VLPs was confirmed by the r_1 relaxivity per particle calculated for **5** (7092 mM⁻¹ s⁻¹), leading to values comparable to other covalently attached Gd³⁺ complex VLPs.

Figure 2.26. a) CuAAC reaction to attach the different functionalities to **1**. b) UV illumination of sucrose gradient following CuAAC between **1** and **2** (upper band, unreacted **2**; lower band, **4**). Adapted with permission from.¹⁹⁰ Copyright 2011 American Chemical Society.

Zeolites. Microporous inorganic scaffolds such as zeolites, characterized by their biocompatibility and high active surface available, have also been explored as platforms for the integration of various imaging modalities. Tsotsalas *et al.*¹⁹¹ used zeolite L crystals as dual optical and paramagnetic

probes. The channels of the zeolite crystals were filled with pyronine molecules by means of exchange of potassium cations. An APTS linker was then reacted with the free Si-OH groups on the zeolite surface and subsequently coupled to DOTA-NHS. The paramagnetic zeolites were obtained by complexation of the DOTA ligand with Gd^{3+} (Figure 2.27). The enhanced paramagnetic properties of these nanocontainers are evidenced by the high ionic r_1 relaxivity measured ($30 \text{ mM}^{-1} \text{ s}^{-1}$, $> 6\times$ larger than Gd^{3+} -DOTA) and the calculated zeolite overall relaxivity (ca. $11000 \text{ mM}^{-1} \text{ s}^{-1}$).

Figure 2.27. Synthesis of Gd^{3+} -DOTA functionalized zeolite L crystals. First, pyronine molecules are introduced into the channels via ion exchange (green ovals). Then, APTS (linker) is bound to the entire surface of the zeolite L crystals (blue dot). Subsequently, the DOTA ligand is covalently attached to the backside of the linker (blue ring). Finally, the lanthanide (Gd^{3+}) ions are complexed to the surface of the zeolite L crystals (red balls). Adapted with permission from.¹⁹¹ Copyright 2008 American Chemical Society.

2.5 Conclusions

Despite the increasing number of imaging modalities available nowadays, the use of more traditional imaging techniques such as MRI, PET, SPECT and optical imaging is still favored. Preference for the combination MR/optical imaging is illustrated by the large number of publications dealing with multimodal IAs for these two techniques shown in this literature survey. Innovative development of multimodal probes is triggered by the rapidly growing knowledge of chemistry of IAs, nanomaterials and molecular self-assembly. In this thesis, new approaches for the development of IAs, inspired by novel nanomaterials and molecular self-assembly, for MR and/or optical imaging are described.

2.6 References

- (1) Galbán, C. J.; Galbán, S.; Van Dort, M. E.; Luker, G. D.; Bhojani, M. S.; Rehemtulla, A.; Ross, B. D. In *Progress in Molecular Biology and Translational Science*; Raymond, W. R., Ed.; Academic Press: **2010**; Vol. Volume 95, p 237.
- (2) Hausner, S. H. In *Nanoplatfrom-Based Molecular Imaging*; Chen, X., Ed.; John Wiley & Sons, Inc.: **2011**, p 1.
- (3) Weissleder, R.; Mahmood, U. *Radiology* **2001**, *219*, 316.
- (4) Cassidy, P. J.; Radda, G. K. *J. Royal Soc. Interface* **2005**, *2*, 133.
- (5) Brindle, K. *Nat. Rev. Cancer* **2008**, *8*, 94.
- (6) Seaman, M. E.; Contino, G.; Bardeesy, N.; Kelly, K. A. *Expert Rev. Mol. Med.* **2010**, *12*, 1.
- (7) Brücher, E. In *Top Curr Chem*; Krause, W., Ed.; Springer Berlin / Heidelberg: **2002**; Vol. 221, p 103.
- (8) Louie, A. *Chem. Rev.* **2010**, *110*, 3146.
- (9) Frullano, L.; Meade, T. *J. Biol. Inorg. Chem.* **2007**, *12*, 939.
- (10) Jennings, L. E.; Long, N. J. *Chem. Commun.* **2009**, 3511.
- (11) Geninatti Crich, S. G.; Biancone, L.; Cantaluppi, V.; Esposito, D. D. G.; Russo, S.; Camussi, G.; Aime, S. *Magn. Reson. Med.* **2004**, *51*, 938.
- (12) Picard, C.; Geum, N.; Nasso, I.; Mestre, B.; Tisnes, P.; Laurent, S.; Muller, R. N.; Vander Elst, L. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 5309.
- (13) Judenhofer, M. S.; Wehrl, H. F.; Newport, D. F.; Catana, C.; Siegel, S. B.; Becker, M.; Thielscher, A.; Kneilling, M.; Lichy, M. P.; Eichner, M.; Klingel, K.;

- Reischl, G.; Widmaier, S.; Rocken, M.; Nutt, R. E.; Machulla, H.-J.; Uludag, K.; Cherry, S. R.; Claussen, C. D.; Pichler, B. J. *Nat. Med.* **2008**, *14*, 459.
- (14) Catana, C.; Wu, Y.; Judenhofer, M. S.; Qi, J.; Pichler, B. J.; Cherry, S. R. *J. Nucl. Med.* **2006**, *47*, 1968.
- (15) Cherry, S. R.; Louie, A. Y.; Jacobs, R. E. *P. IEEE* **2008**, *96*, 416.
- (16) Choi, J. S.; Park, J. C.; Nah, H.; Woo, S.; Oh, J.; Kim, K. M.; Cheon, G. J.; Chang, Y.; Yoo, J.; Cheon, J. *Angew. Chem., Int. Ed.* **2008**, *47*, 6259.
- (17) Culver, J.; Akers, W.; Achilefu, S. *J. Nucl. Med.* **2008**, *49*, 169.
- (18) Cai, W.; Chen, K.; Li, Z.-B.; Gambhir, S. S.; Chen, X. *J. Nucl. Med.* **2007**, *48*, 1862.
- (19) Edwards, W. B.; Xu, B.; Akers, W.; Cheney, P. P.; Liang, K.; Rogers, B. E.; Anderson, C. J.; Achilefu, S. *Bioconjugate Chem.* **2007**, *19*, 192.
- (20) Li, C.; Wang, W.; Wu, Q.; Ke, S.; Houston, J.; Sevic-Muraca, E.; Dong, L.; Chow, D.; Charnsangavej, C.; Gelovani, J. G. *Nucl. Med. Biol.* **2006**, *33*, 349.
- (21) Gore, J. C.; Manning, H. C.; Quarles, C. C.; Waddell, K. W.; Yankeelov, T. E. *Magn. Reson. Imaging* **2011**, *29*, 587.
- (22) Weissleder, R.; Pittet, M. J. *Nature* **2008**, *452*, 580.
- (23) Nolting, D. D.; Gore, J. C.; Pham, W. *Curr. Org. Synth.* **2011**, *8*, 521.
- (24) Damadian, R. *Science* **1971**, *171*, 1151.
- (25) Lauterbur, P. C. *Nature* **1973**, *242*, 190.
- (26) Pekar, J. J. *IEEE Eng. Med. Biol. Mag.* **2006**, *25*, 24.
- (27) Stapf, S.; Han, S.-I. In *NMR Imaging in Chemical Engineering*; Stapf, S., Han, S.-I., Eds.; Wiley-VCH Verlag GmbH & Co. KGaA: **2006**, p 1.
- (28) Bottrill, M.; Kwok, L.; Long, N. J. *Chem. Soc. Rev.* **2006**, *35*, 557.
- (29) Caravan, P.; Ellison, J. J.; McMurry, T. J.; Lauffer, R. B. *Chem. Rev.* **1999**, *99*, 2293.
- (30) Hermann, P.; Kotek, J.; Kubicek, V.; Lukes, I. *J. Chem. Soc., Dalton Trans.* **2008**, 3027.
- (31) Reichert, D. E.; Lewis, J. S.; Anderson, C. J. *Coord. Chem. Rev.* **1999**, *184*, 3.
- (32) Terreno, E.; Castelli, D. D.; Viale, A.; Aime, S. *Chem. Rev.* **2010**, *110*, 3019.
- (33) Laurent, S.; Forge, D.; Port, M.; Roch, A.; Robic, C.; Vander Elst, L.; Muller, R. N. *Chem. Rev.* **2008**, *108*, 2064.
- (34) Bulte, J. W. M.; Kraitchman, D. L. *NMR Biomed.* **2004**, *17*, 484.
- (35) Wang, Y.-X.; Hussain, S.; Krestin, G. *Eur. Radiol.* **2001**, *11*, 2319.
- (36) Viswanathan, S.; Kovacs, Z.; Green, K. N.; Ratnakar, S. J.; Sherry, A. D. *Chem. Rev.* **2010**, *110*, 2960.
- (37) Aime, S.; Fasano, M.; Terreno, E. *Chem. Soc. Rev.* **1998**, *27*, 19.
- (38) Sherry, A. D. *J. Less-Common Met.* **1989**, *149*, 133.
- (39) Gries, H.; Krause, W., Ed.; Springer Berlin / Heidelberg: **2002**; Vol. 221, p 1.
- (40) Tóth, É.; Helm, L.; Merbach, A. In *Top Curr Chem*; Krause, W., Ed.; Springer Berlin / Heidelberg: **2002**; Vol. 221, p 61.
- (41) Cohen, S. M.; Xu, J.; Radkov, E.; Raymond, K. N.; Botta, M.; Barge, A.; Aime, S. *Inorg. Chem.* **2000**, *39*, 5747.

- (42) Datta, A.; Hooker, J. M.; Botta, M.; Francis, M. B.; Aime, S.; Raymond, K. N. *J. Am. Chem. Soc.* **2008**, *130*, 2546.
- (43) Werner, E. J.; Datta, A.; Jocher, C. J.; Raymond, K. N. *Angew. Chem., Int. Ed.* **2008**, *47*, 8568.
- (44) Pierre, V. C.; Botta, M.; Aime, S.; Raymond, K. N. *J. Am. Chem. Soc.* **2006**, *128*, 5344.
- (45) Raymond, K. N.; Pierre, V. C. *Bioconjugate Chem.* **2004**, *16*, 3.
- (46) Werner, E. J.; Kozhukh, J.; Botta, M.; Moore, E. G.; Avedano, S.; Aime, S.; Raymond, K. N. *Inorg. Chem.* **2008**, *48*, 277.
- (47) Datta, A.; Raymond, K. N. *Acc. Chem. Res.* **2009**, *42*, 938.
- (48) Aime, S.; Batsanov, A. S.; Botta, M.; Howard, J. A. K.; Parker, D.; Senanayake, K.; Williams, G. *Inorg. Chem.* **1994**, *33*, 4696.
- (49) Koenig, S. H.; Brown, R. D. *Prog. Nucl. Magn. Reson. Spectrosc.* **1990**, *22*, 487.
- (50) Botta, M. *Eur. J. Inorg. Chem.* **2000**, *2000*, 399.
- (51) Bonnet, C. S.; Fries, P. H.; Crouzy, S.; Sénèque, O.; Cisnetti, F.; Boturyn, D.; Dumy, P.; Delangle, P. *Chem. Eur. J.* **2009**, *15*, 7083.
- (52) Fulton, D. A.; Elemento, E. M.; Aime, S.; Chaabane, L.; Botta, M.; Parker, D. *Chem. Commun.* **2006**, 1064.
- (53) Ali, M. M.; Woods, M.; Caravan, P.; Opina, A. C. L.; Spiller, M.; Fettingner, J. C.; Sherry, A. D. *Chem. Eur. J.* **2008**, *14*, 7250.
- (54) Aime, S.; Gianolio, E.; Longo, D.; Pagliarin, R.; Lovazzano, C.; Sisti, M. *ChemBioChem* **2005**, *6*, 818.
- (55) Jaszberenyi, Z.; Sour, A.; Toth, E.; Benmelouka, M.; Merbach, A. E. *Dalton Trans.* **2005**, 2713.
- (56) Kotek, J.; Lebdušková, P.; Hermann, P.; Vander Elst, L.; Muller, R. N.; Geraldes, C. F. G. C.; Maschmeyer, T.; Lukeš, I.; Peters, J. A. *Chem. Eur. J.* **2003**, *9*, 5899.
- (57) Laus, S.; Ruloff, R.; Tóth, É.; Merbach, A. E. *Chem. Eur. J.* **2003**, *9*, 3555.
- (58) Tei, L.; Botta, M.; Lovazzano, C.; Barge, A.; Milone, L.; Aime, S. *Magn. Reson. Chem.* **2008**, *46*, S86.
- (59) Tei, L.; Gugliotta, G.; Baranyai, Z.; Botta, M. *Dalton Trans.* **2009**, 9712.
- (60) Lebduškova, P.; Hermann, P.; Helm, L.; Toth, E.; Kotek, J.; Binnemans, K.; Rudovsky, J.; Lukes, I.; Merbach, A. E. *Dalton Trans.* **2007**, 493.
- (61) Karfeld, L. S.; Bull, S. R.; Davis, N. E.; Meade, T. J.; Barron, A. E. *Bioconjugate Chem.* **2007**, *18*, 1697.
- (62) Yang, J. J.; Yang, J.; Wei, L.; Zurkiya, O.; Yang, W.; Li, S.; Zou, J.; Zhou, Y.; Maniccia, A. L. W.; Mao, H.; Zhao, F.; Malchow, R.; Zhao, S.; Johnson, J.; Hu, X.; Krogstad, E.; Liu, Z.-R. *J. Am. Chem. Soc.* **2008**, *130*, 9260.
- (63) Abiraj, K.; Jaccard, H.; Kretzschmar, M.; Helm, L.; Maecke, H. R. *Chem. Commun.* **2008**, 3248.
- (64) Botta, M.; Quici, S.; Pozzi, G.; Marzanni, G.; Pagliarin, R.; Barra, S.; Geninatti Crich, S. *Org. Biomol. Chem.* **2004**, *2*.
- (65) Ratnakar, S. J.; Alexander, V. *Eur. J. Inorg. Chem.* **2005**, *2005*, 3918.
- (66) Villaraza, A. J.; Bumb, A.; Brechbiel, M. W. *Chem. Rev.* **2010**, *110*, 2921.

- (67) Aime, S.; Castelli, D. D.; Crich, S. G.; Gianolio, E.; Terreno, E. *Acc. Chem. Res.* **2009**, *42*, 822.
- (68) Caravan, P. *Chem. Soc. Rev.* **2006**, 35.
- (69) Venditto, V. J.; Regino, C. A. S.; Brechbiel, M. W. *Mol. Pharm.* **2005**, *2*, 302.
- (70) Kobayashi, H.; Kawamoto, S.; Jo, S.-K.; Bryant, H. L.; Brechbiel, M. W.; Star, R. A. *Bioconjugate Chem.* **2003**, *14*, 388.
- (71) Wiener, E.; Brechbiel, M. W.; Brothers, H.; Magin, R. L.; Gansow, O. A.; Tomalia, D. A.; Lauterbur, P. C. *Magn. Reson. Med.* **1994**, *31*, 1.
- (72) Langereis, S.; de Lussanet, Q. G.; van Genderen, M. H. P.; Backes, W. H.; Meijer, E. W. *Macromolecules* **2004**, *37*, 3084.
- (73) Langereis, S.; Dirksen, A.; Hackeng, T. M.; van Genderen, M. H. P.; Meijer, E. W. *New J. Chem.* **2007**, *31*, 1152.
- (74) Nwe, K.; Xu, H.; Regino, C. A. S.; Bernardo, M.; Ileva, L.; Riffle, L.; Wong, K. J.; Brechbiel, M. W. *Bioconjugate Chem.* **2009**, *20*, 1412.
- (75) Bryant, L. H.; Brechbiel, M. W.; Wu, C.; Bulte, J. W. M.; Herynek, V.; Frank, J. A. *J. Magn. Reson. Imaging* **1999**, *9*, 348.
- (76) Aime, S.; Frullano, L.; Geninatti Crich, S. *Angew. Chem., Int. Ed.* **2002**, *41*, 1017.
- (77) Caravan, P. *Acc. Chem. Res.* **2009**, *42*, 851.
- (78) Assfalg, M.; Gianolio, E.; Zanzoni, S.; Tomaselli, S.; Lo Russo, V.; Cabella, C.; Ragona, L.; Aime, S.; Molinari, H. *J. Med. Chem.* **2007**, *50*, 5257.
- (79) Tei, L.; Barge, A.; Geninatti Crich, S.; Pagliarin, R.; Negri, V.; Ramella, D.; Cravotto, G.; Aime, S. *Chem. Eur. J.* **2010**, *16*, 8080.
- (80) Opsahl, L. R.; Uzgiris, E. E.; Vera, D. R. *Acad. Radiol.* **1995**, *2*, 762.
- (81) Lucas, R. L.; Benjamin, M.; Reineke, T. M. *Bioconjugate Chem.* **2007**, *19*, 24.
- (82) Prasuhn, J. D. E.; Yeh, R. M.; Obenaus, A.; Manchester, M.; Finn, M. G. *Chem. Commun.* **2007**, 1269.
- (83) Liepold, L. O.; Abedin, M. J.; Buckhouse, E. D.; Frank, J. A.; Young, M. J.; Douglas, T. *Nano Lett.* **2009**, *9*, 4520.
- (84) Liepold, L.; Anderson, S.; Willits, D.; Oltrogge, L.; Frank, J. A.; Douglas, T.; Young, M. *Magn. Reson. Med.* **2007**, *58*, 871.
- (85) Datta, A.; Hooker, J. M.; Botta, M.; Francis, M. B.; Aime, S.; Raymond, K. N. *J. Am. Chem. Soc.* **2008**, *130*, 2546.
- (86) Garimella, P. D.; Datta, A.; Romanini, D. W.; Raymond, K. N.; Francis, M. B. *J. Am. Chem. Soc.* **2011**, *133*, 14704.
- (87) Hooker, J. M.; Datta, A.; Botta, M.; Raymond, K. N.; Francis, M. B. *Nano Lett.* **2007**, *7*, 2207.
- (88) Song, Y.; Kohlmeir, E. K.; Meade, T. J. *J. Am. Chem. Soc.* **2008**, *130*, 6662.
- (89) Sirlin, C. B.; Vera, D. R.; Corbeil, J. A.; Caballero, M. B.; Buxton, R. B.; Mattrey, R. F. *Acad. Radiol.* **2004**, *11*, 1361.
- (90) Overoye-Chan, K.; Koerner, S.; Looby, R. J.; Kolodziej, A. F.; Zech, S. G.; Deng, Q.; Chasse, J. M.; McMurphy, T. J.; Caravan, P. *J. Am. Chem. Soc.* **2008**, *130*, 6025.

- (91) Ye, F.; Jeong, E.-K.; Jia, Z.; Yang, T.; Parker, D.; Lu, Z.-R. *Bioconjugate Chem.* **2008**, *19*, 2300.
- (92) Endres, P. J.; MacRenaris, K. W.; Vogt, S.; Meade, T. J. *Bioconjugate Chem.* **2008**, *19*, 2049.
- (93) Livramento, J. B.; Tóth, É.; Sour, A.; Borel, A.; Merbach, A. E.; Ruloff, R. *Angew. Chem., Int. Ed.* **2005**, *44*, 1480.
- (94) Parac-Vogt, T. N.; Vander Elst, L.; Kimpe, K.; Laurent, S.; Burtea, C.; Chen, F.; Van Deun, R.; Ni, Y.; Muller, R. N.; Binnemans, K. *Contrast Media Mol. Imaging* **2006**, *1*, 267.
- (95) Accardo, A.; Tesauro, D.; Aloj, L.; Pedone, C.; Morelli, G. *Coord. Chem. Rev.* **2009**, *253*, 2193.
- (96) André, J. P.; Tóth, É.; Fischer, H.; Seelig, A.; Mäcke, H. R.; Merbach, A. E. *Chem. Eur. J.* **1999**, *5*, 2977.
- (97) Kielar, F.; Tei, L.; Terreno, E.; Botta, M. *J. Am. Chem. Soc.* **2010**, *132*, 7836.
- (98) Schuhle, D. T.; Polasek, M.; Lukes, I.; Chauvin, T.; Toth, E.; Schatz, J.; Hanefeld, U.; Stuart, M. C. A.; Peters, J. A. *Dalton Trans.* **2010**, *39*, 185.
- (99) Torres, S.; Prata, M. I. M.; Santos, A. C.; André, J. P.; Martins, J. A.; Helm, L.; Tóth, É.; García-Martín, M. L.; Rodrigues, T. B.; López-Larrubia, P.; Cerdán, S.; Geraldes, C. F. G. C. *NMR Biomed.* **2008**, *21*, 322.
- (100) Liu, T.; Qian, Y.; Hu, X.; Ge, Z.; Liu, S. *J. Mater. Chem.* **2012**, *22*, 5020.
- (101) Jin, T.; Yoshioka, Y.; Fujii, F.; Komai, Y.; Seki, J.; Seiyama, A. *Chem. Commun.* **2008**, 5764.
- (102) Mulder, W. J. M.; Koole, R.; Brandwijk, R. J.; Storm, G.; Chin, P. T. K.; Strijkers, G. J.; de Mello Donegá, C.; Nicolay, K.; Griffioen, A. W. *Nano Lett.* **2005**, *6*, 1.
- (103) Yang, H.; Santra, S.; Walter, G. A.; Holloway, P. H. *Adv. Mater.* **2006**, *18*, 2890.
- (104) Gerion, D.; Herberg, J.; Bok, R.; Gjersing, E.; Ramon, E.; Maxwell, R.; Kurhanewicz, J.; Budinger, T. F.; Gray, J. W.; Shuman, M. A.; Chen, F. F. *J. Phys. Chem. C* **2007**, *111*, 12542.
- (105) Manus, L. M.; Mastarone, D. J.; Waters, E. A.; Zhang, X.-Q.; Schultz-Sikma, E. A.; MacRenaris, K. W.; Ho, D.; Meade, T. J. *Nano Lett.* **2009**, *10*, 484.
- (106) Feldmann, V.; Engelmann, J.; Gottschalk, S.; Mayer, H. A. *J. Colloid Interface Sci.* **2012**, *366*, 70.
- (107) Vivero-Escoto, J. L.; Taylor-Pashow, K. M. L.; Huxford, R. C.; Della Rocca, J.; Okoruwa, C.; An, H.; Lin, W.; Lin, W. *Small* **2011**, *7*, 3519.
- (108) Voisin, P.; Ribot, E. J.; Miraux, S.; Bouzier-Sore, A. K.; Lahitte, J. F.; Bouchaud, V.; Mornet, S.; Thiaudiere, E.; Franconi, J. M.; Raison, L.; Labrugere, C.; Delville, M. H. *Bioconjugate Chem.* **2007**, *18*, 1053.
- (109) Taylor, K. M. L.; Kim, J. S.; Rieter, W. J.; An, H.; Lin, W.; Lin, W. *J. Am. Chem. Soc.* **2008**, *130*, 2154.
- (110) Plush, S. E.; Woods, M.; Zhou, Y.-F.; Kadali, S. B.; Wong, M. S.; Sherry, A. D. *J. Am. Chem. Soc.* **2009**, *131*, 15918.
- (111) Richard, C.; Doan, B. T.; Beloeil, J. C.; Bessodes, M.; Toth, E.; Scherman, D. *Nano Lett.* **2008**, *8*, 232.

- (112) Matson, M. L.; Wilson, L. J. *Future Med. Chem.* **2010**, *2*, 491.
- (113) Ananta, J. S.; Wilson, L. J. In *Chemistry of Nanocarbons*; Akasaka, T., Wudl, F., Nagase, S., Eds.; John Wiley & Sons, Ltd: **2010**, p 287.
- (114) Alric, C.; Taleb, J.; Duc, G. L.; Mandon, C.; Billotey, C.; Meur-Herland, A. L.; Brochard, T.; Vocanson, F.; Janier, M.; Perriat, P.; Roux, S.; Tillement, O. *J. Am. Chem. Soc.* **2008**, *130*, 5908.
- (115) Debouttière, P. J.; Roux, S.; Vocanson, F.; Billotey, C.; Beuf, O.; Favre-Réguillon, A.; Lin, Y.; Pellet-Rostaing, S.; Lamartine, R.; Perriat, P.; Tillement, O. *Adv. Funct. Mater.* **2006**, *16*, 2330.
- (116) Moriggi, L. c.; Cannizzo, C.; Dumas, E.; Mayer, C. d. R.; Ulianov, A.; Helm, L. *J. Am. Chem. Soc.* **2009**, *131*, 10828.
- (117) Park, J.-A.; Reddy, P. A. N.; Kim, H.-K.; Kim, I.-S.; Kim, G.-C.; Chang, Y.; Kim, T.-J. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 6135.
- (118) Warsi, M. F.; Chechik, V. *Phys. Chem. Chem. Phys.* **2011**, *13*, 9812.
- (119) Liu, L.; Ding, H.; Yong, K.-T.; Roy, I.; Law, W.-C.; Kopwithaya, A.; Kumar, R.; Erogbogbo, F.; Zhang, X.; Prasad, P. *Plasmonics* **2011**, *6*, 105.
- (120) Sun, H.; Yuan, Q.; Zhang, B.; Ai, K.; Zhang, P.; Lu, L. *Nanoscale* **2011**, *3*, 1990.
- (121) Ntziachristos, V. *Nat. Meth.* **2010**, *7*, 603.
- (122) Wu, J. C.; Sundaresan, G.; Iyer, M.; Gambhir, S. S. *Mol. Ther.* **2001**, *4*, 297.
- (123) Choy, G.; Choyke, P.; Libutti, S. K. *Mol. Imaging* **2003**, *2*, 303.
- (124) Villalobos, V.; Naik, S.; Piwnica-Worms, D. *Annu. Rev. Biomed. Eng.* **2007**, *9*, 321.
- (125) Thorne, S. H.; Contag, C. H. *P. IEEE* **2005**, *93*, 750.
- (126) Jobsis, F. *Science* **1977**, *198*, 1264.
- (127) Licha, K. In *Top Curr Chem*; Krause, W., Ed.; Springer Berlin / Heidelberg: 2002; Vol. 222, p 1.
- (128) Sevick-Muraca, E. M.; Houston, J. P.; Gurfinkel, M. *Curr. Opin. Chem. Biol.* **2002**, *6*, 642.
- (129) Faulkner, S.; Pope, S. J. A.; Burton-Pye, B. P. *Appl. Spectrosc. Rev.* **2005**, *40*, 1.
- (130) Terai, T.; Kikuchi, K.; Iwasawa, S.; Kawabe, T.; Hirata, Y.; Urano, Y.; Nagano, T. *J. Am. Chem. Soc.* **2006**, *128*, 8699.
- (131) Bunzli, J.-C. G.; Piguet, C. *Chem. Soc. Rev.* **2005**, *34*, 1048.
- (132) Beeby, A.; Bushby, L. M.; Maffeo, D.; Gareth Williams, J. A. *J. Chem. Soc., Dalton Trans.* **2002**, 48.
- (133) Faulkner, S.; Carrie, M.-C.; Pope, S. J. A.; Squire, J.; Beeby, A.; Sammes, P. G. *J. Chem. Soc., Dalton Trans.* **2004**, 1405.
- (134) Pal, R.; Parker, D. *Org. Biomol. Chem.* **2008**, *6*.
- (135) Burton-Pye, B. P.; Heath, S. L.; Faulkner, S. *J. Chem. Soc., Dalton Trans.* **2005**, 146.
- (136) Koulourou, T.; Natrajan, L. S.; Bhavsar, H.; Pope, S. J. A.; Feng, J.; Narvainen, J.; Shaw, R.; Scales, E.; Kauppinen, R.; Kenwright, A. M.; Faulkner, S. *J. Am. Chem. Soc.* **2008**, *130*, 2178.

- (137) Imbert, D.; Cantuel, M.; Bünzli, J.-C. G.; Bernardinelli, G.; Piguet, C. *J. Am. Chem. Soc.* **2003**, *125*, 15698.
- (138) Klink, S. I.; Keizer, H.; Veggel, F. C. J. M., van *Angew. Chem., Int. Ed.* **2000**, *39*, 4319.
- (139) Pandya, S.; Yu, J.; Parker, D. *J. Chem. Soc., Dalton Trans.* **2006**, 2757.
- (140) Montgomery, C. P.; Murray, B. S.; New, E. J.; Pal, R.; Parker, D. *Acc. Chem. Res.* **2009**, *42*, 925.
- (141) Bünzli, J.-C. G. *Chem. Rev.* **2010**, *110*, 2729.
- (142) Beeby, A.; S. Dickins, R.; Faulkner, S.; Parker, D.; A. Gareth Williams, J. *Chem. Commun.* **1997**, 1401.
- (143) Werts, M. H. V.; Woudenberg, R. H.; Emmerink, P. G.; van Gassel, R.; Hofstraat, J. W.; Verhoeven, J. W. *Angew. Chem., Int. Ed.* **2000**, *39*, 4542.
- (144) Bünzli, J.-C. G. *Acc. Chem. Res.* **2005**, *39*, 53.
- (145) Smith, A. M.; Gao, X.; Nie, S. *Photochem. Photobiol.* **2004**, *80*, 377.
- (146) Xing, Y. In *Nanoplatfrom-Based Molecular Imaging*; Chen, X., Ed.; John Wiley & Sons, Inc.: 2011, p 159.
- (147) Diez, I.; Ras, R. H. A. *Nanoscale* **2011**, *3*, 1963.
- (148) Patel, S. A.; Richards, C. I.; Hsiang, J.-C.; Dickson, R. M. *J. Am. Chem. Soc.* **2008**, *130*, 11602.
- (149) Xu, H.; Suslick, K. S. *Adv. Mater.* **2010**, *22*, 1078.
- (150) Zheng, J.; Dickson, R. M. *J. Am. Chem. Soc.* **2002**, *124*, 13982.
- (151) Petty, J. T.; Zheng, J.; Hud, N. V.; Dickson, R. M. *J. Am. Chem. Soc.* **2004**, *126*, 5207.
- (152) Richards, C. I.; Choi, S.; Hsiang, J.-C.; Antoku, Y.; Vosch, T.; Bongiorno, A.; Tzeng, Y.-L.; Dickson, R. M. *J. Am. Chem. Soc.* **2008**, *130*, 5038.
- (153) Vosch, T.; Antoku, Y.; Hsiang, J.-C.; Richards, C. I.; Gonzalez, J. I.; Dickson, R. M. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104*, 12616.
- (154) Yu, J.; Choi, S.; Richards, C. I.; Antoku, Y.; Dickson, R. M. *Photochem. Photobiol.* **2008**, *84*, 1435.
- (155) Yu, J.; Patel, S. A.; Dickson, R. M. *Angew. Chem., Int. Ed.* **2007**, *46*, 2028.
- (156) Yu, J.; Choi, S.; Dickson, R. M. *Angew. Chem., Int. Ed.* **2009**, *48*, 318.
- (157) Pellegatti, L.; Zhang, J.; Drahos, B.; Villette, S.; Suzenet, F.; Guillaumet, G.; Petoud, S.; Toth, E. *Chem. Commun.* **2008**, 6591.
- (158) Mamedov, I.; Parac-Vogt, T. N.; Logothetis, N. K.; Angelovski, G. *Dalton Trans.* **2010**, *39*, 5721.
- (159) Mindt, T. L.; Müller, C.; Stuker, F.; Salazar, J.-F. d. r.; Hohn, A.; Mueggler, T.; Rudin, M.; Schibli, R. *Bioconjugate Chem.* **2009**, *20*, 1940.
- (160) Manning, H. C.; Goebel, T.; Thompson, R. C.; Price, R. R.; Lee, H.; Bornhop, D. J. *Bioconjugate Chem.* **2004**, *15*, 1488.
- (161) Li, H.; Gray, B. D.; Corbin, I.; Lebherz, C.; Choi, H.; Lund-Katz, S.; Wilson, J. M.; Glickson, J. D.; Zhou, R. *Acad. Radiol.* **2004**, *11*, 1251.
- (162) Tekade, R. K.; Kumar, P. V.; Jain, N. K. *Chem. Rev.* **2009**, *110*, 2574.
- (163) Bosman, A. W.; Janssen, H. M.; Meijer, E. W. *Chem. Rev.* **1999**, *99*, 1665.
- (164) Menjoge, A. R.; Kannan, R. M.; Tomalia, D. A. *Drug Discov. Today* **2010**, *15*, 171.

- (165) Talanov, V. S.; Regino, C. A. S.; Kobayashi, H.; Bernardo, M.; Choyke, P. L.; Brechbiel, M. W. *Nano Lett.* **2006**, *6*, 1459.
- (166) Xu, H.; Regino, C. A. S.; Koyama, Y.; Hama, Y.; Gunn, A. J.; Bernardo, M.; Kobayashi, H.; Choyke, P. L.; Brechbiel, M. W. *Bioconjugate Chem.* **2007**, *18*, 1474.
- (167) Boswell, C. A.; Eck, P. K.; Regino, C. A. S.; Bernardo, M.; Wong, K. J.; Milenic, D. E.; Choyke, P. L.; Brechbiel, M. W. *Mol. Pharm.* **2008**, *5*, 527.
- (168) Barrett, T.; Choyke, P. L.; Kobayashi, H. *Contrast Media Mol. Imaging* **2006**, *1*, 230.
- (169) Mulder, W. J. M.; Strijkers, G. J.; van Tilborg, G. A. F.; Griffioen, A. W.; Nicolay, K. *NMR Biomed.* **2006**, *19*, 142.
- (170) Mulder, W. J. M.; Strijkers, G. J.; Griffioen, A. W.; van Bloois, L.; Molema, G.; Storm, G.; Koning, G. A.; Nicolay, K. *Bioconjugate Chem.* **2004**, *15*, 799.
- (171) Kamaly, N.; Kalber, T.; Ahmad, A.; Oliver, M. H.; So, P.-W.; Herlihy, A. H.; Bell, J. D.; Jorgensen, M. R.; Miller, A. D. *Bioconjugate Chem.* **2008**, *19*, 118.
- (172) Mulder, W. J. M.; Strijkers, G. J.; Briley-Saboe, K. C.; Frias, J. C.; Aguinaldo, J. G. S.; Vucic, E.; Amirbekian, V.; Tang, C.; Chin, P. T. K.; Nicolay, K.; Fayad, Z. A. *Magn. Reson. Med.* **2007**, *58*, 1164.
- (173) Moriggi, L.; Aebischer, A.; Cannizzo, C.; Sour, A.; Borel, A.; Bunzli, J.-C. G.; Helm, L. *Dalton Trans.* **2009**, 2088.
- (174) Dehaen, G.; Eliseeva, S. V.; Kimpe, K.; Laurent, S.; Vander Elst, L.; Muller, R. N.; Dehaen, W.; Binnemans, K.; Parac-Vogt, T. N. *Chem. Eur. J.* **2012**, *18*, 293.
- (175) SalmanOgli, A. *Cancer Nanotechnology* **2011**, *2*, 1.
- (176) Mulder, W. J. M.; Strijkers, G. J.; van Tilborg, G. A. F.; Cormode, D. P.; Fayad, Z. A.; Nicolay, K. *Acc. Chem. Res.* **2009**, *42*, 904.
- (177) Mulder, W. J. M.; Koole, R.; Brandwijk, R. J.; Storm, G.; Chin, P. T. K.; Strijkers, G. J.; deMelloDonega, C.; Nicolay, K.; Griffioen, A. W. *Nano Lett.* **2006**, *6*, 1.
- (178) van Tilborg, G. A. F.; Mulder, W. J. M.; Chin, P. T. K.; Storm, G.; Reutelingsperger, C. P.; Nicolay, K.; Strijkers, G. J. *Bioconjugate Chem.* **2006**, *17*, 865.
- (179) Prinzen, L.; Miserus, R.-J. J. H. M.; Dirksen, A.; Hackeng, T. M.; Deckers, N.; Bitsch, N. J.; Megens; Douma, K.; Heemskerk, J. W.; Kooi, M. E.; Frederik, P. M.; Slaaf, D. W.; van Zandvoort, M. A. M. J.; Reutelingsperger, C. P. M. *Nano Lett.* **2006**, *7*, 93.
- (180) Bakalova, R.; Zhelev, Z.; Aoki, I.; Masamoto, K.; Mileva, M.; Obata, T.; Higuchi, M.; Gadjeva, V.; Kanno, I. *Bioconjugate Chem.* **2008**, *19*, 1135.
- (181) Park, J.; Bhuniya, S.; Lee, H.; Noh, Y.-W.; Lim, Y. T.; Jung, J. H.; Hong, K. S.; Kim, J. S. *Chem. Commun.* **2012**, *48*, 3218.
- (182) Song, Y.; Xu, X.; MacRenaris, K. W.; Zhang, X.-Q.; Mirkin, C. A.; Meade, T. J. *Angew. Chem., Int. Ed.* **2009**, *121*, 9143.
- (183) Pinho, S. L. C.; Faneca, H.; Geraldles, C. F. G. C.; Delville, M.-H.; Carlos, L. D.; Rocha, J. *Biomaterials* **2012**, *33*, 925.
- (184) Pinho, S. L. C.; Faneca, H.; Geraldles, C. F. G. C.; Rocha, J.; Carlos, L. D.; Delville, M.-H. *Eur. J. Inorg. Chem.* **2012**, 2828.

- (185) Rieter, W. J.; Kim, J. S.; Taylor, K. M. L.; An, H.; Lin, W.; Tarrant, T.; Lin, W. *Angew. Chem., Int. Ed.* **2007**, *46*, 3680.
- (186) Nam, T.; Park, S.; Lee, S.-Y.; Park, K.; Choi, K.; Song, I. C.; Han, M. H.; Leary, J. J.; Yuk, S. A.; Kwon, I. C.; Kim, K.; Jeong, S. Y. *Bioconjugate Chem.* **2010**, *21*, 578.
- (187) Tan, W. B.; Zhang, Y. *J. Nanosci. Nanotechnol.* **2007**, *7*, 2389.
- (188) Anderson, E. A.; Isaacman, S.; Peabody, D. S.; Wang, E. Y.; Canary, J. W.; Kirshenbaum, K. *Nano Lett.* **2006**, *6*, 1160.
- (189) Suci, P. A.; Berglund, D. L.; Liepold, L.; Brumfield, S.; Pitts, B.; Davison, W.; Oltrogge, L.; Hoyt, K. O.; Codd, S.; Stewart, P. S.; Young, M.; Douglas, T. *Chem. Biol.* **2007**, *14*, 387.
- (190) Pokorski, J. K.; Breitenkamp, K.; Liepold, L. O.; Qazi, S.; Finn, M. G. *J. Am. Chem. Soc.* **2011**, *133*, 9242.
- (191) Tsotsalas, M.; Busby, M.; Gianolio, E.; Aime, S.; De Cola, L. *Chem. Mater.* **2008**, *20*, 5888.

CHAPTER 3

MULTIFUNCTIONAL HYBRID SILVER NANOSTRUCTURES (*hAgNSs*) GROWN USING A POLYAMINO CARBOXYLIC ACID SCAFFOLD

Abstract

In this chapter, the aqueous-based, one-step synthesis of hybrid silver nanostructures (*hAgNSs*) is described. *hAgNSs* were grown using a polyamino carboxylic acid scaffold, namely 1,4,7,10-tetraaza-1-(1-aminobutyl)-4,7,10-triacetic acid cyclododecane (AB-DO3A). While the carboxylic moieties of the AB-DO3A scaffold provide nucleation sites for nanoparticle (NP) growth and contribute to the further stabilization of the species formed, the amine group provides both water solubility and an active group (for further conjugation) to the final product. A feasible arrangement for these *hAgNSs* is proposed based on UV-Vis absorbance spectroscopy, transmission electron microscopy, dynamic light scattering, fluorescence (lifetime and steady-state), NMR, infrared spectroscopy and mass spectrometry. The engineered *hAgNSs* consist of two functional units: a "large" AgNP stabilized by the carboxylate moieties of the AB-DO3A scaffold, that can act simultaneously as a template for surface modification and potentially as a therapeutic agent, and "smaller" Ag nanoclusters (AgNCs) that can be used as optical trackers.

3.1 Introduction

Noble metal nanoparticles (NPs), known for their chameleon-like assets, are interesting for solar energy cells^{1,2} and biological³⁻⁵ applications. Due to the large surface areas they offer, in which virtually any functional probe can be integrated via surface modification,⁶ noble metal NPs have found several applications in both therapeutics and optical diagnostics.⁴⁻⁶ Their optical properties, characterized by strong light scattering and absorption due to localized surface plasmon resonance, are strongly dependent on their shape and size.⁷⁻⁹

Silver NPs (AgNPs, Ag > 100 atoms) are characterized by their antiviral, antibacterial and antifungal properties as well as their applications for surface enhanced Raman scattering (SERS) and metal enhanced fluorescence (MEF).^{10,11} While the use of AgNPs in cancer therapy is still limited, these nanostructures are known to inhibit cell proliferation in human cancer cells.^{12,13} In contrast, noble metal nanoclusters (NCs), containing up to only 100 atoms,^{14,15} show very different behavior than their larger NP analogues. In particular, silver NCs (Ag_nNCs), consisting of a limited number of silver atoms ($n \sim 2-10$) exhibit fluorescence¹⁶, electro(chemi)luminescence¹⁷ and two-photon fluorescence;¹⁸ properties that are highly appealing for medical applications.

Despite several fairly established synthetic routes for the fabrication of AgNPs,^{3,8,11} the need for synthetic protocols that contribute to a more controlled fabrication of these nanostructured materials leaves space for improvement. In this chapter, the templating effect of a polyamine carboxylic acid scaffold, namely 1-(4-aminobutyl)-4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecane (AB-DO3A, Figure 3.1A), on the formation of AgNPs is investigated. The formation of AgNPs

of nanometer dimensions is triggered by a single-step, aqueous, chemical reduction of AgNO_3 with NaBH_4 . During and after formation, AB-DO3A can stabilize the AgNPs and, surprisingly, this also leads to the concomitant formation of fluorescent Ag_nNCs . The hybrid silver nanostructures (hAgNSs) formed consist of AgNPs presumably stabilized by the cyclen core and the carboxylic moieties of the pendant arms of the AB-DO3A scaffold where, also, Ag_nNCs have grown (Figure 3.1B).

Figure 3.1. Hybrid silver nanostructures (hAgNSs) formed by the chemical reduction of AgNO_3 in the presence of the 1,4,7,10-tetraaza-1-(1-aminobutyl)-4,7,10-triacetic acid cyclododecane (AB-DO3A) scaffold. The localization of the Ag_nNCs within the AgNP is only a schematic representation.

3.2 Design and Synthesis

Several stabilizing templates/scaffolds have been used for the fabrication of AgNPs, including peptides,¹⁹ aminated β -cyclodextrins,²⁰ polymers,²¹⁻²⁴ macrocycles such as cucurbituril¹² and dendrimers.^{25,26} The various reducing agents used, e.g., citrate, ascorbic acid and sodium borohydride,³ illustrate the extensive work that has been done in this particular field. Moreover, following similar procedures together with scaffolds such as dendrimers,²⁷ oligonucleotides²⁸⁻³⁰ polymers³¹⁻³⁴ and small organosulfur compounds,³⁵⁻³⁸ fluorescent Ag_nNCs have been

successfully synthesized. Despite the wide variety of wet chemical approaches described for the synthesis of AgNPs,^{3,16} there is need for new synthetic methodologies that can, hopefully, contribute to a more controlled fabrication of these nanostructures.

Here, it is expected that the multi-functional AB-DO3A scaffold provides the initial nucleation sites for NP growth by means of structurally flexible α -carboxylic moieties, known to show strong affinity towards Ag^+ ions and previously used for the synthesis of AgNCs in “macromolecular” templates.^{31,32,34} Moreover, the highly polar carboxylic moieties of the acetate pendant arms, as well as the aminobutyl pendant arm, provide water solubility to the scaffold, a vital feature for biological applications. The AB-DO3A scaffold is an interesting, multifunctional platform as other metal functionalities can be included by complexing inside the azacycle and its aminobutyl pendant arm can be used for further functionalization *via* amide formation.

3.2.1 Synthesis of the Polyamino Carboxylic Acid Scaffold AB-DO3A

The synthesis of the polyamino carboxylic acid scaffold 1,4,7,10-tetraaza-1-(4-aminobutyl)-4,7,10-triacetic acid cyclododecane (AB-DO3A, **5**), is described in Scheme 3.1. First, 1,4,7,10-tetraaza-1-(4-butyl phthalimide)-4,7,10-tris(*tert*-butoxycarbonylmethyl)cyclododecane (**3**) was obtained by alkylation of 1,4,7-tris(*tert*-butoxycarbonylmethyl)-1,4,7,10-tetraazacyclododecane (**1**) with *N*-(4-bromobutyl)phthalimide (**2**) in dry acetonitrile using K_2CO_3 (under N_2) for two days at 50°C . The *N*-alkylated phthalimide in (**3**) was cleaved in ethanol using hydrazine. The AB-DO3A ligand (**5**) was obtained by means of Boc-deprotection of (**4**) using $\text{TFA}/\text{CH}_2\text{Cl}_2$ at room temperature overnight. The final product was obtained with an overall yield (after purification) of 37 %.

Scheme 3.1. Synthetic route followed for the synthesis of AB-DO3A (5).

3.2.2 Synthesis of *hAgNSs*.

hAgNSs were grown by adding 50 μL of $AgNO_3$ in water (from a 0.01, 0.02 or 0.03 M stock solution, depending on the desired stoichiometry) to a 4.9 mL solution of AB-DO3A (final concentration of AB-DO3A is 1 mM) in water. The solution was allowed to stir for 30 minutes before 50 μL of $NaBH_4$ in water (from a 0.01, 0.02 and 0.03 M stock solution, depending on the desired stoichiometry) were slowly added. The solution was stirred for 2 minutes and subsequently left to stand, protected from light, for 24 h. All *hAgNS* solutions were analyzed and characterized without further purification.

3.3 Results and Discussion

3.3.1 Spectroscopic Characterization of *AgNPs*

In order to confirm the formation of *AgNPs*, a series of control reactions were performed. In the absence of the scaffold and upon addition of

NaBH_4 , the solution became turbid, with almost no color (Figure 3.2A, left) and an absorption peak with a maximum at 388 nm was observed (Figure 3.2B, black line). This absorption band is attributed to AgNPs of various sizes (> 75 nm, whose extinction is dominated by scattering rather than by absorption),^{39,40} that have grown uncontrollably due to the absence of a “stabilizing” agent. By comparison, in the absence of NaBH_4 the solution is colorless (Figure 3.2A, right) and a relatively weak UV absorption is observed (Figure 3.2B, grey line).

Figure 3.2. A) Image of the solutions of 0.2 mM AgNO_3 /0.2 mM NaBH_4 (left), 1 mM ABDO3A/0.2 mM AgNO_3 /0.2 mM NaBH_4 (center) and 1 mM AB-DO3A/0.2 mM AgNO_3 (right) after 24 h. B) UV-Vis spectra of the solutions of 0.2 mM AgNO_3 /0.2 mM NaBH_4 (black) and 1 mM AB-DO3A/0.2 mM AgNO_3 (grey) after 24 h. Note the maximum for the former solution at 388 nm.

In order to guarantee colloidal stability of the NPs formed over time, the growth of AgNPs in the presence of the AB-DO3A scaffold was studied using a five-fold excess of ligand. The stabilizing effect of the AB-DO3A scaffold is demonstrated in Figure 3.2A (center), as the solution becomes visibly yellow upon addition of NaBH_4 , strongly suggesting the formation of AgNPs of a defined size. In Figure 3.3, the UV spectra of three solutions with different AB-DO3A: AgNO_3 ratios, 24h after being chemically reduced,

are presented. Upon reduction, an absorption peak at 402 nm appears with increasing intensity as the AB-DO3A:AgNO₃ ratio decreases. Previously synthesized AgNPs (< 10 nm) using pseudo-dendritic polymers⁴¹ and surfactants⁴² show strong absorption bands around 400 nm. Hence, we attribute this plasmonic absorption to the formation of AgNPs stabilized by the AB-DO3A scaffold. Remarkably, for the 10:1 AB-DO3A:AgNO₃ solution, a broad shoulder towards the red region of the spectrum is observed (inset Figure 3.3). This peak, which becomes less evident as the relative AgNO₃ amount increases, can either point to a less monodisperse sample where larger Ag aggregates are formed or it can originate from other species formed during or after reduction. The position of the shoulder in the UV/Vis spectrum (> 500 nm) might indicate the formation of Ag_nNCs (n=2-4), known for their electronic transitions between 420 and 520 nm.^{17,27,28}

Figure 3.3. UV-Vis spectra of hAgNSs prepared using stoichiometries 10:1, 10:2 and 10:3 (AB-DO3A:AgNO₃) after 24 h. Inset: normalized spectra.

3.3.2 AgNP Size Characterization

The size distribution of the AgNPs formed was measured using two techniques: dynamic light scattering (DLS) and transmission electron microscopy (TEM). 24 h After reduction of a 10:2 AB-DO3A:AgNO₃ solution, DLS measurements showed that the AgNPs formed had an average size of 3.2 ± 0.7 nm (Figure 3.4A). This size is comparable to previously reported AgNPs synthesized using pseudo-dendritic polymers,⁴¹ with sizes between 2.6 and 4.2 nm and strong absorption bands around 400 nm. A smaller average size, namely 2.3 ± 0.7 nm (Figure 3.4B), was observed by TEM analysis and energy-dispersive X-ray spectroscopy (EDX) analysis showed that these NPs are composed of metallic silver. The difference in measured average size between DLS and TEM (~ 1 nm) is attributed to the nature of the scattering measured by each technique. While TEM images illustrate the electron scattering caused by the AgNP metal core only, DLS measurements are based on light scattering caused by spheres of a certain hydrodynamic radius (with solvent contributions included).

Figure 3.4. Characterization of the AgNP size: A) DLS data of a 10:2 AB-DO3A:AgNO₃ sample (after 24 h) showing an average particle size of 3.2 ± 0.7 nm (SD). B) TE micrograph of the same sample (Scale bar = 10 nm). Inset: corresponding histogram showing an average particle size of 2.3 ± 0.7 nm (SD).

3.3.3 Characterization of Ag_nNCs

Whilst the UV absorbance at ~ 400 nm has been attributed to AgNPs successfully observed by TEM, the shoulder at > 500 nm strongly suggests the formation of smaller species such as Ag_nNCs . However, it is rather difficult to confirm any Ag_nNC absorption due to the strong plasmon resonance peak coming from the AgNPs (which overshadows the signals of the smaller Ag_nNCs)^{40,43} and the sub-nanometric size of Ag_nNCs , which is hard to visualize by TEM. Contrary to larger AgNPs, small Ag_nNCs are known to be fluorescent,^{16,27,44-46} with clear differences in emission spectra when differing in number by only one single atom.^{28,30} Therefore, one way to determine the presence of Ag_nNCs is by identifying their (possible) fluorescence emission upon excitation. In order to do so, the emission properties of all three (reduced) solutions of AB-DO3A:AgNO₃ with ratios 10:1, 10:2 and 10:3 were compared, exciting in the range $\lambda_{exc} = 400$ to 600 nm. As depicted in Figure 3.5, fluorescence emission spectra at 595, 635 and 670 nm, when exciting at 440, 480 and 520 nm respectively, is observed. Moreover, the maximum fluorescence emission for the 10:1 solution (Figure 3.5A) is observed at 595 nm, for the 10:2 solution (Figure 3.5B) at 635 nm and for the 10:3 solution (Figure 3.5C) at 670 nm. None of these emission maxima were observed in either the AgNO₃ solution nor in the AB-DO3A scaffold solution (Figure 3.10, Appendix 3.1). While emission of Ag_nNCs has been previously identified in the regions 610-650 nm ($n=2,3,5$),¹⁷ 650 nm ($n=4,5$),³⁸ and 530-650 nm ($n=1-4$),²⁷ the differences in fluorescence intensity observed for each λ_{exc} show that, as the AB-DO3A:AgNO₃ ratio increases, the emission maximum shifts to the red. One possible explanation for the difference in emission intensities could come from the interaction with the organic scaffold, as it has been previously observed that the absorption and emission properties of few-

atom Ag_nNCs change dramatically according to the chemical environment surrounding them.¹⁷

Figure 3.5. Fluorescence spectra of chemically reduced AB-DO3A:AgNO₃ solutions (after 24 h) at different excitation wavelengths, namely λ_{exc} 440 nm, 480 nm and 520 nm. A) 10:1 AB-DO3A:AgNO₃ solution. B) 10:2 AB-DO3A:AgNO₃ solution. C) 10:3 AB-DO3A:AgNO₃ solution. D) Fluorescence lifetime (black line) of the 10:2 AB-DO3A:AgNO₃ solution (diluted) with excitation at 450 nm. The instrument response (grey line) is also shown.

For all three solutions, shifting of the emission maxima to longer wavelengths upon increase of the excitation wavelength used is observed, which is often associated to a distribution of different NC sizes.^{28,32-34} Interestingly, the highest fluorescence intensity is observed in the 10:1 AB-DO3A:AgNO₃ sample (Figure 3.5A), which might be explained by the fact that, at this ratio, less AgNPs are formed and, therefore, more carboxylate groups are available for Ag_nNC stabilization.³⁴ This hypothesis is further supported by the UV absorption data, as a dramatic increase in the

intensity at 400 nm (attributed to AgNPs) for the 10:2 and the 10:3 AB-DO3A:AgNO₃ samples is observed.

Other optical properties of the Ag_nNCs formed under the previously described conditions were explored using lifetime measurements. In order to remove the AgNPs from the solution, a 10:2 AB-DO3A:AgNO₃ sample was centrifuged at 11000 rpm for 10 minutes. The supernatant (still containing Ag_nNCs) was subsequently diluted 10 times and the lifetime was measured. The decay of this sample could be fitted to a biexponential function with a slow component of τ_1 4.3 ns and a fast component of τ_2 245 ps (Figure 3.5D). Bifunctionally fitted decays with comparable values have previously been observed for multiple population ($n=2-5$) Ag_nNCs grown in both peptides (1.9-2.9 ns and 73-420 ps)⁴⁷ and polymer matrixes (2.4-3.1 ns and 960 ps).¹⁷

A well-established technique used to determine the exact number of Ag atoms in NCs is mass spectrometry (MS).^{28,29,35,36,48} Chemical species containing Ag can be easily identified by the characteristic isotope pattern generated by the two abundant isotopes ¹⁰⁷Ag and ¹⁰⁹Ag. Figure 3.6 shows the matrix-assisted laser desorption ionization (MALDI)-MS spectrum of three solutions of 10:1, 10:2 and 10:3 AB-DO3A:AgNO₃ ratios, obtained in the negative ion mode. Only three sets of peaks centered at m/z 670.8, 894.9 and 1119 were successfully identified as Ag-containing compounds in all three samples (labeled 1, 2 and 3 in Figure 3.6, respectively). The isotope's unity spacing indicates that the corresponding species bear a -1 charge. The highest intensity for all three peaks was observed in the 10:2 sample (Figure 3.6A) and the isotope pattern agrees well with the presence of two Ag atoms embedded in AB-DO3A. The isotopic pattern and m/z values of the set of peaks 1 (m/z = 670.8, theoretical molecular weight: 671.02), corresponding to the ion [Ag₂L - H + K]⁻ (L = AB-DO3A, Figure 3.6B), match the simulated isotopic pattern in Figure 3.6C. The same

isotopic pattern is observed for peaks 2 and 3, but their m/z values (theoretical molecular weights: 895.09 and 1119.16 respectively) are higher due to the presence of one or two molecules of the matrix (sinapic acid, Figure 3.11 in Appendix 3.2).

Figure 3.6. MALDI-MS spectra. A) Chemically reduced 10:1, 10:2 and 10:3 AB-DO3A:AgNO₃ solutions. Ag-containing species: 1) $[\text{Ag}_2\text{L} - \text{H} + \text{K}]^-$; 2) $[\text{Ag}_2\text{L} - \text{H} + \text{K} + \text{M}]^-$; 3) $[\text{Ag}_2\text{L} - \text{H} + \text{K} + 2\text{M}]^-$; L is AB-DO3A, M is the matrix (sinapic acid). B) Amplification of the set of peaks 1 ($m/z = 670.8$). C) Simulated isotopic pattern of 1 showing the characteristic isotopic pattern of Ag.

In all cases, no assignable peaks for Ag₁ or any other Ag containing species with a number of atoms higher than two could be identified. While this observation suggests that (within the scaffold) only Ag₂NCs are formed, the steady-state and lifetime fluorescence data show that the presence of other Ag_nNC populations cannot be excluded.

3.3.4 Interaction AB-DO3A/Ag Before and After Reduction

To determine the initial metal-to-ligand complex formation and its possible stoichiometry, a titration of AB-DO3A with Ag⁺ was carried out and the

chemical shifts (δ) of the different protons of AB-DO3A were monitored by ^1H -NMR. The differences between ^1H -NMR spectra of the scaffold in the presence of Ag^+ at different concentrations are shown in Figure 3.7A-D. The peaks corresponding to $\text{H}_{\text{a,a'},\text{b,c,d,e,f}}$ shift as the concentration of Ag^+ increases, therefore corroborating that indeed Ag^+ ions are captured by the acetate arms ($\text{H}_{\text{a,a'},\text{b}}$) and that the Ag^+ ions are in close vicinity of the cyclen core ($\text{H}_{\text{c,d,e,f}}$). Conversely, the chemical shifts of protons H_i and H_j do not change upon addition of Ag^+ , indicating that the aminobutyl arm does not participate in the initial complexation process to the metal ion precursor. Likewise, protons H_i and H_j remain unaltered after addition of NaBH_4 (Figure 3.7E), thereby strongly suggesting that both the cyclen core and the carboxylic acids of the three acetate pendant arms (and not the aminobutyl arm) contribute to the stabilization of the AgNPs formed.

The chemical shifts corresponding to protons $\text{H}_{\text{a,a'},\text{b,c,d,e,f}}$ of AB-DO3A upon titration of the precursor Ag^+ salt were fitted collectively to several complexation models with different binding stoichiometries (assuming independent binding sites, see Appendix 3.3). AB-DO3A can bind (at least) three Ag^+ atoms with an intrinsic equilibrium constant of $4 \times 10^3 \text{ M}^{-1}$. The data and modeling do not imply any cooperativity or preference for the formation of a complex with a particular stoichiometry. Therefore, any preference for the formation of only one population of Ag_nNCs (e.g., Ag_2NCs , as the MS data suggest) cannot be attributed to any predilection during the preceding Ag^+ complexation step, and hence must occur during or shortly after the reduction step.

Figure 3.7. ^1H -NMR spectra, including peak assignment and structure of AB-DO3A. A-D) Relevant spectra of AB-DO3A in D_2O upon addition of aliquots of 10 mM AgNO_3 solution (see Appendix 3.3). $\text{AgNO}_3/\text{AB-DO3A}$ concentrations (mM/mM): A) 0/10; B) 2.65/7.35; C) 4.60/5.40 and D) 6.60/3.40. E) Spectrum after chemical reduction ($\text{AgNO}_3/\text{AB-DO3A}$ ratio: 0.88). Note that the highest AB-DO3A: AgNO_3 ratio studied (10:3) is reached after the second titration point (B).

The by NMR proposed role of the cyclen core and the carboxylic moieties with respect to the stabilization of the newly formed AgNPs was further reinforced by infrared spectroscopy (Figure 3.8). After reduction, a decrease in the intensity of the $\text{C}=\text{O}$ stretch frequency band corresponding to the carboxylic moieties of AB-DO3A ($1665\text{--}1645\text{ cm}^{-1}$) is observed, as well as two new frequency bands that can be attributed to N-H bending (1595 cm^{-1}) and stretching (3195 cm^{-1}) vibrations. From these observations

it can be concluded that the aminobutyl arm of AB-DO3A does not contribute to the stabilization of the AgNPs formed.

Figure 3.8. IR Spectra of AB-DO3A (black line) and *h*AgNSs (grey line).

3.3.5 The Hybrid AgNP-Ag_nNC System

The potential scaffold-mediated physical connection of the NCs to the NPs or hybrid nature of the AgNSs formed was tested by centrifuging a 10:2 AB-DO3A:AgNO₃ solution after reduction and measuring the UV absorption and fluorescence emission at the maximum emission value (635 nm) before and after centrifugation. This was done by centrifuging the sample at 11000 rpm for 10 minutes and (re)dissolving the pellet in the same water volume as the original solution. Assuming that any independently formed Ag_nNCs (so not physically connected to AgNPs) do not precipitate out of solution after centrifugation, any fluorescence observed in the re-dissolved pellet must be related to species physically connected to the AgNPs. Figure 3.9A and B, which correspond to the absorption and fluorescence of the solution before centrifugation (black line) and of the pellet after centrifugation (red line), respectively, show that the ratio initial:final absorbance and initial:final fluorescence is, in both

cases, the same (~ 2). This is clearly not the case when comparing the initial solution with the supernatant (Figure 3.9C and D, green lines), as the ratios initial:final absorbance and initial:final fluorescence are different (4 and 8 respectively). While still some NPs are observed in the supernatant after centrifugation, very weak fluorescence is detected. Since all of our previous observations corroborate the fact that both Ag_nNCs as well as AgNPs are present in solution, this last observation strongly suggests some degree of attachment between these two species. Hence, we foresee a physical connection between the NPs and the NCs, thereby confirming the formation of $h\text{AgNSs}$. Lastly, while a feasible role of the AB-DO3A in the AgNP stabilization is clearly portrayed in Figure 3.1, how this scaffold stabilizes the Ag_nNCs formed is not fully understood yet.

Figure 3.9. Spectra of chemically-reduced 10:2 AB-DO3A: AgNO_3 solution. The black line corresponds to the spectrum before centrifugation, the red line to the redispersed pellet (the same initial water volume) and the green line to the supernatant. A, C) UV spectra. B, D) Fluorescence emission (λ_{exc} 480 nm) spectra.

Overall, AB-DO3A-like scaffolds are believed to be an interesting platform for engineering fluorescent *h*AgNSs with precise size of both NPs and NCs. This control is thought to arise from the lower amount of degrees of freedom compared to bigger organic scaffolds, where the growth of clusters is governed by a large number of parameters such as pH, shape and conformation in space.^{30,31,33} Nevertheless, proper characterization of such systems proved to be challenging as fairly established techniques such as MS did not seem to provide full information with respect to the Ag_nNC composition.

3.4 Conclusions

Altogether, the chemical reduction of AgNO₃ in the presence of the AB-DO3A scaffold yields AgNPs stabilized by the AB-DO3A scaffold that, at the same time, stabilizes fluorescent Ag_nNCs. While MS is a fairly established, standard technique for the quantification of the number atoms in Ag_nNCs,^{28,29,35,36,48} our steady-state fluorescence studies reveal the presence of other Ag_nNC populations not detected by MS. Therefore, the formation of NCs of various sizes, grown in or outside of the scaffold, cannot be ruled out. While fluorescent AgNPs have previously been described in the literature^{49,50} this is, to our knowledge, the first example of multifunctional *h*AgNSs grown using a polyamino carboxylic acid scaffold. This new class of hybrid nanostructures opens up the door for a simpler, straightforward controlled synthesis of multifunctional materials for molecular imaging and (possibly) therapeutic purposes.

3.5 Experimental

Materials. Commercial grade reagents were purchased from Sigma Aldrich and TCI Europe and were used without further purification unless otherwise stated. N-(4-bromobutyl)phthalimide was obtained from Sigma Aldrich. Solvents were dried over molecular sieves. Deuterated solvents were purchased from Cambridge Isotope Laboratories and NMR tubes from Wilmad LabGlass. All Ultrapure (Milli-Q) water was used in all experiments.

Synthesis of 1,4,7,10-tetraaza-1-(4-butylphthalimide)-4,7,10-tris(*tert*-butoxycarboxymethyl)cyclododecane (Bphth-*t*butylDO3A, **3).**

In a three-neck round-bottom flask, K₂CO₃ (420 mg, 3.04 mmol) was added to a stirring solution of 1,4,7-Tris(*tert*-butoxycarboxymethyl)-1,4,7,10-tetraazacyclododecane **1** (DO3A, synthesized as previously described in literature)⁵¹ (500 mg, 0.97 mmol) in 40 mL dry acetonitrile. The mixture was kept under N₂ for 10 minutes under vigorous stirring. Afterwards, N-(4-bromobutyl)phthalimide (**2**) (274 mg, 0.97 mmol) dissolved in 10 mL dry acetonitrile was added slowly to the reaction mixture and allowed to react for 2 days at 50°C. The mixture was left to cool, filtered to remove inorganic salts and the solvent evaporated under reduced pressure. The desired compound was obtained as a yellow oil after chromatography on alumina (94:6 CH₂Cl₂:MeOH, R_f 0.5). Yield: 70 % (489 mg, 0.683 mmol). ¹H NMR (CDCl₃, 600 MHz) δ (ppm): 7.86 (m, 2H), 7.75 (m, 2H), 3.66 (t, *J* (H, H) = 7.3 Hz, 2H), 3.6-2.1 (bs, 24H), 1.63 (m, *J* (H, H) = 7.3, 7.6 Hz, 2H), 1.45 (bs, 2H), 1.45-1.49 (m, 27H). ¹³C NMR (CDCl₃, 150 MHz) δ (ppm): 172.7, 168.3, 134.1, 132.1, 123.2, 82.7,

82.2, 56.5, 55.8, 53.8, 52.8, 50, 47.95, 37.6, 28, 27.8, 26.8, 23.7.
MS (ESI⁺): m/z : 738.3 [$M^+ + Na$].

Synthesis of 1,4,7,10-tetraaza-1-(4-aminobutyl)-4,7,10-tris(*tert*-butoxycarbonylmethyl)cyclododecane (AB-*t*butylDO3A, 4).

Compound **4** was synthesized using a procedure adapted from literature.⁵² To a stirring solution of **3** (450 mg, 0.63 mmol) in ethanol, 100% hydrazine monohydrate (1.4 mL, 28.35 mmol) was added and the solution was allowed to react at 50°C for 2 days. The mixture was evaporated under reduced pressure and the residue was co-evaporated with ethanol three times in order to remove excess hydrazine. Afterwards, 40 mL of CHCl₃ were added to the residue and the mixture was filtered after sonication. The product was extracted from the organic phase acid water (acetic acid, pH 3, 3 x 20 mL). Subsequently, the pH of the combined aqueous phases was adjusted to 10 with 1M NaOH and the final product extracted from the aqueous phase with chloroform (3 x 30 mL). After drying the combined organic phases over MgSO₄, the solvent was evaporated under reduced pressure and the desired compound was obtained as a yellow oil. Yield: 53 % (197 mg, 0.34 mmol). ¹H NMR (CDCl₃, 600 MHz) δ (ppm): 3.31-3.29 (s, 6H), 2.86-2.48 (bs, 20H) 1.89 (m, 2H), 1.76 (m, 2H), 1.46-1.48 (m, 27H). ¹³C NMR (CDCl₃, 150 MHz) δ (ppm): 171.8, 171.6, 81.9, 81.4, 57.2, 56.9, 54.3, 50.9, 39, 28.1, 28.07, 26.8, 25.8. MS (ESI⁺): m/z : 586.0 [$M^+ + H$].

Synthesis of 1,4,7,10-tetraaza-1-(4-aminobutyl)-4,7,10-triacetic acid cyclododecane (AB-DO3A, 5). In a round-bottom flask, TFA (5 mL, 67 mmol) was added to a solution of **4** (200 mg, 0.34 mmol) in 5 mL CH₂Cl₂ and the reaction mixture was allowed to stir at room temperature overnight. The mixture was evaporated under reduced pressure and the

residue co-evaporated with dichloromethane three times in order to remove excess TFA. Subsequently, the residue was re-dissolved in water and washed with dichloromethane three times. The aqueous solution was freeze dried and subsequently triturated with diisopropyl ether. Compound **5** was obtained as a yellow oil (quantitative yield). ^1H NMR (D_2O , 600 MHz) δ (ppm): 3.84 (H_b , s, 2H), 3.57-3.54 & 3.43-3.40 ($\text{H}_{a, a'}$, d, J (H, H) = 18 Hz, 4H), 3.35 (H_d , bs, 4H), 3.29 (H_f , bs, 4H), 3.16 (H_g , bs, 2H), 3.06 (H_e , bs, 4H), 3 (H_c , bs, 4H), 2.95 (H_j , t, J (H, H) = 7.6 Hz, 2H), 1.75 (H_h , bs, 2H), 1.63 (H_i , m, J (H, H) = 7.6, 7.8 Hz, 2H). ^{13}C NMR (D_2O), 150 MHz) δ (ppm): 174.1, 173.9, 169.4, 55.3, 53.3, 53, 52.8, 51.3, 49.8, 48.4, 48, 38.5, 23.6, 20.1. MS (ESI+): m/z : 418. 3 [$\text{M}^+ + \text{H}$].

UV-Vis Spectroscopy. UV spectra were acquired on a Perkin Elmer Lambda 850 UV-Vis spectrometer. All samples were measured in UV quality quartz cuvettes with a path length of 1 cm.

Transmission Electron Microscopy (TEM). TEM images were obtained using analytical TEM FEI instruments. In order to prepare the samples, 5 μL of the desired sample was applied onto Formvar-carbon coated grids (from electron microscopy sciences). After leaving the sample for 1 minute, the excess of liquid was removed using a piece of filter paper. The size distribution of $h\text{AgNPs}$ was determined by measuring the particle core diameter of more than 80 counts from TEM images using Image J software. Energy-dispersive X-ray analysis was done using a Thermo Noran System 6 with a resolution of 138 eV on a Manganese standard.

Dynamic Light Scattering (DLS). The hydrodynamic sizes of individual *h*AgNPs were characterized by dynamic light scattering using a Zetatrac Microtrac Flex 10.6.1 particle analyzer with a 180° back-scatter (Microtrac Inc., PA, USA). The particle diameter was calculated using a heterodyne technique to obtain a characteristic frequency, which is then related to the particle diameter by the Stokes-Einstein equation. 1 mL *h*AgNPs samples were inserted in a cell inside the DLS-setup. The size distribution of the nanoparticles was determined by measuring their diameter for more than 450 counts. The number-average diameter was recorded for the sample and averaged over 5 measurements.

Nuclear Magnetic Resonance (NMR) Characterization and Titration. All NMR experiments were performed on a Bruker Avance II NMR spectrometer operating at 600.35 MHz for ¹H and 150.09 MHz for ¹³C. Chemical shifts are given in ppm using the residual solvent signal as reference. The multiplicity of the peaks is reported using the following abbreviations: s = singlet, d = doublet, t = triplet, quint = quintuplet, m = multiplet. All NMR spectra were processed using TOPSPIN 2.1 and MestReC software packages.

Mass Spectrometry. Mass spectra were acquired on a Waters Micromass LCT (ESI) mass spectrometer and on a applied Biosystems Voyager-DE RP (MALDI-TOF) mass spectrometer, using sinapic acid as matrix.³⁶

Fluorescence Spectroscopy. All steady-state spectra were recorded using a Perkin Elmer Fluorescence Spectrometer.

Infrared Spectroscopy. All IR spectra were recorded using a Fourier Transform (FT-IR) Thermo Scientific Spectrometer.

Lifetime Measurements. Lifetime measurements were done using a single-molecule confocal microscope consisting of an Olympus IX71 microscope equipped with a PicoQuant Diode Laser, an Acousto Optic tunable filter from Crystal Technologies, a single mode fiber, objectives from Olympus (60x/1.20 water) and Nikon (Plan APO) and an ADP with an active area diameter of 100 μm . Excitation was done at 450 nm and a 488 nm long-pass filter was used during measurements.

3.6 References

- (1) Günes, S.; Sariciftci, N. S. *Inorg. Chim. Acta* **2008**, *361*, 581.
- (2) Saunders, B. R.; Turner, M. L. *Adv. Colloid Interface Sci.* **2008**, *138*, 1.
- (3) García-Barrasa, J.; López-de-Luzuriaga, J.; Monge, M. *Cent. Eur. J. Chem.* **2011**, *9*, 7.
- (4) Bhattacharya, R.; Mukherjee, P. *Adv. Drug Delivery Rev.* **2008**, *60*, 1289.
- (5) Bhattacharyya, S.; Kudgus, R.; Bhattacharya, R.; Mukherjee, P. *Pharm. Res.* **2011**, *28*, 237.
- (6) Thanh, N. T. K.; Green, L. A. W. *Nano Today* **2010**, *5*, 213.
- (7) Banin, U.; Millo, O. In *Nanoparticles: from Theory to Application*; Schmid, G., Ed.; Wiley-VCH Verlag GmbH & Co. KGaA: Weinheim, FRG, **2005**, p 305.
- (8) Bradley, J. S.; Schmid, G.; Talapin, D. V.; Shevchenko, E. V.; Weller, H. In *Nanoparticles: from Theory to Application*; Schmid, G., Ed.; Wiley-VCH Verlag GmbH & Co. KGaA: Weinheim, FRG, **2005**, p 185.
- (9) Jain, P. K.; Huang, X.; El-Sayed, I. H.; El-Sayed, M. A. *Acc. Chem. Res.* **2008**, *41*, 1578.
- (10) Galdiero, S.; Falanga, A.; Vitiello, M.; Cantisani, M.; Marra, V.; Galdiero, M. *Molecules* **2011**, *16*, 8894.
- (11) Nair, L. S.; Laurencin, C. T. *J. Biomed. Nanotechnol.* **2007**, *3*, 301.
- (12) Premkumar, T.; Lee, Y.; Geckeler, K. E. *Chem. Eur. J.* **2010**, *16*, 11563.
- (13) Wu, Q.; Cao, H.; Luan, Q.; Zhang, J.; Wang, Z.; Warner, J. H.; A. R. Watt, A. *Inorg. Chem.* **2008**, *47*, 5882.
- (14) Wilcoxon, J. P.; Abrams, B. L. *Chem. Soc. Rev.* **2006**, *35*, 1162.
- (15) Zheng, J.; Nicovich, P. R.; Dickson, R. M. *Annu. Rev. Phys. Chem.* **2007**, *58*, 409.
- (16) Diez, I.; Ras, R. H. A. *Nanoscale* **2011**, *3*, 1963.

- (17) Díez, I.; Pusa, M.; Kulmala, S.; Jiang, H.; Walther, A.; Goldmann, A. S.; Müller, A. H. E.; Ikkala, O.; Ras, R. H. A. *Angew. Chem., Int. Ed.* **2009**, *48*, 2122.
- (18) Patel, S. A.; Richards, C. I.; Hsiang, J.-C.; Dickson, R. M. *J. Am. Chem. Soc.* **2008**, *130*, 11602.
- (19) Graf, P.; Mantion, A.; Foelske, A.; Shkilnyy, A.; Mašić, A.; Thünemann, A. F.; Taubert, A. *Chem. Eur. J.* **2009**, *15*, 5831.
- (20) Abou-Okeil, A.; Amr, A.; Abdel-Mohdy, F. A. *Carbohydr. Polym.* **2012**, *89*, 1.
- (21) Sun, Y.; Xia, Y. *Science* **2002**, *298*, 2176.
- (22) Huang, H. H.; Ni, X. P.; Loy, G. L.; Chew, C. H.; Tan, K. L.; Loh, F. C.; Deng, J. F.; Xu, G. Q. *Langmuir* **1996**, *12*, 909.
- (23) Tan, Y.; Dai, X.; Li, Y.; Zhu, D. *J. Mater. Chem.* **2003**, *13*, 1069.
- (24) He, R.; Qian, X.; Yin, J.; Zhu, Z. *J. Mater. Chem.* **2002**, *12*, 3783.
- (25) Sun, X.; Dong, S.; Wang, E. *Macromolecules* **2004**, *37*, 7105.
- (26) Lesniak, W.; Bielinska, A. U.; Sun, K.; Janczak, K. W.; Shi, X.; Baker, J. R.; Balogh, L. P. *Nano Lett.* **2005**, *5*, 2123.
- (27) Zheng, J.; Dickson, R. M. *J. Am. Chem. Soc.* **2002**, *124*, 13982.
- (28) Petty, J. T.; Zheng, J.; Hud, N. V.; Dickson, R. M. *J. Am. Chem. Soc.* **2004**, *126*, 5207.
- (29) Ritchie, C. M.; Johnsen, K. R.; Kiser, J. R.; Antoku, Y.; Dickson, R. M.; Petty, J. T. *J. Phys. Chem. C* **2006**, *111*, 175.
- (30) Richards, C. I.; Choi, S.; Hsiang, J.-C.; Antoku, Y.; Vosch, T.; Bongiorno, A.; Tzeng, Y.-L.; Dickson, R. M. *J. Am. Chem. Soc.* **2008**, *130*, 5038.
- (31) Zhang, J.; Xu, S.; Kumacheva, E. *Adv. Mater.* **2005**, *17*, 2336.
- (32) Shen, Z.; Duan, H.; Frey, H. *Adv. Mater.* **2007**, *19*, 349.
- (33) Shang, L.; Dong, S. *Chem. Commun.* **2008**, 1088.
- (34) Xu, H.; Suslick, K. S. *ACS Nano* **2010**, *4*, 3209.
- (35) Udaya Bhaskara Rao, T.; Pradeep, T. *Angew. Chem., Int. Ed.* **2010**, *49*, 3925.
- (36) Wu, Z.; Lanni, E.; Chen, W.; Bier, M. E.; Ly, D.; Jin, R. *J. Am. Chem. Soc.* **2009**, *131*, 16672.
- (37) Cathcart, N.; Kitaev, V. *J. Phys. Chem. C* **2010**, *114*, 16010.
- (38) Adhikari, B.; Banerjee, A. *Chem. Mater.* **2010**, *22*, 4364.
- (39) Evanoff, D. D.; Chumanov, G. *ChemPhysChem* **2005**, *6*, 1221.
- (40) Evanoff, D. D.; Chumanov, G. *J. Phys. Chem. B* **2004**, *108*, 13957.
- (41) Kuo, P.-L.; Chen, W.-F. *J. Phys. Chem. B* **2003**, *107*, 11267.
- (42) Manna, A.; Imae, T.; Iida, M.; Hisamatsu, N. *Langmuir* **2001**, *17*, 6000.
- (43) Schultz, D.; Gwinn, E. *Chem. Commun.* **2011**, *47*, 4715.
- (44) Peyser, L. A.; Vinson, A. E.; Bartko, A. P.; Dickson, R. M. *Science* **2001**, *291*, 103.
- (45) Xu, H.; Suslick, K. S. *Adv. Mater.* **2010**, *22*, 1078.
- (46) Yu, J.; Choi, S.; Richards, C. I.; Antoku, Y.; Dickson, R. M. *Photochem. Photobiol.* **2008**, *84*, 1435.
- (47) Yu, J.; Patel, S. A.; Dickson, R. M. *Angew. Chem., Int. Ed.* **2007**, *46*, 2028.
- (48) Gwinn, E. G.; O'Neill, P.; Guerrero, A. J.; Bouwmeester, D.; Fygenson, D. K. *Adv. Mater.* **2008**, *20*, 279.

- (49) Maretti, L.; Billone, P. S.; Liu, Y.; Scaiano, J. C. *J. Am. Chem. Soc.* **2009**, *131*, 13972.
- (50) Stamplecoskie, K. G.; Scaiano, J. C. *J. Am. Chem. Soc.* **2011**, *133*, 3913.
- (51) Beeby, A.; Bushby, L. M.; Maffeo, D.; Gareth Williams, J. A. *J. Chem. Soc., Dalton Trans.* **2002**, 48.
- (52) Sasaki, T.; Minamoto, K.; Itoh, H. *J. Org. Chem.* **1978**, *43*, 2320.

Appendix 3.1. Control Experiments: Fluorescence of AgNO₃ and AB-DO3A Solutions

Figure 3.10. A) Fluorescence spectra (taken after 24 h) of a 0.2 mM AgNO₃ solution excited at $\lambda_{\text{excitation}}$: 440, 480 and 520 nm (black, red and blue, respectively). B) Fluorescence spectra (taken after 24 h) of a 1 mM AB-DO3A solution excited at $\lambda_{\text{excitation}}$: 440, 480 and 520 nm (black, red and blue, respectively). Note the very small intensity scale.

Appendix 3.2. Amplification of MALDI-MS Regions
2 $[\text{Ag}_2\text{L} - \text{H} + \text{K} + \text{M}]^-$ and 3 $[\text{Ag}_2\text{L} - \text{H} + \text{K} + 2\text{M}]^-$ (L
is AB-DO3A, M is the matrix -sinapic acid-)

Figure 3.11. Amplification of MALDI-MS (-) regions: A) 2, B) 3.

Appendix 3.3. Titration of AB-DO3A with Ag⁺ Followed by ¹H-NMR

In order to study the complexation process, a 10 mM solution of AB-DO3A in D₂O was titrated with Ag⁺. This was done by adding 100 μL of AgNO₃ (10 mM in D₂O) to 600 μL of AB-DO3A in an NMR tube and measuring the spectrum. For each subsequent aliquot addition of 100 μL AgNO₃, 600 μL of the previously measured solution were used and the NMR spectrum was taken. This procedure was repeated 15 times; although for the final fitting only 12 points were used due to overlapping of ¹H NMR signals. The differences in chemical shift (δ) for protons H_{a,a'}, b, c, d, e, f (upon addition of Ag⁺) were quantified and the equilibrium constants were calculated by non-linear least squares fitting all peaks simultaneously to a 1:2 and a 1:3 model, given by:

1:2 Model:

1:3 Model:

Where L is AB-DO3A and M is Ag⁺ and the equilibrium constants are given by:

$$K_1 = \frac{[LM]}{[L][M]} \quad K_2 = \frac{[LM_2]}{[LM][M]} \quad K_3 = \frac{[LM_3]}{[LM_2][M]}$$

1:2 Model

The data was fitted to a 1:2 model assuming independent binding sites (Figure 3.12), where the calculated intrinsic equilibrium constant K_i ($2.4 \times 10^3 \text{ M}^{-1}$) is given by:

$$K_1/2 = K_i = 2K_2$$

Figure 3.12. Titration curves, including experimental (E) and calculated (C) values, non-linear least squares fitted to a 1:2 model assuming independent binding sites ($K_i = 2.4 \times 10^3 \text{ M}^{-1}$), for the peaks $H_{a,a',b,c,d,e,f}$ of AB-DO3A ($[\text{AB-DO3A}]_{\text{initial}} = 10 \text{ mM}$, $[\text{AgNO}_3]_{\text{added}} = 10 \text{ mM}$, 25°C)

The poor fitting of the data to the 1:2 model (Figure 3.12) demonstrates that a 1:2 complex is not the only species formed. Subsequently, the fitting of this data to less restrictive 1:2 models was checked, assuming positive cooperativity ($K_2 > K_1/4$) as well as negative cooperativity ($K_2 < K_1/4$).

Assuming negative cooperativity ($K_1 = 1 \times 10^4 \text{ M}^{-1}$ and $K_2 = 1.3 \times 10^3 \text{ M}^{-1}$), the quality of the fit did not change when compared to the 1:2 (independent binding sites) model, while for positive cooperativity ($K_1 = 7 \times 10^0 \text{ M}^{-1}$ and $K_2 = 4 \times 10^4 \text{ M}^{-1}$) a slightly better fit was obtained.

1:3 Model

Given the poor fitting of the data to the 1:2 model, non-linear least squares fitting to a 1:3 model assuming independent binding sites (Figure 3.13) was performed, where the intrinsic equilibrium constant K_i is given by:

$$K_1/3 = K_i = K_2 = 3K_3$$

Figure 3.13. Titration curves, including experimental (E) and calculated (C) values, non-linear least squares fitted to a 1:3 model assuming independent binding sites ($K_i = 3.9 \times 10^3 \text{ M}^{-1}$), for the peaks $H_{a,a',b,c,d,e,f}$ of AB-DO3A ($[\text{AB-DO3A}]_{\text{initial}} = 10 \text{ mM}$, $[\text{AgNO}_3]_{\text{added}} = 10 \text{ mM}$, 25°C)

The titration data was successfully fitted to this 1:3 model (Figure 3.13), with $K_i = 3.9 \times 10^3 \text{ M}^{-1}$, strongly suggesting that at least three Ag^+ atoms are captured by AB-DO3A.

CHAPTER 4

SELF-ASSEMBLY TRIGGERED BY SELF-ASSEMBLY: PROTEIN CAGE ENCAPSULATED MICELLES VISUALIZED BY MRI

Abstract

In this chapter, the successful encapsulation of paramagnetic micelles inside protein cages of the cowpea chlorotic mottle virus (CCMV) is reported. The stable assemblies formed were engineered by pure hierarchical self-assembly of micelles of the 1,4,7,10-tetraza-1-(1-carboxymethylundecane)-4,7,10-triacetic acid cyclododecane (DOTAC10) ligand inside CCMV. The DOTAC10 ligand was used to either complex with Gd^{3+} in order to form paramagnetic micelles visualized by MRI or to encapsulate amphiphilic Zn^{2+} phthalocyanine (ZnPc) dye molecules, which optically confirmed the encapsulation of the micelles. Subsequent incorporation of the ZnPc dye in the Gd^{3+} -DOTAC10 paramagnetic micelles led to noticeably higher capsid loadings. Protein cage formation was confirmed by transmission electron microscopy, elemental analysis and size exclusion chromatography. These purely self-assembled protein cages containing paramagnetic micelles showed enhanced ionic and capsid r_1 relaxivities, highlighting the potential of these nanostructures as contrast agents for MRI. The enhanced relaxivity properties are attributed to aggregation and altered micelle association kinetics resulting from local confinement inside the protein cage and ZnPc dye encapsulation.

4.1 Introduction

Magnetic resonance imaging (MRI) is a non-invasive technique with a non-ionizing character widely used to distinguish between different types of tissue while offering excellent spatial resolution.^{1,2} To enhance image contrast, extracellular contrast agents (CAs) based on Gd^{3+} complexes³ are extensively used. These CAs shorten the longitudinal relaxation times (T_1) of the water molecules present in the tissues where they accumulate. The capability of a CA to shorten T_1 , and therefore increase the signal, is expressed in terms of the relaxivity r_1 .

A well-established, popular way of improving the relaxometric properties of Gd^{3+} complexes regards the use of amphiphilic molecules in order to make paramagnetic micelles and liposomes.^{4,5} The combination of high Gd^{3+} loadings (per aggregate) and low tumbling rates τ_R (global rotational motion) in these self-assembled nanostructures leads to enhanced contrast resulting from increased r_1 relaxivities.⁶ However, a major drawback of these supramolecular aggregates is their propensity to disaggregate once below the critical aggregation concentration (cac). Yet, one way of overcoming this issue while still benefitting from relaxivity enhancement is well-illustrated by the various nanoparticles that have successfully been encapsulated in micelles and liposomes of paramagnetic amphiphiles.⁷

Viral protein cages have attracted much attention as potential nanometer-sized carriers of CAs for MRI.⁸ To date, the highest (ionic) relaxivity measured is attributed to paramagnetic protein cages consisting of Gd^{3+} ions bound to the metal binding sites of cowpea chlorotic mottle virus (CCMV) capsids.⁹ In order to overcome Gd^{3+} release from such systems, both internal and external covalent functionalization with Gd^{3+} complexes

has been explored.¹⁰⁻¹⁷ However, covalent functionalization of viral capsids still remains a synthetically demanding task.

In this chapter, we work under the hypothesis that the undesired disaggregation of supramolecular aggregates (e.g., micelles) upon dilution can be overcome by means of confinement within CCMV capsid (i.e., protein cage without RNA inside). Herein, we describe the encapsulation of negatively charged paramagnetic micelles inside CCMV capsids by pure hierarchical self-assembly. As CCMV coat proteins (CPs) are known to self-assemble around negatively charged molecules,¹⁸⁻²¹ the negatively charged micelles are expected to trigger capsid CP self-assembly by acting as “nucleation points”, yielding stable protein cages (Figure 4.1).²² MRI was used to visualize the newly formed paramagnetic capsids.

Figure 4.1. Schematic representation of the self-assembly triggered by self-assembly process: micelles are formed when the monomer concentration is higher than the critical aggregation concentration (cac). The negatively charged micelles act as “nucleation points” for self-assembly of CCMV coat proteins (CPs), yielding stable paramagnetic and optically active protein cages. The localization and orientation of the ZnPc molecules within the micelles is only schematic.

4.2 Design and Synthesis

The building blocks of the paramagnetic micelles are Gd^{3+} complexes of the amphiphilic ligand 1,4,7,10-tetraza-1-(1-carboxymethylundecane)-4,7,10-triacetic acid cyclododecane (Gd^{3+} -DOTAC10, **6**), known to show enhanced r_1 relaxivities in its aggregated form (Figure 4.2).⁶ The (increased) local concentration effect due to cage confinement and the reduced molecular exchange of the micelles' building blocks inside the cage are envisaged to contribute to improved relaxivity properties, thereby allowing visualization by MRI. Moreover, the DOTAC10 ligand (**5**) was used to optically confirm encapsulation of the micelles within CCMV by means of entrapment of amphiphilic Zn^{2+} phthalocyanine dye molecules (ZnPc), whose amphiphilic nature favors its inclusion into the DOTAC10 micelles.

Figure 4.2. Chemical structure of Gd -DOTAC10 (**6**), DOTAC10 (**5**) and the amphiphilic ZnPc dye.

4.2.1 Synthesis of the Micelles' Building Blocks

The synthesis of the building blocks of the two types of micelles encapsulated in this study is shown in Scheme 4.1. The ligand 1,4,7,10-tetraaza-1-(1-carboxymethylundecane)-4,7,10-triacetic acid cyclododecane (DOTAC10, **5**) was synthesized as follows. First, *tert*-butyl alcohol, 4-(dimethylamino)pyridine (DMAP), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 2-

bromododecanoic acid (**1**) were allowed to react in CH_2Cl_2 for 2 h at 0°C and at room temperature overnight in order to Boc-protect the carboxylic acid of (**1**). Subsequently, compound (**4**) was obtained by alkylation of 1,4,7-tris(*tert*-butoxycarbonylmethyl)-1,4,7,10-tetraazacyclododecane (**3**) with (**2**) in dry acetonitrile using K_2CO_3 and KI (under N_2) for three days at 50°C . The DOTAC10 ligand (**5**) was obtained by means of Boc-deprotection of (**4**) using TFA/ CH_2Cl_2 at room temperature overnight. Finally, the Gd^{3+} complex of DOTAC10 (Gd^{3+} -DOTAC10, **6**) was prepared by adding one equivalent of GdCl_3 to a solution of (**5**) in water, adjusting the pH to 6 and stirring at 90°C for 1 day. The final product (**6**) was obtained with an overall yield (after purification) of 13 %.

Scheme 4.1. Synthetic route followed for the synthesis of DOTAC10 (**5**) and its Gd^{3+} -complex Gd^{3+} -DOTAC10 (**6**).

4.2.2 Encapsulation in CCMV

The building blocks of the micelles, in the form of the ligand DOTAC10 or its Gd^{3+} complex, were dissolved in milli-Q water to a final concentration of 0.04 M (5.5 times the cac^6 of Gd^{3+} -DOTAC10). The “optically active” DOTAC10/ZnPc micelles were formed by addition (drop wise) of 50 μL of a 5.5×10^{-6} M ZnPc solution in MeOH to **5**. The resulting solution was heated at 80°C for 2 minutes, allowed to cool down and refilled with water to the initial volume. Subsequently, the Gd^{3+} -DOTAC10 and DOTAC10/ZnPc micelle solutions were mixed with a 0.4 mM CCMV CP solution (pH 7.5, 50 mM Tris, 300 mM NaCl, 1 mM DTT) in a 1:1 ratio (v/v). In all solutions, the final ratio was 100 monomeric micelle units per CCMV CP. The mixtures were incubated for 30 min at 4°C and dialyzed to pH 5 for 12 h at the same temperature, in order to allow the CCMV CPs to self-assemble around the negatively charged micelles to form capsids. The non-encapsulated ZnPc molecules that precipitated out of solution were removed by centrifugation. The assembled capsids were purified by means of size exclusion chromatography (fast protein liquid chromatography, FPLC) and monitored at $\lambda = 280$ nm (characteristic of the CP).

4.3 Results and Discussion

4.3.1 Evidence for Capsid Formation and Encapsulation of Micelles

The newly formed, stable capsid assemblies eluted at 1.3 mL, whereas unassembled CCMV CPs eluted at 2.3 mL. In Figure 4.3a (left), which corresponds to the encapsulation of paramagnetic Gd^{3+} -DOTAC10 micelles, the elution peak at around 1.3 mL is only indicative of the formation of a protein cage, as the paramagnetic micelles do not absorb in the UV region. However, successful encapsulation of DOTAC10/ZnPc

micelles within CCMV under the previously described conditions was confirmed by the co-elution peak at 1.3 mL when monitoring at $\lambda = 682$ nm (Figure 4.3b, left). This absorption corresponds to encapsulated ZnPc molecules in DOTAC10 micelles (see Appendix 4.1). In addition, total reflection X-ray fluorescence (TXRF) measurements further confirmed the presence of Gd^{3+} and Zn^{2+} in both the encapsulated Gd^{3+} -DOTAC10 and DOTAC10/ZnPc micelles, respectively (Appendix 4.2, Table 4.1 and Table 4.3).

Figure 4.3. Size exclusion chromatograms (left) and transmission electron micrographs (TEM) (right) of: a) paramagnetic Gd^{3+} -DOTAC10 micelles encapsulated in the CCMV and b) optically active DOTAC10/ZnPc micelles encapsulated in the CCMV. The red trace is characteristic of the capsid CP (monitored at 280 nm), whereas the black trace is characteristic of the ZnPc dye (monitored at 682 nm). Elution volume of the protein cages ~ 1.3 mL. Insets of TEM images: histograms showing the respective diameter distributions of capsids. Scale bar: 50 nm.

TXRF is an excellent alternative to other elemental analysis techniques (e.g., inductively coupled plasma optical emission spectroscopy ICP-OES) as it involves simple sample preparation and requires very little amounts of material. Furthermore, recent studies have demonstrated the validity of this technique for determining Gd^{3+} concentrations in organic matrixes (urine samples), with variable limits of detection ($\text{LOD} = 100 \mu\text{g/L}$) and quantification ($\text{LOQ} = 330 \mu\text{g/L}$).²³ While our TXRF results do confirm the presence of Gd^{3+} in the capsid filled with Gd^{3+} -DOTAC10 micelles ($167 \mu\text{g/L} > \text{LOD}$), quantification is not possible as the Gd^{3+} concentration measured is below the previously reported LOQ. Most likely, the short lifetime of the Gd^{3+} -DOTAC10 micelles results in quick disaggregation. Since the non-aggregated monomers are lost through the pores of the capsid during dialysis, very low micelle encapsulation efficiencies are achieved. For the encapsulated DOTAC10/ZnPc micelles, where TXRF results show an average of 3 ZnPc dye molecules/micelles hosted in a single protein cage, higher encapsulation efficiency is observed. Tentatively, the dynamics and overall charge of the micelles appear to be altered by the inclusion of the ZnPc dye, possibly leading to more stable, easier to encapsulate aggregates.

The formation of stable protein cages was further confirmed by transmission electron microscopy (TEM). Additionally, these images were used to determine the size distribution of the newly formed protein cages (Appendix 4.3, Table 4.5). The results showed that for the CCMV encapsulated paramagnetic Gd^{3+} -DOTAC10 micelles, a mean size of $24.6 \pm 3 \text{ nm}$ was obtained (Figure 4.3a, right). The mean size for the CCMV encapsulated optically active DOTAC10/ZnPc micelles was calculated to be $24.3 \pm 3 \text{ nm}$ (Figure 4.3b, right). Both values are comparable to that of the capsid containing *only* DOTAC10 micelles ($24.8 \pm 3 \text{ nm}$, Appendix 4.3)

and smaller than $T = 3$ CCMV particles (28 nm).^{24,25} The observed size distribution, with an average value between 20 ± 2 nm (“pseudo $T = 2$ ”)^{22,26,27} and 28 nm ($T = 3$), suggests a mixture of filled and empty capsids.

4.3.2 Improved Properties by Means of Encapsulation of Amphiphilic Dyes

Encouraged by the successful encapsulation of the optically active DOTAC10/ZnPc micelles, we investigated a combination of the ZnPc dye with the Gd^{3+} -DOTAC10 monomers in detail (Figure 4.4a). This was done as described for DOTAC10/ZnPc micelles, but using Gd^{3+} -DOTAC10 micelles instead, with subsequent self-assembly of the CPs around them. FPLC results confirmed the formation of a protein cage eluting at 1.3 mL (Figure 4.4b). However, a much lower intensity of the trace recorded at 682 nm was observed when compared to the case of encapsulated DOTAC10/ZnPc micelles (Figure 4.3b, left). Surprisingly, TXRF measurements showed that, on average, a protein cage assembled under these conditions contained 15 dye molecules and 350 Gd^{3+} -DOTAC10 molecules (Appendix 4.2). Assuming that the micelles formed consist of at least 15 monomers (as previously reported for Gd^{3+} -DOTAC14 micelles),⁶ these data imply a maximum of 23 micelles encapsulated per capsid. Furthermore, the newly formed capsids appeared to be somewhat smaller than those containing each “functionality” alone, showing an average mean diameter of 22.4 ± 2 nm (Figure 4.4c). This value is only slightly larger than the previously reported values (20 ± 2 nm) corresponding to protein cages with “pseudo $T = 2$ ” architectures.^{22,26,27} Given the previously discussed assumption that a mixture of both “pseudo $T = 2$ ” and $T = 3$ particles are formed after the encapsulation of Gd^{3+} -DOTAC10 and DOTAC10/ZnPc micelles, the average mean diameter obtained for the

Gd³⁺-DOTAC10/ZnPc loaded micelles points out to the formation of more “pseudo T = 2” particles than in the former cases.

Figure 4.4. a) Schematic representation of the CCMV capsid containing Gd³⁺-DOTAC10/ZnPc micelles. b) Size exclusion chromatogram. The red trace is characteristic of the capsid CP (monitored at 280 nm), whereas the black trace is characteristic of the ZnPc dye (monitored at 682 nm). Elution volume of the protein cages ~ 1.3 mL. c) TEM image. Inset: Histogram showing the diameter distribution of capsids filled with Gd³⁺-DOTAC10/ZnPc micelles. Scale bar: 50 nm.

Noticeably, the initial observation suggesting that the encapsulation efficiency of DOTAC10-based micelles is affected by the presence of the ZnPc dye coincides with the previously presented TEM and TXRF data. Although our EM studies do not provide conclusive data on the actual filled/empty capsid ratio, the smaller size distribution found for the Gd³⁺-DOTAC10/ZnPc encapsulated micelles (Figure 4.4c), together with the significantly higher Gd³⁺ loadings found by TXRF analysis, points towards a considerably improved filled/empty capsid ratio. Moreover, TXRF confirmed the presence of the ZnPc dye and the affected optical properties observed upon encapsulation in Gd³⁺-DOTAC10 micelles might be a consequence of ZnPc dye stacking inside the micelles. Therefore, either a micelle consists of more than 15 Gd³⁺-DOTAC10 monomers, or the

ZnPc dye encapsulation efficiency of the micelles varies. Altogether, the encapsulation of the ZnPc dye seems to lead to a significant change in the overall surface charge of the Gd^{3+} -DOTAC10 micelles, which might be responsible for promoting a stronger interaction between the Gd^{3+} -DOTAC10 micelle surface and the CP, leading to smaller structures with (clearly) higher loadings.

4.3.3 Paramagnetic Properties of the Encapsulated Micelles

The encapsulation of paramagnetic micelles was confirmed by determining their relaxation efficiency using MRI. Here, the longitudinal relaxation times (T_1) of a series of dilutions (of known Gd^{3+} and capsid concentrations) were measured by means of a standard saturation recovery experiment at 600 MHz (14.1 T). By plotting the measured $1/T_1$ values as a function of Gd^{3+} or capsid concentration, the relaxivity r_1 ($\text{mM}^{-1} \text{s}^{-1}$) was obtained as the slope of the curve (Appendix 4.4). For comparison purposes, the ionic r_1 of a 45 μM solution of the free Gd^{3+} -DOTAC10 complex ($[\text{Gd}^{3+}\text{-DOTAC10}] < \text{cac}$) was also calculated ($2.52 \text{ mM}^{-1} \text{s}^{-1}$, Figure 4.5). Owing to the impossible quantification of the Gd^{3+} concentration of the encapsulated paramagnetic Gd^{3+} -DOTAC10 micelles, any changes in the global rotational motion of Gd^{3+} -DOTAC10 monomers upon micellization inside the capsid are not properly assessable.

Figure 4.5. Relaxivity plots at 600 MHz and 25°C of the Gd³⁺-DOTAC10 complex (**6**, $r_1 = 2.52 \text{ mM}^{-1} \text{ s}^{-1}$).

A decrease in the tumbling rate τ_R of the complex due to micellization inside the capsid⁴⁻⁶ is confirmed by the calculated ionic r_1 value for the Gd³⁺-DOTAC10/ZnPc encapsulated micelles ($3.85 \text{ mM}^{-1} \text{ s}^{-1}$, Figure 4.6a), which is higher than that obtained for the free Gd³⁺-DOTAC10 complex in solution ($2.52 \text{ mM}^{-1} \text{ s}^{-1}$). This ionic r_1 value is comparable to previously reported values (measured at high magnetic field strengths) involving covalently attached Gd³⁺-DOTA complexes to wild-type cowpea mosaic virus (CPMV, $3\text{--}4.4 \text{ mM}^{-1} \text{ s}^{-1}$ at 500 MHz).¹⁵ Nevertheless, making a fair comparison between our “paramagnetic capsid” and previously reported values¹⁵ is not straightforward as the enhancement shown by the latter systems is related to the slower tumbling rate of the Gd³⁺ complexes due to *covalent attachment* to the protein cage. In our case, as the micellar structures are (non-covalently) encased inside the capsid, we believe that the differences in relaxivity can be attributed to the altered dynamics and molecular exchange kinetics of the micelle monomers as a result of capsid confinement (e.g., decreased intermicellar distance).^{30,3128,29} Moreover, the dynamic behavior of the Gd³⁺-DOTAC10 micelles might vary as the micelle

robustness and properties change upon encapsulation of the ZnPc dye. Likewise, the higher flexibility of the *non-covalently* attached paramagnetic moieties can also contribute to high relaxivity values, as recent studies suggest that (at high magnetic fields) some degree of flexibility of the Gd^{3+} chelate is vital.³⁰

Figure 4.6. Relaxivity plot of capsid filled with Gd^{3+} -DOTAC10/ZnPc micelles at 600MHz and 25°C: a) based on Gd^{3+} concentration ($r_1 = 3.85 \text{ mM}^{-1} \text{ s}^{-1}$) and b) based on capsid concentration ($r_1 = 1352 \text{ mM}^{-1} \text{ s}^{-1}$).

With respect to the capsid r_1 value for encapsulated Gd^{3+} -DOTAC10/ZnPc micelles ($1352 \text{ mM}^{-1} \text{ s}^{-1}$, Figure 4.6b), the observed enhancement is attributed to the high local Gd^{3+} concentration (confirmed by TXRF) that can be achieved upon encapsulation. Owing to the high amount of Gd^{3+} encapsulated, a decreased ionic r_1 relaxivity is expected as studies on Gd^{3+} -chelate micellar systems have shown that decreased distances between Gd^{3+} -monomers affect the electronic relaxation times.³¹ Therefore, although we are not able to quantify the Gd^{3+} payload of the protein cage encapsulated Gd^{3+} -DOTAC10 micelles, their r_1 value must be higher than $3.85 \text{ mM}^{-1} \text{ s}^{-1}$ (Gd^{3+} -DOTAC10/ZnPc micelles).

4.3.4 Visualization of Encapsulated Micelles by MRI

The encapsulation of paramagnetic Gd^{3+} -DOTAC10 and Gd^{3+} -DOTAC10/ZnPc micelles is illustrated in the T_1 -weighted MR image shown in Figure 4.7. The aggregation inside the capsid of the paramagnetic micelles is demonstrated by the higher contrast both show at low concentrations of Gd^{3+} and far below the cac of Gd^{3+} -DOTAC10 in solution.⁶ Also, the encapsulation of paramagnetic Gd-DOTAC10 micelles ($< 1.1 \mu\text{M}$ Gd^{3+} concentration) is evidenced by the higher contrast it shows when compared to Gd^{3+} -DOTAC10 in solution at higher concentrations ($45 \mu\text{M}$ Gd^{3+}) and their differences in signal-to-noise ratio (83 vs. 65, respectively).

Figure 4.7. T_1 -weighted MR image of encapsulated paramagnetic Gd^{3+} -DOTAC10 and Gd^{3+} -DOTAC10/ZnPc micelles at 600 MHz ($TE = 6$ ms, $TR = 500$ ms). For direct comparison, the contrast given by capsid buffer (50 mM sodium acetate, 1 M sodium chloride and 1 mM sodium azide, pH 5) and a solution containing Gd^{3+} -DOTAC10, at the highest Gd^{3+} concentration measured ($45 \mu\text{M}$), are also shown.

Whereas the encapsulation of Gd^{3+} -DOTAC10 micelles in CCMV is clearly less efficient, inclusion of the ZnPc dye in these micelles leads to improved ionic r_1 relaxivities and high capsid loadings, as shown by the MR image in Figure 4.7.

4.4 Conclusions

In summary, we report the MRI visualization of encapsulated paramagnetic micelles inside protein cages. T_1 -weighted MR images and relaxivity curves showed that, upon encapsulation of Gd^{3+} -DOTAC10 micelles within the CCMV capsid, enhanced r_1 values are obtained. This enhancement is attributed to i) aggregation resulting from local confinement inside the protein cage and ii) altered micelle association kinetics probably due to a decreased intermicellar distance. The higher payloads, altered size distribution and clear enhancement of the ionic r_1 values of encapsulated Gd^{3+} -DOTAC10/ZnPc micelles show that the encapsulation of these “combined” micelles is more successful than that of only Gd^{3+} -DOTAC10 micelles. Furthermore, these paramagnetic protein cages might show therapeutically relevant properties, since ZnPc dyes are known for their use in photodynamic therapy.^{18,32,33} Our approach is based on hierarchical self-assembly and offers a simple, fast, synthetically straightforward methodology that can be extended to micelles with other dyes for (multimodal) optical imaging. While little is known about the behavior of these new protein cages at physiological pH, the present work is a first step towards the potential use of self-assembled nanocages *in vivo*.

4.5 Experimental

Materials. Commercial grade reagents were purchased from Sigma Aldrich and TCI Europe and were used without further purification unless otherwise stated. Solvents were dried over molecular sieves. Deuterated solvents were purchased from Cambridge Isotope Laboratories and NMR and MRI tubes from Wilmad LabGlass. All chemicals used for the preparation of buffers were of analytical quality and were prepared in ultrapure (Milli-Q) water.

2-Bromododecanoic acid *tert*-butyl ester (2). This compound was synthesized following an adapted procedure.³⁴ 2-bromododecanoic acid (1) (2 g, 7.1 mmol), 4-(dimethylamino)pyridine (0.44 g, 3.6 mmol) and *tert*-butanol (0.6 g, 8.1 mmol) were dissolved in 40 mL dry CH₂Cl₂ and cooled in an ice bath. Subsequently, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (1.5 g, 7.8 mmol) was added and the reaction mixture was stirred at 0°C for 2 h and at room temperature overnight. The solution was washed with saturated sodium bicarbonate (2 x 60 mL), water (3 x 60 mL), and dried over MgSO₄. The organic phase was passed through a layer of silica and the solvent was removed under reduced pressure. The desired compound was obtained as a colorless liquid after chromatography on silica gel 60 (hexane/EtOAc 20:1, later 9:1). Yield: 34 % (810 mg, 2.4 mmol). ¹H NMR (600 MHz, CDCl₃, 25°C, TMS): δ (ppm): 4.13 (t, *J*(H,H) = 7.3, 1H; CHBr), 2.04-1.95 (m, 2H; CHBr-CH₂), 1.5 [s, 9H; C(CH₃)₃], 1.28 (m, 16H; CH₂), 0.9 (t, *J*(H,H) = 6.8, 3H; CH₃). ¹³C NMR (150 MHz, CDCl₃, 25°C, TMS) δ (ppm): 169 (COOtBu), 82.2 [C(CH₃)₃], 47.9 (HCB), 34.9 (HCB-CH₂), 31.9 (HCB-CH₂-CH₂), 29.5, 29.5, 29.4, 29.3,

28.9, 27.3, 22.7 (CH_2), 27.8 [$\text{C}(\text{CH}_3)_3$], 14.1 (CH_3). MS (ESI+, $\text{CH}_2\text{Cl}_2/\text{MeOH}$): m/z : 257.5 [$\text{M} - \text{Br} + \text{H}$]⁺, 301.6, (fragm), 311.8 (fragm), 357.8 [$\text{M} + \text{Na}$]⁺.

1,4,7,10-Tetraaza-1-(1-carboxy-*tert*-butoxymethylundecane)-

4,7,10-triacetic acid *tert*-butyl ester cyclododecane (4). In a three-neck round-bottom flask, K_2CO_3 (370 mg, 2.7 mmol) and KI (catalytic amount) were added to a stirring solution of 1,4,7-tris(*tert*-butoxycarboxymethyl)-1,4,7,10-tetraazacyclododecane **3** (DO3A, synthesized as previously described³⁵) (460 mg, 0.9 mmol) in 40 mL dry acetonitrile. The mixture was kept under N_2 for 10 minutes under vigorous stirring. Afterwards, 2-bromododecanoic acid *tert*-butyl ester (**2**) (300 mg, 0.9 mmol) dissolved in 10 mL dry CH_2Cl_2 was added slowly to the reaction mixture which was, subsequently, allowed to react for 3 days at 50°C. The mixture was left to cool down, filtered to remove inorganic salts and the solvent was evaporated under reduced pressure. The desired compound was obtained as a yellow oil after chromatography on silica ($\text{CH}_2\text{Cl}_2/\text{EtOH}$ 9:1, later EtOH/NH_3 95:5). Yield: 37 % (253 mg, 0.3 mmol). ^1H NMR (600 MHz, CDCl_3 , 25°C, TMS) δ (ppm): 3.67 (dd, 1H; CHN), 3.5-2 (m, 22H; CHN- CH_2 , $\text{CH}_2\text{-COOtBu}$), 1.58 (m, 2H; CHN- CH_2), 1.46-1.44 (s, 36H; $\text{C}(\text{CH}_3)_3$), 1.25 (m, 16H; CH_2), 0.88 (t, $J(\text{H,H}) = 6.9$, 3H; CH_3). ^{13}C NMR (150 MHz, CDCl_3 , 25°C, TMS) δ (ppm): 173, 172.9, (COOtBu), 81.6, 81.7 [$\text{C}(\text{CH}_3)_3$], 70.6 (HCNCH_2), 61.2, 55.9, 55.6, 55.3, 53.4, 52.5, 48.9, 48.5, 47.3, 45 (HCNCH_2 , NCH_2 , CH_2COOtBu), 31.9, 31.92, 31, 29.7, 29.7, 29.6, 29.4, 29.4, 29.3, 29.1, 27.94, 27.92, 27.9, 27.8 [CH_2 , $\text{C}(\text{CH}_3)_3$], 14.1 (CH_3). MS (ESI+, $\text{CH}_2\text{Cl}_2/\text{MeOH}$): m/z : 791.4 [$\text{M}^+ + \text{Na}$].

1,4,7,10-Tetraaza-1-(1-carboxymethylundecane)-4,7,10-triacetic acid cyclododecane (DOTAC10, 5). In a round-bottom flask, TFA

(4 mL, 52 mmol) was added to a solution of **4** (100 mg, 0.13 mmol) in 4 mL CH_2Cl_2 and the reaction mixture was allowed to stir at room temperature overnight. The mixture was evaporated under reduced pressure and the residue co-evaporated with CH_2Cl_2 3 times in order to remove excess TFA. **5** was obtained as a solid after precipitation from diethyl ether and it was subsequently used for the next reaction. Yield: 98% (69 mg, 0.127 mmol). MS (ESI+, $\text{H}_2\text{O}/\text{ACN} + 0.1\% \text{TFA}$): m/z : 545.4 $[\text{M}^+ + \text{H}]$.

Gd³⁺-1,4,7,10-Tetraaza-1-(1-carboxymethylundecane)-4,7,10-triacetic acid cyclododecane (Gd³⁺-DOTAC10, 6). In a round-bottom flask, **5** (40 mg, 0.073 mmol) and GdCl_3 (19 mg, 0.073 mmol) were dissolved in 10 mL water. The pH of the mixture was adjusted to 6 using NaOH (1M) and the reaction mixture was allowed to stir at 90°C overnight. The solution was allowed to cool for 30 minutes and it was subsequently stirred for 10 minutes with (previously washed with acetone) DOWEX Monosphere 650C cation exchange resin. After filtering in order to remove the resin, the absence of free Gd^{3+} ions was confirmed by the xylenol orange test. Compound **6** was obtained after the solvent was evaporated under reduced pressure. Yield: 99% (50 mg, 0.072 mmol). MS (MALDI-, β -indoleacrylic acid³⁶): m/z : 698.217 $[\text{M}^-]$. The BMS shift for a 100 μM of Gd^{3+} -DOTAC10 was 0.012 ppm (theoretical value = 0.0109 ppm).

Zinc (II) 2(3),9(10),16(17)-tri-tert-butyl-23(24)-(2-trimethylaminoethylsulfanyl)phthalocyaninato (2-)-N²⁹, N³⁰, N³¹, N³² iodide (ZnPc dye). PhD thesis of Eduardo Anaya-Plaza, Universidad Autónoma de Madrid.

CCMV Synthesis and Characterization. Purification of CCMV, removal of RNA and isolation of the CP were carried out according to literature procedures.^{37,38} The purity of the viral CP was characterized by fast protein liquid chromatography (FPLC; elution volume of CP = 2.3 mL) and UV-Vis absorbance spectroscopy.

Nuclear Magnetic Resonance (NMR) Characterization. All NMR experiments were performed on a Bruker Avance II NMR spectrometer. Chemical shifts are given in ppm using the residual solvent signal as reference. The multiplicity of the peaks is reported using the following abbreviations: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. All NMR spectra were processed using TOPSPIN 2.1 and MestReC software packages. Quality of the Gd³⁺-DOTAC10 complex was assessed by measuring the Bulk Magnetic Susceptibility (BMS) shift (Δ_x) of a 100 μ M solution of the complex (measured according to literature procedures³⁹) and comparing it to the theoretical BMS shift given by:

$$\Delta_x = \frac{4\pi c s}{T} \left(\frac{\mu_{eff}}{2.84} \right)^2 \times 10^3$$

Where c is the concentration of the paramagnetic solute in mol/L, s is dependent on the shape of the sample ($s=1/3$ for a cylinder parallel to the main field), T is the absolute temperature (in K) and μ_{eff} is the effective magnetic moment for Gd³⁺ (7.94⁴⁰).

Mass Spectrometry (MS). Mass spectra were acquired on a Waters Micromass LCT ESI mass spectrometer, an Applied Biosystems QSTAR ESI mass spectrometer, a Bruker Ultraflex III MALDI-TOF mass spectrometer and a Voyager-DE RP mass spectrometer.

UV-Vis Spectroscopy. UV spectra were acquired on a Perkin Elmer Lambda 850 UV-Vis spectrometer, a Jasco V-660 spectrometer and a NanoDrop 1000 spectrophotometer from Thermo Scientific.

Fast Protein Liquid Chromatography (FPLC). Size exclusion chromatography was performed using the following buffers: 50 mM sodium acetate, 1 M sodium chloride and 1 mM sodium azide (pH 5). The FPLC was equipped with a Superose 6 size exclusion column 10/100 GL (GE Healthcare) with a bed volume of 2.4 mL. For each run, the injection volume was 100 μ L.

Transmission Electron Microscopy (TEM). TEM images were obtained using analytical TEM FEI instruments. In order to prepare the samples, 5 μ L of the desired sample was applied onto Formvar-carbon coated grids (from electron microscopy sciences). After leaving the sample for 1 minute, the excess of liquid was removed using a piece of filter paper. Uranyl acetate (5 mL, 1% w/v) was then applied and the drying procedure was repeated. The size distribution of capsids was determined by measuring their diameter, for more than 200 counts, from TEM images.

Total Reflection X-ray Fluorescence (TXRF). TXRF measurements were performed by a Bruker S2 Picofox TXRF (Bruker AXS GmbH, Karlsruhe, Germany) portable system. The primary X rays were generated by an air-cooled low power X-ray tube using a molybdenum anode. The excitation settings were 50 kV and 750 mA. Measurements were performed by signal integration over 1500 seconds. Quartz glass disks were used as sample carriers, which were previously cleaned in 1 N nitric

acid followed by flushing in water for 1 h at 80°C. After that, they were dried, coated with silicon solution for better wettability and dried again. Before measuring the samples, the quartz glass sample carriers were analyzed to ensure the absence of Gd^{3+} contamination. The quantification is performed by the Bruker S2 Picofox TXRF software (Spectra, Version 6.1.5.0), based on the known quantity of the gallium internal standard added. Samples for TXRF analysis were prepared by adding equal amounts of the gallium internal standard (10 mg/L) and the sample to be analyzed (volume). After vortexing, a drop of the solution was placed on the quartz disk and dried under vacuum for 15 minutes. For control purposes, elemental analysis of the empty capsid (in buffer) was conducted and the values obtained, for each capsid sample in question, were corrected.

Magnetic Resonance Imaging (MRI). All experiments were carried out on a 600 MHz (14.1 T) Avance II NMR Spectrometer from Bruker (Karlsruhe, Germany) equipped with a vertical narrow bore magnet (14.1 T), a great B_0 compensation unit (BGU-II) and 3 great 1/60 amplifier units (X, Y and Z) also from Bruker. A micro 5 imaging probe, equipped with a 10 mm diameter saddle coil insert from Bruker (Karlsruhe, Germany) was used. The maximum gradient strength was 4.8 G/cm/A and the temperature inside the probe was kept at ~ 25°C by a gradient water cooling unit BCU20 from Bruker. All NMR/MRI tubes (5/10 mm diameter) were purchased from Wilmad Labglass (New Jersey, U.S.A.). Glass capillary tubes (melting point tubes, inner diameter 1mm) used for MRI were purchased from VWR. T_1 measurements were performed in a 1 cm (outer diameter) glass tube as follows. Stock solutions of the Gd^{3+} -DOTA complex and the encapsulated Gd^{3+} -DOTA and Gd^{3+} -DOTA/ZnPc micelles (with known concentrations of Gd^{3+} and capsid) were diluted 2, 5 and 10

times. For each experiment, capillary tubes of 1 mm inner diameter were filled with these solutions, placed in a custom-made Kel-F capillary holder and subsequently introduced in the 1 cm tube for imaging. Longitudinal relaxation times were measured using a saturation recovery pulse sequence with static TE = 6 ms and 16 TR values, namely 100, 150, 200, 350, 500, 600, 700, 800, 1000, 1500, 2000, 3000, 4000, 6000, 8000, 10000 ms. The measurements were done on 5 subsequent axial slices of 1mm, with a field of view (FOV) of 10 mm x 10 mm, a matrix size of 128 x 128 and 2 averages. T_1 values were obtained by mono-exponentially fitting the intensities of selected regions of interest (ROIs) for each axial slice using the image sequence analysis tool in Paravision 4.0 software. T_1 -weighted images were obtained using a spin echo sequence with the following parameters: TR = 500 ms, TE = 6 ms, 1 axial slice of 6 mm thickness, FOV = 10 mm x 10 mm, matrix size = 256 x 256 and 10 averages. The longitudinal relaxivity r_1 (in mM⁻¹ s⁻¹) was obtained as the slope of the plot $1/T_1$ vs. Gd³⁺ (or capsid) concentration:

$$\left(\frac{1}{T_1}\right)_{obs} = \left(\frac{1}{T_1}\right)_d + r_1[Gd]$$

4.6 References

- (1) Cassidy, P. J.; Radda, G. K. *J. Royal Soc. Interface.* **2005**, *2*, 133.
- (2) Weissleder, R.; Mahmood, U. *Radiology* **2001**, *219*, 316.
- (3) Caravan, P.; Ellison, J. J.; McMurry, T. J.; Lauffer, R. B. *Chem. Rev.* **1999**, *99*, 2293.
- (4) Accardo, A.; Tesauro, D.; Aloj, L.; Pedone, C.; Morelli, G. *Coord. Chem. Rev.* **2009**, *253*, 2193.
- (5) Mulder, W. J. M.; Strijkers, G. J.; van Tilborg, G. A. F.; Griffioen, A. W.; Nicolay, K. *NMR Biomed.* **2006**, *19*, 142.
- (6) Nicolle, G. M.; Tóth, É.; Eisenwiener, K.-P.; Mäcke, H. R.; Merbach, A. E. *J. Biol. Inorg. Chem.* **2002**, *7*, 757.

- (7) Mulder, W. J. M.; Strijkers, G. J.; van Tilborg, G. A. F.; Cormode, D. P.; Fayad, Z. A.; Nicolay, K. *Acc. Chem. Res.* **2009**, *42*, 904.
- (8) Werner, E. J.; Datta, A.; Jocher, C. J.; Raymond, K. N. *Angew. Chem., Int. Ed.* **2008**, *47*, 8568.
- (9) Allen, M.; Bulte, J. W. M.; Liepold, L.; Basu, G.; Zywicke, H. A.; Frank, J. A.; Young, M.; Douglas, T. *Magn. Reson. Med.* **2005**, *54*, 807.
- (10) Datta, A.; Hooker, J. M.; Botta, M.; Francis, M. B.; Aime, S.; Raymond, K. N. *J. Am. Chem. Soc.* **2008**, *130*, 2546.
- (11) Garimella, P. D.; Datta, A.; Romanini, D. W.; Raymond, K. N.; Francis, M. B. *J. Am. Chem. Soc.* **2011**, *133*, 14704.
- (12) Hooker, J. M.; Datta, A.; Botta, M.; Raymond, K. N.; Francis, M. B. *Nano Lett.* **2007**, *7*, 2207.
- (13) Liepold, L.; Anderson, S.; Willits, D.; Oltrogge, L.; Frank, J. A.; Douglas, T.; Young, M. *Magn. Reson. Med.* **2007**, *58*, 871.
- (14) Liepold, L. O.; Abedin, M. J.; Buckhouse, E. D.; Frank, J. A.; Young, M. J.; Douglas, T. *Nano Lett.* **2009**, *9*, 4520.
- (15) Prasuhn, J. D. E.; Yeh, R. M.; Obenaus, A.; Manchester, M.; Finn, M. G. *Chem. Comm.* **2007**, 1269.
- (16) Anderson, E. A.; Isaacman, S.; Peabody, D. S.; Wang, E. Y.; Canary, J. W.; Kirshenbaum, K. *Nano Lett.* **2006**, *6*, 1160.
- (17) Pokorski, J. K.; Breitenkamp, K.; Liepold, L. O.; Qazi, S.; Finn, M. G. *J. Am. Chem. Soc.* **2011**, *133*, 9242.
- (18) Brasch, M.; de la Escosura, A. s.; Ma, Y.; Uetrecht, C.; Heck, A. J. R.; Torres, T.; Cornelissen, J. J. L. M. *J. Am. Chem. Soc.* **2011**, *133*, 6878.
- (19) de la Escosura, A.; Janssen, P. G. A.; Schenning, A. P. H. J.; Nolte, R. J. M.; Cornelissen, J. J. L. M. *Angew. Chem., Int. Ed.* **2010**, *49*, 5335.
- (20) Minten, I. J.; Ma, Y.; Hempenius, M. A.; Vancso, G. J.; Nolte, R. J. M.; Cornelissen, J. J. L. M. *Org. Biomol. Chem.* **2009**, *7*, 4685.
- (21) Sikkema, F. D.; Comellas-Aragones, M.; Fokkink, R. G.; Verduin, B. J. M.; Cornelissen, J. J. L. M.; Nolte, R. J. M. *Org. Biomol. Chem.* **2007**, *5*, 54.
- (22) Kwak, M.; Minten, I. J.; Anaya, D.-M.; Musser, A. J.; Brasch, M.; Nolte, R. J. M.; Müllen, K.; Cornelissen, J. J. L. M.; Herrmann, A. *J. Am. Chem. Soc.* **2010**, *132*, 7834.
- (23) Telgmann, L.; Holtkamp, M.; Kunнемeyer, J.; Gelhard, C.; Hartmann, M.; Klose, A.; Sperling, M.; Karst, U. *Metallomics* **2011**, *3*, 1035.
- (24) Reddy, V. S.; Natarajan, P.; Okerberg, B.; Li, K.; Damodaran, K. V.; Morton, R. T.; Brooks, C. L.; Johnson, J. E. *J. Virol.* **2001**, *75*, 11943.
- (25) Speir, J. A.; Munshi, S.; Wang, G.; Baker, T. S.; Johnson, J. E. *Structure* **1995**, *3*, 63.
- (26) Cadena-Nava, R. D.; Hu, Y.; Garmann, R. F.; Ng, B.; Zelikin, A. N.; Knobler, C. M.; Gelbart, W. M. *J. Phys. Chem. B* **2011**, *115*, 2386.
- (27) Aniagyei, S. E.; Kennedy, C. J.; Stein, B.; Willits, D. A.; Douglas, T.; Young, M. J.; De, M.; Rotello, V. M.; Srisathiyarayanan, D.; Kao, C. C.; Dragnea, B. *Nano Lett.* **2009**, *9*, 393.
- (28) Aniansson, E. A. G.; Wall, S. N.; Almgren, M.; Hoffmann, H.; Kielmann, I.; Ulbricht, W.; Zana, R.; Lang, J.; Tondre, C. *J. Phys. Chem.* **1976**, *80*, 905.
- (29) Patist, A.; Oh, S. G.; Leung, R.; Shah, D. O. *Colloids Surf., A* **2001**, *176*, 3.

- (30) Ferreira, M. F.; Mousavi, B.; Ferreira, P. M.; Martins, C. I. O.; Helm, L.; Martins, J. A.; Geraldès, C. F. G. C. *Dalton Trans.* **2012**, 41, 5472.
- (31) Nicolle, G. M.; Helm, L.; Merbach, A. E. *Magn. Reson. Chem.* **2003**, 41, 794.
- (32) Jori, G.; Coppellotti, O. *Anti-Infect. Agents Med. Chem.* **2007**, 6, 119.
- (33) O'Connor, A. E.; Gallagher, W. M.; Byrne, A. T. *Photochem. Photobiol.* **2009**, 85, 1053.
- (34) Dhaon, M. K.; Olsen, R. K.; Ramasamy, K. *J. Org. Chem.* **1982**, 47, 1962.
- (35) Beeby, A.; Bushby, L. M.; Maffeo, D.; Gareth Williams, J. A. *J. Chem. Soc., Dalton Trans.* **2002**, 48.
- (36) Vetterlein, K.; Bergmann, U.; Büche, K.; Walker, M.; Lehmann, J.; Linscheid, M. W.; Scriba, G. K. E.; Hildebrand, M. *Electrophoresis* **2007**, 28, 3088.
- (37) Comellas-Aragones, M.; Engelkamp, H.; Claessen, V. I.; Sommerdijk, N. A. J. M.; Rowan, A. E.; Christianen, P. C. M.; Maan, J. C.; Verduin, B. J. M.; Cornelissen, J. J. L. M.; Nolte, R. J. M. *Nat. Nanotechnol.* **2007**, 2, 635.
- (38) Verduin, B. J. M. *FEBS Lett.* **1974**, 45, 50.
- (39) Corsi, D. M.; Platas-Iglesias, C.; Bekkum, H. v.; Peters, J. A. *Magn. Reson. Chem.* **2001**, 39, 723.
- (40) Peters, J. A.; Huskens, J.; Raber, D. J. *Prog. Nucl. Magn. Reson. Spectrosc.* **1996**, 283.

Appendix 4.1. Encapsulation of ZnPc Dye

In order to study the behavior (in aqueous solution) of the ZnPc dye, absorption spectra in the absence and presence of ligand **5** were measured. This was done by adding (drop wise) 50 μL of a 5.5×10^{-6} M ZnPc solution in MeOH to 1 mL of water and to a 0.04 M 1 mL solution of **5**. After addition of the dye, both solutions were heated for 2 minutes, allowed to cool down and refilled to 1 mL with water. While a (blue) precipitate was observed in the solution containing only water, the solution containing **5** remained colored (blue) after treatment. This last observation indicates the encapsulation of one ZnPc dye per DOTAC10 micelle, as the optical properties of the ZnPc dye are affected when forming π -stacked aggregates in water. Spectroscopic evidence for this observation is shown in Figure 4.8; in the presence of ligand **5** (black line) a strong absorption peak at around 682 nm appears, while in the absence of **5** (grey line) very weak absorption is observed.

Figure 4.8. UV absorption spectrum of ZnPc before (grey line) and after (black line) encapsulation in a 0.04 M aqueous solution of ligand **5**.

Appendix 4.2. TXRF Data, Capsid Concentration and Encapsulation Efficiency Calculations

Table 4.1. Capsid, Zn^{2+} and Gd^{3+} concentrations for all samples

Protein cage filled with micelles of	[capsid] (nM)	[Zn^{2+}] mg/L / [Gd^{3+}] mg/L*	# Zn^{2+} atoms per capsid / [Zn^{2+}] mM	# Gd^{3+} atoms per capsid / [Gd^{3+}] mM
Gd³⁺-DOTAC10	364	---/0.167	---	3 / 0.001062
ZnPc	125	0.075 (corrected value 0.026)/---	3 / 0.0003975	---
Gd³⁺-DOTAC10/ZnPc (non-diluted)	1022	1.048 (corrected value 0.99)/56.471	15 / 0.015	350 / 0.359
Gd³⁺-DOTAC10/ZnPc**	128	0.124/7.059	15 / 0.0019	350 / 0.0449

* Values as shown in Tables S2, S3 and S4. As Zn^{2+} was detected in capsid buffer, these values are corrected

**This sample was diluted 8 times (after elemental analysis) for MRI/relaxivity measurements

Table 4.2. TXRF analysis data of the capsid filled with Gd^{3+} -DOTAC10 micelles

Element	Line	Energy (keV)	Net	Backgr	σ	[C] (mg/l)	σ [C] (mg/l)	LLD (mg/l)
Cl	K12	2.622	19620461	134674	4460	1100354	10.624	0.617
K	K12	3.314	1232	97895	444	0.29	0.105	0.221
Ca	K12	3.692	18824	96328	460	3.182	0.08	0.157
Fe	K12	6.405	9400	94935	446	0.35	0.017	0.034
Ga	K12	9.251	585008	95556	881	10	0.022	0.016
Ga	L1	1.098	73072	203406	693	---	---	---
Br	K12	11.924	262532	130632	724	3.221	0.011	0.013
Br	L1	1.481	10911	192045	628	---	---	---
Gd	L1	6.053	2815	91840	432	0.167	0.026	0.054

Table 4.3. TXRF analysis data of the capsid filled with DOTAC10/ZnPc micelles

Element	Line	Energy (keV)	Net	Backgr	σ	[C] (mg/l)	σ [C] (mg/l)	LLD (mg/l)
Cl	K12	2.622	10574625	57564	3270	11742.05	14.471	0.799
K	K12	3.314	2843	39852	287	1.326	0.135	0.279
Ca	K12	3.692	4061	38274	284	1.359	0.096	0.196
Fe	K12	6.405	2219	38538	282	0.163	0.021	0.043
Zn	K12	8.637	1922	38133	280	0.075	0.011	0.023
Ga	K12	9.251	295465	39068	611	10	0.03	0.02
Ga	L1	1.098	28078	89470	455	---	---	---
Br	K12	11.924	46203	51933	387	1.122	0.01	0.017
Br	L1	1.481	1	84976	412	---	---	---

Table 4.4. TXRF analysis data of the capsid filled with Gd³⁺-DOTAC10/ZnPc micelles

Element	Line	Energy (keV)	Net	Backgr	σ	[C] (mg/l)	σ [C] (mg/l)	LLD (mg/l)
Cl	K12	2.622	15265615	55336	3921	22817.842	73.901	1.055
K	K12	3.314	4536	35447	275	2.847	0.181	0.354
Ca	K12	3.692	9874	34884	282	4.449	0.14	0.252
Fe	K12	6.405	1884	36742	275	0.187	0.028	0.057
Zn	K12	8.637	20051	36377	305	1.048	0.019	0.03
Ga	K12	9.251	219495	35924	540	10	0.054	0.026
Ga	L1	1.098	32703	87820	456	---	---	---
Br	K12	11.924	93549	45807	430	3.059	0.023	0.021
Br	L1	1.481	3628	84274	415	---	---	---
Gd	L1	6.053	358050	35163	655	56.471	0.272	0.089

Figure 4.9. TXRF spectrum of the capsid filled with Gd^{3+} -DOTAC10/ZnPc micelles.

DOTAC10/ZnPc Encapsulation

Calculations based on a 1 mL sample:

$$\begin{aligned} \# \text{ capsids} &= 1 \text{ mL solution} \times \frac{125 \text{ nmol}}{\text{L}} \times \frac{1 \text{ mol}}{10^9 \text{ nmol}} \times \frac{6.023 \times 10^{23} \text{ capsids}}{1 \text{ mol}} \times \frac{1 \text{ L}}{1000 \text{ mL}} \\ &= 7.53 \times 10^{13} \end{aligned}$$

ZnPc dyes

$$\begin{aligned} &= 1 \text{ mL solution} \times \frac{0.0003975 \text{ mmol Zn}^{2+}}{\text{L}} \times \frac{1 \text{ mol}}{1000 \text{ mmol}} \times \frac{6.023 \times 10^{23} \text{ atoms Zn}^{2+}}{1 \text{ mol}} \times \frac{1 \text{ L}}{1000 \text{ mL}} \\ &= 2.39 \times 10^{14} \end{aligned}$$

Assuming that 1 dye is encapsulated by one micelle:

$$\# \text{ micelles} = \# \text{ ZnPc dyes} = 2.39 \times 10^{14}$$

$$\# \text{ Zn}^{2+} \text{ per capsid} = \frac{2.39 \times 10^{14} \text{ Zn}^{2+} \text{ dyes}}{7.53 \times 10^{13} \text{ capsids}} = 3 \text{ Zn}^{2+} \text{ dyes capsid}$$

Based on these numbers, on average, **three** micelles are present in **one** (T = 3) capsid.

Gd³⁺-DOTAC10/ZnPc Encapsulation

Calculations based on a 1 mL sample:

monomers

$$= 1 \text{ mL solution} \times \frac{0.0449 \text{ mmol Gd}^{3+}}{L} \times \frac{1 \text{ mol}}{1000 \text{ mmol}} \times \frac{6.023 \times 10^{23} \text{ atoms Gd}^{3+}}{1 \text{ mol}} \times \frac{1 L}{1000 \text{ mL}}$$

$$= 2.7 \times 10^{16}$$

ZnPc dyes

$$= 1 \text{ mL solution} \times \frac{0.00191 \text{ mmol Zn}^{2+}}{L} \times \frac{1 \text{ mol}}{1000 \text{ mmol}} \times \frac{6.023 \times 10^{23} \text{ atoms Zn}^{2+}}{1 \text{ mol}} \times \frac{1 L}{1000 \text{ mL}}$$

$$= 1.15 \times 10^{15}$$

$$\# \text{ micelles} = 2.7 \times 10^{16} \text{ monomers} \times \frac{1 \text{ micelle}}{15 \text{ monomers}} = 1.8 \times 10^{15}$$

$$\# \text{ capsids} = 1 \text{ mL solution} \times \frac{128 \text{ nmol}}{L} \times \frac{1 \text{ mol}}{10^9 \text{ nmol}} \times \frac{6.023 \times 10^{23} \text{ capsids}}{1 \text{ mol}} \times \frac{1 L}{1000 \text{ mL}}$$

$$= 7.71 \times 10^{13}$$

$$\# \text{ Zn}^{2+} \text{ per capsid} = \frac{1.15 \times 10^{15} \text{ Zn}^{2+} \text{ dyes}}{7.71 \times 10^{13} \text{ capsids}} = 15 \text{ Zn}^{2+} \text{ dyes capsid}$$

$$\# \text{ Gd}^{3+} \text{ per capsid} = \frac{2.7 \times 10^{16} \text{ Gd}^{3+} \text{ ions or monomers}}{7.71 \times 10^{13} \text{ capsids}} = 350 \text{ Gd}^{3+} \text{ per capsid}$$

$$\# \text{ micelles per capsid} = \frac{1.8 \times 10^{15} \text{ micelles}}{7.71 \times 10^{13} \text{ capsids}} = 23$$

Based on these numbers, on average, a maximum of **23** micelles are present in **one** (T = 3) capsid.

Appendix 4.3. TEM Analysis

For the sake of comparison, the size distribution of the capsid filled with only the ligand (**5**) was assessed (Figure 4.10), showing a mean internal diameter of 24.8 nm. Mean sizes of each capsid are summarized in Table 4.5.

Table 4.5. Average size of the different protein cages

Protein cage filled with micelles of	DOTAC10	Gd ³⁺ - DOTAC10	DOTAC10/ZnPc	Gd ³⁺ - DOTAC10/ZnPc
Mean diameter / nm	24.8	24.6	24.3	22.4
Standard deviation /	3	3	3	2

Figure 4.10. TE micrograph of protein cages filled with only micelles of DOTAC10 (**5**). Inset: particle size distribution histogram showing that the mean internal diameter is 24.8 nm. Scale bar: 50 nm.

Appendix 4.4. MRI Data

Figure 4.11. T_1 -weighted image of solutions of different concentrations of capsid filled with Gd³⁺-DOTAC10/ZnPc micelles (shown as Gd³⁺ concentration) at 600 MHz and 25°C. Slice thickness 6 mm, 10 averages, matrix size: 256 x 256, TE = 6 ms and TR = 500 ms

Table 4.6. T_1 values obtained for the capsid filled with Gd³⁺-DOTAC10/ZnPc micelles at 600 MHz and 25°C

[Gd ³⁺] mM	T_1 Slice 1 (ms)	T_1 Slice 2 (ms)	T_1 Slice 3 (ms)	T_1 Slice 4 (ms)	T_1 Slice 5 (ms)	Average (ms)	Standard Deviation (ms)	1/ T_1 (s ⁻¹)
0.0449	1946.8	2015.6	2037.0	2019.5	2019.5	2007.7	35.0	0.498
0.02245	2294.1	2386.7	2360.4	2385.7	2354.0	2356.2	37.7	0.424
0.00898	2796.3	2835.3	2807.5	2799.9	2755.4	2798.9	28.7	0.357
0.00449	2948.2	2954.7	2878.0	2864.4	2795.6	2888.2	65.7	0.346
0	3125.8	3243.8	3218.4	3301.2	3279.2	3233.7	68.2	0.309

CHAPTER 5

MULTIMODAL GOLD NANORODS FOR MAGNETIC RESONANCE AND FLUORESCENCE IMAGING

Abstract

In this chapter, the synthesis, characterization and study of the cytotoxicity of multimodal gold nanorods (MMAuNRs) for magnetic resonance and optical imaging is described. The surface of these nanostructures was modified by means of thiol-based ligand exchange and the resulting AuNRs were characterized by means of UV-Vis absorption spectroscopy, transmission electron microscopy (TEM) and total reflection x-ray fluorescence (TXRF). Using various incubation percentages of O-[2-(3-mercaptopropionylamino)ethyl]-O'-methylpolyethylene glycol (PEG-SH) and Gd^{3+} -1-(11-sulfanylundecyl)-4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecane (Gd^{3+} -SUDDO3A) a good compromise between biocompatibility/colloidal stability and Gd^{3+} loading is attainable. The second imaging modality was added by incorporating (3aS,4S,6aR)-11-sulfanylundecyl 5-(hexahydro-2-oxo-1H-thieno[3,4-d]imidazol-4-yl) pentanoate (Biotin-SH) during the ligand exchange process. Subsequent incubation with Cy5-streptavidin led to paramagnetic and fluorescent AuNRs, visualized by MRI and fluorescence microscopy. The MMAuNRs obtained using this one-pot synthesis are biocompatible, show median lethal concentration (LC_{50}) values $> 150 \text{ pM}$ AuNR, improved relaxivity properties, high Gd^{3+} loadings and can be optically colocalized with mouse myoblast (C2C12) cells *in vitro*.

5.1 Introduction

Magnetic resonance imaging (MRI) is a well-established technique used in medical diagnostics due to its non-ionizing character and the excellent soft tissue contrast and spatial resolution it offers.^{1,2} This technique takes advantage of the different longitudinal (T_1) relaxation times most body tissues have. However, when different tissues show similar relaxation behavior, a contrast agent (CA, typically a paramagnetic Gd^{3+} complex)^{3,4} is used to aid the image contrast by shortening T_1 . The T_1 -shortening efficiency shown by a CA is known as the relaxivity r_1 .

Due to the inherent lack of sensitivity of MRI and the large amount of water found in the human body, high concentrations of CAs are usually required. In the past, CAs with enhanced paramagnetic properties were developed by: i) increasing the “local” Gd^{3+} concentration and ii) decreasing the global rotational motion (the tumbling rate τ_R) of the Gd^{3+} complexes used. One of the most popular strategies followed is the surface modification of inorganic spherical nanostructures such as fluorescent quantum dots⁵⁻⁷ and gold nanoparticles⁸⁻¹³ with functionalized Gd^{3+} chelates. These nanoparticle-based systems^{16,17} not only show enhanced relaxivity properties but have also been used for other imaging modalities such as optical imaging^{5-7,12} or X-ray computed tomography (CT).⁸

Gold nanoparticles are appealing for biological applications due to their light absorption and scattering properties derived from localized surface plasmon resonance (LSPR), often observed as strong extinction peaks in the visible and near-infrared regions.¹⁴ Fine-tuning of the optical properties of these nanostructures can be achieved by altering their size and morphology. For example, gold nanorods (AuNRs)^{15,16} show two prominent SPR peaks originating from the excitation of surface plasmon oscillations

along both the short axis (transverse band, located around 520 nm) and the long axis (longitudinal band, located in the near-infrared region). The near-infrared absorption band, whose position strongly depends on the aspect ratio (length/width) of the AuNRs, favors their use for biomedical applications,¹⁷⁻¹⁹ e.g, as photothermal therapy agents^{20,21} or for photoacoustic imaging.²²

More recently, AuNR-based CAs for MRI have been synthesized by means of ligand exchange and subsequent covalent²³ and non-covalent²⁴ functionalization with paramagnetic ligands. In both cases, CAs with enhanced relaxivity properties were obtained. While these examples nicely illustrate the potential of AuNRs as multimodal imaging agents (IAs) by combining MRI with CT²⁴ and plasmonic imaging,²³ the integration of a more widespread second modality as optical imaging has not been investigated yet. In this chapter, the use of AuNRs as platforms for the one-pot synthesis of multimodal IAs is proposed. Herein, the facile synthesis of biocompatible, multimodal AuNRs (MMAuNRs) suitable for magnetic resonance and optical imaging is described. The elongated structure of the AuNRs, available for ligand exchange, makes them excellent platforms for the integration of paramagnetic, fluorescent and biocompatible moieties. Consequently, MMAuNRs represent a novel class of IAs with expected improved relaxivity properties due to high Gd³⁺ payloads per particle and interesting optical properties due to the incorporation of fluorescent dyes.

5.2 Design and Synthesis

Whereas one of the major advantages of AuNRs is their straightforward and well-described synthesis, large quantities of cetyl trimethylammonium

bromide (CTAB, which is known to be toxic)^{25,26} are required. However, surface modification of AuNRs allows the incorporation of biocompatible ligands,^{27,28} as well as biomarkers (targeting),²⁰ ligands for enhanced cellular uptake²⁹ and quantum dots³⁰ and organic dyes for optical imaging purposes.³¹

Here, the gold nanostructures are coated, by simultaneous thiol-based ligand exchange, with an optimized combination of the Gd^{3+} complex of 1-(11-sulfanylundecyl)-4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecane (**6**, Gd^{3+} -SUDDO3A) for MRI and O-[2-(3-mercaptopropionylamino)ethyl]-O'-methylpolyethylene glycol (PEG-SH, $M_w = 5000$) in order to increase their biocompatibility and solubility. Furthermore, (3aS,4S,6aR)-11-sulfanylundecyl 5-(hexahydro-2-oxo-1H-thieno[3,4-d]imidazol-4-yl) pentanoate (**8**, Biotin-SH) is used to incorporate (in a later stage) a fluorescent dye (Cy5) for optical imaging by means of the biotin-streptavidin interaction (Figure 5.1).³²

Figure 5.1. Schematic representation of the synthesis of MMAuNRs

5.2.1 Synthesis of Gd^{3+} -SUDDO3A

To synthesize the thiolated Gd^{3+} complex 1-(11-sulfanylundecyl)-4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecane (Gd^{3+} -SUDDO3A, Scheme 5.1), bromo-1-undecanthiol (**1**) was protected by reacting it with dihydropyran and pyridinium *p*-toluene sulfonate in CH_2Cl_2 at room temperature overnight. Compound (**4**) was then synthesized by alkylating 1,4,7-tris(*tert*-butoxycarboxymethyl)-1,4,7,10-tetraazacyclododecane (**3**)

with the protected thiol 11-bromo-1-undecyltetrahydropyranyl sulfide (**2**) in dry acetonitrile using K_2CO_3 (under N_2) for three days at $50^\circ C$. The SUDDO3A ligand (**5**) was obtained by means of Boc-deprotection of (**4**) using TFA/ CH_2Cl_2 at room temperature overnight. Lastly, the Gd^{3+} complex of SUDDO3A (**6**) was prepared by adding one equivalent of $GdCl_3$ to a solution of (**5**) in water, adjusting the pH to 6 and stirring at $60^\circ C$ for 1 day. The final product **6** was obtained with an overall yield (after purification) of 25%.

Scheme 5.1. Synthetic route followed for the synthesis of the Gd^{3+} complex of 1-(11-sulfanylundecyl)-4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecane (Gd^{3+} -SUDDO3A, **6**).

5.2.2 Synthesis of Biotin-SH

The syntheses of (3a*S*,4*S*,6a*R*)-11-sulfanylundecyl 5-(hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazol-4-yl) pentanoate (biotin-SH) and its brominated

precursor (Scheme 5.2, **7** and **8**, respectively) were carried out following a literature procedure (see experimental section).³³ The final product **8** was obtained with an overall yield of 60%.

Scheme 5.2. Structures of (3aS,4S,6aR)-11-sulfanylundecyl 5-(hexahydro-2-oxo-1H-thieno[3,4-d]imidazol-4-yl) pentanoate (biotin-SH, **8**) and the brominated precursor **7**

5.2.3 AuNRs Synthesis and Characterization

The AuNRs were grown following a previously described seed-mediated growth procedure.³⁴ All glassware was cleaned with a 3:1 solution of concentrated H_2SO_4 and 30% H_2O_2 prior to use, rinsed abundantly with Milli-Q water and dried in the oven overnight (60°C). The gold seeds were prepared by placing a (water) solution consisting of 5 mL of 0.0005 M HAuCl_4 and 5 mL of 0.2 M CTAB in an oil bath at 27°C , stirring at 1000 rpm. Rapid addition of a solution of 600 μL ice cold NaBH_4 (0.01 M) led to the formation of seed particles. This *seed solution* was stirred for exactly 2 min and allowed to stand for another 10 min in an oil bath at 27°C . Meanwhile, the *growth solution* was prepared by mixing in the following order 25 μL freshly prepared 0.04 M AgNO_3 , 5 mL 0.2 M CTAB, 20 μL 0.5 M H_2SO_4 , 5 mL 0.001 M HAuCl_4 and 70 μL freshly prepared 0.0788 M ascorbic acid. The solution was gently mixed immediately until the color changed from dark yellow to colorless. Finally, 12 μL of the *seed solution* were slowly added to the *growth solution* in an oil bath at 27°C . The final solution was incubated for at least 2 hours and analyzed without further purification. The synthesized AuNRs were characterized by UV-Vis

absorption spectroscopy and transmission electron microscopy (TEM). Results revealed that the two synthesized batches, Batch #1 and Batch #2, show longitudinal plasmon band maxima at 744 and 771 nm (Figure 5.2, left) and aspect ratios of 3.5 and 3.8 respectively (Figure 5.2, right).

Figure 5.2. UV-Vis spectra (left) and TEM images (right) of Batch #1 and Batch #2 AuNRs. Scale bar for Batch #1 is 50 nm and for Batch #2 100 nm.

The concentration of AuNRs in solution was calculated using the Beer-Lambert law (Equation 5.1):

$$A = \epsilon \times l \times c \quad (5.1)$$

Where A is the absorbance, l the length of the cuvette (1 cm) and ϵ the molar extinction coefficient ($\text{M}^{-1} \text{cm}^{-1}$). The latter parameter was obtained

using a calibration plot,³⁵ where the longitudinal plasmon band peak maximum was used to estimate ϵ . The calculated concentrations of Batch#1 and Batch #2 AuNRs are shown in Table 5.1.

Table 5.1. AuNRs concentrations of Batch #1 and Batch #2

Batch #	Corrected Absorbance	Extinction coefficient ($M^{-1} cm^{-1}$)	Aspect ratio (l/w) in nm	λ_{max} (nm)	[AuNRs] pM (in a 10mL solution)
1	1.86	4.120×10^9	3.5 (63/18)	743	451
2	1.17	4.464×10^9	3.8 (68/18)	771	262

5.3 Results and Discussion

5.3.1 Functionalization of AuNRs using Gd^{3+} -SUDDO3A and PEG-SH

The biocompatibility of AuNRs synthesized using seed-mediated methods is often compromised by the large quantities of CTAB used. However, improved biocompatibility and colloidal stability can be achieved by functionalizing these nanostructures with polyethylene glycol (PEG)-based ligands.^{23,27,28} Here, the biocompatibility and stability of AuNRs coated with various Gd^{3+} -SUDDO3A:PEG-SH percentage ratios were studied (Figure 5.3); thereby allowing us to understand the influence of the Gd^{3+} -SUDDO3A coating on the stability, biocompatibility and attainable Gd^{3+} loading of the AuNRs. This was done by incubating 1 mL of Batch #1 AuNRs ($[AuNR]_{stock\ solution} = 900\ pM$) with 5 mM Gd^{3+} -SUDDO3A (filtered with a 0.2 μm filter before using) and 5 mM PEG-SH at 5 different volume percentages (Table 5.2). For all solutions, tris(2-carboxyethyl)phosphine (TCEP, 5 mg) was added in order to both break any disulfide bonds and to prevent their formation. After incubation for 2 days, all 5 solutions were centrifuged 3 times at 11.000 rpm for 15 minutes (in PBS buffer, pH 7.5), in

order to remove excess Gd^{3+} -SUDDO3A. The final pellets were resuspended in PBS + 5% BSA.

Figure 5.3. AuNRs ligand exchange using different Gd^{3+} -SUDDO3A:PEG-SH (volume) percentages, expressed as % X and % (100 - X), respectively. The localization, size and distribution of the ligands along the AuNRs are only indicative.

Table 5.2. Quantities (volumes) of 5 mM Gd^{3+} -SUDDO3A and 5 mM PEG-SH used to incubate 1 mL of Batch #1 AuNRs

Gd^{3+} -SUDDO3A:PEG-SH (%)	mL Gd^{3+} -SUDDO3A	mL PEG-SH
0:100	0	5
25:75	1.25	3.75
50:50	2.5	2.5
75:25	3.75	1.25
100:0	5	0

The newly coated AuNRs were characterized using UV-Vis absorbance spectroscopy and TEM analysis. In most of the cases, the AuNRs exhibited a red-shifted longitudinal plasmon band peak maximum upon ligand exchange by incubation using various Gd^{3+} -SUDDO3A:PEG-SH percentages (Figure 5.4, left). While the largest red shifts are observed in the AuNRs incubated with the highest Gd^{3+} -SUDDO3A percentage (pink and grey lines), those incubated with 50:50% showed almost no shift in the absorption spectra (green line). In our hands, we have constantly observed this dissimilarity when using this ligand exchange protocol. The

broadened longitudinal plasmon bands observed in all cases indicate modified colloidal stabilities as the coating composition of the AuNRs changes. As expected, the lowest colloidal stability is observed in the AuNRs incubated with only Gd^{3+} -SUDDO3A (grey line), as these nanostructures tend to flocculate/aggregate rapidly. Furthermore, no changes in the morphology of the functionalized AuNRs are observed, as demonstrated by TEM (Figure 5.4, right). Overall, higher colloidal stability was observed at decreasing Gd^{3+} -SUDDO3A incubation percentages. Therefore, under the studied conditions, a 25:75% Gd^{3+} -SUDDO3A:PEG-SH incubation ratio leads to the most stable assemblies.

Figure 5.4. Left: UV-Vis spectrum (in PBS + 5% BSA) of AuNRs incubated with different Gd^{3+} -SUDDO3A:PEG-SH percentage ratios. Dashed line represents the longitudinal plasmon band peak maximum of Batch #1 at 744 nm. Right: TEM images of 25:75 (top) and 50:50 (bottom) % Gd^{3+} -SUDDO3A:PEG-SH incubated AuNRs showing that the morphology of the AuNRs does not change after ligand exchange. Note the background contrast in the 50:50% picture given by the combination of albumin, salts from buffer and Gd^{3+} -SUDDO3A surrounding the AuNRs as the nanostructures collapse upon appliance on the TEM grid. Scale bar is 50 nm and 20 nm respectively.

In previous studies, the Gd^{3+} -to-Au ratio (in weight) of functionalized AuNRs²⁴ and Au nanoparticles¹² has been successfully studied using inductively coupled plasma (ICP)-based techniques, namely ICP-AES and ICP-MS, respectively. In this way, the degree of functionalization of the final nanostructures was quantified. Here, in order to quantify both the Gd^{3+} and Au concentrations, all samples were analyzed using total reflection X-ray fluorescence (TXRF). The average volume of a single AuNR was calculated assuming ideal cylinder nanorods (Equation 5.2).²⁴ The number of Au atoms per AuNR and the number of AuNRs were calculated using Equation 5.3 and Equation 5.4, respectively.

$$V_{NR} = \pi r^2 l \quad (5.2)$$

$$\# \text{ Au atoms per AuNR} = \rho_{Au} V_{NR} \quad (5.3)$$

$$\# \text{ AuNRs} = \frac{\# \text{ Au atoms}}{\# \text{ atoms per AuNR}} \quad (5.4)$$

Where ρ_{Au} is 59 atoms/nm³ (FCC structure)³⁶ and r is half the width and l the length of the NRs. Based on TXRF, the incubation of AuNRs with various Gd^{3+} -SUDDO3A:PEG-SH ratios led to successful thiol-based ligand exchange as increasing Gd^{3+} ions were detected (Table 5.3). Furthermore, as the Gd^{3+} -SUDDO3A incubation percentages increased, higher Gd^{3+} :Au ratios (weight:weight) were obtained. Surprisingly, the calculated number of Gd^{3+} ions per AuNR is 40 to 90 times higher than previously reported AuNRs functionalized with Gd^{3+} -DTPA-based ligands.²⁴ However, this number decreases when calculating the AuNR concentration using the Beer-Lambert law (section 5.2.3, Table 5.3). More specifically, the maximal Gd^{3+} per AuNR loading is reached at an incubation

percentage of 75:25 Gd³⁺-SUDDO3A:PEG-SH. This discrepancy in the estimation of the AuNR concentration, leading to differences in the calculated Gd³⁺ per AuNR loadings, suggests a sensitivity/detection issue when using TXRF for the determination of the Au concentration which might arise from the presence of a large organic matrix (BSA).³⁷

Table 5.3. Elemental analysis data for all incubated AuNRs

Gd ³⁺ -SUDDO3A:PEG-SH (%)	25:75	50:50	75:25	100:0
From TXRF Data				
Ratio Gd³⁺:Au (w/w)	100:6.5	100:4.1	100:3.2	100:2.7
[Gd³⁺] (mM)	1.9	3.6	4.8	4.2
[Au] (M)	9.6×10^{-5}	11.8×10^{-5}	12.5×10^{-5}	8.9×10^{-5}
# atoms Au	5.8×10^{19}	7.1×10^{19}	7.5×10^{19}	5.4×10^{19}
Volume one AuNR (nm)	16023	16023	16023	16023
# atoms per AuNR	945382	945382	945382	945382
# AuNRs	6.1×10^{13}	7.5×10^{13}	8×10^{13}	5.7×10^{13}
# Gd³⁺ atoms	1.1×10^{21}	2.2×10^{21}	2.9×10^{21}	2.5×10^{21}
# Gd³⁺ ions per AuNR	1.8×10^7	2.9×10^7	3.6×10^7	4.4×10^7
From UV Data (AuNR concentration) and TXRF (Gd³⁺ concentration)				
# AuNRs	1.2×10^{14}	1.2×10^{14}	1×10^{14}	1×10^{14}
# Gd³⁺ atoms	1.1×10^{21}	2.2×10^{21}	2.9×10^{21}	2.5×10^{21}
# Gd³⁺ ions per AuNR	9.3×10^6	1.9×10^7	2.9×10^7	2.5×10^7

While high Gd³⁺ loadings are expected when using such nanostructures due to their large surface to volume ratios, the higher values obtained strongly suggests the formation of highly dense “paramagnetic networks” of Gd³⁺-SUDDO3A molecules surrounding the AuNRs and possibly entrapped in the long PEG chains. The formation of such highly dense coatings around the AuNRs is likely to occur, as the amphiphilic Gd³⁺-SUDDO3A ligand used is known to self-assemble in water, with a critical aggregation concentration (cac) of 0.63 mM (Appendix 5.1). Therefore, it is thinkable that multiple layers can form around the AuNRs as the Gd³⁺-

SUDDO3A concentration increases. This observation is in line with previously studied AuNRs, where the formation of thick, non-uniform polymer shells surrounding AuNRs was observed.³¹ In the absence of PEG-SH, these highly dense “paramagnetic packing” around the AuNRs is evident from the low colloidal stability AuNRs show as the water solubility of Gd^{3+} -SUDDO3A is lower than that of PEG-SH. Moreover, the lower Gd^{3+} per AuNR loading obtained for the 100:0% Gd^{3+} -SUDDO3A:PEG-SH incubated sample (when using UV data for the calculation of the AuNR concentration) strongly suggest the need for a PEG-SH in order to entrap more Gd^{3+} -SUDDO3A molecules. However, direct observation of any network of molecules using, e.g., TEM, is challenging since the sample preparation is a critical factor. Most likely, these densely packed paramagnetic networks will tend to collapse during sample preparation thereby leading to dark backgrounds consisting of organic (albumin, Gd^{3+} -SUDDO3A) and inorganic (salts from buffer) matter (as observed in Figure 5.4, right bottom).

The biocompatibility of Gd^{3+} -SUDDO3A:PEG-SH coated AuNRs was evaluated by studying the viability of C2C12 mouse myoblast cells upon incubation for 24 h with 4 serial dilutions of the 5 different Gd^{3+} -SUDDO3A:PEG-SH coated AuNRs ($[\text{AuNRs}]_{\text{stock solution}} = 200 \text{ pM}$) in triplicate. As shown in Figure 5.5, cells incubated with *only* PEG-SH coated AuNRs (pink line) show the highest survival percentage at the concentration range studied, followed by those incubated with 25% Gd^{3+} -SUDDO3A (blue line). On the other hand, cells incubated with 100% Gd^{3+} -SUDDO3A coated AuNRs showed the lowest median lethal concentration (LC_{50} , 50 pM) upon 24 h exposure (black line) while, for all of the other cases, this value was higher than 100 pM. These results confirm that the biocompatibility of the AuNRs studied is lowered as the Gd^{3+} -SUDDO3A incubation percentage increases.

Figure 5.5. Mouse myoblast C2C12 cell response upon exposure to Gd^{3+} -SUDDO3A:PEG-SH coated AuNRs. Cells were incubated for 24 h with different Gd^{3+} -SUDDO3A:PEG-SH percentages at increasing AuNR concentrations. For comparison purposes, response upon exposure to PEG-SH coated AuNRs is also shown (pink line).

5.3.2 MMAuNRs: Functionalization of AuNRs using Gd^{3+} -SUDDO3A, PEG-SH and Biotin-SH

The incorporation of the second imaging modality to AuNRs was achieved using the biotin-streptavidin system. Here, AuNRs were biotinylated by incubation with Gd^{3+} -SUDDO3A (6), PEG-SH and Biotin-SH (8, Figure 5.6) at different ratios. As described in previous sections, the (optimum) Gd^{3+} -SUDDO3A percentage was 25%. The AuNRs were prepared by incubating 1 mL of Batch #2 AuNRs ($[AuNR]_{stock\ solution} = 873\text{ pM}$) with 5 mM Gd^{3+} -SUDDO3A (filtered before using) and 5 mM PEG-SH and 5 mM Biotin-SH (filtered before using) at 3 different volume percentages (quantities shown in Table 5.4). For all solutions, tris(2-carboxyethyl)phosphine (TCEP, 5 mg)

was added in order to both brake any disulfide bonds and to prevent their formation. After incubation for 2 days, the 3 solutions were centrifuged 3 times at 11.000 rpm for 15 minutes (in PBS buffer, pH 7.5) and resuspended in PBS buffer + 5% BSA. The fluorescent dye was incorporated into the system by incubation with 0.6 μM Streptavidin Cy5 for one hour. Once again, the samples were centrifuged 3 times at 11.000 rpm for 15 minutes, in order to remove excess Gd^{3+} -SUDDO3A. The final pellets were resuspended in PBS + 5% BSA.

Figure 5.6. Schematic representation of multimodal gold nanorods (MMAuNRs), prepared by simultaneous thiol-based ligand exchange, using optimal percentages of PEG-SH (x), Gd^{3+} -SUDDO3A (y) and Biotin-SH (z). The localization, size and distribution of the ligands along the AuNRs are only indicative.

Table 5.4. Quantities (volumes) of 5 mM Gd^{3+} -SUDDO3A, 5 mM PEG-SH and 5mM Biotin-SH used to incubate 1 mL of Batch #2 AuNRs

Gd^{3+} -SUDDO3A:PEG-SH:Biotin-SH (%)	mL Gd^{3+} -SUDDO3A	mL PEG-SH	mL Biotin-SH
0:100:0	0	7	0
0:50:50	0	3.5	3.5
25:50:25	2.33	2.33	2.33

After ligand exchange of the AuNRs using various Gd^{3+} -SUDDO3A:PEG-SH:Biotin-SH percentage ratios, a red shift of the peak corresponding to the longitudinal plasmon band occurred (Figure 5.7, left). Furthermore, no changes in this peak are observed after incubation with Streptavidin Cy5 (black line). The higher colloidal stability of these nanostructures, when compared to those incubated with only Gd^{3+} -SUDDO3A and PEG-SH (Figure 5.4, left), is evident from the smaller broadening of the longitudinal plasmon bands observed. No changes in the morphology of these ligand exchanged-AuNRs are observed by TEM either before (top) or after (bottom) incubation with Streptavidin Cy5 (Figure 5.7, right).

Figure 5.7. Left: UV-Vis spectrum (in PBS + 5% BSA) of AuNRs incubated with different percentages of Gd^{3+} -SUDDO3A:PEG-SH:Biotin-SH. Dashed line represents the longitudinal plasmon band peak maximum of Batch #2 at 771 nm. Right: TEM images of 0:50:50 (top) and 25:50:25 (bottom) % Gd^{3+} -SUDDO3A:PEG-SH:Biotin-SH incubated AuNRs showing that the morphology of the AuNRs does not change either after ligand exchange or subsequent incubation with Streptavidin-Cy5. Scale bar is 50 nm and 20 nm, respectively.

Analogous to the AuNRs previously studied, the Gd^{3+} concentration was assessed using TXRF analysis, while the AuNR concentration was obtained from UV data (section 5.2.3). After incubation of AuNRs using 25:50:25 Gd^{3+} -SUDDO3A:PEG-SH:Biotin-SH percentage ratios (and subsequent incubation with Streptavidin Cy5), successful ligand exchange was confirmed by the detection of Gd^{3+} ions. Curiously, the number of Gd^{3+} ions per AuNR observed is only 4 times higher than that reported for non-covalently functionalized paramagnetic AuNRs²⁴ (2.1×10^6 vs. 5.1×10^5) and lower than that measured for the 75:25 Gd^{3+} -SUDDO3A:PEG-SH sample (2.1×10^6 vs. 9.3×10^6 , respectively). Analogous to previously synthesized AuNRs, the formation of a highly dense paramagnetic Gd^{3+} -SUDDO3A layer around the AuNRs is thought to be responsible for the higher Gd^{3+} loadings observed.

The biocompatibility of the biotinylated AuNRs was studied *in vitro* by incubating C2C12 cells with 5 serial dilutions of the 3 different AuNR samples (Table 5.5) in triplicate. As shown in Figure 5.8, the maximum percentage of cell survival after 24 h of exposure corresponded to cells incubated with 0:50:50% Gd^{3+} -SUDDO3A:PEG-SH-Biotin-SH (red line). Moreover, AuNRs incubated with 25 and 50% Biotin-SH showed median lethal concentrations (LC_{50}) higher than 150 pM, both higher than that obtained for AuNRs incubated with *only* Gd^{3+} -SUDDO3A and PEG-SH (green/red and blue lines, respectively). Whereas previous cell viability studies showed that AuNRs incubated with 25% Gd^{3+} -SUDDO3A showed an LC_{50} value higher than 100 pM (blue line), the incorporation of the biotin moiety results in an even higher LC_{50} value (> 150 pM). This increase in cell viability percentage is not surprising, since biotin is natively found in many biological systems with negligible toxicity. Moreover, the LC_{50} values obtained are comparable to previously reported PEGylated AuNRs (> 165 pM).²⁸

Figure 5.8. Mouse myoblast C2C12 cell response upon exposure to Gd^{3+} -SUDDO3A:PEG-SH:Biotin-SH coated AuNRs. Cells were incubated for 24 h with different Gd^{3+} -SUDDO3A:PEG-SH:Biotin-SH percentages at increasing AuNR concentrations. For comparison purposes, cell response upon exposure to Gd^{3+} -SUDDO3A:PEG-SH and PEG-SH coated AuNRs is also shown (pink line).

5.3.3 Properties of MMAuNRs

Cy 5 fluorescence quenching after functionalization of the AuNRs is expected, as previous reports using fluorescein have shown a dramatic decrease of its quantum yield after AuNR functionalization.³¹ Nevertheless, the residual fluorescence proved to be sufficient for imaging applications. Therefore, the fluorescent MMAuNRs were visualized *in vitro* using C2C12 cells incubated with 150 pM MMAuNRs. After exposure for 24 h to these nanostructures, the cells were stained with Alexa Fluor 488 (green) and DAPI (blue) in order to elucidate their location in the wells. As shown in Figure 5.9 (top), fluorescence linked to both the cells (left image) and the MMAuNRs (center image) was successfully observed. While cell

internalization of the MMAuNRs remains inconclusive, the overlay of these two images (right image) shows that the MMAuNRs are colocalized in the surroundings of the cells. For control purposes, wells without incubated cells were also treated with the same staining procedure and the same MMAuNR solution. As Figure 5.9 (bottom) shows, faint red fluorescence (corresponding to the MMAuNRs) was observed. This lower fluorescence intensity observed (even at higher exposure times) strongly suggests that the MMAuNRs are not colocalized and clustered due to the absence of cells.

Figure 5.9. Top: fluorescence microscopy images of C2C12 cells incubated with MMAuNRs. Cells were stained using Alexa Fluor® 488 (cytoskeleton, green, exposure time 50 ms) and DAPI (cell nuclei, blue, exposure time 1 ms). Left: Overlay of blue + green filters, showing the location of the cells in the well. Center: red emission observed corresponding to Cy5-labeled MMAuNRs (exposure time 2000 ms). Right: overlay of the previously described images. Bottom: control experiments in wells where no cells were incubated (blue, red and green filter). The blue and green filter images confirm the absence of cells, while the red fluorescence confirms the presence of MMAuNRs all over the well (exposure time 15000 ms).

Finally, the MR contrast provided by the MMAuNRs is evident from the T_1 -weighted MR image shown in Figure 5.10, highlighting the differences in contrast observed at different Gd^{3+} concentrations (and with respect to PBS buffer). In addition, the relaxation efficiency of MMAuNRs was assessed by measuring the longitudinal relaxation times (T_1) of a series of dilutions (of known Gd^{3+} concentration determined by TXRF) using a standard saturation recovery experiment at 600 MHz (14.1 T). By plotting the measured $1/T_1$ values as a function of Gd^{3+} concentration, the relaxivity r_1 ($\text{mM}^{-1} \text{s}^{-1}$) was obtained as the slope of the curve. For control purposes, the ionic relaxivity of the Gd^{3+} -SUDDO3A complex in solution was also calculated ($r_1 = 0.81 \text{ mM}^{-1} \text{s}^{-1}$, Appendix 5.2).

Figure 5.10. T_1 -weighted MR image of MMAuNRs at different Gd^{3+} concentrations (600 MHz, TE = 4 ms, TR = 500 ms). The plot shows ionic r_1 relaxivity corresponding to the MMAuNRs. The inset plot corresponds to a linear fitting of the 0-0.13 mM Gd^{3+} concentration range ($r_1 = 1.8 \text{ mM}^{-1} \text{s}^{-1}$).

The non-linearity observed in the $1/T_1$ vs. $[\text{Gd}^{3+}]$ concentration plot strongly suggest T_1 saturation,^{8,38} as the former value approaches a plateau at Gd^{3+}

concentrations between 0.13 and 0.6 mM (Figure 5.10). This behavior has previously been observed in AuNRs functionalized with Gd^{3+} -DTPA complexes at Gd^{3+} concentrations higher than 1.17 mM.²⁴ Furthermore, a relaxivity plot in the Gd^{3+} concentration range 0-0.13 mM (inset Figure 5.10) shows enhanced ionic r_1 relaxivity for the MMAuNRs when compared to the Gd^{3+} -SUDDO3A complex in solution ($1.8 \text{ mM}^{-1} \text{ s}^{-1}$ vs. $0.81 \text{ mM}^{-1} \text{ s}^{-1}$, respectively). While an increase in ionic r_1 relaxivity is expected due to slower global rotational motion (τ_R) upon attachment to the AuNRs, the rather modest enhancement observed may be due to the molecular crowding resulting from the formation highly dense “paramagnetic networks” of attached and non-attached Gd^{3+} -SUDDO3A molecules (in the form of free, non-aggregated molecules, micelles, etc) around the surface of the AuNRs (Figure 5.11). While an increase in relaxivity is expected when Gd^{3+} -SUDDO3A molecules are attached to the AuNR or in an aggregated form, this is not the case for the relaxivity of non-aggregated, entrapped by the long PEG chains, Gd^{3+} -SUDDO3A molecules. Moreover, in these densely packed networks, relaxivity is likely to be affected by the limited water exchange and diffusion due to low accessibility of the paramagnetic moieties, as previous studies in hybrid silica nanoparticles have shown.³⁹

Figure 5.11. Schematic representation of the suspected highly dense paramagnetic network formed around the AuNRs, showing both attached and unattached Gd^{3+} -SUDDO3A molecules.

With respect to the relaxivity per AuNR, defined as the ionic relaxivity r_1 times number of calculated Gd^{3+} ions per AuNR, this value is estimated to be $4 \times 10^6 \text{ mM}^{-1} \text{ s}^{-1}$, which is lower than previously reported values for paramagnetic labeled AuNRs.²⁴ However, this value is much lower when calculated as the slope of the $1/T_1$ vs. [AuNR] curve, i.e. $3881 \text{ mM}^{-1} \text{ s}^{-1}$ (Figure 5.14, Appendix 5.2). This observation reinforces the hypothesis of the formation of highly dense paramagnetic networks in which *mostly* the Gd^{3+} -SUDDO3A molecules attached to AuNRs contribute to the relaxivity enhancement observed.

5.4 Conclusions

In conclusion, MMAuNRs for magnetic resonance and fluorescence imaging have successfully been synthesized by simultaneous thiol-based ligand-exchange using three different moieties for

biocompatibility/colloidal stability (PEG-SH) and MRI (Gd^{3+} -SUDDO3A)/optical (Biotin-SH) detection. This one-pot synthesis, using optimum ratios of Gd^{3+} -SUDDO3A, PEG-SH and Biotin-SH (+ Streptavidin Cy5), yielded biocompatible MMAuNRs with high Gd^{3+} loadings. The MMAuNRs were visualized in solution by MR imaging and *in vitro* by fluorescence microscopy, respectively. Cell viability studies in (mouse myoblast) C2C12 cells showed median lethal concentration (LC_{50}) values for these MMAuNRs higher than 150 pM, comparable to *only* PEGylated AuNRs.²⁸ Furthermore, MMAuNRs showed a two-fold increased ionic r_1 relaxivity, thereby suggesting the formation of densely packed “paramagnetic networks” around these nanostructures as a result of entrapment of Gd^{3+} -SUDDO3A in the long PEG-SH (MW 5000) chains. Overall, these results show that AuNRs are excellent platforms for merging several imaging modalities. Moreover, MMAuNRs can be used for other imaging modalities, as AuNRs are known to exhibit intrinsic imaging behavior, e.g., for photoacoustic²² and CT²⁴ imaging. The already known therapeutic properties of AuNRs^{20,21} reassure their versatility and potential as agents for theranostics.

5.5 Experimental

Materials. Commercial grade reagents were purchased from Sigma Aldrich and TCI Europe and were used without further purification unless otherwise stated. Solvents were dried over molecular sieves. Deuterated solvents were purchased from Cambridge Isotope Laboratories and NMR and MRI tubes from Wilmad LabGlass. All AuNRs were synthesized using 18.2 M Ω /cm² water. Streptavidin-Cy5 from was purchased from Anova. O-

[2-(3-mercaptopropionylamino)ethyl]-O'-methylpolyethylene glycol, MW=5000 (PEG-SH) was purchased from Aldrich.

11-Bromo-1-undecyltetrahydropyranyl sulfide (2). This compound was synthesized following an adapted procedure from literature.⁴⁰ 11-bromo-1-undecanethiol (**1**) (250 mg, 0.94 mmol), dihydropyran (85 μ L, 0.94 mmol) and pyridinium *p*-toluene sulfonate (23.5 mg, 0.094 mmol) were dissolved in 25 mL dry CH_2Cl_2 and the reaction mixture was stirred at room temperature overnight. The solution was washed with 10% NaOH (3 x 20 mL), water (3 x 20 mL), and dried over MgSO_4 . The solvent was removed under reduced pressure and the desired compound was obtained as a colorless liquid after chromatography on silica gel 60 (pre-treated with 0.1% TEA in hexane) using toluene as eluent ($r_f \sim 0.5$). Yield: 43% (143 mg, 0.41 mmol). ^1H NMR (600 MHz, CDCl_3 , 25°C, TMS) δ (ppm): 4.86 (m, 1H; SCH_2O), 4.1 & 3.5 (m, 2H; OCH_2), 3.43 [t, $J(\text{H,H}) = 6.87$ Hz, 2H; CH_2Br], 2.66 & 2.6 (m, 2H; CH_2S), 1.95 & 1.69 (m, 2H; SCH-CH_2), 1.86 (m, 2H; $\text{CH}_2\text{Br-CH}_2$), 1.83 & 1.58 (m, 2H; $\text{SCH-CH}_2\text{CH}_2$), 1.6 & 1.59 (m, 2H; $\text{OCH}_2\text{-CH}_2$), 1.61 (m, 2H, $\text{CH}_2\text{S-CH}_2$), 1.44 (m, 2H; $\text{CH}_2\text{Br-CH}_2\text{-CH}_2$), 1.39 (m, 2H, $\text{CH}_2\text{S-CH}_2\text{-CH}_2$), 1.29 (m, 10H, CH_2). ^{13}C NMR (150 MHz, CDCl_3 , 25°C, TMS) δ (ppm): 82.3 (SCH_2O), 64.7 (OCH_2), 34.1 (CH_2Br), 32.9 ($\text{CH}_2\text{Br-CH}_2$), 31.5 ($\text{CH}_2\text{S-CH}_2$), 30.4 (SCH-CH_2), 30 (CH_2S), 29.5 29.4, 29.2, 29, 28.8 (CH_2), 28.2 ($\text{CH}_2\text{Br-CH}_2\text{-CH}_2$), 25.6 ($\text{OCH}_2\text{-CH}_2$), 21.8 ($\text{SCH-CH}_2\text{CH}_2$). MS (ESI+, $\text{CH}_2\text{Cl}_2/\text{MeOH}$): m/z : 351.3, 353.3 [$\text{M}^+ + \text{H}$].

1,4,7,10-Tetraaza-1-(11-undecyltetrahydropyranyl sulfide)-4,7,10-triacetic acid *tert*-butyl ester cyclododecane (4). In a three-neck round-bottom flask, K_2CO_3 (483 mg, 3.5 mmol) was added to a stirring solution of 1,4,7-tris(*tert*-butoxycarbonylmethyl)-1,4,7,10-

tetraazacyclododecane **3** (DO3A, synthesized as previously described in literature⁴¹) (600 mg, 1.12 mmol) in 70 mL dry acetonitrile. The mixture was kept under N₂ for 10 minutes under vigorous stirring. Afterwards, 11-bromo-1-undecyltetrahydropyranyl sulfide (**2**) (410 mg, 1.12 mmol) dissolved in 15 mL dry CH₂Cl₂ was added slowly to the reaction mixture which was, subsequently, allowed to react for 3 days at 50°C. The mixture was left to cool down, filtered to remove inorganic salts and the solvent was evaporated under reduced pressure. The desired compound was obtained as a yellow oil after chromatography on silica gel 60 (pre-treated with 0.1% TEA in hexane) using CH₂Cl₂/MeOH 9:1 as eluent (rf ~ 0.5). Yield: 57% (525 mg, 0.67 mmol). ¹H NMR (600 MHz, CDCl₃, 25°C, TMS) δ (ppm): 4.83 (m, 1H; SCHO), 4.08 & 3.5 (m, 2H; OCH₂), 3.48-2.1 [m, 24H; NCH₂ (cyclen core), NCH₂ (4th arm), CH₂-COOtBu], 2.63 & 2.58 (m, 2H; CH₂S), 1.92 & 1.66 (m, 2H; SCH-CH₂), 1.83 & 1.59 (m, 2H; SCH-CH₂CH₂), 1.59 & 1.58 (m, 2H; OCH₂-CH₂), 1.59 (m, 2H, CH₂S-CH₂), 1.6 [m, 6H; OCH₂-CH₂, CH₂S-CH₂, NCH₂-CH₂ (4th arm)], 1.5-1.45 (s, 27H; C(CH₃)₃), 1.39 [m, 4H; NCH₂-CH₂-CH₂ (4th arm), CH₂S-CH₂-CH₂], 1.22 (m, 10H, CH₂). ¹³C NMR (150 MHz, CDCl₃, 25°C, TMS) δ (ppm): 173.5, 172.8 (COOtBu), 82.4 [C(CH₃)₃], 82.2 (SCHO), 64.8 (OCH₂), 55.8, 54.4, 53.4, 53.1, 52.1, 49.8, 45.6 [NCH₂ (all), NCH₂-CH₂, CH₂COOtBu], 31.5 (CH₂S-CH₂), 30.4 (SCH-CH₂), 29.9 (CH₂S), 29.7, 29.5, 29.3, 29, 28.2, 28.1, 27.6 (CH₂), 27.8, 28 [C(CH₃)₃], 25.6 (OCH₂-CH₂), 21.9 (SCH-CH₂CH₂). MS (ESI+, CH₂Cl₂/MeOH): *m/z*: 809.2 [M⁺+Na].

1,4,7,10-Tetraaza-1-(11-sulfanylundecyl)-4,7,10-triacetic acid cyclododecane (SUDDO3A, 5). In a round-bottom flask, TFA (5 mL) was added to a solution of **4** (200 mg, 0.25 mmol) in 5 mL CH₂Cl₂ and the reaction mixture was allowed to stir at room temperature overnight. The

mixture was evaporated under reduced pressure and the residue co-evaporated with CH_2Cl_2 3 times in order to remove excess TFA. **5** was obtained as a solid after precipitation from diethyl ether and was subsequently used for the next reaction. Yield: quantitative. MS (MALDI+, DHB): m/z : 533.5 [$\text{M}^+ + \text{H}$], 1066.8 [$2\text{M}^+ + \text{H}$].

Gd³⁺-1,4,7,10-Tetraaza-1-(11-sulfanylundecyl)-4,7,10-triacetic acid cycloddodecane (Gd³⁺-SUDDO3A, 6). In a round-bottom flask, **5** (120 mg, 0.23 mmol) and GdCl_3 (92 mg, 0.025 mmol) were dissolved in 50 mL water. The pH of the mixture was adjusted to 6 using NaOH (1M) and the reaction mixture was allowed to stir at 60°C overnight. The solution was allowed to cool for 30 minutes and subsequently stirred for 10 minutes with (acetone washed) DOWEX Monosphere 650C cation exchange resin. After filtering in order to remove the resin, the absence of free Gd^{3+} ions was confirmed by the xylenol orange test. Compound **6** was obtained after the solvent was removed by lyophilization. Yield: 99% (156 mg, 0.23 mmol). MS (ESI+, $\text{H}_2\text{O}/\text{ACN} + 0.1\% \text{TFA}$): m/z : 687.3 [$2\text{M}^{2+} + \text{H}$], 1218 [$2\text{M}^+ - 1\text{Gd} + \text{H}$], 1372.8 [$2\text{M}^+ + \text{H}$].

(3aS,4S,6aR)-11-Bromoundecyl 5-(Hexahydro-2-oxo-1H-thieno[3,4-d]imidazol-4-yl)pentanoate (7). This compound was synthesized following an adapted procedure.³³ A mixture biotin (1.00 g, 4.09 mmol), 11-bromo-1-undecanol (5.15 g, 20.50 mmol), p-toluenesulfonic acid (76.9 mg, 0.4 mmol) and toluene (34 mL) was stirred and refluxed at 120 °C for 72 h under argon. The precipitated unreacted biotin was immediately separated by filtration and the solvent was removed *in vacuo*. The residue was purified by flash chromatography ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 95:5) to give **7** as a white solid. Yield: 68% (1.33 g,

2.78 mmol). MS (ESI+, CHCl₃/MeOH): m/z: 477.35 [M⁺+H]. ¹NMR data were obtained in agreement with the reference.³³

(3aS,4S,6aR)-11-Sulfanylundecyl 5-(Hexahydro-2-oxo-1H-thieno[3,4-d]imidazol-4-yl)pentanoate (biotin-SH, 8). A stirred solution **7** (1.33 g, 2.78 mmol) in THF (100 mL) was cooled down to -10°C. Subsequently, hexamethyldisilathiane (2.92 mL, 13.95 mmol) and TBAF (1.0 M solution in THF with 5% water, 3.29 mL, 13.95 mmol) were added. The reaction mixture was allowed to reach room temperature while stirring under argon and under protection from ambient light in order to avoid photoinduced side reactions. After 20 min, the reaction mixture was washed with 20 mL of aqueous ammonium chloride (saturated) and with of CH₂Cl₂ (2 x 10 mL). Recrystallization from CH₂Cl₂ and hexane gave the desired compound **8** as a white solid. Yield: 89% (1.07 g, 2.48 mmol). MS (ESI+, CHCl₃/MeOH): m/z: 430.3 [M⁺+H]. ¹NMR data were obtained in agreement with the reference.³³

Nuclear Magnetic Resonance (NMR) Characterization. All NMR experiments were performed on a Bruker Avance II NMR spectrometer operating at 600.16 MHz for ¹H and 150.91 MHz for ¹³C. Chemicals shifts are given in ppm using the residual solvent signal as reference. The multiplicity of the peaks is reported using the following abbreviations: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. All NMR spectra were processed using TOPSPIN 2.1 and MestReC software packages.

Mass Spectrometry (MS). Mass spectra were acquired on a Waters Micromass LCT ESI or a Voyager-DE RP MALDI-TOF mass spectrometer.

UV-Vis Spectroscopy. UV spectra were acquired on a Perkin Elmer Lambda 850 UV-Vis spectrometer using Plastibrand 1.5 mL semi-micro cuvettes.

Fluorescence Spectroscopy. All steady-state spectra were recorded using a Perkin Elmer Fluorescence Spectrometer and 1cm quartz cuvettes.

Magnetic Resonance Imaging (MRI). All experiments were carried out on a 600 MHz (14.1) Avance II NMR Spectrometer from Bruker (Karlsruhe, Germany) equipped with a vertical narrow bore magnet (14.1 T), a great B_0 compensation unit (BGU-II) and 3 great 1/60 amplifier units (X, Y and Z) also from Bruker. A micro 5 imaging probe, equipped with a 10 mm diameter saddle coil insert from Bruker (Karlsruhe, Germany) was used. The maximum gradient strength was 4.8 G/cm/A and the temperature inside the probe was kept at $\sim 25^\circ\text{C}$ by a gradient water cooling unit BCU20 from Bruker. All NMR/MRI tubes (5/10 mm diameter) were purchased from Wilmad Labglass (New Jersey, U.S.A.). Glass capillary tubes (melting point tubes, inner diameter 1mm) used for MRI were purchased from VWR. T_1 measurements were performed in a 1 cm (outer diameter) glass tube as follows. Stock solutions of the Gd^{3+} -SUDDO3A complex and the MMAuNRs (with known concentrations of Gd^{3+} and AuNRs) were diluted 2, 5 and 10 times. For each experiment, capillary tubes of 1 mm inner diameter were filled with these solutions, placed in a custom-made Kel-F capillary holder and subsequently introduced in the 1 cm tube for imaging. Longitudinal relaxation times were measured using a saturation recovery pulse sequence with static $\text{TE} = 4$ ms and 16 TR values, namely 100, 150, 200, 350, 500, 600, 700, 800, 1000, 1500, 2000, 3000, 4000, 6000, 8000, 10000 ms. The

measurements were done on 5 subsequent axial slices of 1mm, with a field of view (FOV) of 10 mm x 10 mm, a matrix size of 128 x 128 and 2 averages. T_1 values were obtained by mono-exponentially fitting the intensities of selected regions of interest (ROIs) for each axial slice using the image sequence analysis tool in Paravision 4.0 software. T_1 -weighted images were obtained using a spin echo sequence with the following parameters: TR = 500 ms, TE = 4 ms, 1 axial slice of 6 mm thickness, FOV = 10 mm x 10 mm, matrix size = 256 x 256 and 10 averages. The longitudinal relaxivity r_1 (in $\text{mM}^{-1} \text{s}^{-1}$) was obtained as the slope of the plot $1/T_1$ vs. Gd^{3+} (or AuNR) concentration:

$$\left(\frac{1}{T_1}\right)_{obs} = \left(\frac{1}{T_1}\right)_d + r_1[Gd]$$

Transmission Electron Microscopy (TEM) and Energy-Dispersive X-Ray (EDX). TEM images were obtained using analytical TEM FEI instruments. In order to prepare the samples, 10 μL of the desired sample was applied onto Formvar-carbon coated grids (from electron microscopy sciences). After leaving the sample for 1 minute, the excess of liquid was removed using a piece of filter paper. The average aspect ratio (length/width) of the different AuNRs was determined by measuring their length and width, for more than 50 counts, from TEM images.

Cell Culture. C2C12 (Mouse myoblast) cells were plated in a 96 well plate at a density of 5000 cells/well in Dulbecco's modified eagle medium (DMEM) media (Sigma-Aldrich). The medium was supplemented with 4.5 g/L glucose, L-glutamine and NaHCO_3 ; 1% (v/v) Penicillin-Streptomycin (with 10,000 units penicillin and 10 mg streptomycin/mL, Sigma-Aldrich). The cells were cultured at 37°C in a humidified 5% CO_2 atmosphere for 16

hours. Subsequent to equilibration, adhered cells were carefully washed twice with sterile PBS buffer to remove loosely attached cells.

Cell Viability Assay. Cell viability as a response to AuNRs (supplemented in the culture media) was studied using the CellTiter 96® AQueous One Solution Cell Proliferation Assay Test (Promega, The Netherlands). After removing loosely attached cells, the test AuNRs were supplemented (50 µL) in fresh culture media in four or five serial dilutions (in triplicate, see Table 5.5). The same series of dilutions were added without any cells (in duplicate) to be used as sample background (SB) control. Culture medium without the test compounds and cells was used as a medium-only control (LC_0) and C2C12 cells were used at the same density as cell-only control (LC_{100}). Cells were exposed to the AuNRs for 24 h in the same culture conditions described above. Subsequently, 20 µL of CellTiter 96® AQueous One Solution Reagent ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) and an electron coupling reagent (phenazine ethosulfate, PES) were added to all wells. The plates were shielded from ambient light and incubated for another 4 h at the culture conditions. The absorbance was then recorded at 490 nm using a Tecan plate reader. A reference wavelength of 630 nm was used to subtract the background contributed by excess cell debris, fingerprints and other nonspecific absorbance.

Table 5.5 AuNRs concentrations used for cell viability studies

Incubation conditions	[AuNRs] pM	0	10	50	100	150	200
100% PEG-SH		x	x	x	x		x
75% PEG-SH / 25% Gd ³⁺ -SUDDO3A		x	x	x	x		x
50% PEG-SH / 50% Gd ³⁺ -SUDDO3A		x	x	x	x		x
25% PEG-SH / 75% Gd ³⁺ -SUDDO3A		x	x	x	x		x
100% Gd ³⁺ -SUDDO3A		x	x	x	x		x
50% PEG-SH / 50% Biotin-SH*		x	x	x	x	x	
25% PEG-SH / 25% Gd ³⁺ -SUDDO3A / 25% Biotin-SH*		x	x	x	x	x	

* These AuNRs were incubated with Streptavidin-Cy5 before use

Data processing and analysis was done following an adapted procedure from literature.²⁸ Briefly, the average values of the absorbance readings for the triplicates were calculated and corrected by deducting the average absorbance from the sample background control for each case. The standard deviation of the corrected values (σ) was calculated from the linear combination of errors.

The corrected absorbance for the cell-only control was calculated by subtracting the average absorbance of the medium-only control. This is taken as the absorbance associated with living cells. The percentage of surviving cells is then calculated dividing the corrected sample absorbance by the corrected cell-only absorbance and multiplied by 100. The corresponding standard deviation was calculated following the quotient combination of errors.

Cell Staining. After removal of the cell culture medium, cells were washed with sterile PBS once and subsequently fixed with a 4% formaldehyde solution in PBS for 10 minutes at room temperature. Afterwards, C2C12 cells were stained for F-actin with Alexa Fluor® 488

phalloidin (Invitrogen) and DAPI, dilactate (Invitrogen) following the protocol from the provider. Finally, the substrates were washed two times with PBS, water and were carefully blown-dry with N₂.

Fluorescence Microscopy. Fluorescence microscopy images were taken using an Olympus inverted research microscope IX71 equipped with a mercury burner U-RFL-T as light source and a digital Olympus DR70 camera for image acquisition using the following Olympus filter cubes: $300\text{ nm} \leq \lambda_{\text{ex}} \leq 400\text{ nm}$ for blue emission ($410\text{ nm} \leq \lambda_{\text{em}} \leq 510\text{ nm}$), $460\text{ nm} \leq \lambda_{\text{ex}} \leq 490\text{ nm}$ for green emission ($\lambda_{\text{em}} \geq 520$) and $630\text{ nm} \leq \lambda_{\text{ex}} \leq 650\text{ nm}$ for Cy5 emission ($665\text{ nm} \leq \lambda_{\text{ex}} \leq 695\text{ nm}$). All fluorescence microscopy images were acquired in air.

Total Reflection X-ray Fluorescence (TXRF). TXRF measurements were performed by a Bruker S2 Picofox TXRF (Bruker AXS GmbH, Karlsruhe, Germany) portable system. The primary X rays were generated by an air-cooled low power X-ray tube using a molybdenum anode. The excitation settings were 50 kV and 750 mA. Measurements were performed by signal integration over 1500 seconds. Quartz glass disks were used as sample carriers, which were previously cleaned in 1 N nitric acid followed by flushing in water for 1 h at 80°C. After that, they were dried, coated with silicon solution for better wettability and dried again. The quantification is performed by the Bruker S2 Picofox TXRF software (Spectra, Version 6.1.5.0), based on the known quantity of the gallium internal standard added. Samples for TXRF analysis were prepared by adding equal amounts of the gallium internal standard (10 mg/L) and the sample to be analyzed (volume). After vortexing, a drop of the solution was placed on the quartz disk and dried under vacuum for 15 minutes. For

control purposes, elemental analysis of the empty capsid (in buffer) was conducted and the values obtained, for each capsid sample in question, were corrected.

5.6 References

- (1) Cassidy, P. J.; Radda, G. K. *J. R. Soc. Interface* **2005**, *2*, 133.
- (2) Weissleder, R.; Mahmood, U. *Radiology* **2001**, *219*, 316.
- (3) Caravan, P.; Ellison, J. J.; McMurry, T. J.; Lauffer, R. B. *Chem. Rev.* **1999**, *99*, 2293.
- (4) Bottrill, M.; Kwok, L.; Long, N. J. *Chem. Soc. Rev.* **2006**, *35*, 557.
- (5) Jin, T.; Yoshioka, Y.; Fujii, F.; Komai, Y.; Seki, J.; Seiyama, A. *Chem. Commun.* **2008**, 5764.
- (6) Mulder, W. J. M.; Koole, R.; Brandwijk, R. J.; Storm, G.; Chin, P. T. K.; Strijkers, G. J.; de Mello Donegá, C.; Nicolay, K.; Griffioen, A. W. *Nano Lett.* **2005**, *6*, 1.
- (7) Yang, H.; Santra, S.; Walter, G. A.; Holloway, P. H. *Adv. Mater.* **2006**, *18*, 2890.
- (8) Alric, C.; Taleb, J.; Duc, G. L.; Mandon, C.; Billotey, C.; Meur-Herland, A. L.; Brochard, T.; Vocanson, F.; Janier, M.; Perriat, P.; Roux, S.; Tillement, O. *J. Am. Chem. Soc.* **2008**, *130*, 5908.
- (9) Debouttière, P. J.; Roux, S.; Vocanson, F.; Billotey, C.; Beuf, O.; Favre-Réguillon, A.; Lin, Y.; Pellet-Rostaing, S.; Lamartine, R.; Perriat, P.; Tillement, O. *Adv. Funct. Mater.* **2006**, *16*, 2330.
- (10) Moriggi, L. c.; Cannizzo, C.; Dumas, E.; Mayer, C. d. R.; Ulianov, A.; Helm, L. *J. Am. Chem. Soc.* **2009**, *131*, 10828.
- (11) Park, J.-A.; Reddy, P. A. N.; Kim, H.-K.; Kim, I.-S.; Kim, G.-C.; Chang, Y.; Kim, T.-J. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 6135.
- (12) Song, Y.; Xu, X.; MacRenaris, K. W.; Zhang, X.-Q.; Mirkin, C. A.; Meade, T. J. *Angew. Chem., Int. Ed.* **2009**, *48*, 9143.
- (13) Warsi, M. F.; Chechik, V. *Phys. Chem. Chem. Phys.* **2011**, *13*, 9812.
- (14) Murphy, C. J.; Gole, A. M.; Stone, J. W.; Sisco, P. N.; Alkilany, A. M.; Goldsmith, E. C.; Baxter, S. C. *Acc. Chem. Res.* **2008**, *41*, 1721.
- (15) Huang, X.; Neretina, S.; El-Sayed, M. A. *Adv. Mater.* **2009**, *21*, 4880.
- (16) Pérez-Juste, J.; Pastoriza-Santos, I.; Liz-Marzán, L. M.; Mulvaney, P. *Coord. Chem. Rev.* **2005**, *249*, 1870.
- (17) Tong, L.; Wei, Q.; Wei, A.; Cheng, J.-X. *Photochem. Photobiol.* **2009**, *85*, 21.
- (18) Thakor, A. S.; Jokerst, J.; Zavaleta, C.; Massoud, T. F.; Gambhir, S. S. *Nano Lett.* **2011**, *11*, 4029.
- (19) Giljohann, D. A.; Seferos, D. S.; Daniel, W. L.; Massich, M. D.; Patel, P. C.; Mirkin, C. A. *Angew. Chem., Int. Ed.* **2010**, *49*, 3280.

- (20) Zhou, W.; Shao, J.; Jin, Q.; Wei, Q.; Tang, J.; Ji, J. *Chem. Commun.* **2010**, 46, 1479.
- (21) Yi, D. K.; Sun, I.-C.; Ryu, J. H.; Koo, H.; Park, C. W.; Youn, I.-C.; Choi, K.; Kwon, I. C.; Kim, K.; Ahn, C.-H. *Bioconjugate Chem.* **2010**, 21, 2173.
- (22) Manohar, S.; Ungureanu, C.; Van Leeuwen, T. G. *Contrast Media Mol. I.* **2011**, 6, 389.
- (23) Liu, L.; Ding, H.; Yong, K.-T.; Roy, I.; Law, W.-C.; Kopwiththaya, A.; Kumar, R.; Erogbogbo, F.; Zhang, X.; Prasad, P. *Plasmonics* **2011**, 6, 105.
- (24) Sun, H.; Yuan, Q.; Zhang, B.; Ai, K.; Zhang, P.; Lu, L. *Nanoscale* **2011**, 3, 1990.
- (25) Cortesi, R.; Esposito, E.; Menegatti, E.; Gambari, R.; Nastruzzi, C. *Int. J. Pharm.* **1996**, 139, 69.
- (26) Takahashi, H.; Niidome, Y.; Niidome, T.; Kaneko, K.; Kawasaki, H.; Yamada, S. *Langmuir* **2005**, 22, 2.
- (27) Kopwiththaya, A.; Yong, K.-T.; Hu, R.; Roy, I.; Ding, H.; Vathy, L. A.; Bergey, E. J.; Prasad, P. N. *Nanotechnology* **2010**, 21, 315101.
- (28) Rayavarapu, R. G.; Petersen, W.; Hartsuiker, L.; Chin, P.; Janssen, H.; van Leeuwen, F. W. B.; Otto, C.; Manohar, S.; van Leeuwen, T. G. *Nanotechnology* **2010**, 21.
- (29) Vigdeman, L.; Manna, P.; Zubarev, E. R. *Angew. Chem., Int. Ed.* **2012**, 51, 636.
- (30) Basiruddin, S. K.; Maity, A. R.; Saha, A.; Jana, N. R. *J. Phys. Chem. C* **2011**, 115, 19612.
- (31) Basiruddin, S. K.; Saha, A.; Pradhan, N.; Jana, N. R. *Langmuir* **2010**, 26, 7475.
- (32) Weber, P.; Ohlendorf, D.; Wendoloski, J.; Salemme, F. *Science* **1989**, 243, 85.
- (33) Reichmuth, P.; Sigrist, H.; Badertscher, M.; Morf, W. E.; de Rooij, N. F.; Pretsch, E. *Bioconjugate Chem.* **2001**, 13, 90.
- (34) Castellana, E. T.; Gamez, R. C.; Russell, D. H. *J. Am. Chem. Soc.* **2011**, 133, 4182.
- (35) Orendorff, C. J.; Murphy, C. J. *J. Phys. Chem. B* **2006**, 110, 3990.
- (36) Link, S.; El-Sayed, M. A. *J. Phys. Chem. B* **1999**, 103, 8410.
- (37) Telgmann, L.; Holtkamp, M.; Kunнемeyer, J.; Gelhard, C.; Hartmann, M.; Klose, A.; Sperling, M.; Karst, U. *Metallomics* **2011**, 3, 1035.
- (38) Kobayashi, H.; Kawamoto, S.; Bernardo, M.; Brechbiel, M. W.; Knopp, M. V.; Choyke, P. L. *J. Controlled Release* **2006**, 111, 343.
- (39) Rieter, W. J.; Kim, J. S.; Taylor, K. M. L.; An, H.; Lin, W.; Tarrant, T.; Lin, W. *Angewandte Chemie, International Edition* **2007**, 46, 3680.
- (40) Block, E.; Eswarakrishnan, V.; Gernon, M.; Ofori-Okai, G.; Saha, C.; Tang, K.; Zubieta, J. *J. Am. Chem. Soc.* **1989**, 111, 658.
- (41) Beeby, A.; Bushby, L. M.; Maffeo, D.; Gareth Williams, J. A. *J. Chem. Soc., Dalton Trans.* **2002**, 48.
- (42) Wilhelm, M.; Zhao, C. L.; Wang, Y.; Xu, R.; Winnik, M. A.; Mura, J. L.; Riess, G.; Croucher, M. D. *Macromolecules* **1991**, 24, 1033.

Appendix 5.1. Calculation of the Critical Aggregation Concentration (cac) of Gd^{3+} -SUDDO3A

The critical aggregation concentration of Gd^{3+} -SUDDO3A, calculated to be 0.63 mM, was obtained using a previously reported pyrene-based procedure.⁴² In short, a known amount of pyrene in acetone was added to nine Gd^{3+} -SUDDO3A sample solutions (concentrations ranging from 0.0025 to 0.0001 M in water), to give a final pyrene concentration of 6×10^{-7} M (saturation solubility in water). After heating and cooling overnight to guarantee micelle formation, the emission spectra of all nine solutions was taken ($\lambda_{\text{exc}} = 332$ nm). In a plot of the fluorescence intensity at 387 nm vs. $\log [\text{Gd}^{3+}\text{-SUDDO3A}]$ (Figure 5.12), the intersection of the two tangents (before and after the inflection point) corresponds to the critical aggregation concentration (cac).

Figure 5.12. Plot of the fluorescence intensity of pyrene ($\lambda_{\text{exc}} = 332$ nm) as a function of the Gd^{3+} -SUDDO3A concentration.

Appendix 5.2. MRI Data

Figure 5.13. Relaxivity plot of the Gd³⁺-SUDDO3A complex at 600 MHz and 25°C.

Figure 5.14. Relaxivity plot of the MMAuNRs (with respect to the AuNR concentration) at 600 MHz and 25°C

Table 5.6. T_1 values obtained for the Gd^{3+} -SUDDO3A complex at 600 MHz and 25°C

$[\text{Gd}^{3+}]$ mM	T_1 Slice 1 (ms)	T_1 Slice 2 (ms)	T_1 Slice 3 (ms)	T_1 Slice 4 (ms)	T_1 Slice 5 (ms)	Average (ms)	Stand. Dev. (ms)	$1/T_1$ (s^{-1})
0.65	1153.2	1206.9	1198	1202.3	1177.5	1187.6	22.2	0.842
0.325	1594.3	1695.6	1659.8	1686	1625.9	1652.3	42.2	0.605
0.130	2193.7	2298.7	2336.3	2327.2	2268.7	2284.9	57.5	0.438
0.065	2732.7	2793.2	2798.5	2777.2	2760.3	2772.4	26.7	0.361

Table 5.7. T_1 values obtained for the MMAuNRs at 600 MHz and 25°C

$[\text{Gd}^{3+}]$ mM	T_1 Slice 1 (ms)	T_1 Slice 2 (ms)	T_1 Slice 3 (ms)	T_1 Slice 4 (ms)	T_1 Slice 5 (ms)	Average (ms)	Stand. Dev. (ms)	$1/T_1$ (s^{-1})
0.643	840.5	943.5	914.4	915.7	909.3	904.7	38.3	1.105
0.129	1570.2	1628.7	1604.5	1548	1518.2	1573.9	43.9	0.635
0.064	1797.2	1930.3	1938.3	1981.9	1937.7	1917.1	70.0	0.522
0	2372.8	2484.6	2534.9	2556.6	2472.4	2484.3	71.3	0.403

*“Et il est un jour arrive / Marteler le ciel/ Et marteler la mer
Et la mer avait embrassé moi/ Et la délivré moi de ma caille
Rien ne peut m'arrêter maintenant”
La mer, Nine Inch Nails*

CHAPTER 6

FUTURE PERSPECTIVES: METAL COMPLEX-BASED TEMPLATES IN CARBON-BASED SCAFFOLDS AS MAGNETIC RESONANCE/OPTICAL MULTIMODAL IMAGING AGENTS

Abstract

The future perspectives of the metal complex-based templates and the multimodal imaging agents described in this thesis are discussed here. The versatility of the 1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid (DO3A)-based ligands described in previous chapters is demonstrated by their integration in carbon-based scaffolds such as small organic molecules and carbon nanotubes (CNTs). The preliminary characterization and evaluation of their properties presented here show promising results, thereby highlighting their potential for multimodal purposes. Finally, challenges ahead for the metal complex-based templates and magnetic resonance/optical multimodal imaging agents presented in this thesis are briefly discussed with an outlook toward the future.

6.1 Introduction

Molecular imaging has developed as a diagnostic discipline dedicated to the visualization of biological processes in the human body in an, ideally but not always attainable, non-invasive manner. Throughout the years, several strategies and methodologies for the synthesis of multimodal imaging agents (IAs), needed in order to aid the visualization of region of interest (ROIs), have been followed. While many of them are based on pure synthetic chemistry,¹⁻⁴ the combination of existing chromophores and metal complexes with nanomaterials is currently a highly explored approach.⁵⁻¹¹ Inspired by the latter, the results presented in this thesis show that 1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid (DO3A)-based ligands are highly suitable candidates for (multimodal) molecular imaging probes either by i) acting as templates for the growth of nanomaterials (as shown in Chapter 4), or ii) as chelates for metal complexation in nanomaterials (e.g., protein cages and gold nanorods, Chapters 5 and 6 respectively).

Lanthanides have been widely used for the fabrication of IAs as these elements show strikingly different electronic and magnetic properties.¹² Whereas gadolinium (Gd^{3+}) is known for its excellent paramagnetic properties,¹³⁻¹⁵ europium (Eu^{3+}) and terbium (Tb^{3+}) are highly attractive owing to their fluorescent properties.^{16,17} In the latter cases, an antenna or sensitizer (e.g., a chromophore¹⁸⁻²² or a transition metal)²³⁻²⁶ is needed for energy transfer to the lanthanide as its electric dipole f-f transitions have very low extinction coefficients and are Laporte forbidden.¹⁶ Remarkably, lanthanides can be chelated by polydentate ligands in a rather comparable manner since they are, chemically, very similar.^{14,16,17} The previously mentioned features have led to the development of bimodal molecular

probes that can be used for either optical or magnetic resonance imaging (MRI).^{4,23,27-30}

In this chapter, the use of functionalized carbon-based organic scaffolds as platforms for multiple lanthanide complexation is proposed. First, a small, water-soluble carbon-based scaffold, consisting of an anthracene core where two identical DO3A-based chelators (similar to the one presented in Chapter 5) are integrated, is presented. Preliminary results show the promising paramagnetic and optical properties of the final metal complex. Subsequently, the covalent attachment of the AB-DO3A template (shown in Chapter 4) to a bigger carbon-based scaffold, i.e. carbon nanotubes (CNTs), is debated together with some preliminary characterization. The scaffolds discussed here are envisaged as excellent candidates for multimodal molecular imaging applications. Finally, the challenges ahead are briefly discussed.

6.2 An Anthracene-Core Multimodal Agent for Magnetic Resonance and Fluorescence Imaging

The successful synthesis and implementation of bimodal imaging probes paved the way for the development of IAs where various imaging modalities are combined within one molecule. Initially, the first di- and trinuclear lanthanide complexes described in the literature consisted of combinations of only fluorescent lanthanides for sensing purposes.^{31,32} The first example of a potential di-nuclear lanthanide complex for multimodal imaging was described by Mamedov *et al.*,² who linked two different chelating moieties using a benzene-based antenna. The formation of bimetallic species was confirmed by means of luminescence and NMR diffusion studies.

Here, a small, water-soluble ligand suitable for multiple lanthanide complexation is presented. The bis-macrocyclic ligand ANT-2DO3A consists of an anthracene core where two (identical) 1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid (DO3A)-based chelators are integrated. Therefore, the ANT-2DO3A ligand is expected to show comparable affinity to various lanthanide ions. The characterized ligand was subsequently complexed with a 50% Gd^{3+} / 50% Eu^{3+} mixture, thereby leading to the formation of a multimodal IA, as suggested by its fluorescent and paramagnetic properties. This approach serves as a proof-of-principle for further development of small molecule, multimodal IAs by exploiting the chemically similar, but electronically/magnetically different, properties lanthanides show.

6.2.1 Design and Synthesis

Within this study the model ligand serving as proof-of-principle is a symmetrical bis-macrocycle, consisting of two identical chelating units for lanthanide complexation tethered by an anthracene-base core. The choice for identical chelating units is based on the hypothesis that all lanthanides are complexed by a single chelator in a similar fashion.^{14,16,17} Consequently, equal affinity to any lanthanide upon reaction is assumed. Moreover, the symmetrical, equally substituted anthracene core is an excellent platform for integration of chelators using the same chemistry, thereby reducing the number of synthetic steps and simplifying the characterization by NMR. More importantly, the anthracene core fulfills a second function by serving as an antenna for energy transfer to the fluorescent lanthanide.

The synthesis of the bis-macrocyclic ligand for lanthanide complexation (ANT-2DO3A) was carried out in three steps, namely: i) integration of a propyl alkylated maleimide linker to the anthracene core (Scheme 6.1), ii)

integration of a thiolated propyl linker to the 1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid (DO3A) chelator (Scheme 6.2) and iii) reaction of the DO3A-derivative with the anthracene core via a Michael-type addition reaction (Scheme 6.3).

6.2.2 Synthesis of the Building Blocks

The synthesis of the anthracene-core building block 9,10-bis[methyl-4-*N*-(butanamide)maleimide]anthracene (**3**) is shown in Scheme 6.1. The starting material, 9,10-bis(aminomethyl)anthracene (**1**) was synthesized by bromoalkylation of anthracene³³ and subsequent nucleophilic substitution³⁴ using adapted literature procedures. The bromopropyl linker was incorporated on both sides of (**1**) by the formation of an amide in chloroform using 4-bromobutyryl chloride (under N₂) in the presence of *N,N'*-diisopropylethylamine (DIPEA). Finally, the substituted anthracene core **3** was obtained by reacting maleimide with (**2**) using K₂CO₃ in acetonitrile (at 50°C) for 24 hours. Compound **3** was obtained with an overall yield (after purification) of 52%.

Scheme 6.1. Reaction scheme showing the synthetic pathway for the synthesis of the anthracene-core building block 9,10-bis[methyl-4-*N*-(butanamide)maleimide]anthracene (**3**).

The synthesis of the second building block of the bis-macrocyclic ligand ANT-2DO3A, the thiolated 1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid (DO3A) chelator (**8**), is shown in Scheme 6.2. First, 3-chloro-1-propanethiol (**4**) was protected by reaction with dihydropyran in the presence of pyridinium *p*-toluene sulfonate in CH₂Cl₂ overnight at room temperature. To obtain **7**, 1,4,7-tris(*tert*-butoxycarbonylmethyl)-1,4,7,10-tetraazacyclododecane (**6**) was alkylated with the protected thiol (**5**) in dry acetonitrile using K₂CO₃ and KI (under N₂) for three days at 50°C. The chelator 1-(3-mercaptopropyl)-4,7,10-triacetic acid-1,4,7,10-tetraazacyclododecane (**8**) was formed by deprotection of (**7**) at room temperature using TFA/CH₂Cl₂ overnight with an overall yield (after purification) of 24%.

Scheme 6.2. Reaction scheme showing the synthetic pathway followed for the synthesis of 1-(3-mercaptopropyl)-4,7,10-triacetic acid-1,4,7,10-tetraazacyclododecane (**8**).

6.2.3 Synthesis of the ANT-2DO3A Ligand and its Lanthanide Complexes

The building blocks **3** and **8** were allowed to react in methanol overnight (Scheme 6.3), leading to the formation of the ligand **9** via a thiol-maleimide Michael-type addition. After dialysis in water for three days, ANT-2DO3A (**9**) was obtained with a yield of 5%.

Scheme 6.3. Reaction scheme showing the formation of the bis-macrocyclic ligand ANT-2DO3A (**9**) by means of a thiol-maleimide Michael-type addition.

The paramagnetic and fluorescent properties were integrated to the ANT-2DO3A (**9**) ligand by simultaneous complexation with Gd^{3+} and Eu^{3+} , respectively. This reaction was carried out in the presence of 50% GdCl_3 and 50% EuCl_3 (Scheme 6.4) in water (pH 6, adjusted with 1M NaOH) at 50°C for 24 hours. Owing to the assumed equal lanthanide affinity by the ANT-2DO3A ligand, the formation of three complexes, i.e., the paramagnetic 2Gd^{3+} -ANT-2DO3A, the fluorescent 2Eu^{3+} -ANT-2DO3A and the multimodal $\text{Gd}^{3+}/\text{Eu}^{3+}$ -ANT-2DO3A, is expected.

Scheme 6.4. Reaction scheme showing the three possible complexes formed after complexation of **9** with 50% GdCl_3 and 50% EuCl_3 .

6.2.4 Characterization of the Final Bis-Macrocylic ANT-2DO3A Ligand and its Lanthanide Complexes

All intermediates of the two building blocks shown in Scheme 6.1 and Scheme 6.2 were characterized using ^1H and ^{13}C nuclear magnetic resonance (NMR) and electrospray (ESI)/matrix-assisted laser desorption ionization (MALDI) mass spectrometry (MS) (data can be found in the experimental section and Appendix 6.1).

Interestingly, the molecular weight of the water-soluble ANT-2DO3A ligand (1406.6 g/mol) facilitates its purification by dialysis. When using a 1000 Da dialysis membrane, low percentages of 1) the mono-macrocylic ligand (anthracene-core building block **3** with only one DO3A-based ligand **8**

attached to it) and 2) the DO3A-based ligand **8** dimer (resulting from possible disulfide bridge formation) are likely to be present in the final solution as both species have molecular weights below (but close) the molecular cut-off of the membrane (988.4 and 838.4 g/mol respectively.). Full characterization of the final bis-macrocyclic ligand ANT-2DO3A (**9**, Scheme 6.3) proved to be challenging due to various constraints: MS data remain inconclusive (as no m/z ratio peak could be assigned to the final molecule or to any fragment) and the water solubility of ANT-2DO3A was lower than that required for ^{13}C NMR spectroscopy. Still, characterization of ANT-2DO3A was possible using ^1H NMR spectroscopy. However, the restricted rotational motion of the full molecule, together with the rigidity of the DO3A cyclen core made it difficult to identify and assign all proton signals properly. As shown in Figure 6.1 (top), the ^1H NMR spectrum shows signals on both the aromatic and the aliphatic region. Whereas the aromatic peaks correspond to the anthracene core, those in the aliphatic region agree with typical DO3A ^1H NMR spectra, where broad signals are often observed due to hindered rotation.³⁵ This observation is supported by experiments at higher temperatures (50°C, Figure 6.1 bottom), where *only* the resolution of the peaks corresponding to the DO3A-based ligand increases (highlighted area). No changes in the ^1H NMR spectra were observed after 8 months, thereby confirming the stability of ANT-2DO3A in solution.

Figure 6.1. ^1H NMR spectrum of ANT-2DO3A (**9**) in D_2O at 25 and 50°C.

Further studies using 2D NMR techniques (COSY AND HMQC) led to the assignment of several of the proton signals observed in Figure 6.1. As shown in Figure 6.2, the aromatic (**a**, **b**), amide (**d**), and DO3A protons (**i**, **h**, **m**, **n**, **o**, **p**, **q**, **r**) were identified at 7.42–7.31, 8 and 4–2.75 ppm, respectively. Also, protons **e**, **f** and **g** were assigned to the propyl alkylated maleimide linker and the signal corresponding to the **c** protons was found to coincide with the water signal (intentionally suppressed in this spectrum). While little is known about the spatial location of the DO3A building block (**8**), upon reaction with **3**, the signals corresponding to the thiolated propyl linker (**j**, **k** and **l**) protons are expected to show splitting (non-equivalency) due to the presence of two chiral centers (highlighted with black circles in Figure 6.2). The proton signals observed at 2.18, 1.5, 1.38, 1.23 and 0.78 ppm are thought to correspond to the thiolated propyl

linker (j, k and l). Interestingly, the splitting observed for these signals show that the symmetry in the molecule is lost only after the chiral centers. Furthermore, integration of the assigned signals revealed that the value obtained for the aromatic signals (8 protons, 7.42-7.31 ppm) matches those obtained for the propyl alkylated maleimide linker (2.70 and 2.02 ppm, 4 protons each, 3.10 cannot be integrated) and the thiolated propyl linker (total of 12 protons).

Figure 6.2. ^1H NMR spectrum and peak assignment of ANT-2DO3A at 25°C. Signals from dialysis membrane residual are indicated with an asterisk (*) and chiral centers are indicated with black circles.

Matrix-assisted laser desorption ionization (MALDI) MS characterization of the $\text{Gd}^{3+}/\text{Eu}^{3+}$ -ANT-2DO3A complex suggests the formation of a Gd^{3+} -containing complex. As shown in Figure 6.3, the peak with a m/z value of

976.52 was successfully identified as a fragment of the final compound, namely one resulting from cleavage of one of the amide bonds connecting the propyl alkylated maleimide to the anthracene core (theoretical molecular weight: 976.28). Furthermore, this peak shows a Gaussian-like isotope pattern consistent with lanthanide-containing species (due to the various abundant isotopes) (Figure 6.3, inset), most likely Gd^{3+} as it has more isotopes than Eu^{3+} (7 vs. 2).

Figure 6.3. MALDI-MS spectrum of $\text{Gd}^{3+}/\text{Eu}^{3+}$ -ANT-2DO3A. The peak with the highest intensity was observed at m/z 976.52. Inset: amplification of the region between m/z 969 and 982.

Despite the various challenges faced in order to fully characterize the ANT-2DO3A ligand and its $\text{Gd}^{3+}/\text{Eu}^{3+}$ complex, and encouraged by the promising MS results, the paramagnetic and optical properties of the suspected $\text{Gd}^{3+}/\text{Eu}^{3+}$ -ANT-2DO3A complex were studied.

6.2.5 Optical Properties

The optical properties of the ANT-2DO3A ligand and its $\text{Gd}^{3+}/\text{Eu}^{3+}$ complex were investigated using UV/Vis absorbance and steady-state fluorescence spectroscopy. As shown in Figure 6.4 (black line), a broad absorption band with maximum at 312 nm was observed for the ANT-2DO3A ligand in H_2O . Note that in H_2O , the fine structure (vibronic peaks corresponding to different vibrational sub-levels) usually seen for anthracene in non-polar solvents is not observed. Upon lanthanide complexation, the absorption spectrum shows a hypsochromic (blue) shift with a maximum at 270 nm (green line), thereby suggesting a change in the absorption properties of the ANT-2DO3A ligand. In particular, the π -electron system of the anthracene core is strongly affected by the incorporation of the lanthanide.³⁶ For comparative purposes, the absorption spectrum of $\text{Gd}^{3+}/\text{Eu}^{3+}$ -ANT-2DO3A in $\text{H}_2\text{O}/\text{ACN}$ is also shown (red line). In this case, the anthracene-like fine structure is observed, with maxima at 269 and 262 nm.

Figure 6.4. UV absorption spectra of the ligand ANT-2DO3A in H_2O (black line) and the complex $\text{Gd}^{3+}/\text{Eu}^{3+}$ -ANT-2DO3A in H_2O (green line) and $\text{H}_2\text{O}/\text{ACN}$ (red line).

In case of Eu^{3+} complexation by the ANT-2DO3A ligand, energy transfer from the anthracene core to the chelated lanthanide is expected. However, how efficient this process will be is unknown since little is understood about the final conformation of the complex (how close the chelates are to the antenna) and if the length of the linkage between building blocks 3 and 8 affects it. Therefore, possible fluorescence emission resulting from energy-transfer from the anthracene to the Eu^{3+} complex was studied by steady-state fluorescence spectroscopy. In Figure 6.5, the emission spectrum of the ANT-2DO3A ligand in H_2O (grey line) is presented, showing anthracene emission (when exciting at $\lambda = 312$ nm) with a maximum at 442 nm. Moreover, anthracene-like emission was also observed in the $\text{Gd}^{3+}/\text{Eu}^{3+}$ -ANT-2DO3A complex sample ($\text{H}_2\text{O}/\text{ACN}$, black line), when exciting at 262 nm, with maxima at 397, 419 and 561 nm. A broad peak, with maxima at 600 nm and two shoulders at 590 and 615 nm, was also observed. Although the peaks could not be resolved, this region is consistent with bands observed for sensitized Eu^{3+} complexes and associated to $^5\text{D}_0 \rightarrow 7\text{F}_1$ (591 nm), $^5\text{D}_0 \rightarrow 7\text{F}_2$ (615 nm) and $^5\text{D}_0 \rightarrow 7\text{F}_3$ (658 nm) transitions.^{26,36,37} Thus, these results strongly suggest the formation of an Eu^{3+} complex with a conformation of the final ANT-2DO3A complex such that, despite the distance between the anthracene moiety and the Eu^{3+} complex, energy transfer occurs.

Figure 6.5. Emission spectra of ANT-2DO3A (grey line, H_2O , $\lambda_{\text{exc}} = 312 \text{ nm}$) and $\text{Gd}^{3+}/\text{Eu}^{3+}$ -ANT-2DO3A (black line, $\text{H}_2\text{O}/\text{ACN}$, $\lambda_{\text{exc}} = 262 \text{ nm}$).

6.2.6 Paramagnetic Properties

Shortened longitudinal relaxation (T_1), resulting from the formation of a Gd^{3+} complex, was studied using MRI. As shown in Figure 6.6, enhanced T_1 contrast for a series of dilutions of $\text{Gd}^{3+}/\text{Eu}^{3+}$ -ANT-2DO3A was observed, thereby confirming the paramagnetic properties of the complex. While the two lowest concentrations (0.4 and 0.8 g/L $\text{Gd}^{3+}/\text{Eu}^{3+}$ -ANT-2DO3A) appear to be within the detection limit, the highest two clearly show contrast differences when compared to the control (H_2O).

Figure 6.6. T_1 -weighted MR Image showing various concentrations (4, 2, 0.8 and 0.4 g/L) of Gd^{3+}/Eu^{3+} -ANT-2DO3A complex and H_2O (control).

6.3 Carbon Nanotubes (CNTs) for Multimodal Molecular Imaging: a Preliminary Study

To demonstrate the versatility of the 1,4,7,10-tetraaza-1-(1-aminobutyl)-4,7,10-triacetic acid cyclododecane (AB-DO3A, Chapter 4) ligand, the nanoparticle growth-mediator template AB-DO3A was attached to carbon nanotubes (CNTs) and used for metal (lanthanide) complexation.

6.3.1 Design and Synthesis

Given the wide variety of current approaches for covalent functionalization of CNTs,³⁸ the incorporation of AB-DO3A to SWCNTs was explored. Under the hypothesis that the free amine of the 4th pendant arm of AB-DO3A can be incorporated to any nanostructure, CNTs are an advantageous archetype nanomaterial.³⁹⁻⁴¹ In the past ten years, the increased water solubility (and stability) of oxidized SWCNTs (ox-SWCNTs)⁴² has opened

up the doors for various biological applications, including multimodal imaging.^{39,41,43,44} Indeed, while known for their intrinsic T_2 relaxation effect,⁴⁴ one of the first examples of CNT-based T_1 relaxation agents were the gadonanotubes described by Hartman *et al.*⁴³ These single-walled carbon nanotubes (SWCNTs) contained small paramagnetic clusters of Gd^{3+} ions residing in the sidewall defects of the nanotubes. In another example, Richard *et al.*⁴⁴ reported non-covalently functionalized multi-walled carbon nanotubes (MWCNTs) using amphiphilic Gd^{3+} -DTPA-based chelates. Encouraged by their high surface-to-volume ratio, making them excellent platforms for the attachment of large loads of (various) molecular imaging probes, SWCNTs were functionalized with AB-DO3A, whose water solubility is enhanced by the three carboxylic acids of this ligand. Subsequently, AB-DO3A-SWCNTs were used as chelates for lanthanide complexation. Interestingly, since lanthanides are characterized by their differences in properties (paramagnetic Gd^{3+} vs. fluorescent Eu^{3+}),¹³⁻¹⁷ AB-DO3A-SWCNTs are excellent platforms for magnetic resonance/optical multimodal imaging. The final SWCNTs were characterized by means of 1H NMR and infrared (IR) spectroscopy, vibrating sample magnetometry (VSM), transmission electron microscopy (TEM) and energy-dispersive X-ray (EDX) spectroscopy.

6.3.2 Covalent Functionalization of CNTs

The reaction scheme followed for the synthesis of Ln^{3+} -AB-DO3A SWCNTs is shown in Scheme 6.5. Here, the free amine of the AB-DO3A ligand serves as an excellent handle for amide formation with ox-SWCNTs using standard protocols (in water).³⁹ The AB-DO3A-SWCNTs obtained were dialyzed and allowed to bind to Gd^{3+} (for MRI) and Eu^{3+} (for optical imaging) in order to form the desired complexes. The water solubility of all

the intermediates and the final product allows the use of simple dialysis protocols for purification of the final nanomaterial.

Scheme 6.5. Synthesis of Ln³⁺-AB-DO3A-SWCNTs.

The AB-DO3A functionalized SWCNTs were characterized using ¹H NMR and IR spectroscopy. The ¹H NMR spectrum corresponding to the functionalized AB-DO3A-SWCNTs (Figure 6.7, top) shows the presence of various resonance peaks in the aliphatic region. These peaks are shifted and broadened when compared those observed in the ¹H NMR spectrum of AB-DO3A (Figure 6.7, bottom). This last feature can be attributed to a slower molecular rotation due to attachment to bigger scaffolds (in this case the ox-SWCNTs), making it difficult to fully assign all protons.

Additionally, the peaks corresponding to the closest protons to the free amine (3 ppm) are not observed after functionalization (highlighted area). These two observations strongly suggest the successful functionalization of ox-SWCNTs with the AB-DO3A ligand.

Figure 6.7. ^1H NMR spectra of functionalized AB-DO3A-SWCNTs (top) and AB-DO3A (bottom) in D_2O at 25°C .

IR spectroscopic studies further supported the apparent surface functionalization of ox-SWCNTs. As shown in Figure 6.8, ox-SWCNTs (grey line) show vibrational bands at 1715 cm^{-1} ($\text{C}=\text{O}$ stretch, carboxylic acids on the ox-SWCNTs surface) and 1565 cm^{-1} ($\text{C}=\text{C}$ bend, SWCNTs). After functionalization, the vibrational band at 1715 cm^{-1} (black line) corresponds to remaining (non-functionalized) carboxylic acids. However, the appearance of a more intense, broad vibrational band at 1534 cm^{-1} with a shoulder at 1633 cm^{-1} strongly suggests changes in the structure of

the SWCNTs⁴⁵ due to amide formation ($1690\text{--}1630\text{ cm}^{-1}$) resulting from the functionalization with AB-DO3A.

Figure 6.8. IR- spectra of ox-SWCNTs (grey line) and AB-DO3A-SWCNTs (black line).

6.3.3 Lanthanide Complexation using AB-DO3A-SWCNTs

The AB-DO3A-SWCNTs were subsequently allowed to react with Gd^{3+} and Eu^{3+} salts in order to provide paramagnetic and optical properties, respectively, to them. Changes in the magnetic properties of the Gd^{3+} -AB-DO3A-SWCNTs were measured using a vibrating sample magnetometer (VSM). Pristine SWCNTs are super paramagnetic, most likely due to the presence of transition metals (Fe and Ni) used in catalysts for their synthesis (spectrum not shown). A significant decrease in magnetization is observed upon oxidation of the SWCNTs (Figure 6.9, left), showing that the catalyst debris is removed during the oxidation step. The very low saturation magnetization of the ox-SWCNTs facilitates the observation of the diamagnetic component (due to the carbon of the SWCNTs): a negative slope (at saturation) is observed. The positive slope of the

magnetization (at saturation) observed, as well as the disappearance of the diamagnetic component, is indicative of paramagnetic Gd^{3+} -AB-DO3A-SWCNTs (Figure 6.9, right). The manifestation of a paramagnetic component strongly suggests the presence of Gd^{3+} complexes on the surface of the ox-SWCNTs.

Figure 6.9. Magnetization curves of Ox-SWCNTs (left), and Gd^{3+} -AB-DO3A-SWCNTs (right).

Finally, the resulting Ln^{3+} -AB-DO3A-SWCNTs were visualized by TEM (Figure 6.10), and the presence of Gd^{3+} and Eu^{3+} on the surface of the SWCNTs was studied using EDX spectroscopy. The insets of Figure 6.10 show the EDX spectra of Gd^{3+} -AB-DO3A-SWCNTs (left) and Eu^{3+} -AB-DO3A-SWCNTs (right). In both cases, X-ray emission at the appropriate lanthanide energy was observed, thereby confirming the presence of these species on the surface of the functionalized SWCNTs. In each TEM image, X-ray emission at the energy corresponding to Eu^{3+} or Gd^{3+} was not observed for selected background regions.

Figure 6.10. TEM-images of Gd³⁺-AB-DO3A-SWCNTs (left) and Eu³⁺-AB-DO3A-SWCNTs (right) (scale bar left = 50 nm and right = 20 nm). Inset in both pictures is the EDX spectrum of the regions highlighted in white.

While this example highlights the versatility of the metal complex-based templates developed in previous chapters, there are several concerns and challenges to be overcome. Furthermore, the incorporation of extra features such as a targeting/therapeutic moiety or simply other combinations of imaging modalities is, intuitively, the next step to follow.

6.4 Challenges in Metal Complex-Based Templates for Magnetic Resonance/Optical Multimodal Imaging Agents

6.4.1 Characterization

Proper characterization of these imaging probes remains a difficult task. Typically, a combination of routine techniques for small organic molecules

(e.g., NMR, IR, MS, HPLC) and state-of-the-art techniques for nanomaterials (e.g., TEM, SEM, XPS, etc.) is used. However, the former techniques not always provide full information (as shown in Chapter 3) and the latter ones strongly depend on, e.g., sample preparation (AuNRs in Chapter 5). Furthermore, several of these probes suffer from solubility issues, making their characterization even more problematic. The challenge remains finding the right set of techniques that offer sufficient accurate and valuable information.

6.4.2 Other Imaging Techniques

The ligands presented in this thesis show great flexibility as they can be used to complex other metal ions and isotopes, resulting in new properties for other modalities such as nuclear imaging. Moreover, the metal nanoparticles described here are known for their use as IAs for X-ray computed tomography (CT), various optical imaging modalities and photo acoustic imaging.⁴⁶⁻⁵² This versatility makes several of the IAs shown here suitable for alternative multimodal imaging modalities (e.g., PET-MRI).^{51,53-59}

6.4.3 Targeting

The molecular imaging probes described in previous chapters are non-specific and, therefore, suffer from a lack of specificity as intracellular IAs. A straightforward approach to overcome this issue is by incorporating cell-targeting moieties to these probes in order to “direct them” towards cells.⁶⁰ Throughout the years, cellular targeting has been achieved using peptides,^{1,6,61-64} antibodies^{7,61,62} and proteins^{62,65-69} that bind to receptors at cell membrane surfaces, e.g., somatostatin/transferrin receptors.⁷⁰ Several examples of such systems are illustrated in Chapter 2.

6.5 What's (Really) Next?

Upon identification of potential IAs (those that deliver proper contrast for visualization and exhibit cell-specificity by highlighting targeted areas), the next intuitive step is to provide some degree of therapeutical properties to them. In this way, IAs are not only used for visualization of ROIs, but can also serve as carriers for drug delivery or for therapeutical purposes (e.g, ultrasound). This field, known as theranostics, plays a major role in early-stage drug design and development and is expected to contribute to personalized medicine.^{71,72}

6.6 Conclusions

The preliminary results presented here show the potential of the functionalization of carbon-based scaffolds for multimodal imaging purposes. Although the aim was to synthesize a small molecule multifunctional imaging ligand for complexation with 2 different lanthanides, the full characterization of the final molecule by means of 1D and 2D NMR turned out to be inconclusive. However, MS data pointed to the formation of the aimed ligand. While the exact stoichiometry of the final complex could not be elucidated, the studied properties of this new multimodal IA strongly suggest the formation of both complexes (either in one molecule and/or separated). Therefore, future efforts should focus on the full characterization of such ligands/complexes, as they can be the first step towards a lanthanide-controlled synthesis of heteronuclear multimodal IAs. On the other hand, $\text{Gd}^{3+}/\text{Eu}^{3+}$ -AB-DO3A-SWCNTs are expected to be excellent candidates for high-payload multimodal imaging purposes. Intuitively, the next step is the assessment of the fluorescent

properties of these SWCNTs and their visualization by MRI and fluorescence imaging.

Lastly, the combination of simple modifications of widely known polydentate ligands for lanthanide complexation with nanomaterials has opened up the door for new synthetic routes for (multimodal) IAs. The current knowledge of metal complexation, together with commercial availability of many nanomaterials, has made this modest approach popular. Present efforts are directed towards a more comprehensive characterization of these systems, their cell targeting ability and their use for other state-of-the-art imaging modalities.

6.7 Experimental

Materials. Commercial grade reagents were purchased from Sigma Aldrich and TCI Europe and were used without further purification unless otherwise stated. The CNTs used were pristine high purity SWCNTs from Arrynano (purity > 90% according to supplier). The outer diameter of the SWCNTs ranges between 1 nm and 2 nm and their length from 5 μm to 20 μm . Solvents were dried over molecular sieves. Deuterated solvents were purchased from Cambridge Isotope Laboratories and NMR and MRI tubes from Wilmad LabGlass. All CNTs were oxidized and functionalized using 18.2 $\text{M}\Omega/\text{cm}^2$ water. The 1000 Da and 12000-14000 Da Spectra/Por® dialysis membranes were purchased from Aldrich.

9,10-Bis(aminomethyl)anthracene (1). This compound was synthesized following an adapted procedure from reference.³³ Hexamethylenetetramine (1720 mg, 12.3 mmol) was added to a solution of 9,10-bis(bromomethyl)anthracene (synthesized as described in reference)³⁴

(2 g, 5.6 mmol) in 100 mL CHCl_3 . After refluxing overnight, the precipitate was removed by filtration and washed with CHCl_3 . The resulting solid was added to 400 mL of a 8:1 $\text{CH}_3\text{CH}_2\text{OH}:\text{HCl}$ solution, which was subsequently refluxed for two days. The solution was cooled to 0°C , filtered and the remaining solid was washed with cold $\text{CH}_3\text{CH}_2\text{OH}$ (3 x 80 mL) and dried under vacuum. The product was dispersed in 200 mL water ($\text{pH} > 11$, adjusted using 1M NaOH) and extracted with CHCl_3 (3 x 120 mL). Finally, the organic phase was dried over MgSO_4 , the solvent was removed under reduced pressure and the remaining yellow solid was dried overnight in vacuum over P_2O_5 , yielding **1**. Yield: 86% (1.14 g, 4.8 mmol). ^1H NMR (600 MHz, CDCl_3 , 25°C , TMS) δ (ppm): 8.4 [dd, $J_1(\text{H,H}) = 3.7$ Hz, $J_2(\text{H,H}) = 3.3$ Hz, 4H; Ar-*H*], 7.6 [dd, $J_1(\text{H,H}) = 3.7$ Hz, $J_2(\text{H,H}) = 3.3$ Hz, 4H; Ar-*H*], 4.9 (s, 4H, $\text{NH}_2\text{-CH}_2\text{-Ar}$). ^{13}C NMR (150 MHz, CDCl_3 , 25°C , TMS) δ (ppm): 134.7, 129.3, 125.7, 124.6 (Ar-C), 38.4 ($\text{NH}_2\text{-CH}_2\text{-Ar}$).

9,10-Bis(methyl-4-bromobutanamide)anthracene (2).

4 equivalents of *N,N'*-diisopropylethylamine (DIPEA) were added to 200 mL dry CHCl_3 containing 9,10-bis(aminomethyl)anthracene (**1**) (500 mg, 2.1 mmol) under N_2 atmosphere. Subsequently, 4-bromobutyryl chloride (1180 mg, 6.4 mmol) was slowly added to the solution, which was stirred overnight in the dark. The precipitate was filtered, washed with CHCl_3 and dried under vacuum, yielding **2** as a yellow solid. Yield: 75% (852 mg, 1.6 mmol). ^1H NMR (600 MHz, $\text{DMSO-}d_6$, 25°C , TMS) δ (ppm): 8.4 [dd, $J_1(\text{H,H}) = 3.4$ Hz, $J_2(\text{H,H}) = 3.4$ Hz, 4H; Ar-*H*] & (t, $J(\text{H,H}) = 5.2$ Hz, 2H, $\text{CONH-CH}_2\text{-Ar}$), 7.6 [dd, $J_1(\text{H,H}) = 3$ Hz, $J_2(\text{H,H}) = 3.7$ Hz, 4H; Ar-*H*], 5.3 (s, 4H, $\text{CONH-CH}_2\text{-Ar}$), 3.5 (t, $J(\text{H,H}) = 6.6$ Hz, 4H; $\text{NHCO-CH}_2\text{-CH}_2\text{-CH}_2\text{Br}$), 2.2 (t, $J(\text{H,H}) = 7.1$ Hz, 4H; $\text{NHCO-CH}_2\text{-CH}_2\text{-CH}_2\text{Br}$), 2 (m, $J_1(\text{H,H}) = 7$ Hz, $J_2(\text{H,H}) = 7$ Hz, 4H; $\text{NHCO-CH}_2\text{-CH}_2\text{-CH}_2\text{Br}$). ^{13}C NMR (150 MHz,

DMSO-*d*₆, 25°C, TMS) δ (ppm): 171.1 (CONH-Ar), 130.4, 127.5, 126.5, 125.3 (Ar-C), 35.6 (CONH-CH₂-Ar), 35 (NHCO-CH₂-CH₂-CH₂Br), 33.9 (NHCO-CH₂-CH₂-CH₂Br), 29.1 (NHCO-CH₂-CH₂-CH₂Br). MS (ESI+, CH₂Cl₂/MeOH): *m/z*: 455.5, 457.5 [*M*⁺-1Br+H], 555.5, 557.5, 559.5 [*M*⁺+Na].

9,10-Bis[methyl-4-*N*-(butanamide)maleimide]anthracene (3).

Maleimide (36.3 mg, 0.37 mmol) and K₂CO₃ (44 mg, 0.32 mmol) were added to a 10 mL dry ACN solution containing 9,10-bis(methyl-4-bromobutanamide)anthracene (**2**) (100 mg, 0.19 mmol). The mixture was heated at 50°C overnight and left to cool to room temperature. After filtration in order to remove inorganic salts, the solvent was removed under reduced pressure. Recrystallization from MeOH yielded **3** as a white solid. Yield: 80% (95.4 mg, 0.17 mmol). ¹H NMR (600 MHz, CDCl₃, 25°C, TMS) δ (ppm): 7.6.-7.1 [dd, 12H; Ar-*H* & CH=CH maleimide], 4.4 (t, *J*(H,H) = 7 Hz, 4H; NHCO-CH₂-CH₂-CH₂Br), 2.7 (m, 4H; NHCO-CH₂-CH₂-CH₂Br), 2.2 (m, 4H; NHCO-CH₂-CH₂-CH₂Br). ¹³C NMR (150 MHz, CDCl₃, 25°C, TMS) δ (ppm): 176.2 (CONH-Ar), 163.9 (C=O maleimide), 143.9, 141.2, 126.3, 125.6, 123.6 (Ar-C & CH=CH maleimide), 70.4 (NHCO-CH₂-CH₂-CH₂Br), 45.3 (CONH-CH₂-Ar), 28.9 (NHCO-CH₂-CH₂-CH₂Br), 23.6 (NHCO-CH₂-CH₂-CH₂Br). MS (ESI+, CH₂Cl₂/MeOH): *m/z*: 235.6 [bisaminoanthracene+H] (frag), 470.2 [*M*⁺-1maleimide+H] (frag), 567.2 [*M*⁺+H]. Elemental analysis, found: C 68.7, H 5.96, N 8.22, O 17.12. Expected for C₃₂H₃₀N₄O₆•0.4 CH₃CH₂OH: C 67.34, H 5.58, N 9.58, O 17.5.

2-((3-Chloropropyl)thio)tetrahydro-2*H*-pyran (5). This compound was synthesized following an adapted procedure from literature.⁷³ 3-chloro-1-propanethiol (**4**) (500 mg, 4.5 mmol), 3,4-dihydro-2*H*-pyran (410 μ L, 4.5 mmol) and pyridinium *p*-toluene sulfonate (113 mg, 0.45

mmol) were dissolved in 30 mL dry CH_2Cl_2 and the reaction mixture was stirred at room temperature overnight. The solution was washed with 10% NaOH (3 x 20 mL), water (3 x 20 mL), and dried over MgSO_4 . The solvent was removed under reduced pressure and the desired compound was obtained as a yellow liquid after chromatography on silica gel 60 (pre-treated with 0.1 % TEA in hexane) using toluene as eluent ($r_f \sim 0.4$). Yield: 47 % (414 mg, 2.1 mmol). ^1H NMR (600 MHz, CDCl_3 , 25°C , TMS) δ (ppm): 4.9 (m, 1H; SCHO), 4.1 & 3.5 (m, 2H; OCH_2), 3.7 [t, $J(\text{H,H}) = 6.2$ Hz, 2H; CH_2Cl], 2.9 & 2.8 (m, 2H; CH_2S), 2.1 (m, $J(\text{H,H}) = 6.8, 6.7$ Hz, 2H; $\text{CH}_2\text{-CH}_2\text{Cl}$), 2 & 1.7 (SCH-CH_2), 1.8 & 1.6 (m, 2H; $\text{SCH-CH}_2\text{CH}_2$), 1.6 (m, 2H; $\text{OCH}_2\text{-CH}_2$). ^{13}C NMR (150 MHz, CDCl_3 , 25°C , TMS) δ (ppm): 82.1 (SCHO), 64.4 (OCH_2), 43.4 (CH_2Cl), 32.2 ($\text{CH}_2\text{Cl-CH}_2$), 30.6 (SCH-CH_2), 27.1 (CH_2S), 25.4 ($\text{OCH}_2\text{-CH}_2$), 21.5 ($\text{SCH-CH}_2\text{CH}_2$). MS (ESI+, $\text{CH}_2\text{Cl}_2/\text{MeOH}$): m/z : 195.2, 197.2 [$\text{M}^+ + \text{H}$], 217.2, 219.2 [$\text{M}^+ + \text{Na}$].

1-(3-((Tetrahydro-2H-pyran-2yl)thio)propyl)-4,7,10-tris(*tert*-butoxy carbonylmethyl)-1,4,7,10-tetraazacyclododecane (7). In a three-neck round-bottom flask, K_2CO_3 (621 mg, 4.5 mmol) and a catalytic amount of KI were added to a stirring solution of 1,4,7-tris(*tert*-butoxy carbonylmethyl)-1,4,7,10-tetraazacyclododecane **6** (DO3A, synthesized as previously described in literature¹⁸) (790 mg, 1.5 mmol) in 80 mL dry acetonitrile. The mixture was kept under N_2 for 10 minutes while vigorously stirring. Subsequently, 3-chloro-1-propyltetrahydropyranyl sulfide (**5**) (300 mg, 1.5 mmol) dissolved in 20 mL dry CH_2Cl_2 was added slowly to the reaction mixture and allowed to react for 3 days at 50°C . The mixture was left to cool to room temperature, filtered to remove inorganic salts and the solvent was evaporated under reduced pressure. The desired compound was obtained as a yellow oil after chromatography on silica gel 60 (pre-

treated with 0.1 % TEA in hexane) using $\text{CH}_2\text{Cl}_2/\text{MeOH}$ 9:1 as eluent ($R_f \sim 0.5$), followed by precipitation of impurities with ether, filtration and solvent evaporation. Yield: 50 % (538 mg, 0.8 mmol). ^1H NMR (600 MHz, CDCl_3 , 25°C , TMS) δ (ppm): 4.8 (m, 1H; SCH_2O), 4 & 3.6 (m, 2H; OCH_2), 3.5-2.7 [m, 24H; NCH_2 (cyclen core), NCH_2 (4th arm), $\text{CH}_2\text{-COOtBu}$], 2.73 (m, 2H; CH_2S), 2.1 (m, 2H, $\text{CH}_2\text{S-CH}_2$), 1.9 & 1.61 (m, 2H; SCH-CH_2), 1.82 & 1.56 (m, 2H; $\text{SCH-CH}_2\text{CH}_2$), 1.56 (m, 2H; $\text{OCH}_2\text{-CH}_2$), 1.46 [s, 27H; $\text{C}(\text{CH}_3)_3$]. ^{13}C NMR (150 MHz, CDCl_3 , 25°C , TMS) δ (ppm): 170.7, 170.3, 170, (COOtBu), 83.4 (SCH_2O), 82 [$\text{C}(\text{CH}_3)_3$], 66.2 (OCH_2), 56.9, 55.3, 54.7, 53.2, 52.7, 51.8, 51, 50.2, 47.9 [NCH_2 (all)], $\text{NCH}_2\text{-CH}_2$, CH_2COOtBu], 31.7 (SCH-CH_2), 28.3 ($\text{CH}_2\text{S-CH}_2$), 28.1 [$\text{C}(\text{CH}_3)_3$], 25.1 (CH_2S), 23.5 ($\text{SCH-CH}_2\text{CH}_2$), 22.5 ($\text{OCH}_2\text{-CH}_2$). MS (ESI+, $\text{CH}_2\text{Cl}_2/\text{MeOH}$): m/z : 695.2 [$\text{M}^+ + \text{Na}$].

1-(3-Mercaptopropyl)-4,7,10-triacetic acid-1,4,7,10-tetraazacyclododecane (8). In a round-bottom flask, TFA (5 mL) was added to a solution of **7** (200 mg, 0.3 mmol) in 5 mL CH_2Cl_2 and the reaction mixture was allowed to stir at room temperature overnight. The mixture was evaporated under reduced pressure and the residue co-evaporated with CH_2Cl_2 3 times in order to remove excess TFA. **8** was obtained as a solid after precipitation from diethyl ether and it was subsequently used for the next reaction. Yield: quantitative. MS (ESI+, 1:1 ACN:0.1%TFA): m/z : 421.2 [$\text{M}^+ + \text{H}$].

9,10-Bis[methyl-1-(4-(N-butanamide-2,5-dioxopyrrolidin-3-yl)thio)propyl]-4,7,10-triacetic acid-1,4,7,10-tetraazacyclododecane]anthracene (9). **3** (50 mg, 0.09 mmol) and **8** (74 mg, 0.18 mmol) were dissolved in 20 mL MeOH. Subsequently, the solution was flushed with N_2 and allowed to stir overnight. After solvent

removal under reduced pressure, the remaining powder was redissolved in water and dialyzed (against H₂O) for three days using a 1.000 Da dialysis membrane. After water removal under reduced pressure and drying (overnight) under vacuum over P₂O₅, **9** was obtained as a yellow solid. Yield: 5 % (6 mg, 0.0043 mmol). ¹H NMR (600 MHz, D₂O, 25°C, TMS) δ (ppm): 8 (amide), 7.42-7.31 (m, 8H, Ar-H), 4.6 (CONH-CH₂-Ar), 4-2.75, [m, NCH₂ (cyclen core), CH₂-COOH and CH₂-CH maleimide], 3.10 (m, CH₂-CH₂-CH₂-maleimide), 2.7 (t, *J*(H,H) = 6.7 Hz, 4H, CH₂-CH₂-CH₂-maleimide), 2.02 (m, 4H, CH₂-CH₂-CH₂-maleimide), 2.18, 1.5, 1.38, 1.23, 0.78 (m, 12H, CH₂-CH₂-CH₂-S-). The final molecule (as well as possible fragments) could not be identified by MS. Due to the little amount of material elemental analysis was not possible.

Gd³⁺/Eu³⁺-{9,10-Bis [methyl-1-((4-(*N*-butanamide-2,5-dioxopyrrolidin-3-yl)thio)propyl)-4,7,10-triacetic acid-1,4,7,10-tetraazacyclododecane]anthracene} (10). EuCl₃ and GdCl₃ (2.4 mg, 0.0064 mmol) were added to a solution of **9** (6 mg, 0.0043 mmol) in 15 mL H₂O. The pH of the solution was adjusted to 6 using 1M NaOH and the final solution was stirred at 50°C for one day. Excess salts were removed by dialysis against H₂O for three days using a 1.000 Da dialysis membrane. After solvent removal under reduced pressure, **10** was obtained as a pale yellow solid. Yield: quantitative. MS⁺ (MALDI, H₂O/ACN + 0.1%TFA, β-indoleacrylic acid⁷⁴): *m/z*: 976.52 [M⁺-(C=O-propylalkylated maleimide-thiolated propyl linker-DO3Acomplex)+H] (frag).

Synthesis of AB-DO3A. As described in Chapter 4.

Oxidation procedure for SWCNTs. Full procedure is described in the PhD thesis of Martin Sobik (UTwente).

AB-DO3A-SWCNTs.³⁹ AB-DO3A (50 mg, 0.12 mmol), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide HCl (EDC×HCl, 76.5 mg, 0.4 mmol) and diisopropylethylamine (DIPEA, 160 μ l, 1 mmol) were added to a suspension of 10 mg of ox-SWNT in 25 ml of water. The reaction mixture was allowed to stir at room temperature overnight and dialyzed against H₂O for 3 days using a 12000-14000 Da membrane in order to remove the excess of unreacted reactants.

Gd³⁺/Eu³⁺-AB-DO3A-SWCNTs. EuCl₃ or GdCl₃ (8 mg, 0.04 mmol) was added to a solution of **AB-SWCNTs** (5 mg each solution) in 10 mL H₂O. The pH of the solution was adjusted to 6 using 1M NaOH and the final solution was allowed to stir for two days at room temperature. Excess salts were removed by dialysis (against H₂O) for three days using a 12000-14000 Da dialysis membrane.

Nuclear Magnetic Resonance (NMR) Characterization. All NMR experiments were performed on a Bruker Avance II NMR spectrometer operating at 600.16 MHz for ¹H and 150.91 MHz for ¹³C. Chemicals shifts are given in ppm using the residual solvent signal as reference. The multiplicity of the peaks is reported using the following abbreviations: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. All NMR spectra were processed using TOPSPIN 2.1 and MestReC software packages.

Mass Spectrometry (MS). Mass spectra were acquired on a Waters Micromass LCT ESI mass spectrometer and a Waters MALDI Synapt mass spectrometer.

Elemental Analysis. Element analyses were done using an InterScience Flash 2000 organic elemental analyzer.

UV-Vis Spectroscopy. UV spectra were acquired on a Perkin Elmer Lambda 850 UV-Vis spectrometer using 1 cm quartz cuvettes.

Fluorescence Spectroscopy. All steady-state spectra were recorded using a Perkin Elmer Fluorescence Spectrometer.

Infrared Spectroscopy. All IR spectra were recorded using a Nicolet 6700/8700 Fourier Transform (FT-IR) spectrometer from Thermo Scientific. All samples (except AB-DO3A-SWCNTs) were measured using KBr pellets.

Vibrating Sample Magnetometry (VSM). Magnetization of the different SWCNTs was measured using a 10 Vibrating Sample Magnetometer from Microsense.

Transmission Electron Microscopy (TEM) and Energy-Dispersive X-Ray (EDX). TEM images were obtained using analytical TEM FEI instruments. In order to prepare the samples, 10 μL of the desired sample was applied onto Formvar-carbon coated grids (from electron microscopy sciences). After leaving the sample for 1 minute, the excess of liquid was removed using a piece of filter paper. Energy-dispersive X-ray (EDX)

analysis was performed using a Thermo Noran System 6 with a resolution of 138 eV on a Mn standard.

Magnetic Resonance Imaging (MRI). All experiments were carried out on a 14.1 T (600 MHz) Avance II NMR Spectrometer from Bruker (Karlsruhe, Germany) equipped with a vertical narrow bore magnet (14.1 T), a great B_0 compensation unit (BGU-II) and 3 great 1/60 amplifier units (X, Y and Z) also from Bruker. A micro 5 imaging probe, equipped with a 10 mm diameter saddle coil insert from Bruker (Karlsruhe, Germany) was used. The maximum gradient strength was 4.8 G/cm/A and the temperature inside the probe was kept at $\sim 25^\circ\text{C}$ by a gradient water-cooling unit BCU20 from Bruker. All NMR/MRI tubes (5/10 mm diameter) were purchased from Wilmad Labglass (New Jersey, U.S.A.). Glass capillary tubes (melting point tubes, inner diameter 1mm) used for MRI were purchased from VWR. T_1 -weighted images were obtained using a spin echo sequence with the following parameters: TR = 800 ms, TE = 6 ms, 1 axial slice of 6 mm thickness, FOV = 10 mm x 10 mm, matrix size = 256 x 256 and 10 averages. The measurements were performed in a 1 cm (outer diameter) glass tube as follows. A stock solution of the Gd^{3+} -containing complex was diluted 2, 5 and 10 times. For each experiment, capillary tubes of 1 mm inner diameter were filled with these solutions, placed in a custom-made Kel-F capillary holder and subsequently introduced in the 1 cm tube for imaging. Images were analyzed using Paravision 4.0 software.

6.8 References

- (1) Boswell, C. A.; Eck, P. K.; Regino, C. A. S.; Bernardo, M.; Wong, K. J.; Milenic, D. E.; Choyke, P. L.; Brechbiel, M. W. *Mol. Pharm.* **2008**, *5*, 527.

- (2) Mamedov, I.; Parac-Vogt, T. N.; Logothetis, N. K.; Angelovski, G. *Dalton Trans.* **2010**, 39, 5721.
- (3) Manning, H. C.; Goebel, T.; Thompson, R. C.; Price, R. R.; Lee, H.; Bornhop, D. J. *Bioconjugate Chem.* **2004**, 15, 1488.
- (4) Mindt, T. L.; Müller, C.; Stuker, F.; Salazar, J.-F. d. r.; Hohn, A.; Mueggler, T.; Rudin, M.; Schibli, R. *Bioconjug Chem* **2009**, 20, 1940.
- (5) Kamaly, N.; Kalber, T.; Ahmad, A.; Oliver, M. H.; So, P.-W.; Herlihy, A. H.; Bell, J. D.; Jorgensen, M. R.; Miller, A. D. *Bioconjugate Chem.* **2008**, 19, 118.
- (6) Mulder, W. J. M.; Koole, R.; Brandwijk, R. J.; Storm, G.; Chin, P. T. K.; Strijkers, G. J.; deMelloDonega, C.; Nicolay, K.; Griffioen, A. W. *Nano Lett.* **2006**, 6, 1.
- (7) Mulder, W. J. M.; Strijkers, G. J.; Griffioen, A. W.; van Bloois, L.; Molema, G.; Storm, G.; Koning, G. A.; Nicolay, K. *Bioconjugate Chem.* **2004**, 15, 799.
- (8) Mulder, W. J. M.; Strijkers, G. J.; van Tilborg, G. A. F.; Cormode, D. P.; Fayad, Z. A.; Nicolay, K. *Acc. Chem. Res.* **2009**, 42, 904.
- (9) Pokorski, J. K.; Breitenkamp, K.; Liepold, L. O.; Qazi, S.; Finn, M. G. *J. Am. Chem. Soc.* **2011**, 133, 9242.
- (10) Song, Y.; Xu, X.; MacRenaris, K. W.; Zhang, X.-Q.; Mirkin, C. A.; Meade, T. J. *Angew. Chem.* **2009**, 48, 9143.
- (11) Yang, H.; Santra, S.; Walter, G. A.; Holloway, P. H. *Adv. Mater.* **2006**, 18, 2890.
- (12) Cotton, S. *Lanthanide and Actinide Chemistry*; John Wiley & Sons, Ltd, 2006.
- (13) Bottrill, M.; Kwok, L.; Long, N. J. *Chem. Soc. Rev.* **2006**, 35, 557.
- (14) Caravan, P.; Ellison, J. J.; McMurry, T. J.; Lauffer, R. B. *Chem. Rev.* **1999**, 99, 2293.
- (15) Reichert, D. E.; Lewis, J. S.; Anderson, C. J. *Coord. Chem. Rev.* **1999**, 184, 3.
- (16) Bunzli, J.-C. G.; Piguet, C. *Chem. Soc. Rev.* **2005**, 34, 1048.
- (17) Faulkner, S.; Pope, S. J. A.; Burton-Pye, B. P. *Appl. Spectrosc. Rev.* **2005**, 40, 1.
- (18) Beeby, A.; Bushby, L. M.; Maffeo, D.; Gareth Williams, J. A. *J. Chem. Soc., Dalton Trans.* **2002**, 48.
- (19) Terai, T.; Kikuchi, K.; Iwasawa, S.; Kawabe, T.; Hirata, Y.; Urano, Y.; Nagano, T. *J. Am. Chem. Soc.* **2006**, 128, 8699.
- (20) Faulkner, S.; Carrie, M.-C.; Pope, S. J. A.; Squire, J.; Beeby, A.; Sammes, P. G. *Journal of the Chemical Society, Dalton Transactions* **2004**, 1405.
- (21) Pal, R.; Parker, D. *Org. Biomol. Chem.* **2008**, 6.
- (22) Burton-Pye, B. P.; Heath, S. L.; Faulkner, S. *Journal of the Chemical Society, Dalton Transactions* **2005**, 146.
- (23) Koulourou, T.; Natrajan, L. S.; Bhavsar, H.; Pope, S. J. A.; Feng, J.; Narvainen, J.; Shaw, R.; Scales, E.; Kauppinen, R.; Kenwright, A. M.; Faulkner, S. *J. Am. Chem. Soc.* **2008**, 130, 2178.
- (24) Imbert, D.; Cantuel, M.; Bünzli, J.-C. G.; Bernardinelli, G.; Piguet, C. *J. Am. Chem. Soc.* **2003**, 125, 15698.

- (25) Klink, S. I.; Keizer, H.; Veggel, F. C. J. M., van *Angew. Chem., Int. Ed.* **2000**, *39*, 4319.
- (26) Coppo, P.; Duati, M.; Kozhevnikov, V. N.; Hofstraat, J. W.; De Cola, L. *Angew. Chem., Int. Ed.* **2005**, *44*, 1806.
- (27) Andrews, M.; Amoroso, A. J.; Harding, L. P.; Pope, S. J. A. *Dalton Trans.* **2010**, *39*, 3407.
- (28) Geninatti Crich, S. G.; Biancone, L.; Cantaluppi, V.; Esposito, D. D. G.; Russo, S.; Camussi, G.; Aime, S. *Magn. Reson. Med.* **2004**, *51*, 938.
- (29) Pellegatti, L.; Zhang, J.; Drahos, B.; Villette, S.; Suzenet, F.; Guillaumet, G.; Petoud, S.; Toth, E. *Chem. Commun.* **2008**, 6591.
- (30) Picard, C.; Geum, N.; Nasso, I.; Mestre, B.; Tisnes, P.; Laurent, S.; Muller, R. N.; Vander Elst, L. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 5309.
- (31) Harte, A. J.; Jensen, P.; Plush, S. E.; Kruger, P. E.; Gunnlaugsson, T. *Inorg. Chem.* **2006**, *45*, 9465.
- (32) Plush, S. E.; Gunnlaugsson, T. *Dalton Trans.* **2008**, 3801.
- (33) Gassensmith, J. J.; Arunkumar, E.; Barr, L.; Baumes, J. M.; DiVittorio, K. M.; Johnson, J. R.; Noll, B. C.; Smith, B. D. *J. Am. Chem. Soc.* **2007**, *129*, 15054.
- (34) Gunnlaugsson, T.; Davis, A. P.; O'Brien, J. E.; Glynn, M. *Org. Biomol. Chem.* **2005**, *3*, 48.
- (35) Kovacs, Z.; Sherry, A. D. *Synthesis* **1997**, *7*, 759.
- (36) Osawa, M.; Hoshino, M.; Wada, T.; Hayashi, F.; Osanai, S. *J. Phys. Chem. A* **2009**, *113*, 10895.
- (37) Borel, A.; Bean, J. F.; Clarkson, R. B.; Helm, L.; Moriggi, L.; Sherry, A. D.; Woods, M. *Chemistry - A European Journal* **2008**, *14*, 2658.
- (38) Karousis, N.; Tagmatarchis, N.; Tasis, D. *Chem. Rev.* **2010**, *110*, 5366.
- (39) Dumortier, H.; Lacotte, S.; Pastorin, G.; Marega, R.; Wu, W.; Bonifazi, D.; Briand, J. P.; Prato, M.; Muller, S.; Bianco, A. *Nano Lett.* **2006**, *6*, 1522.
- (40) Kostarelos, K.; Lacerda, L.; Pastorin, G.; Wu, W.; Wieckowski, S.; Luangsivilay, J.; Godefroy, S.; Pantarotto, D.; Briand, J.-P.; Muller, S.; Prato, M.; Bianco, A. *Nat. Nanotechnol.* **2007**, *2*, 108.
- (41) Wu, H.-C.; Chang, X.; Liu, L.; Zhao, F.; Zhao, Y. *J. Mater. Chem.* **2010**, *20*, 1036.
- (42) Li, Y.; Zhang, X.; Luo, J.; Huang, W.; Cheng, J.; Luo, Z.; Li, T.; Liu, F.; Xu, G.; Ke, X.; Li, L.; Geise, H. J. *Nanotechnology* **2004**, *15*, 1645.
- (43) Hartman, K. B.; Laus, S.; Bolskar, R. D.; Muthupillai, R.; Helm, L.; Toth, E.; Merbach, A. E.; Wilson, L. J. *Nano Lett.* **2008**, *8*, 415.
- (44) Richard, C.; Doan, B. T.; Beloeil, J. C.; Bessodes, M.; Toth, E.; Scherman, D. *Nano Lett.* **2008**, *8*, 232.
- (45) Zhang, J.; Zou, H.; Qing, Q.; Yang, Y.; Li, Q.; Liu, Z.; Guo, X.; Du, Z. *J. Phys. Chem. B* **2003**, *107*, 3712.
- (46) Banin, U.; Millo, O. In *Nanoparticles: from Theory to Application*; Schmid, G., Ed.; Wiley-VCH Verlag GmbH & Co. KGaA: Weinheim, FRG, 2005, p 305.
- (47) Giljohann, D. A.; Seferos, D. S.; Daniel, W. L.; Massich, M. D.; Patel, P. C.; Mirkin, C. A. *Angew. Chem., Int. Ed.* **2010**, *49*, 3280.
- (48) Hou, Y.; Hao, R. In *Nanoplatfrom-Based Molecular Imaging*; Chen, X., Ed.; John Wiley & Sons, Inc.: 2011, p 529.
- (49) Huang, W.-Y.; Davis, J. J. *Dalton Trans.* **2011**, *40*, 6087.

- (50) Manohar, S.; Ungureanu, C.; Van Leeuwen, T. G. *Contrast Media Mol. Imaging* **2011**, *6*, 389.
- (51) Alric, C.; Taleb, J.; Duc, G. L.; Mandon, C.; Billotey, C.; Meur-Herland, A. L.; Brochard, T.; Vocanson, F.; Janier, M.; Perriat, P.; Roux, S.; Tillement, O. *J. Am. Chem. Soc.* **2008**, *130*, 5908.
- (52) Sun, H.; Yuan, Q.; Zhang, B.; Ai, K.; Zhang, P.; Lu, L. *Nanoscale* **2011**, *3*, 1990.
- (53) Judenhofer, M. S.; Wehrl, H. F.; Newport, D. F.; Catana, C.; Siegel, S. B.; Becker, M.; Thielscher, A.; Kneilling, M.; Lichy, M. P.; Eichner, M.; Klingel, K.; Reischl, G.; Widmaier, S.; Rocken, M.; Nutt, R. E.; Machulla, H.-J.; Uludag, K.; Cherry, S. R.; Claussen, C. D.; Pichler, B. J. *Nat. Med.* **2008**, *14*, 459.
- (54) Cai, W.; Chen, K.; Li, Z.-B.; Gambhir, S. S.; Chen, X. *J. Nucl. Med.* **2007**, *48*, 1862.
- (55) Catana, C.; Wu, Y.; Judenhofer, M. S.; Qi, J.; Pichler, B. J.; Cherry, S. R. *J. Nucl. Med.* **2006**, *47*, 1968.
- (56) Cherry, S. R.; Louie, A. Y.; Jacobs, R. E. *P. IEEE* **2008**, *96*, 416.
- (57) Choi, J. S.; Park, J. C.; Nah, H.; Woo, S.; Oh, J.; Kim, K. M.; Cheon, G. J.; Chang, Y.; Yoo, J.; Cheon, J. *Angew. Chem., Int. Ed.* **2008**, *47*, 6259.
- (58) Culver, J.; Akers, W.; Achilefu, S. *J. Nucl. Med.* **2008**, *49*, 169.
- (59) Morawski, A. M.; Lanza, G. A.; Wickline, S. A. *Curr. Opin. Biotechnol.* **2005**, *16*, 89.
- (60) Kim, E. E. *Korean J. Radiol.* **2003**, *4*, 201.
- (61) Accardo, A.; Tesauro, D.; Aloj, L.; Pedone, C.; Morelli, G. *Coord. Chem. Rev.* **2009**, *253*, 2193.
- (62) Torchilin, V. P. *AAPS J.* **2007**, *9*, E128.
- (63) Overoye-Chan, K.; Koerner, S.; Looby, R. J.; Kolodziej, A. F.; Zech, S. G.; Deng, Q.; Chasse, J. M.; McMurry, T. J.; Caravan, P. *J. Am. Chem. Soc.* **2008**, *130*, 6025.
- (64) Ye, F.; Jeong, E.-K.; Jia, Z.; Yang, T.; Parker, D.; Lu, Z.-R. *Bioconjugate Chem.* **2008**, *19*, 2300.
- (65) Caravan, P. *Acc. Chem. Res.* **2009**, *42*, 851.
- (66) Xu, H.; Regino, C. A. S.; Koyama, Y.; Hama, Y.; Gunn, A. J.; Bernardo, M.; Kobayashi, H.; Choyke, P. L.; Brechbiel, M. W. *Bioconjugate Chem.* **2007**, *18*, 1474.
- (67) van Tilborg, G. A. F.; Mulder, W. J. M.; Chin, P. T. K.; Storm, G.; Reutelingsperger, C. P.; Nicolay, K.; Strijkers, G. J. *Bioconjugate Chem.* **2006**, *17*, 865.
- (68) Prinzen, L.; Miserus, R.-J. J. H. M.; Dirksen, A.; Hackeng, T. M.; Deckers, N.; Bitsch, N. J.; Megens; Douma, K.; Heemskerk, J. W.; Kooi, M. E.; Frederik, P. M.; Slaaf, D. W.; van Zandvoort, M. A. M. J.; Reutelingsperger, C. P. M. *Nano Lett.* **2006**, *7*, 93.
- (69) Li, H.; Gray, B. D.; Corbin, I.; Lebherz, C.; Choi, H.; Lund-Katz, S.; Wilson, J. M.; Glickson, J. D.; Zhou, R. *Acad. Radiol.* **2004**, *11*, 1251.
- (70) Edwards, W. B.; Xu, B.; Akers, W.; Cheney, P. P.; Liang, K.; Rogers, B. E.; Anderson, C. J.; Achilefu, S. *Bioconjugate Chem.* **2007**, *19*, 192.
- (71) Kelkar, S. S.; Reineke, T. M. *Bioconjugate Chem.* **2011**, *22*, 1879.

- (72) Kim, J.; Piao, Y.; Hyeon, T. *Chem. Soc. Rev.* **2009**, *38*, 372.
- (73) Block, E.; Eswarakrishnan, V.; Gernon, M.; Ofori-Okai, G.; Saha, C.; Tang, K.; Zubieta, J. *J. Am. Chem. Soc.* **1989**, *111*, 658.
- (74) Vetterlein, K.; Bergmann, U.; Büche, K.; Walker, M.; Lehmann, J.; Linscheid, M. W.; Scriba, G. K. E.; Hildebrand, M. *Electrophoresis* **2007**, *28*, 3088.

Appendix 6.1. Characterization of the Building Blocks 3 and 8

Owing to the symmetry of the propyl alkylated anthracene (**2**) (Figure 6.11, top), a single set of ^1H NMR peaks were observed. Upon alkylation of the maleimide with the propyl alkylated anthracene core (**2**), a shift in all proton signals was observed. As expected, all the signals corresponding to the propyl alkyl linker (**a**, **b** and **c**) are downfield shifted, with **a** (corresponding to the methylene closest to the substituted group) showing the largest shift. This observation indicates changes in the chemical environment due to the presence of electron-withdrawing functional groups such as maleimides. Moreover, splitting of **a**, **b** and **c** into two identical, overlapping peaks was observed; thereby suggesting that (for each set of protons) full equivalency was lost. Despite various purification attempts, several small signals (labeled as **a'**, **b'** and **c'**), were also observed. These signals could arise from either as impurities (as elemental analysis showed a deviation of 6 %) or as conformers. However, the latter statement is contradicted by ^1H NMR data obtained at 50°C (Figure 6.11, bottom), which shows that these signals (**a'**, **b'** and **c'**) do not shift after increasing the temperature.

Figure 6.11. ^1H NMR spectra of **2** and **3** (at 25°C and 50°C) in d_6 -DMSO. Residual solvents are indicated with an asterisk (*).

^1H NMR spectra at 50°C also revealed that the rotational freedom around **e** (methylene protons connecting the amide to the anthracene) was restricted after the incorporation of the maleimide (**3**). This is illustrated by the broadening of this signal (**e**) after the maleimide alkylation (Figure 6.11, top and center), followed by sharpening as the temperature was increased (Figure 6.11, bottom). This restricted rotational motion, together with the shifting to higher field of the aromatic protons **f** and **g** strongly suggest a “rigid” conformation most likely resulting from π -stacking of the maleimide with the anthracene. The second building block used for the synthesis of the ANT-2DO3A ligand (**8**, Scheme 6.2) was successfully characterized using the above-mentioned techniques.

SUMMARY

Molecular imaging is a discipline in charge of the rapid and accurate visualization of the human body in a, preferably, non-invasive way. Despite the wide variety of imaging modalities available today, precise differentiation of physiologically/pathologically different tissues remains a challenge. While the existing imaging agents (IAs), used to aid image contrast without perturbing metabolic processes, have contributed to a more accurate visualization using different molecular imaging modalities, shortcomings to be overcome still remain. Many of them are related to the well-known limited spatial resolution and/or low sensitivity that most imaging techniques suffer from (Figure 1).

Figure 1. Sensitivity vs. spatial resolution of selected imaging modalities. PET: positron emission tomography. SPECT: single-photon emission computed tomography. NIR: near-infrared. MR: magnetic resonance. GFP: green fluorescent protein.

A common way to tackle with the low sensitivity and/or resolution issue is by combining two (or more) imaging modalities. This emerging field, known as multimodal imaging, is dedicated to the optimization of the synergy of different imaging modalities in order to combine “the best of both worlds”. Despite the various strategies and combinations described in the literature, one of the most preferred ones regards the use of Gadolinium (Gd^{3+}) complexes for magnetic resonance imaging (MRI), combined with fluorescent probes for optical imaging. In this thesis, new approaches directed towards simple and functional IAs for MR and fluorescence multimodal imaging are proposed. The multimodal IAs described here are based on combinations of (novel) metal complex-based ligands and nanostructures, where the organic ligands not only serve as chelators for metal complexation, but also act as templates/scaffolds for the growth of nanostructures.

In Chapter 3, the aqueous-based, one-step synthesis of hybrid silver nanostructures (*hAgNSs*), grown using a polyamino carboxylic acid scaffold, namely 1,4,7,10-tetraaza-1-(1-aminobutyl)-4,7,10-triacetic acid cyclododecane (AB-DO3A, Figure 2A), is described. The nanostructures formed are characterized and a feasible arrangement for these *hAgNSs* is proposed using a wide range of techniques, including UV-Vis absorbance spectroscopy, transmission electron microscopy (TEM), dynamic light scattering (DLS), fluorescence spectroscopy (lifetime and steady-state), mass spectrometry (MS), NMR spectroscopy and infrared spectroscopy. The engineered *hAgNSs* consist of two functional units: a “large” silver nanoparticle (AgNP, stabilized by the carboxylate moieties and the cyclen core of the AB-DO3A scaffold), which can act both as a template for surface modification and potentially as a therapeutic agent, and “smaller”

Ag nanoclusters (AgNCs) that can be used as optical trackers due to their fluorescent properties (Figure 2B).

Figure 2. Hybrid silver nanostructures (*hAgNSs*), formed by the chemical reduction of AgNO₃ in the presence of AB-DO3A. The localization of the Ag_nNCs within the AgNP is only a schematic representation.

The use of (similar) aggregated organic ligands as nucleation points for the formation of protein cages of the cowpea chlorotic mottle virus (CCMV) is described in Chapter 4. Stable assemblies are engineered by pure hierarchical self-assembly of micelles of the 1,4,7,10-tetraza-1-(1-carboxymethylundecane)-4,7,10-triacetic acid cyclododecane (DOTAC10) ligand inside CCMV (Figure 3). The DOTAC10 ligand is used to either complex with Gd³⁺ in order to form paramagnetic micelles visualized by MRI or to encapsulate amphiphilic Zn²⁺ phthalocyanine (ZnPc) dye molecules, in order to confirm (optically) the encapsulation of the micelles. Subsequent incorporation of the ZnPc dye in the Gd³⁺-DOTAC10 paramagnetic micelles leads to noticeably higher capsid loadings, as observed by MRI and elemental analysis. These purely self-assembled protein cages (as confirmed by TEM and size exclusion chromatography) show enhanced ionic and capsid r_1 relaxivities which are attributed to aggregation and altered micelle association kinetics resulting from local confinement inside the protein cage and ZnPc dye encapsulation.

Figure 3. Schematic representation of the self-assembly triggered by self-assembly process: micelles are formed when the monomer concentration is higher than the critical aggregation concentration (cac). The negatively charged micelles act as “nucleation points” for self-assembly of CCMV coat proteins (CPs), yielding stable paramagnetic and optically active protein cages. The localization and orientation of the ZnPc molecules within the micelles is only schematic.

In Chapter 5, the synthesis, characterization and study of the cytotoxicity of multimodal gold nanorods (MMAuNRs) for magnetic resonance and optical imaging is described. The gold nanostructures are modified by thiol-based ligand exchange using an optimized O-[2-(3-mercaptopropionylamino)ethyl]-O'-methylpolyethylene glycol (PEG-SH), Gd^{3+} -1-(11-sulfanylundecyl)-4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecane (Gd^{3+} -SUDDO3A) and (3aS,4S,6aR)-11-sulfanylundecyl 5-(hexahydro-2-oxo-1H-thieno[3,4-d]imidazol-4-yl) pentanoate (Biotin-SH) ratio. Subsequent incubation with Cy5-streptavidin leads to paramagnetic and fluorescent AuNRs, visualized by MRI and fluorescence microscopy (Figure 4) and characterized by means of UV-Vis absorption spectroscopy, TEM and total reflection x-ray fluorescence (TXRF). The MMAuNRs obtained using this one-pot synthesis are biocompatible, show median lethal concentration (LC_{50}) values $> 150 \text{ pM}$

AuNR, improved relaxivity properties, high Gd^{3+} loadings and can be optically colocalized with mouse myoblast (C2C12) cells *in vitro*.

Figure 4. Schematic representation of the synthesis of MMAuNRs. (Upper) Fluorescence image: red emission observed corresponding to Cy5-labeled MMAuNRs (exposure time 2000 ms). (Lower) MR Image: T_1 -weighted MR image of MMAuNRs at different Gd^{3+} concentrations (600 MHz, TE = 4 ms, TR = 500 ms). The plot shows ionic r_1 relaxivity corresponding to the MMAuNRs. The inset plot corresponds to a linear fitting of the 0-0.13 mM Gd^{3+} concentration range ($r_1 = 1.8 \text{ mM}^{-1} \text{ s}^{-1}$).

In Chapter 6, functionalized carbon-based organic scaffolds are proposed as platforms for multiple lanthanide complexation by integration of some of the metal complex-based templates described in previous chapters. First, a small, water-soluble carbon-based scaffold, consisting of an anthracene core (ANT) where two identical 1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid (DO3A)-based chelators (similar to the one presented in Chapter 5) are integrated, is presented. The promising optical and paramagnetic properties observed for this water soluble ANT-2DO3A bis-macrocyclic ligand (Figure 5) show its potential as a proof-of-principle

probe for further development of small molecule, multimodal IAs by exploiting the chemically similar, but electronically/magnetically different, properties lanthanides show.

Figure 5. Left: emission spectra of ANT-2DO3A (grey line, H₂O, $\lambda_{\text{exc}} = 312$ nm) and Gd³⁺/Eu³⁺-ANT-2DO3A (black line, H₂O/ACN, $\lambda_{\text{exc}} = 262$ nm). Right: T_1 -weighted MR image showing various concentrations (4, 2, 0.8 and 0.4 g/L) of Gd³⁺/Eu³⁺-ANT-2DO3A complex and H₂O (control).

Later, the covalent attachment of the AB-DO3A template (shown in Chapter 4) to carbon nanotubes (CNTs) is explored. CNTs are attractive platforms for the incorporation of large payloads of (various) molecular imaging probes due to their high surface-to-volume ratio. The covalent functionalization of SWCNTs with the AB-DO3A ligand, whose water solubility is enhanced by the three carboxylic acids of this ligand, followed by lanthanide complexation, leads to the formation of functionalized SWCNTs (Figure 6).

Figure 6. TEM-images of Gd³⁺-AB-DO3A-SWCNTs (left) and Eu³⁺-AB-DO3A-SWCNTs (right) (scale bar left = 50 nm and right = 20 nm). Inset in both pictures is the EDX spectrum of the regions highlighted in white.

The preliminary characterization and evaluation of the properties of these functionalized carbon-based scaffolds show promising results, thereby highlighting their potential for multimodal imaging purposes. Finally, the future perspectives and challenges ahead for these metal complex-based templates, nanostructures and the multimodal IAs described in this thesis are briefly discussed.

SAMENVATTING

Moleculaire beeldvorming is een discipline die verantwoordelijk is voor de snelle en nauwkeurige visualisatie van het menselijk lichaam middels een, bij voorkeur, niet-invasieve manier. Ondanks het aantal beeldvormende technieken die tegenwoordig beschikbaar zijn, blijft een nauwkeurige onderscheid tussen fysiologisch/pathologisch verschillende weefsels een uitdaging. Hoewel de bestaande beeldvormingsagenten (BAs), welke gebruikt worden om het contrast te verbeteren zonder daarbij de verschillende metabolische processen te verstoren, hebben bijgedragen aan een betere visualisatie zijn er nog beperkingen en tekortkomingen. Deze beperkingen zijn gerelateerd aan zaken als de beperkte resolutie en/of lage gevoeligheid van de meeste technieken (Figuur 1).

Figuur 1. Gevoeligheid vs. resolutie van geselecteerde beeldvormende technieken. PET: positronenemissietomografie. SPECT: *single-photon emission computed tomography*. NIR: nabij-infrarood. MR: magnetische resonantie. GFP: groen fluorescente proteïne.

Een veel voorkomende manier om de lage gevoeligheid en/of resolutie kwestie te verbeteren is door het combineren van twee (of meer) beeldvormende technieken. Dit nieuwe begrip, bekend als multimodale beeldvorming, behelst het optimaliseren van de synergie tussen verschillende beeldvormende technieken met als doel om “het beste van beide werelden” te verenigen. Van de in literatuur beschreven veelheid aan strategieën en combinaties, is (één van) de meest voorkomende multimodale beeldvormingsmethode(s) het gebruik van Gadolinium (Gd^{3+}) complexen (voor beeldvorming met magnetische resonantie, MRI) in combinatie fluorescerende probes (voor optische beeldvorming). In dit proefschrift worden nieuwe multimodale beeldvormingsbenaderingen beschreven die gericht zijn op eenvoudige en functionele BAs voor MR en fluorescentie beeldvorming. De beschreven multimodale BAs zijn gebaseerd op combinaties van (nieuwe) metaalcomplex-gebaseerde liganden en nanostructuren, waarbij de organische liganden niet alleen als chelaten voor metaalcomplexvorming worden gebruikt, maar ook als malplaatje/bouwsteiger voor de groei van nanostructuren.

In hoofdstuk 3 wordt de water-gebaseerde synthese van hybride zilver nanostructuren (*hAgNSs*) gegroeid met een polyamino carbonzuur bouwsteiger, te weten 1,4,7,10-tetraaza-1-(1-aminobutyl)-4,7,10-triacetic acid cyclododecane (AB-DO3A, Figuur 2A), beschreven. De gevormde nanostructuren zijn gekarakteriseerd en een mogelijke structuur voor deze *hAgNSs* is voorgesteld op basis van verschillende analyse technieken, waaronder UV-Vis absorptie spectroscopie, transmissie-elektronenmicroscopie (TEM), dynamische lichtverstrooiing (DLS), fluorescentie spectroscopie (levensduur en steady-state), massaspectrometrie (MS), kernspinresonantie (NMR) en infrarood spectroscopie. De gevormde *hAgNSs* bestaan uit twee functionele

eenheden: een "groot" zilver nanodeeltje (AgNP, gestabiliseerd door de carboxylaat groepen en cyclen kern van de AB-DO3A bouwsteiger), die kan dienen als zowel malplaatje voor oppervlakte modificatie alsmede als therapeutisch middel, en "kleinere" Ag nanoclusters (AgNCs) die gebruikt kunnen worden als optische "trackers" door hun fluorescerende eigenschappen (Figuur 2B).

Figuur 2. Hybride zilver nanostructuren (hAgNSs), gevormd door de chemische reductie van AgNO_3 in aanwezigheid van AB-DO3A. De lokalisatie van de Ag_nNC s in de AgNP is slechts schematisch weergegeven.

Het gebruik van (soortgelijke) geaggregeerde organische liganden als nucleatiepunten voor de vorming van eiwitkooien van het *cowpea chlorotic mottle* virus (CCMV) wordt beschreven in Hoofdstuk 4. Stabiele assemblages zijn ontwikkeld door puur hiërarchische zelf-assemblage van micellen van het 1,4,7,10-tetraza-1-(1-carboxymethylundecane)-4,7,10-triacetic acid cyclododecane (DOTAC10) ligand binnen CCMV (Figuur 3). Het DOTAC10 ligand is gebruikt voor complexatie met Gd^{3+} om paramagnetische micellen te vormen welke gevisualiseerd zijn met MRI, of om amfifiele Zn^{2+} phthalocyanine (ZnPc) kleurstofmoleculen in te kapselen, die gebruikt zijn om de inkapseling van de micellen optisch te verifiëren. Opname van ZnPc in de Gd^{3+} -DOTAC10 paramagnetische micellen leidt tot aanzienlijk hogere inkapseling, zoals geanalyseerd met MRI en

elementaire analyse. De zuivere zelf-geassembleerde eiwitkooien (bevestigd door TEM en *size exclusion* chromatografie) hebben verbeterde r_1 relaxatietijden die toegewezen worden aan de aggregatie en veranderde micel kinetiek als gevolg van lokale opsluiting in de eiwitkooi en de ZnPc kleurstof inkapseling.

Figuur 3. Schematische weergave van het “zelf-assemblage geactiveerd door zelf-assemblage” proces: micellen worden gevormd wanneer de monomeer concentratie hoger is dan de kritische aggregatie concentratie (cac). De negatief geladen micellen dienen als “nucleatiepunten” voor zelf-assemblage van CCMV eiwitten (CP), wat stabiele paramagnetische en optisch actieve eiwit kooien oplevert. De lokalisatie en oriëntatie van de moleculen in de ZnPc micellen is slechts schematisch.

In hoofdstuk 5 wordt de synthese, karakterisatie en toxiciteitsstudie beschreven van multimodale goud nanostaafjes (MMAuNRs) voor magnetische resonantie en optische beeldvorming. De goud nanostructuren zijn gemodificeerd middels thiol-gebaseerde liganduitwisseling met een geoptimaliseerde verhouding van O-[2-(3-mercaptopropionylamino)ethyl]-O'-methylpolyethylene glycol (PEG-SH), Gd^{3+} -1-(11-sulfanylundecyl)-4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecane (Gd^{3+} -SUDDO3A) en (3aS,4S,6aR)-11-sulfanylundecyl 5-(hexahydro-2-oxo-1H-thieno[3,4-d]imidazol-4-yl) pentanoate (Biotin-SH). Incubatie met Cy5-streptavidine resulteert in

paramagnetische en fluorescerende AuNRs, welke zijn gevisualiseerd met MRI en fluorescentie microscopie (Figuur 4) en gekarakteriseerd door middel van UV-Vis absorptie spectroscopie, TEM en totale reflectie X-ray fluorescentie (TXRF). De MMAuNRs, verkregen met behulp van deze “one-pot” synthese, zijn biocompatibel, hebben *median lethal concentration* (LC_{50}) waarden > 150 pM AuNR, verbeterde relaxatie eigenschappen, hogere Gd^{3+} hoeveelheden en kunnen optisch worden “colocalized” met muis myoblast (C2C12) cellen *in vitro*.

Figuur 4. Schematische weergave van de synthese van MMAuNRs. (Bovenste) Fluorescentie beeld: de waargenomen rode emissie komt overeen met Cy5-gelabelde MMAuNRs (belichtingstijd 2000 ms). (Onderste) MR Afbeelding: T_1 -gewogen MR afbeelding van MMAuNRs bij verschillende Gd^{3+} concentraties (600 MHz, TE = 4 ms, TR = 500 ms). De grafiek toont de ionische r_1 relaxatietijd die hoort bij de MMAuNRs. De inzet-plot correspondeert met de raaklijn van het 0-0.13 mM Gd^{3+} concentratiebereik ($r_1 = 1.8 \text{ mM}^{-1} \text{ s}^{-1}$).

In hoofdstuk 6 worden gefunctionaliseerde koolstof-gebaseerde organische bouwsteigers voorgesteld als platforms voor (meerdere)

lanthanide complexatie(s) door integratie van een aantal van de metaalcomplex-gebaseerde malplaatjes beschreven in voorgaande hoofdstukken. Als eerste wordt een kleine, water oplosbare koolstof-gebaseerde bouwsteiger beschreven, bestaande uit een anthraceen (ANT) kern waarin twee identieke 1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid (DO3A)-based liganden (vergelijkbaar met die beschreven in hoofdstuk 5) zijn geïntegreerd. De veelbelovende optische en paramagnetische eigenschappen voor het water oplosbare ANT-2DO3A bis-macrocyclische ligand (Figuur 5) laten het potentieel zien als *proof-of-principle probe* en als basis dienen voor de verdere ontwikkeling van kleine, multimodale BAs door gebruik te maken van de chemisch vergelijkbare, maar toch elektronisch/magnetisch verschillende, eigenschappen die lanthaniden bezitten.

Figuur 5. Links: emissiespectra van ANT-2DO3A (grijze lijn, H₂O, $\lambda_{\text{exc}} = 312$ nm) en Gd³⁺/Eu³⁺-ANT-2DO3A (zwarte lijn, H₂O/ACN, $\lambda_{\text{exc}} = 262$ nm). Rechts: T₁-gewogen MR afbeelding met verschillende concentraties (4, 2, 0,8 en 0,4 g/L) van Gd³⁺/Eu³⁺-ANT-2DO3A complex en H₂O (controle).

Vervolgens is de covalente binding van het AB-DO3A malplaatje (zie hoofdstuk 4) aan koolstofnanobuisjes (CNTs) onderzocht. Enkelwandige CNTs (SWCNTs) zijn aantrekkelijke platforms voor het bevestigen van grote hoeveelheden van (verschillende) moleculaire BAs vanwege hun

hoge oppervlakte-volumeverhouding. De covalente functionalisering van SWCNTs met het AB-DO3A ligand, welke de wateroplosbaarheid van de CNTs verhoogt door de drie carboxylen van dit ligand, gevolgd door lanthanide complexatie, leidt tot de vorming van gefunctionaliseerde SWCNTs (Figuur 6).

Figuur 6. TEM-beelden van Gd^{3+} -AB-DO3A-SWCNTs (links) en Eu^{3+} -AB-DO3A-SWCNTs (rechts) (schaalbalk links = 50 nm en rechts = 20 nm). Inzet in beide foto's is het EDX spectrum van de wit gemarkeerde regio's.

De eerste karakterisering en evaluatie van de eigenschappen van deze gefunctionaliseerde koolstof-gebaseerde malplaatjes laat veelbelovende resultaten zien en bevestigen/accentueren hun potentieel voor multimodale beeldvorming. Tenslotte worden de toekomstperspectieven en de uitdagingen voor de metaalcomplex-gebaseerde malplaatjes/bouwsteigers, nanostructuren en de multimodale BA's zoals beschreven in dit proefschrift kort besproken.

SUMARIO

Imagenología molecular es una disciplina a cargo de la visualización rápida y precisa del cuerpo humano de manera, preferiblemente, no invasiva. A pesar de la gran variedad de modalidades disponibles en la actualidad, la diferenciación precisa de tejidos fisiológicamente/patológicamente diferentes sigue siendo un reto. Mientras que los agentes de imagen (AI) molecular existentes, utilizados para mejorar el contraste en una imagen sin perturbar los procesos metabólicos, han contribuido a una mejor visualización usando diferentes técnicas de imagenología, aún existen deficiencias que superar. Muchas de ellas están relacionadas a la conocida limitada resolución espacial y/o sensibilidad de la que sufren estas técnicas (Figura 1).

Figura 1. Sensibilidad vs. resolución espacial de algunas modalidades de imagenología. PET: tomografía por emisión de positrones. SPECT: tomografía computarizada por emisión de fotones individuales. NIR: infrarrojo cercano. MR: resonancia magnética. GFP: proteína verde fluorescente.

Una forma común de enfrentar el problema de la baja sensibilidad y/o resolución espacial es mediante la combinación de dos (o más) modalidades de imagenología. Este campo emergente, conocido como imagenología multimodal, se dedica a la optimización de la sinergia de diferentes modalidades en un intento por obtener "lo mejor de ambos mundos". A pesar de las diferentes estrategias y combinaciones descritas en la literatura, una de las más populares emplea complejos de Gadolinio (Gd^{3+}) para resonancia magnética (MRI), combinados con sondas fluorescentes para su uso en imágenes ópticas. En esta tesis, nuevos enfoques dirigidos a AI simples y funcionales apropiados para imagenología multimodal empleando resonancia magnética y/o óptica, son propuestos. Los AI multimodales descritos aquí se basan en combinaciones de (nuevos) complejos metálicos a base de ligandos y nanoestructuras, donde los ligandos orgánicos no sólo sirven para la formación de complejos de metales, sino que también actúan como templados/andamios para el crecimiento de nanoestructuras.

En el Capítulo 3, la síntesis (en un paso) acuosa de nanoestructuras híbridas de plata (*hAgNSs*), formadas utilizando el andamio 1,4,7,10-tetraaza-1-(1-aminobutil)-4,7,10-ciclododecano triacético (DO3A AB, Figura 2A), es descrita. Las nanoestructuras formadas se caracterizan y una estructura factible para estas *hAgNSs* es propuesta usando una amplia gama de técnicas, incluyendo espectroscopía UV-Vis, microscopía electrónica de transmisión (TEM), dispersión de luz dinámica (DLS), espectroscopía de fluorescencia (tiempo de vida y el estado de equilibrio), espectrometría de masas (MS), resonancia magnética nuclear (NMR) y espectroscopia infrarroja. Las *hAgNSs* formadas contienen dos unidades funcionales: una "gran" nanopartícula de plata (AgNP, estabilizada por los ácidos carboxílicos y el núcleo cyclen del andamio AB-DO3A), que puede

actuar como una plantilla para modificación de superficies y potencialmente como un agente terapéutico, y "pequeños" Ag nanoclusters (Ag_nNCs) que pueden ser utilizados como rastreadores ópticos debido a sus propiedades fluorescentes (Figura 2B).

Figura 2. Nanoestructuras híbridas de plata ($h\text{AgNSs}$), formadas tras la reducción química de AgNO_3 en presencia de AB-DO3A. La localización de los Ag_nNCs dentro de la AgNP es sólo una representación esquemática.

El uso de (similares) ligandos orgánicos agregados como puntos de nucleación para la formación de jaulas de proteínas del cowpea chlorotic mottle virus (CCMV) se describe en el Capítulo 4. Jaulas moleculares estables fueron diseñadas usando puro auto-ensamblaje jerárquico de micelas del ligando 1,4,7,10-tetraza-1-(1-carboxymetilundecano) ciclododecano-4,7,10-triacético (DOTAC10) dentro del CCMV (Figura 3). DOTAC10 se utilizó para formar complejos de Gd^{3+} , así formando micelas paramagnéticas visualizadas por MRI, o para encapsular ftalocianinas anfífilas Zn^{2+} (ZnPc) y así confirmar ópticamente la encapsulación de las micelas. Incorporación de ZnPc en las micelas paramagnéticas de Gd^{3+} -DOTAC10 resulta en cargas notablemente altas dentro de las jaulas formadas, como se observó por MRI y análisis elemental. Estas jaulas auto-ensambladas (como se confirmó por TEM y cromatografía por exclusión de tamaño) muestran altas relaxividades r_1 , atribuidas a la

agregación y alteración de la cinética de la asociación de las micelas por el confinamiento local dentro de la jaula y por la encapsulación de ZnPc.

Figura 3. Representación esquemática del proceso de auto-ensamblaje desencadenado por auto-ensamblaje: micelas se forman cuando la concentración del monómero es mayor a la concentración de agregación crítica (cac). La cargas negativas de las micelas actúan como "puntos de nucleación" de auto-ensamblaje de proteínas de la cubierta (CP) del CCMV, así generando jaulas de proteínas paramagnéticas y ópticamente activas. La localización y orientación de las moléculas ZnPc dentro de las micelas es sólo esquemática.

En el capítulo 5, la síntesis, caracterización y estudio de la citotoxicidad de nanorods multimodales (MMAuNRs) para resonancia magnética y óptica de imagen son descritas. Las nanoestructuras de oro son modificadas por intercambio de ligandos (tioles) utilizando una relación optimizada de O-[2-(3-mercaptopropionylamino)-etil]-O'-metilpolietilenglicol (PEG-SH), Gd^{3+} -1-(11-sulfanylundecyl)-4,7,10-tris(carboximetil)-1,4,7,10-tetraaza ciclododecano (Gd^{3+} -SUDDO3A) y (3aS, 6aR 4S,-)11-sulfanylundecyl 5-(hexahidro-2-oxo-1H-tieno[3,4-d]imidazol-4-il) pentanoato (Biotina-SH). La posterior incubación con Cy5-estreptavidina conlleva a la formación de AuNRs paramagnéticos y fluorescentes, visualizados por microscopía de fluorescencia y resonancia magnética (Figura 4) y caracterizados usando espectroscopia UV-Vis, TEM y fluorescencia de rayos X por reflexión total

(TXRF). Los MMAuNRs obtenidos con esta síntesis son biocompatibles, muestran valores de concentración letal media (LC_{50}) > 150 pM AuNR, mejores relaxividades, altas cargas de Gd^{3+} y puede ser ópticamente colocalizados con células C2C12 mioblastos de ratón *in vitro*.

Figura 4. Representación esquemática de la síntesis de MMAuNRs. Imagen (superior) de fluorescencia: emisión roja observada, la cual corresponde a MMAuNRs etiquetados con Cy5 (tiempo de exposición 2000 ms). Imagen (baja) de MR: Imagen potenciada en T_1 de MMAuNRs a diferentes concentraciones de Gd^{3+} (600 MHz, TE = 4 ms, TR = 500 ms). La gráfica muestra la relaxividad iónica r_1 correspondiente a los MMAuNRs. El grafico interno muestra el ajuste lineal del rango de concentración 0-0.13 mM Gd^{3+} ($r_1 = 1.8 mM^{-1} s^{-1}$).

En el capítulo 6, andamios orgánicos funcionalizados a base de carbono son propuestos como plataformas para la formación de múltiples complejos de lantánidos utilizando algunos de los complejos metálicos descritos en capítulos anteriores. En primer lugar, un pequeño andamio soluble en agua que consta de un núcleo de antraceno (ANT), donde dos unidades idénticas de ligandos de 1,4,7,10-tetraazaciclododecano-1 ,4,7-

triacético (DO3A, similar al presentado en el capítulo 5) se integran, es presentado. Las propiedades ópticas y paramagnéticas observadas en este ligando bis-macro cíclico (ANT-2DO3A, Figura 5) muestran su potencial como prueba de principio para el desarrollo de pequeños Als aprovechando las químicamente similares, pero electrónicamente/magnéticamente diferentes, propiedades de los lantánidos.

Figura 5. Izquierda: Espectros de emisión de ANT-2DO3A (línea gris, H_2O , $\lambda_{\text{exc}} = 312 \text{ nm}$) y $\text{Gd}^{3+}/\text{Eu}^{3+}$ -ANT-2DO3A (línea negra, $\text{H}_2\text{O}/\text{ACN}$, $\lambda_{\text{exc}} = 262 \text{ nm}$). Derecha: imagen potenciada en T_1 de RM que muestra varias concentraciones (4, 2, 0,8 y 0,4 g/L) de $\text{Gd}^{3+}/\text{Eu}^{3+}$ -ANT-2DO3A y H_2O (control).

Seguidamente, la unión covalente del templado AB-DO3A (Capítulo 4) a nanotubos de carbono (CNT) es explorada. CNT de pared simple (SWCNTs) son plataformas atractivas para la fijación de un gran número de Als debido a su alta relación superficie/volumen. El enlace covalente entre AB-DO3A y los SWCNTs, el cual refuerza su solubilidad en agua con los tres ácidos carboxílicos de este ligando, seguido por la formación de complejos de lantánidos, conduce a la formación de SWCNTs funcionalizados (Figura 6).

Figura 6. TEM imágenes de Gd³⁺-AB-DO3A-SWCNTs (izquierda) y Eu³⁺-AB-DO3A-SWCNTs (derecha) (barra de escala = 50 nm izquierda y derecha = 20 nm). Inserción en ambas imágenes es el espectro EDX de las regiones resaltadas en color blanco.

La caracterización y evaluación preliminar de las propiedades de estos andamios orgánicos a base de carbono funcionalizados muestra resultados prometedores, poniendo de manifiesto su potencial para fines de imagenología multimodal. Por último, las perspectivas de futuro y retos para estos complejos metálicos a base de templados, nanoestructuras y los AI multimodales descritos en esta tesis se discuten brevemente.

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“Happiness only real when shared”

Alexander Supertramp

ABOUT THE AUTHOR

Jealemy Galindo Millán was born on March 9th, 1981 in Caracas (Venezuela). After finishing high school, she started her studies in chemical engineering at the Universidad Central de Venezuela (UCV), where she studied for three years and worked as an assistant for programming courses at the faculty of engineering. At the end of 2001, she moved to the Netherlands and enrolled at the University of Twente in 2003 to continue her studies. During this period she lost her interest in programming and became interested in chemical analysis and biomedical technology, where she minored in 2005. In September of that same year she obtained her bachelor degree at the same university after working at the Chemical Analysis group of Prof. Uwe Karst, where she studied the screening of carbonyl compounds in automobile exhausts using active sampling methods and LC-MS. In November 2007 she obtained her degree (cum laude) in chemistry and technology of materials at the University of Twente. During her master thesis she studied the possible limitations and applications of high-field (micro) magnetic resonance imaging (μ MRI). This work was carried out at the Supramolecular Chemistry and Technology (SMCT) group of Prof. David N. Reinhoudt, under the supervision of Dr. Aldrik Velders. In the same period she received the Unilever Research Prize 2007 for her master thesis. In December 2007 she started her PhD research in the SMCT group, and later became a member of the Biomolecular Nanotechnology (BnT) group of Prof. Jeroen Cornelissen at the University of Twente. The results of this research are presented in this thesis. From November 2012 she is a postdoctoral fellow in the group of Prof. Leonard Prins at the Università degli studi di Padova, Italy.