

**Phosphonate-Anchored Ligands for Upconversion
Imaging Nanoprobes**

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of Doctor of Philosophy in Chemistry

Department of Chemistry

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Declaration of Originality

I hereby declare that the work described in this thesis is entirely my own and was completed at Imperial College London and King's College London between October 2017 and June 2021. When others have contributed, they have been clearly acknowledged within the text.

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Abstract

Upconversion nanoparticles (UCNPs) are a lanthanide-doped inorganic nanomaterial which absorb near-infrared radiation and emit visible light. For the last 20 years, researchers have been interested in using UCNPs as biomedical imaging agents. Their unique optical properties make them suitable for imaging within tissue, with UCNPs exhibiting resistance to photobleaching and having long luminescence lifetimes.

In this work, novel UCNP-ligand systems were synthesised. Phosphonate ligands were designed and synthesised with the aim of achieving particular physical or photophysical properties. Work in this thesis begins with studying the interaction between the upconversion nanomaterial $\text{NaYF}_4\text{:Yb,Er}$ and the bound surface ligands. The stability of the nanoparticle-ligand interaction in an imaging agent *in vivo* determines the resistance to aggregation. The results presented in this work show bisphosphonic acids bind more strongly to the surface of UCNPs than carboxylic acids and this was determined by ^1H NMR spectroscopy and by dynamic light scattering (DLS).

Analyte sensing *in vivo* has the potential to provide information about biological systems in both healthy and disease environments. Phosphonate functionalised, pH-sensitive rhodamine ligands were synthesised and appended to the surface of UCNPs to create UCNP-based pH nanosensors. The nanoprobe was phase transferred using water soluble PEG-bisphosphonate ligands. It was found that poor emission intensity of UCNPs in aqueous systems was a common issue. To mitigate this, sensitizing cyanine ligands were synthesised. Nanosystems of $\text{NaYF}_4\text{:Yb,Er}$ UCNPs functionalised with cyanines were assembled and emission enhancement in these systems was achieved.

In the past few years, there has been increasing interest in the synthesis of multimodal imaging agents. Finally, MR functionality was introduced to the nanoparticle imaging agents. Superparamagnetic iron oxide nanoparticles (SPIONs) were synthesised and various attempts were made at synthesising dual-modal MR/optical imaging agents by creating core-shell SPIONs-UCNP imaging agents.

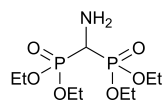
List of Abbreviations

a.u.	arbitrary units
Boc	<i>tert</i> -butoxycarbonyl
BODIPY	boron-dipyrromethene
BP	bisphosphonate
CR	cross relaxation
CT	computer tomography
Cy	cyanine
DCM	dichloromethane
DLS	dynamic light scattering
DMF	dimethylformamide
DO3A	1,4,7-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecane
EdA	ethylenediamine
EDS / EDX	energy-dispersive X-ray spectroscopy
EELS	electron energy loss spectroscopy
EPR	enhanced permeability and retention
ESA	excited state absorption
ESI	electrospray ionisation
ETU	energy transfer upconversion
EtOH	ethanol
FDA	food and drugs administration
FRET	Förster resonance energy transfer
FMHM	full width at half maximum
g	grams
GMP	good manufacturing practice
GSA	ground state absorption
h	hours
HAADF	high-angle annular dark-field
HBTU	2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HRMS	high-resolution mass spectrometry
ICDD	International Centre for Diffraction Data
ICG	indocyanine green
ICP-MS	inductively coupled plasma mass spectrometry
IR	infrared
ISC	intersystem crossing
JCPDS	Joint Committee on Powder Diffraction Standards
λ	wavelength
LCMS	liquid chromatography mass spectrometry
Ln	lanthanide
MALDI	matrix assisted laser desorption ionisation

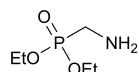
MeCN	acetonitrile
MeOH	methanol
NEt ₃	triethylamine
NMR	nuclear magnetic resonance
mg	milligram
MHz	megahertz
MRI	magnetic resonance imaging
MS	mass spectrometry
NC	nanocrystal
NIR	near-infrared
NMR	nuclear magnetic resonance
NP	nanoparticle
OA	oleic acid
ODE	1-octadecene
OPA	n-octylphosphonate
PA	photon avalanche
PBS	phosphate-buffered saline
Pd/C	palladium on carbon
PDT	photodynamic therapy
PEG	polyethylene glycol
PET	positron emission tomography
ppm	parts per million
QD	quantum dot
r_1	transverse relaxivity
r_2	longitudinal relaxivity
RET	resonance energy transfer
r.t	room temperature
SAED	selected area diffraction
SILAR	sequential ionic layer adsorption and reaction
SPECT	single photon emission computed tomography
SPION	superparamagnetic iron oxide nanoparticle
SQUID	superconducting quantum interference device
STEM	scanning transmission electron microscopy
T ₁	longitudinal relaxation time
T ₂	transverse relaxation time
TEM	transmission electron microscopy
TEOS	tetraethoxysilane
TFA	trifluoroacetic acid
TGA	thermogravimetric analysis
TLC	thin layer chromatography
TMSBr	bromotrimethylsilane

TOPO	trioctylphosphine oxide
UC	upconversion
UCNP	upconversion nanoparticle
XPS	X-ray photoelectron spectroscopy
UV	ultraviolet
UV-Vis	ultraviolet-visible
VR	vibrational relaxation
XAS	X-ray absorption spectroscopy
XPS	X-ray photoelectron spectroscopy
XRD	X-ray diffraction

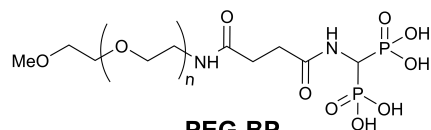
List of Key Compounds



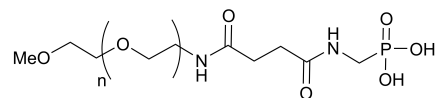
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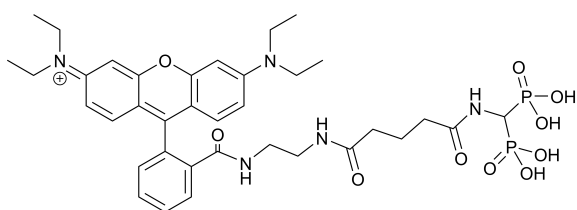
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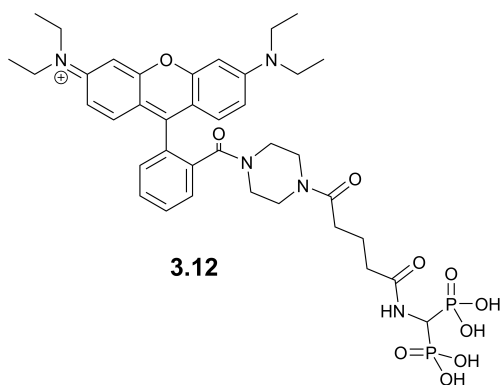
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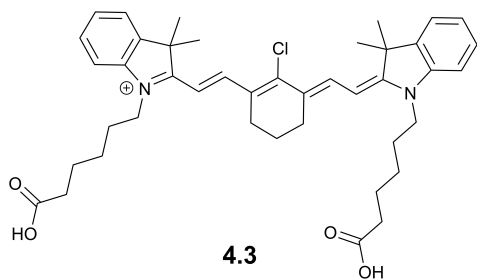
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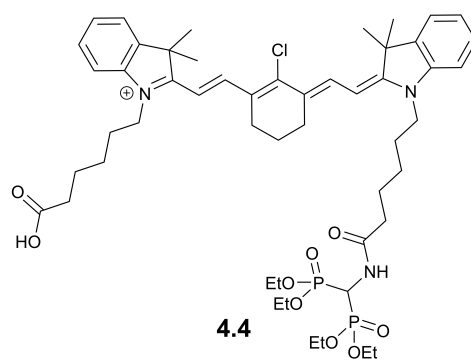
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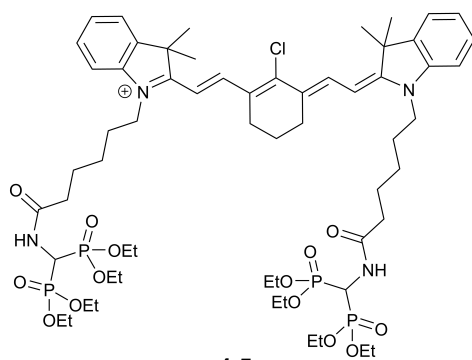
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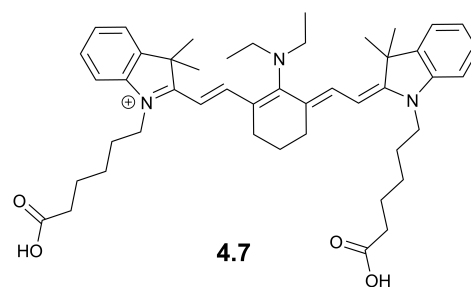
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Chapter 1

Introduction

In the Introduction to this thesis, concepts and work carried out in the field of medical imaging will be discussed and reviewed. This aims to lay a foundation for all the work which follows. Results chapter introductions are more focussed, covering specific relevant work which underpins the research carried out in that particular chapter. This chapter aims to put medical research into context. The Introduction starts with a broad discussion of medical imaging modalities, how the field of medical imaging has developed and the impact this has had on the detection and monitoring of disease. It then moves on to discuss optical imaging in detail, first with a focus on the use of organic fluorophores and then upconversion nanoparticles as optical imaging agents.

The use of upconversion nanoparticles as imaging agents is the fundamental premise of this thesis. The structure and photophysics of upconversion nanomaterials is discussed, with a particular focus on the advantages of using these materials as imaging probes. Methods for the synthesis, modification and characterisation of these materials are summarised, providing a foundation for the work discussed in the Results chapters.

1.1 Molecular Imaging

Medical imaging has become a fundamental tool for clinicians in the diagnosis of a wide range of diseases. Historically, the only way many diseases could be visualised was by biopsy. This invasive technique requires resection of a section of tissue or organ, followed by exogenous analysis. There are a number of issues with this approach. It requires cutting into the body tissue, which is an inherently risky process. Successful biopsy requires knowledge of the location of the primary disease site. This could be missed without additional anatomical or physiological information from the patient. There are also issues with tissue heterogeneity; the resected tissue may not be representative of all the tissue types in the area of the disease.

MRI, PET, SPECT, CT and ultrasound are all examples of routine imaging techniques which are performed in hospitals worldwide, every single day. Developments in scanner hardware, image analysis software and imaging probe formulations have led to clinicians being able to

detect diseases earlier, leading to more effective treatments and ultimately improved clinical outcomes for their patients. By being able to non-invasively peer inside the body, we are able to detect not only the anatomical changes associated with a particular disease state, but also the physiological changes. Medical imaging now sits alongside biopsy as a complementary clinical technique to visualise and diagnose disease.

In recent years, research in the field of medical imaging has been focused on molecular imaging.^{1,2} That is, detecting the subtle biochemical changes which occur during a disease's development and progression. Understanding these changes will lead to better understanding of how and why diseases progress. By having a better understanding of the disease, it is more likely that effective treatments will be developed and any treatments may be able to be implemented at a much earlier stage.^{3,4}

In the clinic, there are a multitude of different imaging techniques available as no individual technique is perfect for every use. There is often a trade-off between technique sensitivity and resolution. The development of new imaging hardware, such as the PET-MRI scanner has driven forward the development of multimodal imaging agents.^{5,6} These imaging agents display contrast in more than one imaging mode. In the case of molecular imaging, it is thought that multimodal contrast agents could allow the detection of multiple biochemical disease markers.^{7,8}

	EM radiation type	Spatial resolution	Sensitivity	Depth	Scan time	Examples
Optical	visible to NIR	15 - 1000 nm	high	<1 cm	seconds	rhodamine, fluorescein
MRI	radiowaves	4 - 100 μm	low	no limit	minutes to hours	Magnevist®, Resovist®
PET	511 keV γ	1 - 2 mm	high	no limit	minutes	^{18}F -fluorodeoxyglucose, ^{11}C -methionine
SPECT	typically low energy γ	1 - 2 mm	high	no limit	minutes	$^{99\text{m}}\text{Tc}$ -sestamibi, $^{99\text{m}}\text{Tc}$ -tetrofosmin
CT (X-ray)	X-rays	50 - 200 μm	low	no limit	minutes	barium sulphate
Ultrasound	not EM - high frequency sound	50 - 500 μm	high	mm to cm	seconds	microbubbles, micelles

Table 1.1: Comparison of imaging modalities and their associated properties.

Contrast agents were originally introduced to improve the sensitivity of anatomical imaging modalities, for example the use of barium sulphate in X-rays of the digestive tract. Their use expanded to also include functional imaging, where signal from an exogenous agent can be used to gain physiological information about the tissues of interest. These complementary imaging methods provide a cornerstone for disease detection in the clinic and contrast agents sit at the heart of this process.

The following sections will focus on the field of optical imaging. Specifically discussed will

be imaging with nanoparticles and the development of the field of upconversion nanoparticles. Other imaging techniques are beyond the scope of this thesis and readers are directed to read other sources for a summary of these techniques.¹

1.2 Optical Imaging

The use of exogenous chromophores for direct staining of intracellular components *in vitro* continues to be the most common microscopic imaging practice in biomedical research.¹ There is a diverse range of optical probes which have been developed: from natural plant extracts to synthetic organic dyes, organometallic complexes and nanoparticle-based materials. This has enabled molecular imaging to develop rapidly in the last twenty years and previously indistinguishable biological processes can now be visualised using fluorescence imaging.⁹

A particularly interesting emerging field for molecular imaging is fluorescence image guided surgery. In the past few years, there has been particular interest in developing targeted fluorophores for cancer imaging, which can be visualised during surgery.^{9–11} In order for a patient to be cured of cancer, all the cancer cells need to be removed from the body. Primarily this means removing all of the cancerous tissue from the primary cancer site. For some cancers this process is relatively straight-forward. For example in early-stage skin cancers, the tumours are well delineated i.e. the boundary of healthy and cancerous tissue is clear and easy to see during surgery. During cancer surgery, the eyes and touch of the surgeon are essential in determining how much tissue to resect. However, palpation and visual inspection are not always sufficient for discriminating between malignant and normal tissue types.¹¹ This can lead to either incomplete resections or the unnecessary removal of healthy tissue. Fluorophores, bound to cancerous tissue, can be used as a visual aid during surgery to help the surgeon to achieve complete tumour resection.¹⁰

1.2.1 Principles of Luminescence

To understand how fluorophores can be used in fluorescence image guided surgery, it is first important to understand the mechanisms producing the optical signal. Luminescence is a general term which describes emission of light by a substance. This is not a result of the substance being heated, it is instead a form of cold-body radiation. Luminescence can be generated by a variety of methods, including: chemical reactions (chemiluminescence), forces applied to a solid (mechanoluminescence) and the absorption of photons (photoluminescence).

The Jablonski diagram in figure 1.1 shows the process of photoluminescence. A substance absorbs a photon, exciting it from the ground state (S_0) to the first excited state (S_1). From here, a number of different processes can occur. Vibrational relaxation (VR) will take place. This is where the substance will redistribute energy from its high lying vibrational states to the surroundings in a rapidly occurring non-radiative emission process (10^{-12} s). Emission can occur from the singly excited state (S_1) to the ground state (S_0). The process is termed fluorescence and occurs rapidly (10^{-9} s). In fluorescence, the spin of the system does not change, both (S_0)

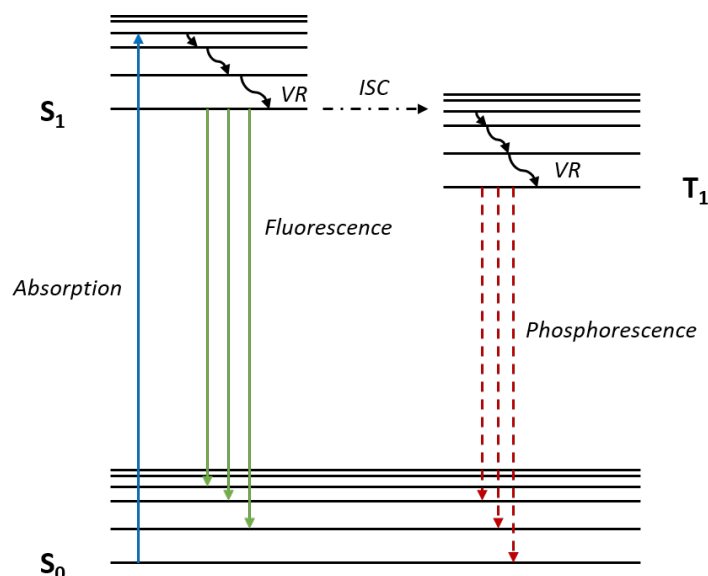


Figure 1.1: Simplified Jablonski diagram for an organic fluorophore. S_0 = singlet ground state, S_1 = excited singlet state, T_1 = excited triplet state, ISC = intersystem crossing, VR = vibrational relaxation.

and (S_1) are singlet states. Alternatively, the system can transition between the excited singlet state (S_1), to the triplet state (T_1). This process is termed intersystem crossing (ISC). From the triplet excited state, emission can occur and is termed phosphorescence ($T_1 \rightarrow S_0$). This emission process breaks the Spin Selection Rule ($\Delta S=0$) and therefore the rate of phosphorescence emission is comparatively slow (10^{-6} - 10^{-3} s and can be up to seconds).¹²

1.2.2 Fluorophores

There are a large and diverse range of chromophores which have been developed over the last 150 years. Initially, they were used in exogenous tissue staining and more recently for applications such as fluorescence image guided surgery. These chromophores can be categorised into a number of groups by their structure and examples of these are shown in figure 1.2.

A number of the fluorophores shown in figure 1.2 have been clinically approved to be used to assist intra-operative dissection. Fluorescein has been used since the 1960s for angioscopy of the retina and iris vasculature.¹³ More recently, there has been a focus on developing fluorophores for cancer surgery. Indocyanine green (ICG) is the only clinically approved fluorophore which displays NIR fluorescence. This is particularly advantageous due to the increased tissue penetration which NIR light (750–1500 nm) can achieve. The fluorophore is hydrophobic and so is typically bound to blood plasma proteins to facilitate *in vivo* use.¹⁴ ICG has been used for the intra-operative identification of liver metastases and hepatocellular carcinomas.^{15,16} Figure 1.3 shows ICG localisation in a hepatic colorectal carcinoma (CRC) metastasis. Heptamethine cyanine dyes will be discussed in more detail in chapter 4.

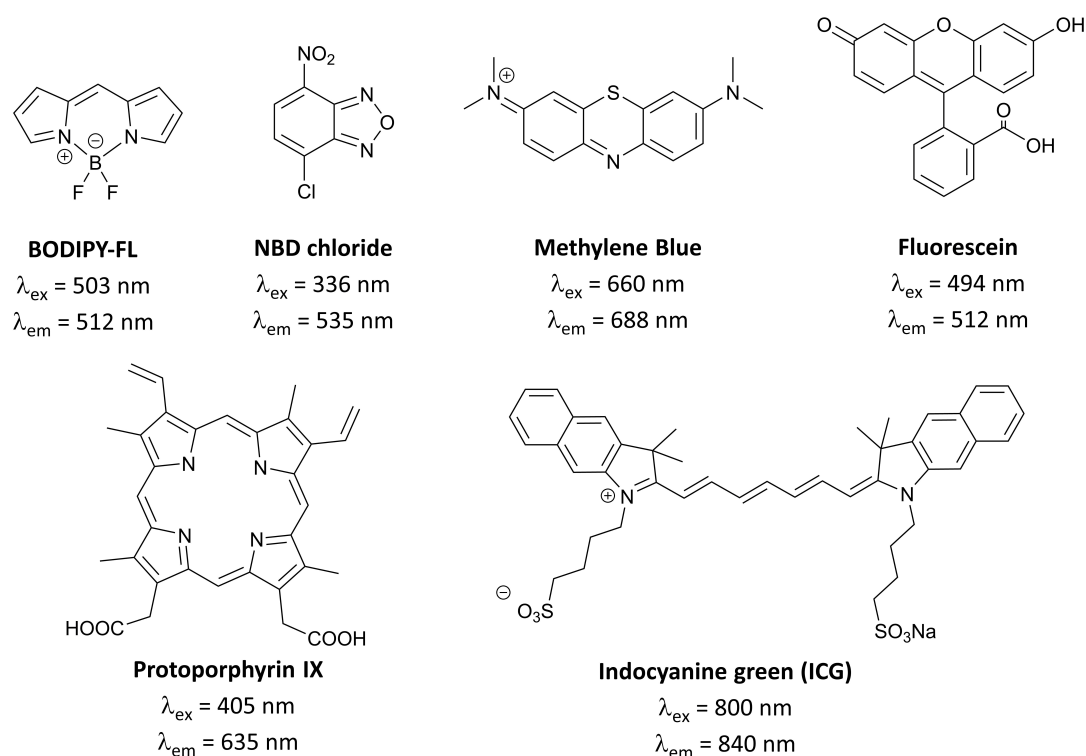


Figure 1.2: Example structures of a members of a number of families of fluorophores, including their peak excitation and emission wavelengths.

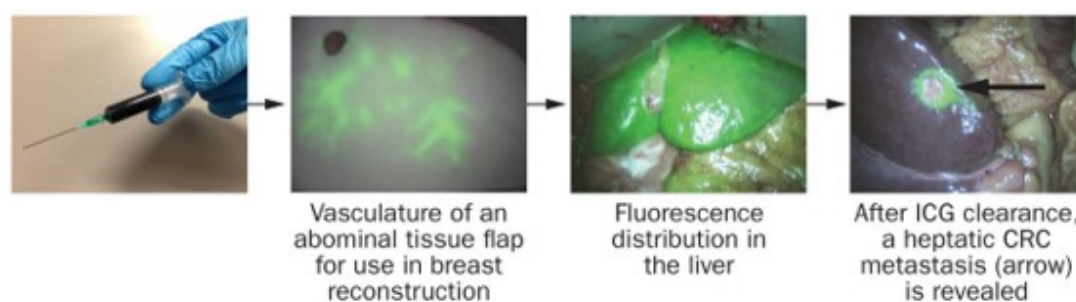


Figure 1.3: 4 key stages in imaging a hepatic colorectal carcinoma (CRC) metastasis with ICG. The fluorophore is administered intravenously and then the dye is visible in the vasculature. The imaging agent is non-targeted and distributes through the body. After allowing clearance time, the metastasis is visible. Reprinted with permission from reference 11. Copyright 2013 Springer Nature.

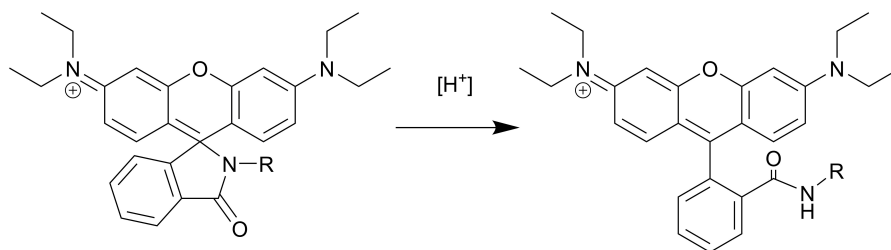
Methylene blue is a fluorophore which has been clinically approved for use in cancer imaging. The structure of the fluorophore leads to methylene blue adopting a variety of charged states in solution. This gives the dye unique biodistribution properties and it has been exploited to achieve visualisation of neuro endocrine insulinomas, fibrous pancreatic tumours and parathyroid adenomas.¹⁷ The precise mechanism by which methylene blue is able to target these specific cancerous is still not well understood.⁹

5-aminolevulinic acid has been clinically approved for fluorescence image guided surgery in detection and removal of glioblastoma.¹⁸ The non-proteinogenic amino acid is a precursor to heme, with protoporphyrin IX produced in an intermediary step. In cancer cells, the ferrochelatase activity needed to chelate iron to protoporphyrin IX, to produce heme, is reduced. Therefore there is an accumulation of protoporphyrin IX in cancerous cells and therefore the emission of this species can be visualised during cancer surgery.⁹

1.2.2.1 Rhodamine

Rhodamine belongs to the same family of xanthene based dyes as fluorescein; its synthesis was first reported in 1905.¹⁹ Compared to fluorescein-based fluorophores, the absorption and emission of rhodamines are red-shifted. Their excellent photostability (compared with other organic-based dyes) high extinction coefficients and long emission wavelengths are some of the features of rhodamine dyes which have made their use prolific over a number of fields. Rhodamines are now used as laser dyes, fluorescence standards for quantum yield measurements, in analyte detection and in living cell imaging.²⁰

Rhodamines attracted attention from organic chemists in 1997 after Dujols et al. showed that an N-substituted rhodamine derivative could be used to detect Cu(II) ions in water.²¹ It was shown that rhodamine spirolactam or spirolactone derivatives are non-fluorescent, whereas the ring-opened structure is highly fluorescent. Scheme 1.1 shows the acid-catalysed ring-opening process, with the fluorophore displaying strong pink fluorescence in acidic conditions. This spiro-ring-opening has been used to detect a number of analytes including Cu²⁺, Hg²⁺, Fe³⁺, Zn²⁺ and Cr³⁺.²²



Scheme 1.1: pH catalysed ring-opening of n-substituted rhodamine-B derivatives.²³

The ability for rhodamine dyes to switch between a highly fluorescent and non-fluorescent form, depending on the environmental pH, has attracted much interest. It has been found that the pH at which this pH switching occurs i.e. the pK_a, can be modified by altering the R group

substituent. Both steric and electronic effects have been shown to contribute to the pK_a and so the R group can be altered to tune the rhodamine ‘switch on’ pH value. For example Yuan et al. showed that when $R = 2$ -hydroxyethylamine the pK_a of the rhodamine-6G dye was 2.8, whereas upon substitution with a larger R group of 1-adamantanamine, the pK_a increased to 6.5.^{24,25}

1.3 Nanoparticles for Imaging

Up to this point, all optical probes discussed in this thesis have been untargeted, simple molecular structures. During the past twenty years, there has been an increasing interest in using nanoparticles for medical imaging.^{26,27} These are materials comprising single units which are between 1 and 100 nm in size. The use of nanomaterials for medical imaging provides a versatility which is much more challenging to achieve with small molecule imaging probes. The surface of the nanomaterial can be functionalised with a variety of ligands, thereby introducing functionality to the imaging probe. This is synthetically more complex for small molecule imaging probes as it would require a number of synthetic steps. Another important consideration is that nanomaterials provide a platform to study processes on the same scale as they occur biologically. In future, it is hoped that this will contribute to the earlier detection of disease and also advance disease characterisation and management.

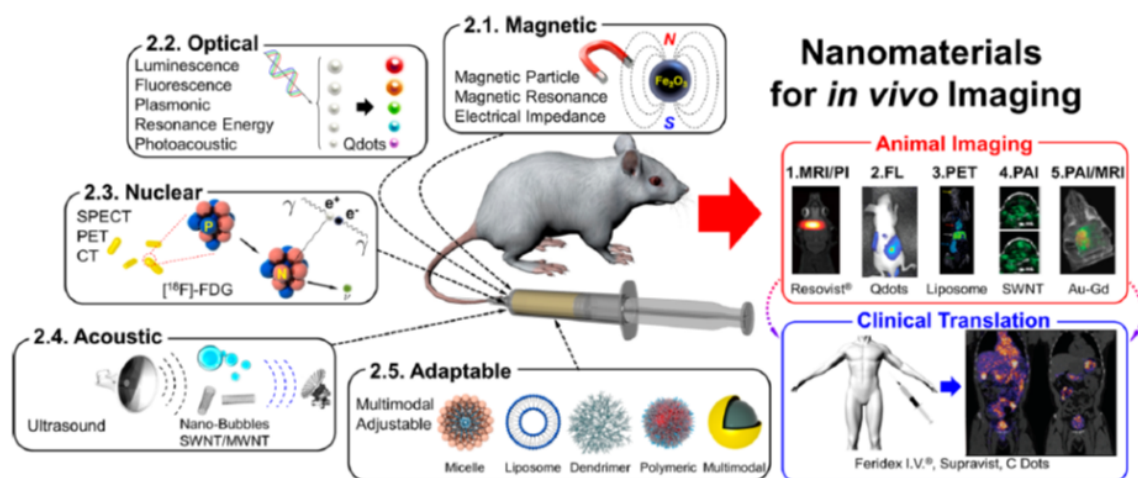


Figure 1.4: Schematic illustrating the functionality versatility of nanoparticle imaging probes, including the ease of incorporation of multiple imaging modalities in one imaging probe.

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A variety of different nanomaterials have been used for imaging including: quantum dots (QDs), gold nanoparticles, carbon nanotubes, iron oxide nanoparticles and upconversion nanoparticles.³ There are a number of reasons as to why nanomaterials are particularly advantageous compared to small molecular imaging probes and these are discussed in the following sections. Figure 1.4 summarises the functionality versatility of nanomaterials with respect to their use as medical imaging agents.

1.3.1 Large Surface Area

The large surface area of a nanoparticle provides a scaffold for attachment of a wide variety of ligands, which can be designed specifically to fit the intended use of the nanomaterial. Ligands can be designed to impart the required properties to the imaging probe e.g. water solubility, biocompatibility, specific disease targeting, environmental sensing and to introduce further imaging functionality. In designing and synthesising small molecule imaging probes, the process of introducing further functionality is often complex. It requires multiple synthetic steps, which can then have dramatic effects on other properties of the probe e.g. the water solubility and biocompatibility. Nanomaterial imaging probes have a modular structure and so it is synthetically straightforward to introduce new functionality to an imaging probe, without many additional steps and without necessarily affecting other properties of the probe.

1.3.2 Incorporation of Multiple Imaging Modalities

The core nanoparticle can exhibit contrast in a number of imaging modalities, depending upon the chosen material. For example, superparamagnetic iron oxide nanoparticles (SPIONs) are used as MR contrast agents and upconversion nanoparticles (UCNPs) made from lanthanide-doped inorganic materials are used as optical imaging agents. Further functionality can be easily introduced to the nanomaterial by incorporating ligands with the required functionality. For example, fluorophores can be bound to the surface of a nanomaterial, introducing optical functionality to the imaging probe. The use of nanomaterials for multimodal imaging will be discussed further in chapter 5.

1.3.3 Controllable Size and Shape

The size and shape of nanoparticles are important factors in determining the circulation time of the imaging agent *in vivo*. Smaller nanoparticles typically have longer circulation times than larger nanoparticles.²⁸ The size of a nanoparticle is a main factor determining its clearance route. Nanoparticles below 10 nm pass through the glomerular basement membrane and are filtered out of the blood by the kidneys. Particles of sizes above 1 μm are filtered by the capillary bed of the lungs.²⁹ In between these sizes, where most nanomaterials sit, the nanoparticles are cleared by the reticuloendothelial system (involved in the immune response). The nanomaterials tend to be phagocytosed and broken down in the liver.

The shape of nanomaterials synthesised can also be carefully controlled by optimising synthesis conditions. The shape can be tuned to promote targeting of the nanomaterial to a specific biological site. Work by Frankenberg et al. compared the biodistribution of 50 nm gold nanospheres and similarly sized gold nanorods (45 nm x 10 nm). They found that by manipulating the shape of the nanomaterial i.e. using gold nanorods rather than spheres, the clearance of the nanomaterial could be slowed down. Nanorods were present at 11 % injected dose per gram after 6 hours, compared to the nanospheres at only 1 % injected dose per gram.³⁰ Synthesis of nanomaterials can be tuned to reliably produce nanoparticles which are highly monodisperse, have a small size distribution and have a well-defined size and shape. This allows nanomaterials to be designed

to achieve the biodistribution required for the imaging agent.

1.3.4 Disease Targeting

As cancer imaging probes, nanoparticles have an advantage over small molecule-based probes as nanoparticles can accumulate in the leaky vasculature commonly associated with solid tumours, due to the enhanced permeability and retention (EPR) effect.³¹ Figure 1.5 shows how this process can occur. The hyper-permeable vasculature in cancerous tissue, along with reduced lymphatic clearance means that nanoparticles are retained within cancerous tissue. This retention enables the nanoparticles to be detected by the appropriate imaging modality and therefore the tumour to be visualised. The EPR effect is particular to larger particles such as nanoparticles, micelles and proteins. Simple molecular imaging agents will not be retained in the tissue as they are small enough to pass into the interstitial space and be cleared through the renal system.

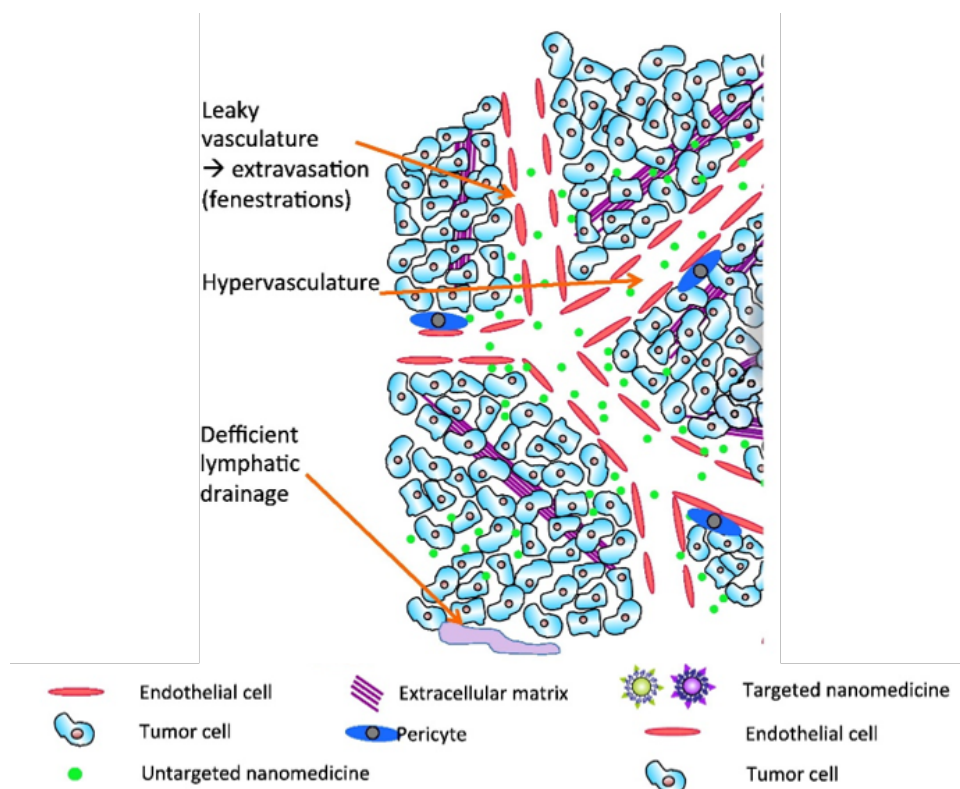


Figure 1.5: Schematic representation of the EPR effect, showing how untargeted nanoparticles can accumulate in cancerous tissue. Reprinted from reference 32. Copyright 2016, with permission from Elsevier.

1.4 Upconversion Nanoparticles

Upconversion nanoparticles are a category of optical nanomaterials which are capable of converting low energy, NIR light to higher energy visible and UV emission. This absorption and emission process is facilitated by the presence of trivalent lanthanide dopants, with long-lived metastable energy states and ladder-like energy levels. The energy levels of lanthanides in the Ln(III) series is shown in figure 1.6. Two or more lower energy photons are absorbed by the material, in an anti-Stokes process, leading to one high energy photon being emitted. 4f-4f transitions in lanthanide(III) ions are weak, partially allowed induced electric dipole transitions, with luminescence lifetimes on the order of microseconds. The upconversion process is non-linear, meaning that the optical properties of the material are dependent upon the radiant flux density of the excitation light and therefore the upconversion intensity is dependent on the excitation density.³³

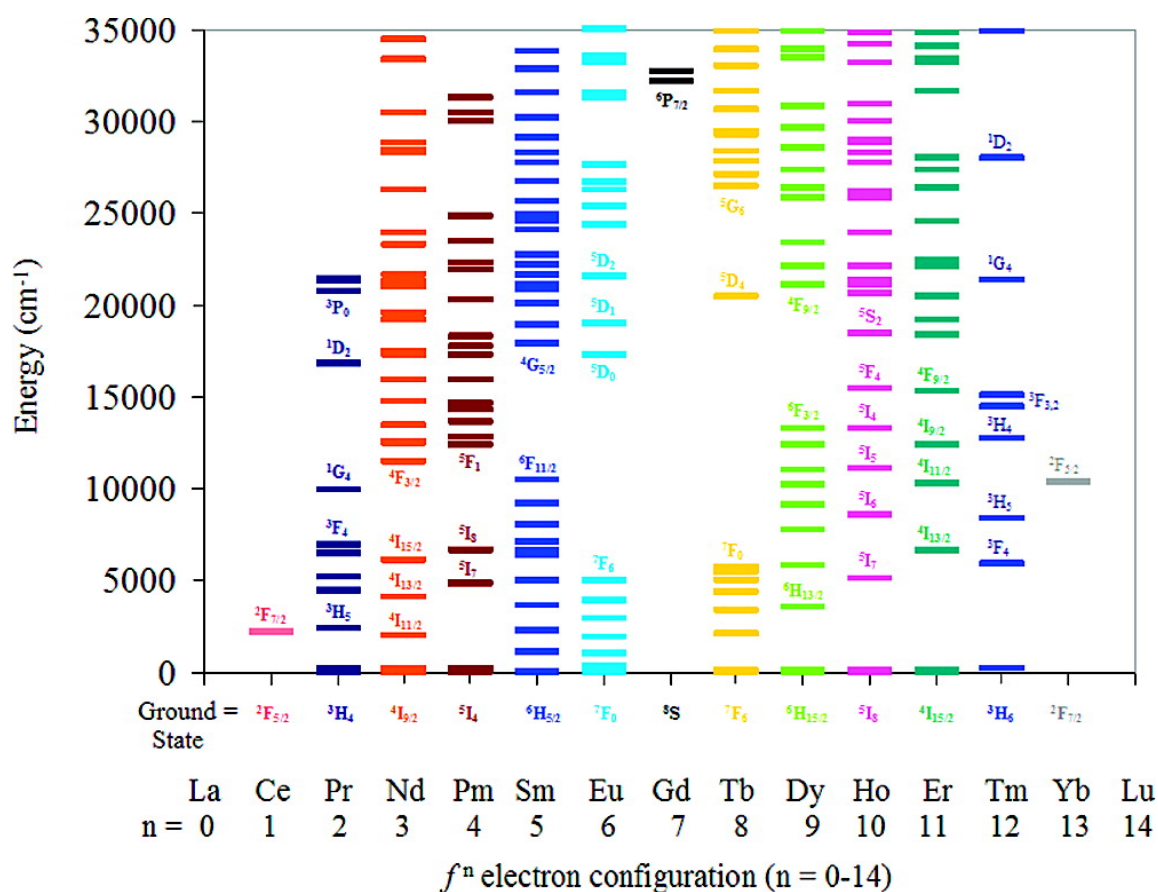


Figure 1.6: The excited state energy levels of the Ln(III) series. Reprinted with permission from reference 34. Copyright 2009 American Chemical Society.

The first reported study of the process of upconversion was published in 1966.^{35–37} Francois Auzel has written a very interesting personal summary of the development of the field, to which readers of this thesis are directed.³⁸ The discovery of highly efficient ‘anti-Stokes emission’ as it was referred to at the time, or upconversion as it is now termed, was achieved by the study of co-doped Yb and Er glasses. Previous to this, two photon excitation had been observed but with the caveat that emission was extremely weak due to the very low probability of the process occurring. Auzel used Yb as a sensitizer to increase the probability of Er absorbing two photons, by transferring energy from the excited state of ytterbium to erbium, also in an excited state. He demonstrated that energy transfer could occur between two rare-earth ions both in their excited state. Before this work, all energy transfers were thought to take place with one ion in its excited state and the other in its ground state.³⁷ Auzel then went on to show the upconversion process in Yb and Tm co-doped glass, with IR to blue conversion observed in a three photon upconversion process.

The field remained relatively small for decades due to limitations with laser excitation power leading to very low emission from bulk UC materials. Menyuk et al. synthesised $\text{NaYF}_4\text{:Yb,Er}$, which was shown to be the most efficient phosphor to date but a quick glance at the synthesis technique demonstrates why the field of upconversion phosphors remained relatively small.³⁹ The $\text{NaYF}_4\text{:Yb,Er}$ material was synthesised by heating YF_3 , YbF_3 and ErF_3 to 750 °C in HF gas, a technique many would be hesitant to attempt!

In the late 1990s, nanoparticle research became of interest and was a rapidly expanding field. In comparison to bulk material, nanoparticles show a comparably poor luminescence intensity, largely attributed to surface quenching and therefore high quality NP preparation techniques were needed to synthesise UCNPs with efficient upconversion.⁴⁰ In the early 2000s, increasing interest in the field of bioimaging, development and refinement of nanoparticle synthesis techniques and advances in laser technology gave renewed interest in the field of upconversion (UC). Heer et al. published the first example of photon upconversion in transparent lanthanide-doped LuPO_4 and YbPO_4 colloidal solution.⁴¹ This work directly led on to the synthesis of $\text{NaYF}_4\text{:Yb,Er}$ UCNPs, previously found to be the most efficient UC bulk material.^{42,43} The UC efficiency of the nanoparticle system was found to be just 1 % of the bulk material.⁴³

Since the early 2000s, the field of upconversion nanoparticles has rapidly expanded, with particular interest in using UCNPs for bioimaging due to a number of interesting properties:

- **Excitation in NIR:** This is particularly advantageous over organic fluorophores which are typically excited by short wavelength light (UV or blue/green visible). This short wavelength excitation of organic fluorophores has a number of disadvantages. The tissue penetration of this wavelength light is poor, only to a depth of 0.5–2 mm and with 15–40 % of the incident radiation reflected.⁴⁴ This means that it is impossible to excite fluorophore deep within the body from an external light source. UV/visible excitation is also problematic as it simultaneously excites many endogenous fluorophores (termed autofluorescence), leading to a high background signal. There is also the possibility of

damage to biomolecules (especially with UV excitation). There is therefore a significant advantage to excitation in the NIR region, especially in the tissue optical windows at 650-1100 nm, where light has its maximum penetration in tissue. The maximum penetration through skin has been shown to be at 1090 nm, with a depth of 3.5 mm.⁴⁴

- **Narrow emission bands:** Lanthanide ions have characteristic sharp emission bands due to the shielding of core-like 4f electrons by outer 5s and 5p electrons, resulting in weak electron-phonon coupling. The 4f-4f transitions are therefore barely effected by the surrounding environment, leading to the sharp emission bands observed. This is particularly useful in UCNP nanosystems in sensing applications as donors / acceptors can be chosen which selectively overlap with one particular UCNP emission band. This leads to the possibility of ratiometric sensing, using the other UCNP emission bands as internal standards. This will be discussed in more detail in chapter 3 and 4.
- **Low photobleaching:** Photobleaching is a phenomenon which describes the irreversible loss of optical properties from fluorophores due to exposure to repeat cycles of excitation and emission. Organic fluorophores exhibit much lower photostability than inorganic materials and typically exhibit photobleaching due to photon induced covalent modification, trapping the fluorophore in a dark (non-emissive) state.⁴⁵ UCNPs are inorganic lattices, which mean they have an intrinsic resistance to photodegradation and excitation in the NIR, rather than the UV/visible region for conventional fluorophores also contributes to UCNPs having a resistance to photobleaching.

Due to the breadth of the field of upconversion nanoparticles in bioimaging, it is not possible to cover every detail in this thesis introduction. Instead, a broad summary is outlined here and readers are directed to other excellent reviews for a more in-depth discussion of specific aspects of the field. Table 1.2 provides a summary of 58 of the most relevant reviews in the field, covering topics such as: fundamentals of the upconversion processes, UCNP synthesis, UCNPs as nanosensors, sensitizing UCNP emission and reviews with a particular focus on *in vivo* applications of UCNPs.^{33,40,46-101}

Author & date	Fundamental Principles	Synthesis and Characterisation	Sensitising upconversion emission	Sensing	UCNPs in biological settings	Reference no.	Author & date	Fundamental Principles	Synthesis and Characterisation	Sensitising upconversion emission	Sensing	UCNPs in biological settings	Reference no.
Auzel, 2004	✓					36	Wang, 2017		✓				71
Wang, 2009		✓				45	Resch, 2017	✓					72
Haase, 2011		✓				32	Xie, 2017		✓				73
Wang, 2011		✓				46	Gu, 2018			✓			74
Kar, 2012	✓	✓				47	Duan, 2018	✓	✓	✓		✓	75
Feng, 2013	✓					48	Wang, 2018		✓	✓			76
Chen, 2013	✓	✓		✓		49	Wen, 2018	✓					77
Johnson, 2013		✓				50	Wang, 2018	✓					78
Dong, 2013	✓					51	Liu, 2018	✓					79
Li, 2013	✓					52	Lyu, 2018		✓	✓			80
Gai, 2014	✓	✓		✓		53	Li, 2018	✓				✓	81
Sun, 2014		✓				54	Wiesholler, 2018	✓	✓				82
Han, 2014			✓			55	Sun, 2018			✓	✓		83
Wang, 2014	✓					56	Fan, 2019	✓					84
Chen, 2014	✓	✓		✓	✓	39	Hong, 2019	✓			✓		85
Singh, 2014	✓					57	Liang, 2019		✓				86
Chen, 2015		✓				58	Zhu, 2019	✓	✓		✓		87
Li, 2015	✓	✓		✓		59	Wu, 2019	✓					88
Chan, 2015	✓					60	Xu, 2019				✓		89
Zheng, 2015	✓		✓	✓		61	Rafique, 2019			✓	✓		90
Zhou, 2015	✓					62	Qin, 2019	✓					91
Li, 2015		✓	✓		✓	63	Hundry, 2019	✓	✓				92
Tu, 2015	✓					64	Liang, 2020	✓			✓		93
Sedlmeier, 2015		✓				65	Ovais, 2020			✓			94
Liu, 2015		✓				66	Li, 2020	✓	✓		✓		95
Wang, 2016				✓		67	Jouyban, 2020			✓			96
Nadort, 2016	✓					68	Xin, 2020		✓		✓		97
Chen, 2016		✓		✓	✓	69	Zhu, 2020	✓	✓				98
Bagheri, 2016		✓	✓			70	Yao, 2020		✓		✓	✓	99

Table 1.2: Summary of UCNP reviews from the last twenty years detailing: the first author, date published and main topics covered in the review.

1.4.1 Upconversion Nanocrystal Structure

An inorganic upconversion nanophosphor comprises several elements: a host lattice and multiple dopant ions which act as sensitizers and activators.

Host lattice: The properties of the host material are important as they have a strong influence on the upconversion process. The lattice spacing determines the distance between dopant ions, optimising this allows the maximum upconversion efficiency to be obtained for the system. The lattice crystal phase determines the position of the lanthanide ions in the material and therefore the distance between the dopant species. It is important that the host lattice has a single crystal phase, to precisely control the position of the dopant ions, thereby achieving the highest possible upconversion efficiency. The crystal phase itself is an important consideration and it has been shown that hexagonal phase $\text{NaYF}_4:\text{Yb,Er}$ has an upconversion efficiency ten times greater than cubic phase $\text{NaYF}_4:\text{Yb,Er}$.^{102,103} This significant difference in upconversion efficiency of the two crystal phases is thought to be due to their differences in symmetry. In the lower symmetry hexagonal-phase $\text{NaYF}_4:\text{Yb,Er}$, the crystal field exerted around the lanthanide dopants will be more uneven than the field in the cubic-phase material. The lower symmetry system allows mixing of the 4f-energy levels in the lanthanides with other energy states. This makes the f-f transition partially allowed and the f-f transition probabilities increase for a lower symmetry lattice, such as $\beta\text{-NaYF}_4:\text{Yb,Er}$.^{40,104}

It is also important that the the phonon energy of the lattice is low, minimising non-radiative emission and therefore maximising the upconversion efficiency of the system. Fluoride based materials are commonly used as host lattices, with fluorides exhibiting low phonon energies of $\sim 350 \text{ cm}^{-1}$.⁴⁷ Fluoride-based host lattices have produced some of the brightest UCNPs to date, notably hexagonal-phase $\text{NaYF}_4:\text{Yb,Er}$ as discussed above. Chloride, bromide and iodide exhibit even lower phonon energies ($\sim 300 \text{ cm}^{-1}$) but the materials are highly hygroscopic, making them unsuitable for many applications. Oxides have also been explored as host lattices due to their high chemical stability but their relatively high phonon energy ($\sim 500 \text{ cm}^{-1}$) makes them less advantageous than fluoride-based lattices.

Activators: These are the emitting lanthanide species in UCNPs, typically erbium(III) or thulium(III) species. These have ladder-like energy states, suitable for absorption of more than one photon of excitation light. In erbium(III), the energy gap between $^4\text{I}_{11/2}$ and $^4\text{I}_{15/2}$ is very close to the energy difference between $^4\text{F}_{7/2}$ and $^4\text{I}_{11/2}$ ($10,350 \text{ cm}^{-1}$ and $10,370 \text{ cm}^{-1}$ respectively), shown in figure 1.7.⁴⁷ Er^{3+} can be excited by 980 nm light from its ground state to $^4\text{I}_{15/2}$ and then further excited to $^4\text{F}_{7/2}$, non-radiative energy transfer takes place to relax to the $^2\text{H}_{11/2}$ energy level, from which green upconversion emission can occur. Alternatively after excitation from the ground state to $^4\text{I}_{15/2}$, relaxation can occur to the metastable state $^4\text{I}_{13/2}$ and then further excitation can occur to $^4\text{F}_{9/2}$. Emission from this energy state can be observed as red emission.

The concentration of the activator species doping is limited by concentration quenching. If the activator concentration is too high, the close proximity can cause the higher energy states to

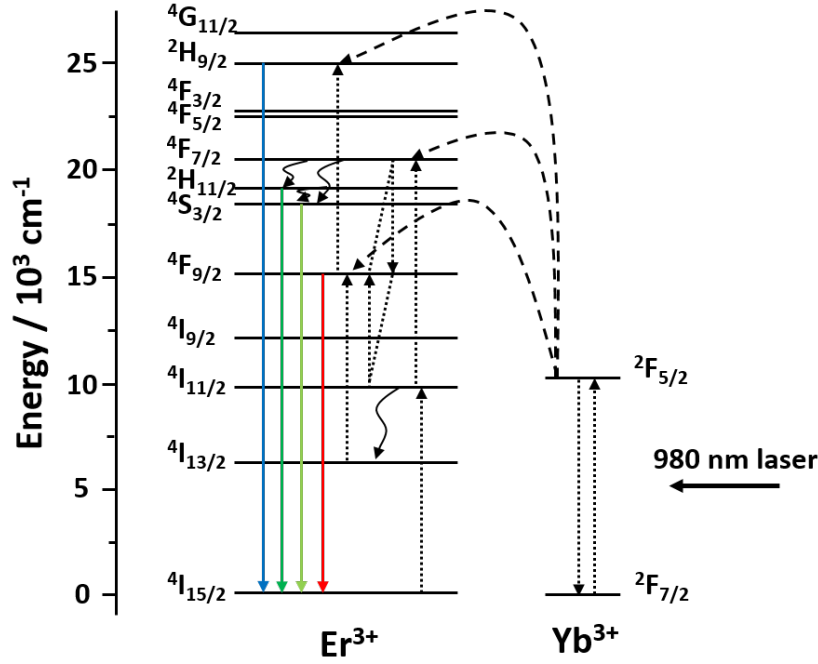


Figure 1.7: Energy level diagram showing the anti-Stoke's emission processes in NaYF₄:Yb,Er UCNPs.⁴⁶

be depopulated by interaction with another lanthanide ion. The higher energy states transfer energy to a lower energy state of a neighbouring lanthanide ion. To prevent deleterious cross-relaxation from occurring, the concentration of activator is typically kept low (<2 mol %) and a sensitizer species is used to enhance the upconversion luminescence efficiency. The mechanism for the cross relaxation process is discussed in section 1.4.2.

Sensitizers: Ytterbium(III) is typically used as the sensitizing species in UCNPs. The ion absorbs 980 nm NIR light and can excite activators through energy transfer. Yb³⁺ has a very simple energy level scheme, with only one excited 4f energy state of ²F_{5/2}, as shown in figure 1.7. The ²F_{7/2} → ²F_{5/2} transition has the highest absorption cross-section of any lanthanide ion transition ($\sim 10^{-20}$ cm²). The absorption cross section is a measure of the probability of a photon being absorbed; a higher absorption cross section leads to more efficient excitation of the lanthanide. The absorption transition of a sensitizer must be resonant with the transition of an activator species in the crystal, to facilitate energy transfer occurring. The ²F_{7/2} → ²F_{5/2} is resonant with many f-f transition in lanthanides, such as the Er³⁺ transitions shown in figure 1.7. The concentration of Yb³⁺ is limited to 20 mol %. This is because energy transfer through the Yb³⁺ ions in the crystal to the surface of the nanoparticle must be limited, to prevent surface quenching occurring.^{97,105}

1.4.2 Upconversion Photophysics

There are a number of different upconversion mechanisms by which the process of upconversion can proceed. These can be broadly categorised into the four processes shown in figure 1.8.

The mechanisms are: excited state absorption (ESA), energy transfer upconversion (ETU) (historically referred to as APTE (addition de photon par transferts d'énergie)), cross relaxation (CR) and photon avalanche (PA). ESA, ETU and PA are based on the sequential absorption of two or more photons by long-lived excited states. Cross relaxation is integral to understanding the process of PA and also underpins the process of concentration quenching - as discussed in the **Activators** section above.

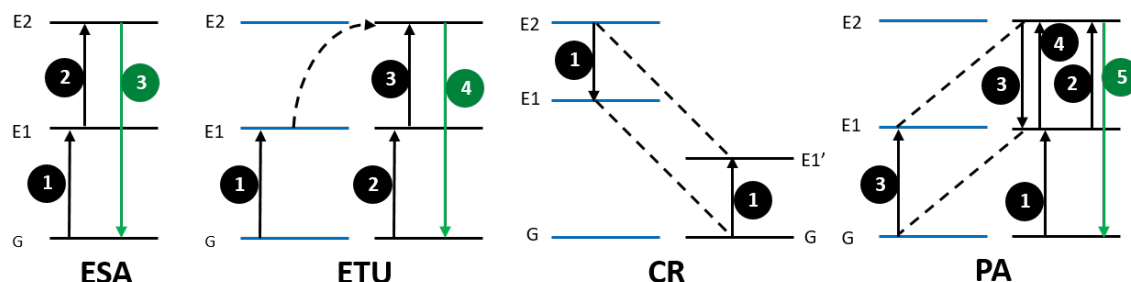


Figure 1.8: Upconversion mechanism steps, with the numbers on the arrows representing the order of electronic transitions. ESA: Excited state absorption, ETU: Energy transfer upconversion, CR: Cross relaxation and PA: Photon avalanche.

ESA: Excited state absorption can occur in lanthanide ions due to their ladder-like energy levels. Figure 1.8 shows a simplified three-level system. If the excitation energy matches the energy gap between the ground (G) and first excited state (E1), the ion can be excited to the higher energy level G1. This is called ground state absorption (GSA). These excited states are long-lived in lanthanides, due to the forbidden nature of f-f transitions. In this time, the system can absorb another photon and be promoted to the higher excited state E2. Emission from this state $E2 \rightarrow G$ is termed upconversion emission. The upconversion efficiency is not dependent on the concentration of lanthanide ions, but will be limited at high concentration due to a higher probability of cross-relaxation.

ETU: Energy transfer upconversion occurs by a similar mechanism to ESA. The main difference is that excitation from E1 to E2 occurs by energy transfer from a neighbouring lanthanide ion. The ETU mechanism occurs by the following steps. First neighbouring lanthanide ions are excited from their ground state to the first excited state $G \rightarrow E1$. For a sensitizer lanthanide ion such as ytterbium(III), this process is more efficient than for activators such as erbium(III). The long-lived intermediate states in ytterbium provide an energy store, ready to transfer energy to populate the emitting states of the activator. Energy transfers from E1 of the sensitizer to E2 of the activator and the sensitizer ion relaxes back to its ground state, G. The energy transfer process in ETU is a non-radiative process where, unlike ESA, upconversion efficiency is dependent on the concentration of lanthanide dopant ions. This is because the energy transfer process from sensitizer to activator can only occur if the two ions are spatially close enough. The average distance between dopant ions is determined by the concentration of the lanthanides in the crystal.

CR: Cross relaxation is an energy transfer process resulting from the close proximity of lanthanide ions in an upconversion crystal. The ions can be either the same or different. The ion-ion interaction can lead to a non-radiative energy transfer process, where energy is transferred from one lanthanide ion to another. This process is responsible for the ‘concentration quenching’ phenomenon which is observed in lanthanide-doped UCNPs. This has led to a significant body of work studying the optimum lanthanide spacing within the nanocrystal, aiming to achieve the brightest possible upconversion nanoparticles.^{106,107}

PA: Photon avalanche is a looping process which encompasses both ESA and CR. Photon avalanche only produces significant upconversion above a threshold excitation power. Below this threshold, little upconversion emission is observed from the system. Above the threshold, the intensity of the emitted light increases dramatically. This is due to the mechanism by which PA occurs, two ions in the E1 state go on to produce four in the E1 energy state. These four can then go on to produce eight in the E1 in an ‘avalanche-like’ process. PA occurs by a looping mechanism. The process starts by ground state absorption (GSA), followed by ESA, populating the E2 energy state. CR then occurs via a neighbouring lanthanide ion so that both ions then occupy the E1 state. This creates a saturated E1 state across the lanthanide ions in the crystal, acting as an energy storage reservoir. ESA follows populating the E2 state, from which upconversion emission occurs.⁴⁶

1.4.3 Synthesis of UCNPs

The luminescent properties of UCNPs are dependent upon their size, shape and crystallinity. Controlled methods to synthesise these nanoparticles are needed so that specific properties can be reliably achieved, each time nanocrystals are synthesised. In the past, it was this that has held back the development of using nanoparticles for imaging. For medical applications, nanoparticles with predictable size and properties must be able to be synthesised in a uniform batch as it is these properties which determine the behaviour of nanoparticles *in vivo*.

In the last twenty years, soft chemical techniques have been developed to synthesise high quality UCNPs with well-defined properties. There are three main techniques used: coprecipitation, hydrothermal / solvothermal and thermal decomposition. These three techniques will be discussed in the following sections.

1.4.3.1 Coprecipitation

Coprecipitation was one of the earliest techniques developed to synthesise UCNPs. It is a simple and cheap method and does not require the use of specialist equipment. The earliest example of the synthesis of high quality, sub-10 nm, $\text{LaF}_3\text{:Eu/Er/Nd/Ho}$ UCNPs was reported by the group of van Veggel in 2002.^{108,109} The authors used RE^{3+} and F^- ions, assisted by ammonium di-n-octadecyldithiophosphate, in an ethanol/water solution at 75 °C. This resulted in UCNPs capped with di-n-octadecyldithiophosphate, used to control particle growth and help prevent particle aggregation. $\text{NaYF}_4\text{:Yb,Er}$ nanoparticles were synthesised in a similar aqueous method using: rare-earth oxides as the lanthanide precursors, NaF as the fluoride source

and ethylenediaminetetraacetic acid (EDTA) as the surface-capping ligand.⁴³ The method did not result in high quality UCNPs. The authors found that the upconversion emission from the sample was negligible and vacuum annealing (heating to 400-600 °C) was necessary to observe upconversion emission. However, this annealing had the detrimental affect of causing aggregation of the sample.

Coprecipitation is rarely the chosen route to synthesise high quality, monodisperse UCNPs. This is because it is challenging to control the size and shape of the nanoparticles produced, with syntheses often resulting in nanoparticles with a large size distribution and a wide range of shapes.⁵⁵

1.4.3.2 Thermal Decomposition

The thermal decomposition route is the most widely used method to produce monodisperse UCNPs, with a small size distribution. The technique commonly uses lanthanide-organic precursors as the source of rare-earth ions. Organic ligands such as oleic acid (OA) are used as surface capping moieties and the syntheses are carried out in high boiling point and non-coordinating solvents, for example 1-octadecene (ODE). The precursors are heated to temperatures above 300 °C, in an oxygen-free environment, causing decomposition of the starting materials and subsequent formation of nanocrystals.

The first example of inorganic lanthanide fluoride nanocrystals synthesised by a thermal decomposition technique was reported by Zhang et al. in 2005.¹¹⁰ In this heat-up method, the reaction mixture was prepared at room temperature and then heated to 300 °C. This method differs from the hot-injection technique, where the lanthanide precursors are injected into pre-prepared, hot reaction mixture.¹¹¹ The heat-up method is more convenient for larger scale syntheses and lends itself to better reproducibility and greater control of the size of the nanoparticles produced. This is because in a hot-injection synthesis, the time taken to inject a reagent often varies between batches. This can have a dramatic effect on reaction kinetics. A controlled single ‘burst’ nucleation event is necessary to synthesise highly uniform nanocrystals, with distinct separation between nucleation and growth steps.⁶¹ This is achieved more easily with a heat-up method.¹¹²

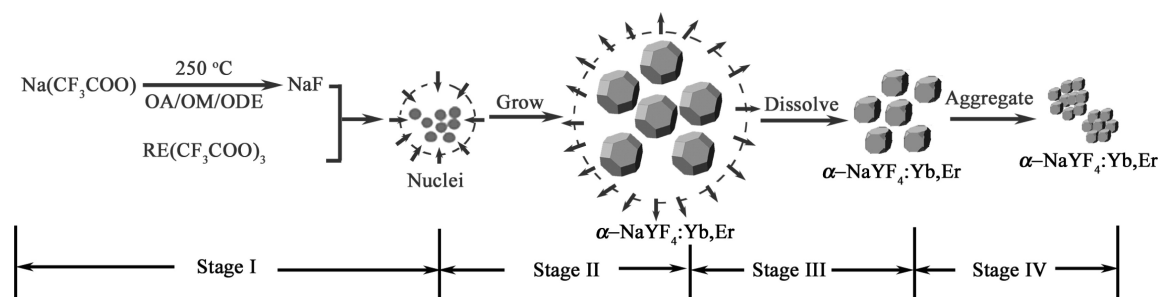


Figure 1.9: Schematic showing the growth process of NaYF₄:Yb,Er UCNPs. Reprinted with permission from reference 113. Copyright 2007 American Chemical Society.

In 2006, Boyer et al. demonstrated the thermal decomposition technique could be used to

synthesise UCNPs.¹¹⁴ The authors were able to synthesise $\text{NaYF}_4\text{:Yb,Er/Tm}$ UCNPs from lanthanide trifluoroacetate precursors. Mai et al. studied the growth process of UCNPs when synthesised by thermal decomposition, as shown in figure 1.9.¹¹³ They showed that the synthesis of UCNPs takes place in four stages. In the first stage, the trifluoroacetate precursors dissolve and nucleation begins. Stage II is the nanocrystal growth process and occurs until the precursors are depleted. The smaller NPs are thermodynamically less stable and so stage III sees the smaller particles redissolving and larger stable particles continue to grow. This is known as Ostwald ripening. Continued heating was found to result in stage IV, the formation of aggregates.

The shape and size of the nanoparticles formed by thermal decomposition synthesis can be controlled by adjusting the ratio of OA and ODE in the reaction mixture, changing the ratio of lanthanide precursors to solvent and by tuning the reaction time.^{115–117} This makes thermal decomposition a reliable and reproducible method to produce high quality and highly tunable UCNPs.¹¹⁸

1.4.3.3 Hydrothermal / Solvothermal

In hydrothermal and solvothermal methods, Teflon-lined autoclaves are used to perform the syntheses under high pressure.⁵⁵ Hydrothermal synthesis is an aqueous technique. The reaction mixture is heated, under pressure, above the boiling point of water. Solvothermal synthesis describes the analogous non-aqueous technique. The high temperature and pressure achieved in the autoclave synthesis enable the precursors to dissolve and allow crystallisation of the nanomaterial product.⁷⁷ As the solvent mixture is heated under pressure, the choice of solvent is not limited by its boiling point. This means that the technique is extremely versatile and a large range of precursors can be used to synthesise various nanoparticles e.g. $\text{LaVO}_4\text{:Yb,Er}$ ¹¹⁹, $\text{Sr}_2\text{ScF}_7\text{:Ln}^{3+}$ ¹²⁰, $\text{Y}_2\text{O}_3\text{:Yb,Er}$ ¹²¹. Typically hydrothermal / solvothermal syntheses use: rare-earth precursors (usually nitrates or chlorides), a solvent, and an organic additive (controlling the crystal growth). The ratio of reaction components can be adjusted to control crystal growth. This gives the hydrothermal and solvothermal synthesis methods the ability to finely control the size and shape of the nanocrystals synthesised. The advantages of these techniques are that lower temperatures are needed than the thermal decomposition synthesis route. However, these methods do require the use of specialist autoclave equipment.

1.4.4 Surface Functionalisation Strategies

As discussed in the previous section, highly monodisperse upconversion nanoparticles are typically synthesised by a thermal decomposition route. This results in UCNPs with hydrophobic ligands on the surface, such as oleic acid. In order to render these systems biocompatible, a number of techniques have been developed including: hydrophobic interaction with amphiphilic molecules, ligand exchange, addition of a silica shell and ligand oxidation. A schematic representing these techniques is shown in figure 1.10. The methods have been discussed in detail in excellent reviews by Sedlmeier et al. and Andresen et al. and will be summarised in the following sections.^{67,122,123}

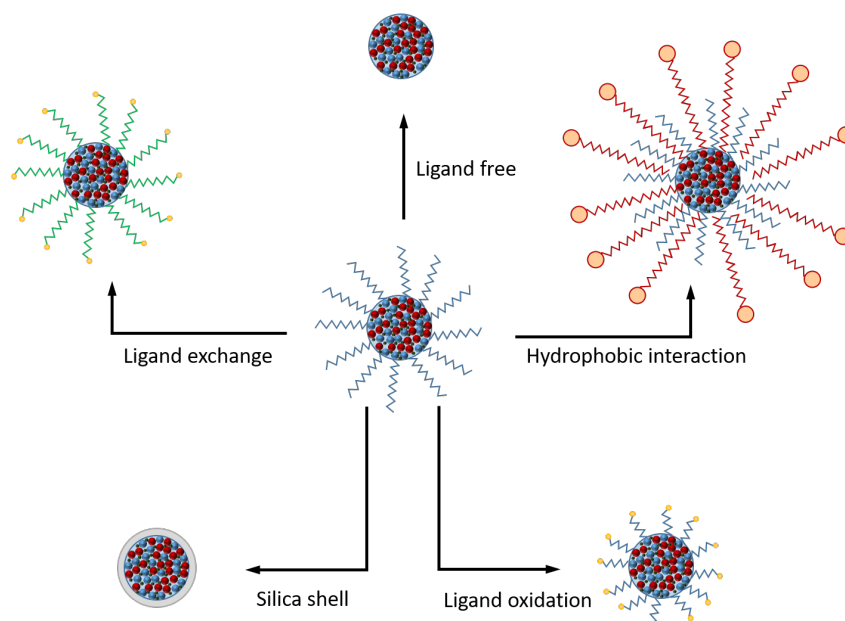


Figure 1.10: Common methods use to functionalise the surface of hydrophobic UCNP.

1.4.4.1 Hydrophobic Interaction

Amphiphilic ligands can be used to encapsulate UCNP, with the non-polar alkyl chains interacting in a hydrophobic interaction with the ligands on the surface of the UCNP. This leaves the polar head groups on the amphiphilic molecule exposed, therefore rendering the UCNP water soluble. The strong van-der-Waals interactions between the alkyl chains on the amphiphilic ligand and the hydrophobic ligands bound to the nanoparticle surface form a stable bilayer.

Phospholipids are often the amphiphilic ligand of choice. This is because they are inherently biocompatible, leading to UCNP which are readily internalised by cells and have long circulation times *in vivo*.¹²⁴ Commonly, polyethylene glycol (PEG) groups are incorporated in the phospholipid as spacer units between the fatty acid tails and the hydrophilic head group, as shown in figure 1.11.¹²⁴ This further increases the biocompatibility of the nanosystem as PEG has been shown to prevent protein adsorption, therefore helping the nanomaterial evade the immune system and prolonging circulation time.^{125–127}

The main issue with using phospholipids to encapsulate UCNP is the cost. Calculations by Muhr et al. suggested that to completely coat 1 μmol of 20 nm diameter UCNP would require 4.5 g of phospholipid. This could cost in the region of \$30,000.¹²²

Another important consideration is that the addition of a phospholipid makes the UCNP nanomaterial considerably larger, including a large distance between the core of nanomaterial and any functionality at the head group of the phospholipid. This is worth considering if any functionality in the nanoprobe relies on energy transfer between the UCNP core and a moiety on the outside of the bilayer. Förster resonance energy transfer (FRET) has a strong distance dependence ($\frac{1}{r^6}$) and so by increasing the distance between the donor and acceptor, the efficiency of energy transfer is dramatically decreased. The limit for FRET-based systems is 10 nm.

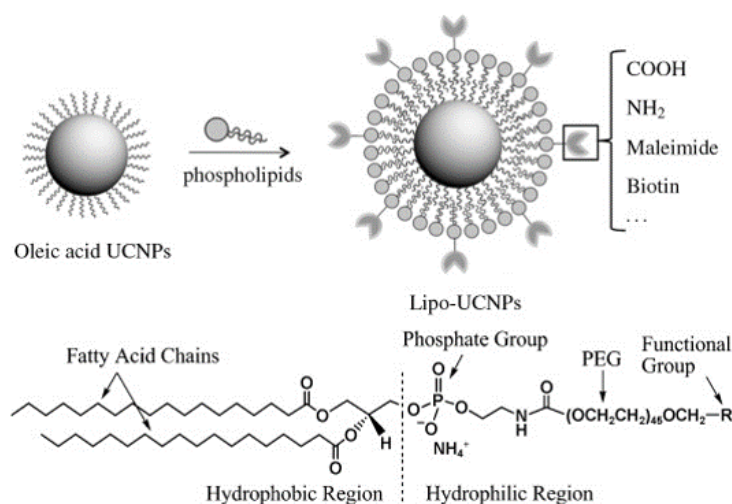


Figure 1.11: Phospholipid encapsulation of oleic-acid capped UCNPs, forming a bilayer. The technique can be used to introduce various functionalities onto the UCNPs surface. Reprinted with permission from reference 124. Copyright 2012 by Wiley and Sons, Inc.

1.4.4.2 Ligand Exchange

The native hydrophobic ligands on the surface of the UCNPs can be replaced by hydrophilic ligands. There are two strategies which can be used to achieve this: either a one-step direct exchange method, or a two-step method via ligand-free UCNPs.

Direct Ligand Exchange

In a one-step ligand exchange process, the native ligand on the surface of the nanoparticle is almost completely replaced. The new ligand is chosen to be more hydrophilic, to confer water solubility to the system. The ligand introduced to the nanoparticle must contain a suitable group for surface binding, typically -COOH, -NH₂, -PO₃H or -SH.¹²² Depending on the nature of the material of the nanoparticle, this binding strength will vary and this will have direct affect on the colloidal stability of the nanoparticle dispersion. Studying the interaction between the nanoparticle and bound ligand will be discussed in more detail in chapter 2.

The new ligand must also possess functionality which induces water dispersibility for the system. Ligands such as citrate and hexanedioic acid have been used, where one carboxylate moiety interacts with the surface of the nanoparticle and another is free to interact with the solvent.^{128,129} It is also common to introduce PEG ligands to the surface of UCNPs during a ligand exchange procedure. The PEG units help impart water solubility to the system and increase biocompatibility. The ligand exchange reactions can be challenging as non-optimal reaction conditions can lead to either no ligand exchange, only partial exchange or aggregation of the nanomaterial.¹²²

Ligand Exchange via Ligand Free UCNPs

Rather than directly exchanging the ligands on the surface of the UCNPs, the hydrophobic ligands can be removed from the surface by treatment with an acid. The method was first developed by the group of Capobianco in 2011; the authors used dilute hydrochloric acid to

liberate the oleic acid ligands from the surface of NaYF₄:Yb,Er UCNP. ¹³⁰ This resulted in nanoparticles with a positively charged surface. The positive charge enables the ligand-free UCNP to be dispersed in aqueous media. In a separate second step new ligands can be introduced to the ligand-free, positively charged UCNP surface.

1.4.4.3 Silica Shell

Water dispersibility of UCNP can also be achieved by depositing a silica shell on the nanoparticle surface. The silica shell provides a coating to UCNP which is transparent, biocompatible, non-toxic and has controllable pore size, giving the option to load small molecules into the shell of UCNP. ⁷⁷ Silica is chemically inert and hydrophilic and so coating on the surface of UCNP produces NPs that are chemically stable and dispersible in aqueous solution. Silica coating has been a popular method to render a number of different types of nanoparticles biocompatible including: gold NPs, iron oxide NPs and quantum dots. ⁶⁷

Depositing a silica shell on the surface of a nanoparticle is usually achieved by either the Stöber process or by reverse microemulsion. The Stöber process is used for UCNP which already have hydrophilic ligands on the surface as the method requires dispersion of the uncoated UCNP in aqueous media. It uses precursors such as tetraethoxysilane (TEOS) as a silicon precursor in a polycondensation reaction to coat the UCNP surface with layers of silica. ¹²³ The required surface functionality can be achieved by reaction of the silica surface with organosilanes or by directly functionalising the UCNP surface in the presence of functionalised silanes. ⁶⁷

Typically, the preferred route for synthesis of UCNP is the thermal decomposition route, which produces NPs with hydrophobic surface ligands. For this reason, the reverse microemulsion method is the most common method used to achieve UCNP surface silanization. In this method, a mixture of ammonia, cyclohexane, TEOS and a surfactant creates micelles within a non-polar solvent system. The silization reaction occurs within these aqueous pockets, resulting in the steady growth of a silica shell on the surface of the UCNP. ^{116,131,132}

There are some issues with using a silica shell to render UCNP biocompatible. Polydispersity of the sample can occur due to multiple UCNP being incorporated into the same silica shell. Recently, Yang et al. showed that stability in biological media of silica shell nanoparticles is limited. ¹³³ Although silica-shell nanoparticles are relatively stable in water and phosphate-buffered saline (PBS), the silica was found to be eroded in biological media, raising questions about the use of silica-shell nanoparticles in biological applications.

1.4.4.4 Ligand Oxidation

After synthesis of UCNP by the thermal decomposition method, typically the resultant NPs are capped with oleic acid surface ligands. The double bond in the centre of the oleic acid ligand provides a site for modification to increase the hydrophilicity of the nanoparticles. Oleic acid bound to the nanoparticle surface can be oxidised by the Lemieux-Rudloff reagent, a catalytic mixture of equal amounts of permanganate (MnO₄⁻) and periodate (IO₄⁻). ¹³⁴ The reaction

leads to 1,9-nonanedioic acid bound to the surface of UCNPs, with the exposed carboxylic acid increasing the water solubility of the system, see figure 1.12.

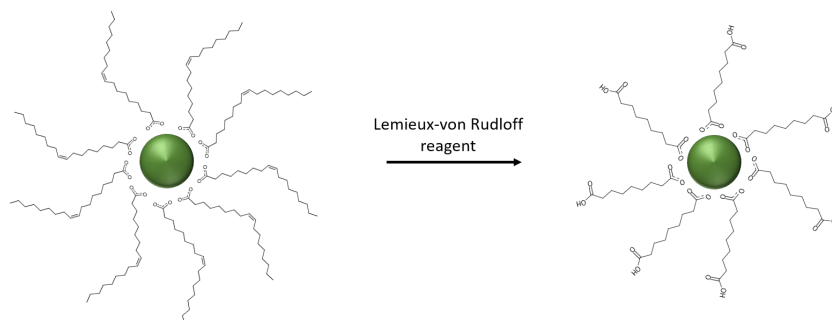


Figure 1.12: Oxidation of oleic acid-capped UCNPs with the Lemieux-Von Rudloff reagent, generating free carboxylic acid groups on the surface ligands.

This technique has limited use as the colloidal stability of the resulting 1,9-nonanedioic acid-capped UCNPs is often found to be poor. There are also issues with versatility as the method is only able to produce free carboxylic acid ligands on the surface of UCNPs and it relies on a suitable double bond for oxidation.

1.4.5 Characterisation Techniques

In developing nanomaterials for bioimaging applications, it is imperative that the properties of the imaging probe are fully understood. This requires comprehensive characterisation of the nature of the nanomaterials, both in their structural and optical properties. There is a requirement that the imaging nanoprobe synthesis is reliably repeatable and that the material synthesised is consistent across the sample. For example the size distribution of the nanomaterials synthesised must be as minimal as possible, as nanomaterials of different sizes could have a vastly different biodistribution *in vivo*. There need to be comprehensive characterisation techniques readily available to screen the nanomaterials after synthesis for the quality of the sample to be assessed. During the imaging probe development stage, precise characterisation is required so that the properties of the nanomaterial can be tuned to achieve the required optical or structural properties.⁶⁸

Determining the structure, composition and properties of nanomaterials requires a variety of different characterisation techniques. Some techniques are recognisable from their routine use in molecular synthetic chemistry, such as NMR and IR spectroscopy. Others are more applicable to materials science applications and deal with the characterisation of larger crystalline solids, studying the morphology and composition of the synthesised materials (e.g. TEM and powder XRD).

In this section, characterisation tools used for probing the nature of nanomaterials are outlined, describing the techniques with a focus on how the methods are used in characterising UCNPs. Many of these techniques are used later in the thesis and this section aims to provide a reference

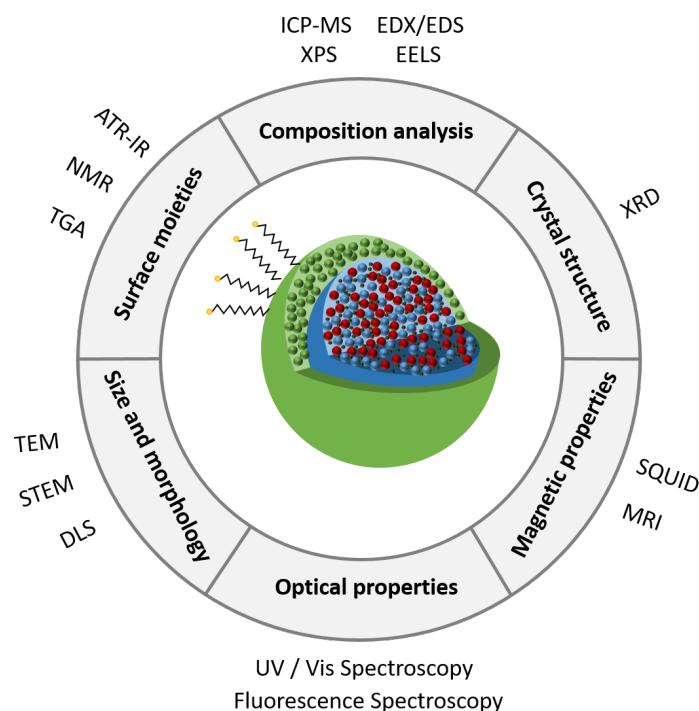


Figure 1.13: Characterisation techniques routinely used to probe the properties of UCNPs.

point for the fundamentals of the techniques described and how these techniques can be used to determine properties of UCNPs. Figure 1.13 provides a summary of the instruments routinely used to characterise UCNPs.

The characterisation techniques described below are sorted by the information they provide: size and morphology, compositional analysis, crystal structure, analysis of surface moieties, optical properties and magnetic properties.

1.4.5.1 Size and Morphology

The size and morphology of UCNPs can affect their optical properties, including both the overall and relative intensity of the lanthanide emissions.^{135–137} These characteristics also affect the distribution *in vivo*, including clearance routes and cell uptake.⁶⁸ The following characterisation techniques can be used to study the size and morphology of UCNPs:

TEM / STEM: Transmission electron microscopy (TEM) and scanning transmission electron microscopy (STEM) are microscopy techniques in which a beam of electrons is passed through a solid sample deposited onto a sample grid. Variation in sample thickness, atomic number (Z) and crystal structure produces contrast in the image. STEM imaging works by converging the beam to a single focal point and scanning it across the sample in what is described as ‘raster illumination’. This describes the process of the electron beam moving across a line of the sample, left to right and then blanking to start scanning left to right on the line below. Both TEM and STEM can be used to image nanoparticles, providing information about their size and shape (as shown in figure 1.14). STEM can be used in the high-angle annular dark-field imaging mode

(HAADF), where the electrons scattered by the sample are collected by the detector. Contrast is reversed compared to standard TEM images and this technique is commonly paired with energy-dispersive X-ray spectroscopy (EDX).

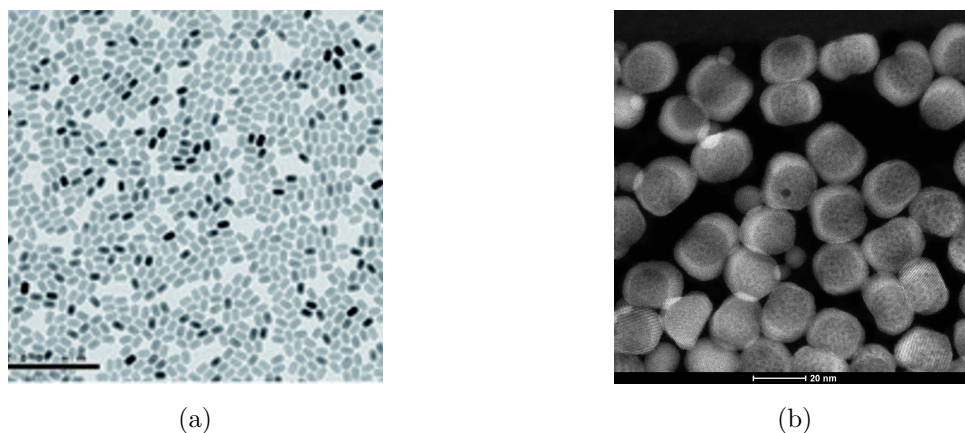


Figure 1.14: (a) TEM image of Ca^{2+} -doped $\text{NaGdF}_4\text{:Yb,Er}$ core UCNPs. Reproduced from reference 138 with permission from The Royal Society of Chemistry. (b) STEM image of $\text{NaYF}_4/\text{NaGdF}_4$ core-shell nanoparticles. Reprinted with permission from reference 139.

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DLS: Dynamic light scattering (DLS) is a technique which can be used to assess the size of colloids in solution and measure the colloidal stability of a dispersion. It provides a method to measure the hydrodynamic diameter of a nanomaterial by measuring the scattering angle of a laser beam passing through a dispersion of the NPs.¹⁴⁰

1.4.5.2 Compositional Analysis

Accurate determination of the concentration and position of lanthanides in the UCNP crystal structure is essential in understanding the optical properties of nanomaterials and designing and tuning UCNP synthesis to achieve optimal emission properties.

ICP-MS: Inductively coupled plasma mass spectrometry (ICP-MS) provides a method to determine the concentration of each ion in a sample of UCNPs. The technique relies on the digestion of a sample of UCNPs, breaking down the nanoparticles into their constituent ions. The concentration of each element can then be determined by mass spectrometry and the technique provides a method to analyse the average concentration of lanthanide ions across a bulk sample, rather than analysis of individual nanocrystals.

XPS: X-ray photoelectron spectroscopy (XPS) can be used to determine the elements at the surface of a nanoparticle and is based on the photoelectric effect. XPS uses a focused X-ray beam to liberate electrons from a material. Ejected electrons have a limited ability to escape the crystal and so only electrons from atoms on the surface of the nanoparticle are able to escape and to reach the detector.

Every element has a characteristic binding energy:

$$E_{\text{binding}} = E_{\text{photon}} - (E_{\text{kinetic}} + \phi) \quad (1.1)$$

By measuring the kinetic energy of the ejected electrons, the elements in the nanocrystal can be identified. The technique is especially useful for confirming elemental composition of core-shell nanoparticle structures.¹⁴¹

EDX / EELS: Energy dispersive X-ray spectroscopy (EDX) and electron energy loss spectroscopy (EELS) are techniques which are routinely used in combination with TEM / STEM imaging. Both techniques rely on the bombardment of a nanoparticle sample with an electron beam. In STEM EDX, the characteristic X-rays ejected from the nanoparticle sample are detected. The electron beam moves across the sample point-by-point and so elemental composition of each small sample area can be determined. EELS works similarly. The technique measures the energy loss of the electron beam as it passes through and is inelastically scattered. The spatial resolution of EELS is higher than STEM EDX due to the presence of an aperture at the entrance of the EELS spectrometer, limiting the volume from which the scattering events are measured, thereby improving the spatial resolution of the technique.¹⁴²

Figure 1.15 shows an example EDX spectrum across a core-shell UCNP sample. The technique can be used to distinguish between the yttrium-doped core and the gadolinium-doped shell.

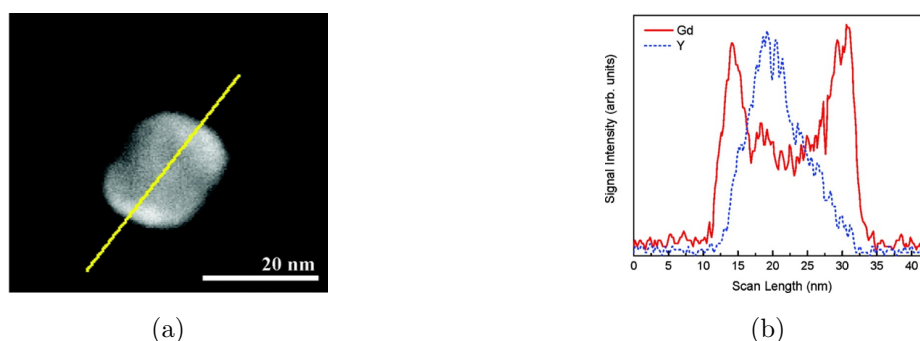


Figure 1.15: (a) HAADF image of core-shell $\text{NaYF}_4/\text{NaGdF}_4$ UCNPs, showing the line scan from which the corresponding EDX data was captured. (b) EDX line scan across the single UCNP. Both images reprinted with permission from reference 139. Copyright 2011 American Chemical Society.

1.4.5.3 Crystal Structure

As discussed in section 1.4.1, the crystal structure is important in determining the efficiency of upconversion in UCNPs. The most commonly used technique to determine crystal structure in a NP sample is powder X-ray diffraction (XRD).

XRD: Powder X-ray diffraction is a straightforward technique which is routinely used in nanoparticle characterisation. It can be used to determine the crystal structure of the nano-

material. In this technique, the sample is prepared as a powder and is bombarded by an X-ray beam, producing characteristic diffracted X-rays. The X-ray beam moves around the sample and the intensity of the diffracted X-rays are recorded with respect to this angle. Bragg's Law (equation (1.2)) describes the fundamental physics underpinning the technique. It describes the interference of X-ray reflection after interaction with a material; if the reflected X-ray constructively interfere, then an intense peak of reflected radiation is detected.

$$n\lambda = 2d\sin\theta \quad (1.2)$$

where n is an integer, λ is the wavelength of the incident X-ray radiation, d is the spacing between the lattice planes and θ is the angle of diffraction between the incident and diffracted X-ray beam.

The pattern of diffracted X-rays (where the X-ray intensity is plotted against the scattering angle (2θ)) is unique to a particular crystal structure. The observed pattern can then be compared to a standard powder diffraction card in the database of the Joint Committee on Powder Diffraction Standards (referred to as JCPDS no.) to determine the crystal structure of the material.

1.4.5.4 Surface Moieties

All the characterisation techniques discussed up to this point describe methods to analyse the UCNP core material. Another important consideration is characterisation of the ligands bound to the surface of the nanoparticles. This is especially important for bioimaging probes as the surface ligands will be selected to impart various functionality onto the nanoprobe. For example, ligands may be introduced to render the UCNP water soluble, targeting ligands may be appended to the surface or other organic ligands introducing other optical functionality. It is important that these ligands can be fully characterised. In this section, general techniques to characterise ligands bound to a nanoparticle surface will be discussed:

IR: Infra-red spectroscopy (IR) can be used to identify chemical groups present in surface ligands. The technique gives valuable structural and qualitative information about the nature of the ligands bound to the surface of the UCNPs. Quantitative analysis is not possible, therefore it cannot be used to calculate the quantity of surface ligand on a nanoparticle.^{68,143}

TGA: Thermogravimetric analysis (TGA) can be used to calculate the ligand surface coverage of nanoparticles and relies on measuring mass loss during heating of the nanomaterial. TGA is useful for quantifying the number of ligands bound to each nanoparticle. There are limitations associated with its use as it is unable to distinguish between different ligands on the surface.¹⁴⁴

NMR: NMR spectroscopy is a tool which allows the electronic structure of a molecule to be determined based on the interaction of an atomic nuclei with a magnetic field and a radio-frequency pulse. The technique can be a powerful method to characterise the nature of species immobilised on the surface of nanoparticles but can suffer from line-broadening of the NMR

signals due to a slower tumbling rate of the ligands on the nanoparticle surface.^{143,145} The use of NMR is also limited if the nanomaterial contains paramagnetic species, as this can lead to line-broadening.

1.4.5.5 Optical Properties

Directly measuring the optical properties of UCNPs is essential to characterise their luminescence. The f-f transitions in lanthanides are partially parity forbidden and so their extinction coefficients are small (typically $<1 \text{ M}^{-1}\text{cm}^{-1}$). Under UV/Vis absorption spectra, the peak is very weak. Excitation in UCNP systems is typically achieved by a continuous-wave laser to provide the excitation power necessary to observe upconversion.

Upconversion Emission Spectroscopy: UCNPs can be excited with relatively low excitation power, compared to upconversion in quantum dots or organic fluorophores. Under excitation power of $<10 \text{ W cm}^{-2}$, the following exponential relationship holds:

$$I_{\text{UC}} = (I_P)^n \quad (1.3)$$

where I_{UC} is the intensity of the upconversion emission, I_P is the excitation power density and n is the number of NIR photons absorbed per photon emitted in the UV-to-visible region.

From this relationship, the number of photons involved in each upconversion process, producing an emission signal, can be determined for an UC nanomaterial. In contrast to conventional fluorescence imaging, upconversion fluorescence imaging cannot be measured with the typical commercial fluorometer excitation source of a xenon or tungsten lamp.⁶³ Instead, the spectrometer is paired with an external NIR laser diode, typically of wavelength 980 nm to match the absorption of ytterbium. This excitation source provides the excitation power density required to observe the upconversion process.

1.4.5.6 Magnetic Properties

The unpaired electrons in lanthanides, such as Gd^{3+} , gives rise to the possibility of using UCNPs in magnetic resonance imaging. Dual-modal MR/optical imaging probes have been developed, based upon UCNPs.⁶⁸ In these dual-modal systems, the magnetic properties of the imaging agents need to be characterised.

SQUID: Superconducting quantum interference device (SQUID) magnetometers measure the magnetic field of a material. They can be used to characterise the magnetism and magnetic mass susceptibility of UCNPs.¹⁴⁶

MRI: The suitability of UCNP-based materials to be used as MR contrast agents can be determined by measuring the materials using an MRI scanner. The relaxivity of these materials, i.e their ability to modulate the relaxation rate of the surrounding water molecules, can be determined using this instrumentation. Both the longitudinal relaxivity (r_1) and transverse relaxivity (r_2) of the system can be measured.

1.4.6 Bioimaging with UCNPs

The first example of UCNPs being imaged *in vivo* was published by Chatterjee et al. in 2008.¹⁴⁷ The authors injected $\text{NaYF}_4:\text{Yb,Er}$ UCNPs subcutaneously into the groin and upper legs of rats. They demonstrated that visible fluorescence could be observed up to 10 mm below the surface of the skin. Work by Nyk et al. made further progress by injecting UCNPs ($\text{NaYF}_4:\text{Yb,Tm}$) intravenously into mice, in a solution of 5 % glucose in water.¹⁴⁸ In doing this, the UCNPs were circulating in the blood stream of the animals. This moves closer to true UCNPs delivery conditions in a clinical bioimaging setting. The authors found that imaging 2h post-injection allowed visualisation of NIR emission (800 nm) from the UCNPs, with excitation at 975 nm.

Intravenous injection followed by upconversion bioimaging, where NIR excitation is converted to visible emission, has been difficult to achieve *in vivo* due to the limited tissue penetration of visible light. More recent work using UCNPs for bioimaging has focused on detection of emission from UCNPs in the NIR window I (650-950 nm). This is because at this wavelength, the tissue penetration is much greater than that of visible light. Figure 1.16 shows work carried out by Chen et al. in 2012.¹⁴⁹ They showed that injection of core-shell $\text{NaYbF}_4:\text{Tm}@ \text{CaF}_2$ UCNPs intravenously enabled detection of 800 nm emission, after excitation at 980 nm. From the merge images, the location of the UCNPs is easily visualised. The signal to background ratio is high, giving good contrast in the images. Importantly, the authors reported no toxicity effects in the mice tested. There are many other examples of *in vivo* bioimaging in mice, with several recent reviews providing comprehensive summaries of the field.^{87,95,150}

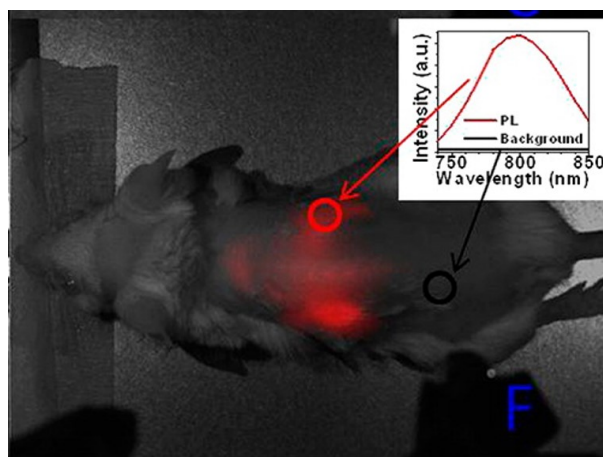


Figure 1.16: Merge bright-field and UC photoluminescence images of mouse injected with $\text{NaYbF}_4:\text{Tm}@ \text{CaF}_2$ core-shell UCNPs. Inset is the UC spectra taken from the circled areas (excitation at 980 nm). Reprinted with permission from reference 149. Copyright 2012 American Chemical Society.

In 2018, Wang et al. published a study they had undertaken using $\text{NaGdF}_4:\text{Nd}@ \text{NaGdF}_4$ NPs in image-guided surgery for treatment of metastatic ovarian cancer.¹⁵¹ In this work, they used an excitation wavelength of 808 nm (for a more in-depth discussion of Nd-doping and excitation at 808 nm, refer to chapter 4) and measured emission in the 2nd NIR window (1000–1700 nm).

They termed these particles downconversion nanoparticles (DCNPs). The NPs were targeted to metastatic ovarian cancer tumour cells by the inclusion of follicle-stimulating hormone (FSH β) peptide on the surface of the nanoprobe. They employed a two-step targeting method, where first DCNPs were injected into the mice intravenously. After 8h, further nanoparticles were injected as this was found to result in increased targeting to the tumour via preferential assembly of the injected DCNPs with those already at the tumour site. The authors investigated the potential to use the technique in intra-operative detection of metastases. They found that the DCNPs could identify tumours of <1 mm diameter, smaller than the human eye could identify. Figure 1.17 shows the photoluminescence images taken, identifying metastatic lesions.

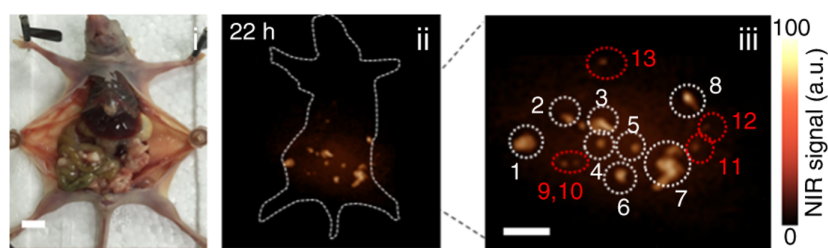


Figure 1.17: (i) Optical photo of the mouse model of metastatic ovarian cancer, before surgery, (ii) corresponding NIR-II photoluminescence images at 22 h post-injection, (iii) enlargement of the abdominal region showing the tumours identified - large peritoneal metastatic tumours (Nos. 1–8) and ultrasmall lesions (Nos. 9–13). Scale bar, 1 cm. Reprinted with permission from reference 151. Copyright 2018 Springer Nature.

The work shows the potential nanomaterials have to impact the field of image-guided surgery. It would not be necessary for the emitted light to penetrate through centimetres of skin; the light would only have to penetrate through a few mm of organ tissue into the body cavity, to be detected by a human eye or camera. This unlocks the potential of using the whole visible region for UCNP emission and does not limit the scope to nanomaterials with emission in the NIR. The use of nanomaterials in image-guided surgery gives the versatility to introduce further functionality to an imaging probe, for example MR. The work by Wang et al. used MR to validate their results from photoluminescence imaging.¹⁵¹

1.4.7 Future Perspectives: The Use of UCNPs in Molecular Imaging

The use of nanoparticle imaging probes in preclinical research is widespread however, the translation from preclinical research into routine clinical has not been smooth and nanoparticle-based imaging probes are yet to see widespread clinical use.²⁹ This is for a number of reasons, but an important consideration is that nanoparticle imaging agents have not yet been shown to be diagnostically superior to the small molecule probes typically used in the clinic.³ Large scale clinical uptake of imaging nanomaterials is likely to only take place if development of these materials is matched with clinical need. This means that research today, even at the earliest stages, needs to have clinical applications in mind.

There are a number of requirements which need to be fulfilled to help pave the way for UCNPs

to make the transition from preclinical research to clinical use.^{152,153}

- **Reproducible control over the core nanomaterial size and shape:** This practical synthesis consideration is important when considering the use of UCNPs for bioimaging. As previously discussed, the shape and size of a nanomaterial affects the biodistribution *in vivo*. The nanomaterial injected must be uniform across the sample to maximise delivery of the imaging probe to the site of interest. The synthesis of UCNP core nanomaterial must therefore be reliable in producing high quality samples and characterisation techniques need to be readily available to assess the uniformity of the sample.
- **Detectable signal:** UCNP imaging probes must be readily excited *in vivo* and have a detectable signal to be useful as an imaging probe. Quenching of the optical signal of UCNPs is often an issue encountered when UCNPs are phase transferred into water. For clinical translation of UCNPs as imaging probes, innovative methods must be developed to increase the brightness of UCNPs *in vivo*.
- **Biocompatibility:** There are a number of factors to consider when assessing the biocompatibility of an imaging probe. Firstly, the material as injected must be non-toxic. Sikora et al. carried out toxicity studies on NaYF₄:Yb,Er,Gd UCNPs, using three cell lines.¹⁵⁴ They observed no cytotoxicity, up to a concentration of 50 µg mL⁻¹. The toxicity of the imaging probe after metabolism must also be considered. Work by Li et al. showed that UCNPs could be broken down into Ln³⁺ ions in lysosomal compartments and these Ln³⁺ ions had toxic effects.¹⁵⁵ The authors did discover that by selecting UCNPs with highly stable surface coatings, for example phosphates, this breakdown of the UCNPs into component ions could be avoided. The findings show the importance of developing UCNPs with strongly bound surface groups, to resist degradation *in vivo*.

1.5 Aims and Objectives

This project aimed to keep the requirements discussed above at the forefront of any work carried out. The target nanomaterial imaging agents were intended to be of high quality (predictable size and small size distribution), have a detectable upconversion signal and be biocompatible (including resistant to aggregation). The objective was to produce a range of nanomaterials which could be suitable for applications in fluorescence image-guided cancer surgery.

The large surface area of nanomaterials allows the design and synthesis of new upconversion nanoparticle imaging probes with a modular approach; both the ligands and the core nanoparticle material can be selected specifically for the bioimaging target. This is the approach taken in this thesis, with the research focusing on using UCNPs as the core nanomaterial. Ligands have been designed and synthesised with the aim of achieving certain physical or photophysical properties for the imaging probe.

The work in this thesis starts with a focus on studying the interaction between the nanomaterial and the bound surface ligand. The motivation behind this work was to understand in detail the affinity of various surface binding groups to the core nanomaterial, helping to guide the design of nanomaterials suitable for *in vivo* imaging applications. The stability of the nanoparticle-ligand interaction in an imaging agent *in vivo* determines the resistance to aggregation. For an imaging agent to reach its target in the body, this aggregation prevention is vitally important.

Analyte sensing *in vivo* has the potential to provide vast quantities of information about biological systems in both healthy and disease environments. By combining UCNPs with analyte responsive fluorophores, nanosensing systems can be created which have the potential to reveal detail about biological environments, by analysing the emission of the nanomaterial. The work in chapter 2 influenced the design of the nanosensors presented in chapter 3. Rhodamine ligands were chosen, due to the spectral overlap between the emission band of NaYF₄:Yb,Er UCNPs and the absorption band of the rhodamine ligands. This gives the potential for energy transfer to occur between the two and the possibility of using these nanosystems as pH sensors.

Chapter 4 describes work undertaken in order to try and mitigate the common issue of poor emission intensity of UCNPs in aqueous environments. It was thought that introduction of an organic dye as sensitizer would improve the fluorescence emission intensity of the system. This chapter describes the synthesis and assembly of cyanine-functionalised UCNPs.

In the past ten years, there has been increasing interest in the synthesis of multimodal imaging agents. In chapter 5, the attempts to introduce MR functionality are described, including the use of super paramagnetic iron oxide nanoparticles (SPIONs) and Gd-doping.

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Chapter 2

Nanoparticle-Ligand Binding

2.1 Introduction

Thermal decomposition provides a method to synthesise UCNPs which are monodisperse nanoparticles and have a small size distribution, as discussed in section 1.4.3.2.^{1,2} However, this route produces UCNPs capped with oleic acid, which are not water soluble and are therefore unsuitable for biological applications. A common method to overcome this issue is to replace the hydrophobic ligands with hydrophilic ligands, in a ligand exchange reaction. This was discussed briefly in section 1.4.4.2.³ There are a number of factors to consider when designing surface ligands for biological applications. The ligands must be robustly anchored to the nanoparticle surface, to prevent desorption of the ligand and aggregation of the UCNPs.¹ There must be effective repulsion of blood proteins. This stops large protein coronas forming around the nanoparticles, thereby preventing rapid clearance by the endorecticular system.⁴ There is also a benefit for the ligands to be bifunctional as this can allow facile attachment of targeting, therapeutic or sensing groups to the nanoparticle surface.⁵

Figure 2.1 provides an overview of the extensive range of ligands which can coordinate to the surface of nanoparticles. Ligands have a variety of functions including: as stabilizers, tethering ligands or providing further functionality to the system. The point at which the ligand coordinates to the NP surface, the anchor point, can be determined by the synthesis method. For example, the amphiphilic ligand oleic acid in thermal decomposition synthesis of UCNPs. Alternatively, these native ligands can be replaced with more strongly coordinating species (such as thiols or phosphines in the case of ligand coordination to the surface of quantum dots). The choice of the anchoring group often depends on the constituent material of the nanoparticle.

A detailed understanding of the organic-inorganic interface is essential in developing imaging agents suitable for use *in vivo*. The nature of ligand coordination, ligand density and lateral expansion define properties such as hydrodynamic diameter, effective charge and long-term colloidal stability. These properties contribute to determining the biocompatibility of the nanomaterial.⁶

The binding strength of an organic ligand to an inorganic surface directly relates to the colloidal stability of the dispersions.⁷ This is a particularly important consideration for *in vivo* applications as the size of a nanomaterial is an important factor which determines biodistribution. The biodistribution is likely to be considerably different for a monodisperse nanomaterial to an aggregated material. If the nanoparticles are prone to aggregation in physiological conditions, this may affect the targeting ability of the probe or the excretion route.⁸

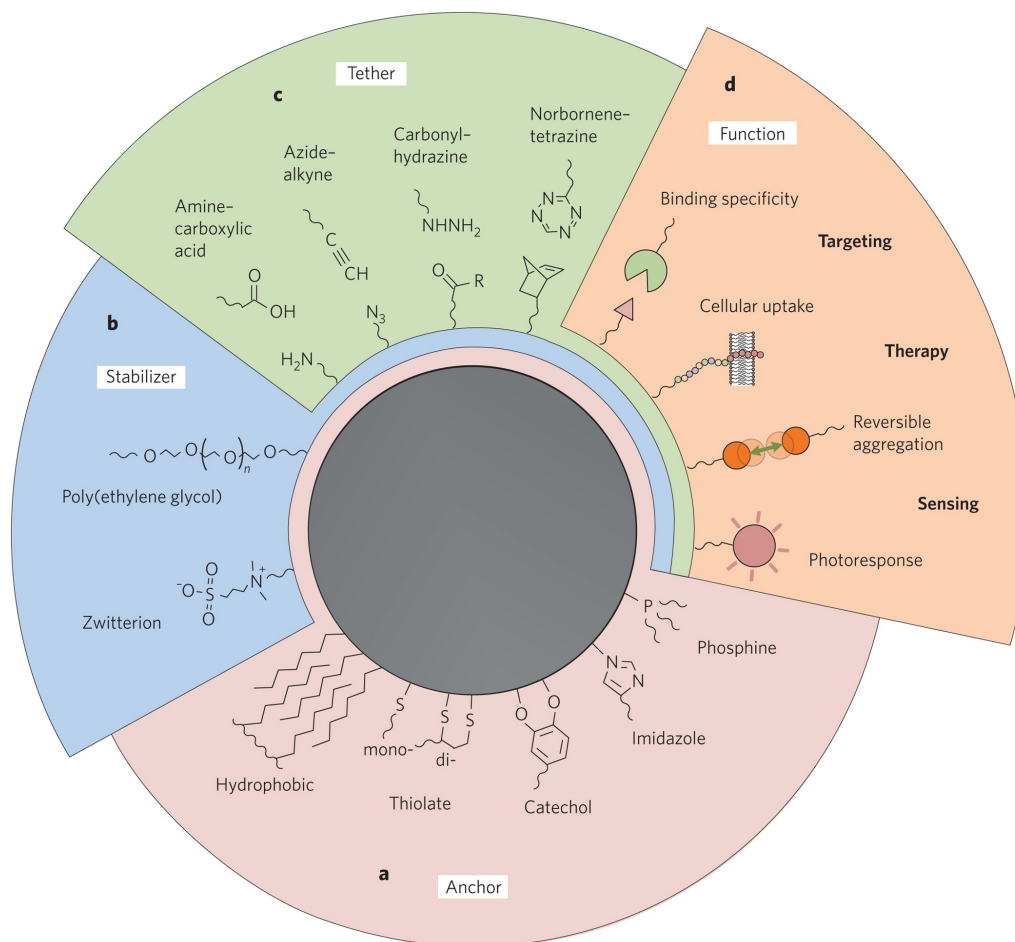


Figure 2.1: Representation of the modular design of surface ligands for nanomaterials. There are a number of aspects to consider when designing surface ligands including: **a** anchor group, **b** biological stabilizing group to enhance biocompatibility, **c** tether for covalent conjugation **d** bifunctional ligand to provide further functionality. Reprinted with permission from Springer Nature: Nature Publishing Group, Nature Materials, reference 5. Copyright 2016.

When considering ligand binding affinity, the strength of the ligand-nanoparticle bond can be rationalised in terms of electronic, entropic and steric effects:⁵

- **Electronic:** Electronic effects have a significant influence on the stability of the surface-capping layer interaction. An example of this is the use of thiols as capping ligands for gold nanoparticles. Thiols bind strongly to gold nanoparticles due to the soft-soft gold-sulphur interaction. The hard-soft acid base principle can be applied to rationalise the strength

of the ligand-nanoparticle interaction in many different nanoparticle-ligand systems.

- **Entropic:** The chelate effect significantly increases the stability of the ligand-nanoparticle bond, in an analogous manner to molecular chemistry. Ligands with two or more binding groups are more stable than those with only one site of interaction with the nanoparticle surface.
- **Steric:** The bulkiness of the ligand will affect how it aligns on the surface of the nanoparticle and how that ligand interacts with other surface ligands and this has a direct effect on the stability of the nanoparticle-ligand bonding.

Assessing the surface-ligand binding strength is not straightforward for nanoparticles and it is not sufficient to simply apply metal coordination theory to rationalise the binding. Nanoparticles have a number of different crystal facets and the metals exposed vary across the crystal surface. This opens up various binding modes of the ligand to the nanoparticle.

There are a number of techniques that have been explored extensively in an effort to characterise ligand-nanoparticle binding. Often a combination of traditional surface science techniques are used alongside traditional coordination chemistry techniques so that a picture of the surface-ligand interface can be pieced together. A selection of the most widely used of these techniques are ATR-IR, thermogravimetric analysis (TGA) and NMR spectroscopy.⁹ A brief summary of using the techniques with a focus on nanomaterial characterisation is given in the introduction chapter, section 1.4.5.3.

NMR spectroscopy is a particularly powerful technique because the spectra obtained can allow the determination of both the identity of the species present and the quantity of the surface ligands. It also allows the system to be studied *in situ* without perturbing the ligand environment by drying out the sample during a preparative step. Importantly the technique is non-destructive, unlike TGA. NMR spectroscopy is particularly sensitive to the chemical environment of the species and this allows ligands immobilised on the surface of nanoparticles to be distinguished from free ligands. Spectral features such as line broadening and chemical shift have enabled the characterisation of a number of ligand-nanoparticle systems.¹⁰

2.1.1 NMR Spectroscopy as a Technique for Assessing Ligand Binding

NMR spectroscopy has been used as a technique to study nanoparticle-ligand binding strength most extensively in the field of quantum dots. Studies in the early 1990s by Sachleben¹¹, Becerra¹² and Majetich¹³ demonstrated the power of using NMR spectroscopy to study ligand exchange on the surface of quantum dots; 1D ¹H NMR was used to calculate ligand surface coverage and in the work by Majetich et al., the authors provided evidence for multiple ligand species binding to the nanoparticle surface.¹³ In more recent years, NMR spectroscopy has been used to determine the composition of a ligand shell, to study ligand binding modes and to determine relative ligand binding strengths.¹⁴

Initial NMR spectroscopy studies of QDs were motivated by the drive to understand the chem-

istry of the surface ligand layer of CdSe QDs. The desire to be able to rationally design surface functionalisation methods relies on understanding what the native surface ligands are and how they interact with the surface of the nanoparticles. In 2008, Kopping et al. used $^{31}\text{P}\{\text{H}\}$ and ^1H NMR spectroscopy to show that the dominating surface species on CdSe QDs was dihydrogen pyrophosphonic acid and n-octylphosphonate (OPA) rather than the previously assumed tri-n-octylphosphine oxide (TOPO).¹⁵ The results relied on the observation that the chemical shift and broadening of the ^1H NMR signal could be used to identify the species coordinating to the nanoparticle studies. The work by Kopping et al. led to a discovery that alkylphosphonate ligands are bound to the surface of CdSe QDs by an X-type interaction and this has helped design ligand exchange reactions which preserve the optical properties of the system.^{16–20}

1D ^1H NMR spectroscopy has also enabled the analysis of how oleic acid is coordinated to the surface of QDs, with Cass et al. proving that chelating and bridging coordinated oleate species are in rapid exchange.²¹ Figure 2.2 shows the different coordination modes the oleate species can adopt when binding to PbS QDs. Similar studies have been undertaken with amine capping species on ZnO nanoparticles and phosphonic acids on CdSe quantum rods.^{22,23}

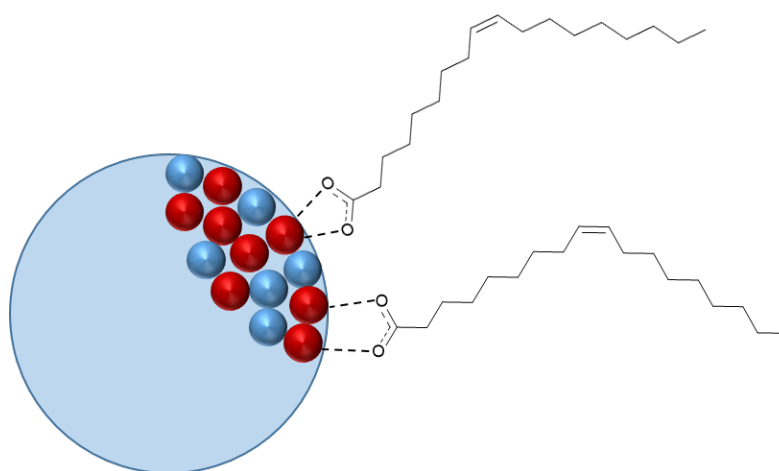


Figure 2.2: Oleic acid ligand interaction with ZnO nanoparticles, blue = zinc: red = oxygen. Two binding modes of oleic acid shown, top = chelating mode: bottom = bridging mode.

The studies described above have shown that NMR spectroscopy can be a powerful tool in probing the interface between nanoparticle and the native ligands adsorbed to the surface during nanoparticle synthesis. However, it is also important to understand the ligand exchange process, as this allows the design of nanoparticle systems suitable for their target applications. When considering the design of nanoparticles for bioimaging, the ligand-nanoparticle interaction must be stable in biological conditions. There are many examples of NMR spectroscopy being used to understand relative binding strengths of ligands to QDs and gold nanoparticles and to follow ligand exchange reactions.^{17,23–31}

During the ligand exchange procedure, surface ligands can be replaced with ligands with different

surface binding groups, varying chain lengths or chain identity. How this ligand exchange reaction proceeds is determined by the identity of the ligand initially bound to the nanoparticle surface and the incoming ligand. There have been a number of studies which have shown that this ligand exchange process is often dynamic and an equilibrium constant for the ligand exchange can be determined by ^1H NMR spectroscopy. Figure 2.1.1 shows an example of work carried out by Ritchhart et al. in 2019.³² NMR titrations were carried out where 10-eneundecylphosphonic acid (UDPA) was added in increasing concentrations to a dispersion of oleate-capped InP QDs. The ^1H NMR spectrum showed that oleate bound to the InP surface could be differentiated from free oleate as the two moieties have different chemical shifts. As 10-eneundecylphosphonic acid was added, the concentration of bound oleate decreased and the concentration of free oleic acid increased. This data was then used to calculate the equilibrium constant of the ligand exchange.

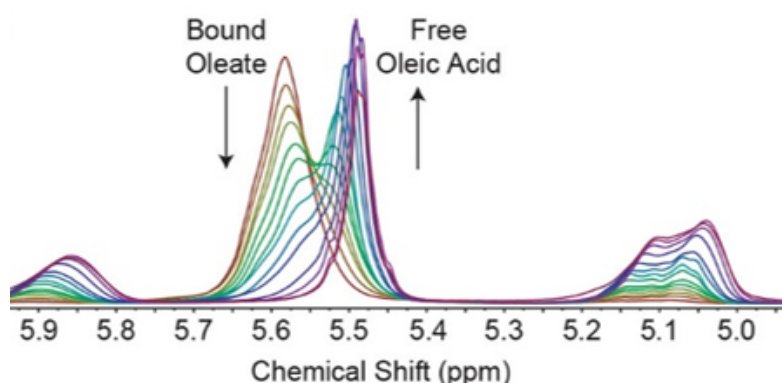


Figure 2.3: ^1H NMR spectra of oleic acid-capped $\text{In}_{37}\text{P}_{30}$ clusters. The alkene region of the spectrum is shown as 10-eneundecylphosphonic acid is gradually added, red = 0 eq UDPA; violet = 51 eq UDPA. Reprinted with permission from reference 32. Copyright 2019 American Chemical Society.

A study by Knuauf et al. in 2016 established that the native oleate ligands on the surface of CdSe QDs undergo an exchange reaction with undec-10-enoic acid with the equilibrium constant of this reaction calculated to be $K_{\text{eq}} = 0.83$ (at 25 °C), demonstrating that the binding of the native oleate species is more favourable.²⁵ Other groups have studied the same exchange reaction on PbS QDs and work by Nolf et al. calculated a very similar equilibrium constant of 0.84 (at 25 °C).³³ The core nanomaterial is an important factor in determining the strength of the nanoparticle-oleate bond and Smock et al. showed that undec-10-enoic acid binds more favourably to CsPbBr_3 QDs ($K_{\text{eq}} = 1.97$ at 25 °C).³¹

The nature of the binding moiety and the composition of the nanoparticle surface are not the only factor affecting the ligand exchange process. ^1H NMR studies have shown that the degree of branching in the ligand also affects the equilibrium constant, with a greater degree of branching resulting in a weaker binding affinity.^{17,30,34} Ligand chain length has been shown to affect binding affinity, with shorter ligands more favourably binding to the nanoparticle surface.³⁴ The degree of ligand-ligand interaction can also affect the position of the equilibrium.

Kroupa et al. in 2016 demonstrated favourable π - π interaction between the aliphatic chains in the *trans*-2,6-difluorocinnamic acid ligand and this promoted favourable further addition of the ligand.¹⁸

A study by Gomes et al. in 2011 showed that not all ligand exchange reactions are in equilibrium and the binding of phosphonate is strong enough that the native oleate species are irreversibly displaced on CdSe QDs.²⁴ There are a number of papers reporting similar results, with phosphonate binding species showing a strong binding affinity to InP³², CdSe^{25,30,35} and CsPbBr₃ QDs.³¹

2.1.2 Phosphonates as Nanoparticle Surface Capping Ligands

Phosphonates are a highly versatile family of ligands. They contain two readily labile protons and both the anion (RPO₃H)⁻ and dianion (RPO₃)²⁻ can readily form by deprotonation. The pKa of a number of phosphonic acids have been determined, with the pKa of the most acidic proton found to be ~ 2 and the second ~ 7.5 .³⁶ Phosphonic acids are increasingly used in the synthesis of nanomaterials due to the good stability of P-C bonds and the versatile coordination chemistry of phosphonate ligand.³⁷ They have been demonstrated to be particularly useful for biological applications due to the absence of polycondensation and the good hydrolytic stability of M-O-P bonds.³⁷

Phosphonate ligands have been used extensively in the synthesis of QDs and there is now a detailed understanding of how phosphonate ligands coordinate to the nanocrystal surface. There are an increasing number of examples of phosphonates being used as surface passivating ligands for UCNPs but there is a current gap in knowledge about exactly how they interact with the nanoparticle surface.³⁸⁻⁴⁰ The literature published to date mainly applies molecular coordination chemistry theory to justify the assumption that there is strong coordination of phosphonate ligands to lanthanide ions on the surface of UCNPs.³⁸⁻⁴⁰ A more detailed understanding of the UCNP-phosphonate ligand interface is needed to aid the development of imaging probes which are stable in biological conditions.

2.1.3 DLS as a Technique to Assess Colloidal Stability

The wide-ranging merits of using NMR spectroscopy as a method to study QD-ligand binding have been discussed above, but there are few examples of the technique being applied to upconversion nanoparticle - ligand binding.^{9,41,42} The main reason for this is due to the line broadening effects which result from measuring samples containing paramagnetic species. As the concentration of paramagnetic lanthanide ions such as Gd³⁺ and Er³⁺ in UCNPs is high, the line broadening effect is extensive and the signals from the organic species are not visible.

The technique most widely used to study UCNP-ligand interaction is dynamic light scattering (DLS). This enables the colloidal stability of the nanoparticle dispersions to be determined, thereby inferring information about the strength of the nanoparticle-ligand bond. A strong UCNP-ligand interaction reduces nanoparticle aggregation and therefore results in a stable

dispersion; a weaker interaction can lead to ligand desorption, nanoparticle aggregation and therefore an unstable colloid.

Figure 2.4 shows how DLS was used to compare the binding strength of monodentate, bidentate and tetradentate PEG phosphonate ligands to NaHoF_4 nanoparticles.⁴³ The photographs in figure 2.4b demonstrate that in phosphate-buffered saline (PBS), the monophosphonate-capped UCNPs aggregated. The authors suggest this is due to phosphonate ligands in the buffer exchanging with the bound monophosphonate PEG ligands. The clear dispersions of the bidentate and tetradentate species demonstrate a greater binding affinity and these nanomaterials are less prone to aggregation.

This can be measured quantitatively by DLS, where the hydrodynamic size of the particles in the dispersion are determined. The distributions can be seen in figure 2.4a. The figure shows larger particle sizes and therefore aggregation of the monodentate PEG-phosphonate species at pH 3 and pH 9. The bisphosphonate and tetraphosphonate species form stable coordination over a wider pH range. The result has been corroborated by work from Hou et al. and Nsubuga et al.^{44,45}

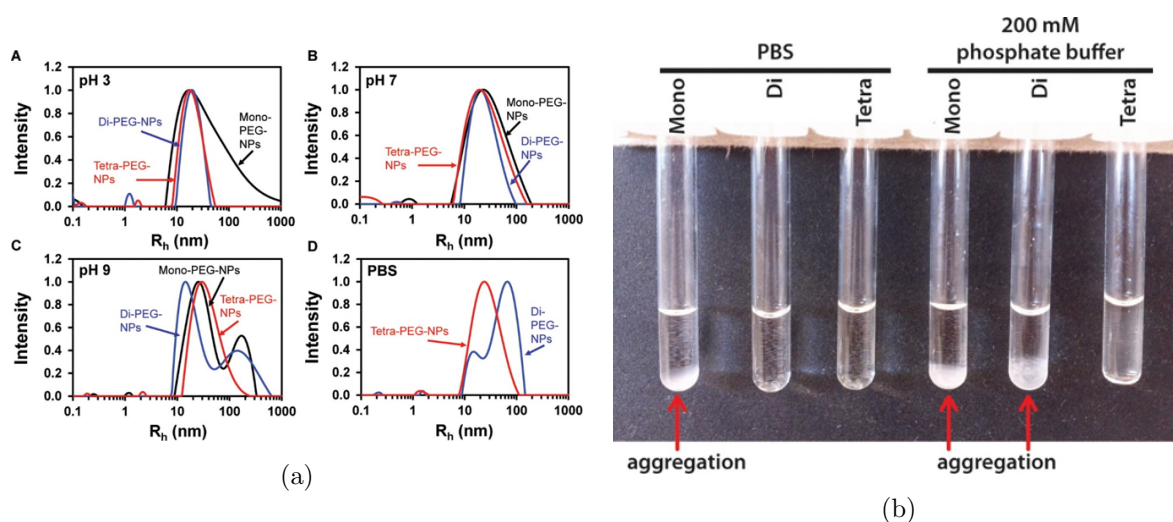


Figure 2.4: (a) DLS plots of monophosphonate-PEG, diphosphonate-PEG and tetraphosphonate-PEG capped NaHoF_4 NPs. Colloidal stability of the UCNPs dispersions compared in water at various pH values and in PBS. (b) Photographs of the dispersions in PBS and 200 mM phosphate buffer, showing greater colloidal stability of the multidentate phosphonate ligands. Reprinted with permission from reference 43. Copyright 2012 American Chemical Society.

DLS has also been used as a technique to show the stability of the phosphonate-UCNP bond, compared to unstable colloids formed with silane, catechol, sulphonate or carboxylate coordinating groups.^{23,46,47} Kostiv et al. report that “phosphonate moieties are considered the most effective anchoring agents to passivate the particle surface and prevent particle dissolution under biological conditions due to the high binding affinity of Ln ions to phosphonate groups.”⁴⁸

2.2 Aims and Objectives

The aim of this chapter was to study the interaction between upconversion nanoparticles and surface ligands, in particular the difference between carboxylic acid and phosphonic acid ligand coordination. By better understanding the nanoparticle-ligand binding, this can be used to inform the design of strongly binding surface ligands, suitable for *in vivo* applications. The two methods chosen to probe this interaction were NMR spectroscopy and DLS. Up to this point, NMR has been an underutilized tool to study UCNP surface ligands. An objective of this chapter was to use ^1H NMR spectroscopy to compare carboxylate and bisphosphonate surface binding affinity by studying ligand exchange on the nanoparticle surface. These results were used to inform the design of water solubilising PEG ligands, suitable for phase transfer of nanoparticles. A second objective was to use DLS to study the colloidal stability of UCNP-ligand dispersions over a range of pH conditions.

2.3 Results and Discussion

The abundance of literature exploiting that phosphonate ligands bind more strongly to UCNPs than carboxylate ligands provided the starting point for NMR studies.^{1,48} The results of these will be discussed in section 2.3.1. Section 2.3.2 describes the synthesis of biocompatible PEG ligands and the DLS studies undertaken in which the colloidal stability of dispersions of these ligands appended to UCNPs was established.

2.3.1 Studying the NP-Ligand Interface by ^1H NMR Spectroscopy

In order to study the binding affinity of phosphonate and carboxylate ligands to the surface of UCNPs, a model system needed to be developed. The thermal decomposition synthesis route to UCNPs results in nanoparticles capped with hydrophobic ligands such as oleic acid. This ligand interacts with the surface of the nanoparticle via a carboxylate group. The central alkene moiety in the ligand provides a distinctive ^1H NMR shift of ~ 5 ppm. Previous quantum dot studies, discussed in section 2.1.1, show that oleic acid bound to nanoparticles and free oleic acid in solution can be differentiated by ^1H NMR spectroscopy. This is because the chemical shifts of the protons are sufficiently different. It was postulated that carrying out NMR titrations of oleic acid-capped UCNPs with a phosphonate ligand should result in quantitative displacement of the oleic acid ligand; the phosphonate ligand should bind preferentially to the UCNP surface. In the reverse system, adding oleic acid to phosphonate-capped UCNPs should result in oleic acid remaining free in solution.

When choosing an appropriate phosphonate ligand to use for ligand binding studies there were a number of points to consider:

- The ligand and ligand-nanoparticle structures must be readily soluble.
- The ligand must maintain the colloidal stability of the dispersion when added in excess.

- There must be a suitable NMR handle so that ligand displacement can be followed.

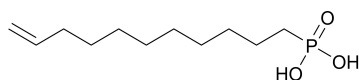


Figure 2.5: Structure of 10-undecenylphosphonic acid, the phosphonic acid ligand used for ^1H NMR spectroscopy binding studies.

The phosphonate ligand chosen for this purpose is shown in figure 2.5. The long aliphatic chain ensures solubility in organic solvents such as chloroform and the terminal alkene provides an NMR handle to follow the ligand exchange. The alkene region of a ^1H NMR spectrum is relatively uncrowded, allowing the integration of the proton signals. This prevents the complications associated with overlapping NMR signals, enabling the ligand exchange to be followed. The ^1H NMR alkene regions of oleic acid and 10-undecenylphosphonic acid are shown in figure 2.6, demonstrating that there would be no overlap in signals in this region during a titration experiment.

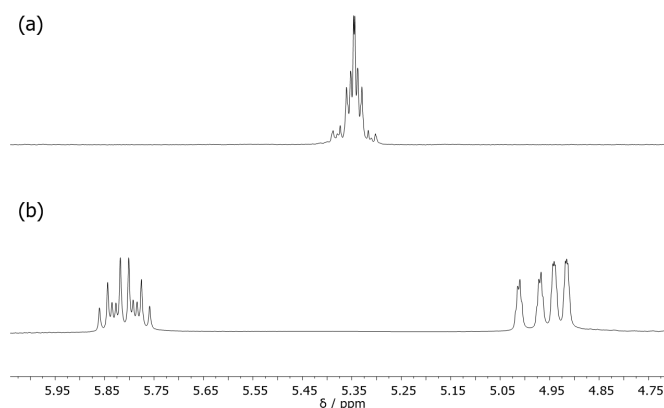
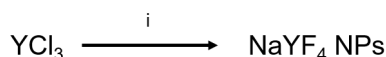


Figure 2.6: ^1H NMR spectra of the alkene signals in (a) oleic acid and (b) 10-undecenylphosphonic acid. (CDCl_3 , 400 MHz, 295 K).

2.3.1.1 Synthesis of NaYF_4 NPs

To be able to follow ligand exchange reactions by NMR spectroscopy, the core nanomaterial had to be carefully chosen so that paramagnetic species were not present. Attempts at using lanthanide-doped UCNPs were unsuccessful due to the extent of line broadening in the spectra.

There are a handful of examples in the literature where NaYF_4 was used as a diamagnetic replacement for $\text{NaYF}_4:\text{Yb,Er}$.^{9,39,41,42} This is not a perfect replacement; the material is not capable of upconversion due to the absence of the lanthanide dopants. However, if the crystal structures of NaYF_4 and $\text{NaYF}_4:\text{Yb,Er}$ are compared, it is possible to see that they are almost identical (see figures 2.8 and 2.22). This suggests that NaYF_4 provides a suitable surrogate for studying ligand binding to the surface of the nanocrystal, as there is negligible difference between the structure on the surface of the two nanomaterials.



Scheme 2.1: Synthesis of NaYF₄ NPs. Reagents and reaction conditions: (i) 1-octadecene, oleic acid, sodium trifluoroacetate, 300 °C.

The nanoparticles were synthesised a thermal decomposition method, as shown in scheme 2.1, based on a synthesis of NaGdF₄:Yb,Er UCNPs by Ryu et al.⁴⁹ Yttrium chloride was added to 1-octadecene and oleic acid, at a ratio of 15:6 mL. The role of oleic acid is the surface-capping ligand and 1-octadecene is a high boiling point solvent. In the first step, this mixture was heated to 120 °C, under reduced pressure (1×10^{-2} mbar), for 1 hour. This results in formation of yttrium oleate in solution, the precursor for NP formation. The step is also important in removing any water from the system. Contamination of the reaction with water can result in a poor quality nanomaterial product.⁵⁰

The reaction mixture was cooled to room temperature and sodium trifluoroacetate added; this provides the source of fluoride for the NPs. The reaction was then rapidly heated to 300 °C, under nitrogen. The reaction was left heating at 300 °C for 2 hours, in which time the NPs formed. After cooling to room temperature, purification was achieved by precipitation of the NPs with ethanol and centrifugation. This acts to remove any free oleic acid, as OA is soluble in ethanol. The step was repeated three times and the resulting solid pellet was redispersed in hexane. It was found that it was important that the NPs were kept in hexane as a dispersion. If the NPs were left to dry out as a solid, this resulted in aggregation. It was then not possible to redisperse the nanomaterial. To further increase the shelf-life of the nanoparticles, they were stored at 4 °C.

Figure 2.7 shows a representative transmission electron microscopy (TEM) image of the sample. The samples were prepared for TEM by addition of 10 µL of the nanomaterial dispersion in hexane to a holey carbon on copper TEM grid. Image analysis shows that the NPs synthesised were monodisperse with a size of 15.4 ± 2.9 nm, as shown in figure 2.7b.

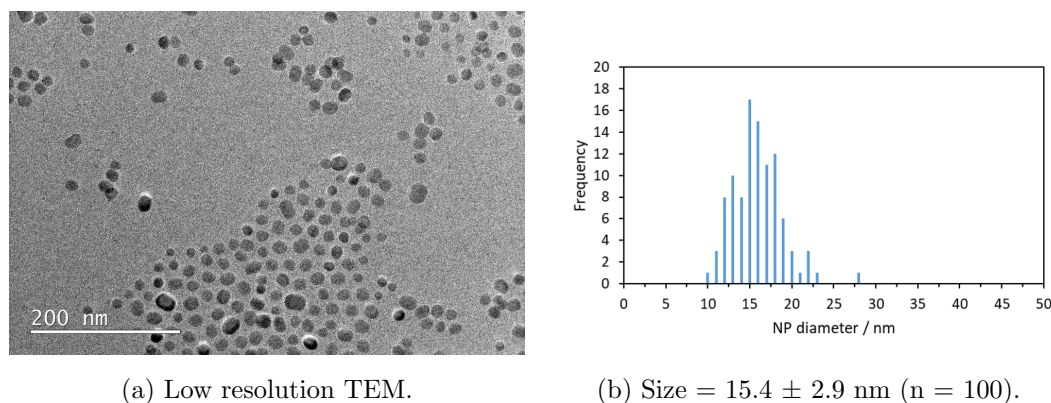


Figure 2.7: (a) Low resolution TEM and (b) Size distribution of NaYF₄ NPs.

Power X-ray diffraction (XRD) was carried out to validate the synthesis of NaYF_4 NPs. The X-ray pattern produced is shown in figure 2.8. The standard analysis method is comparison of the measured diffraction pattern to the International Centre for Diffraction Data (ICDD) database of known samples. In the bottom of figure 2.8, a standard sample of NaYF_4 is shown. There is good correlation between the peaks in the standard sample and the sample of NaYF_4 NPs synthesised, confirming synthesis of the target nanomaterial.

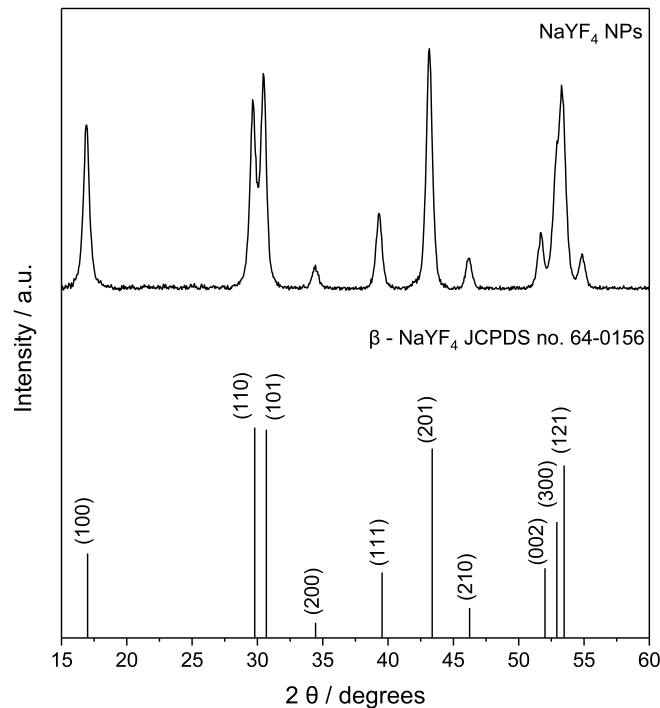


Figure 2.8: XRD diffraction pattern of: (a) synthesised NaYF_4 NPs and (b) ICDD database sample of NaYF_4 . The Miller indices are shown for each diffraction peak.

The line broadening that is visible in the sample is due to the small crystallite size in the samples. When the crystallite size reduces from bulk to nanomaterial size, the diffraction peaks broaden.⁵¹ This broadening was described in 1918 paper by Paul Sherrer and the Sherrer equation is given below. It describes inverse proportional relationship between the peak full width at half maximum (FWHM) and the crystalline domain size.

$$D = \frac{\kappa\lambda}{\beta \cos\theta} \quad (2.1)$$

Where κ is the Sherrer constant (0.9 for spherical nanoparticles), λ is the wavelength of the incident X-ray radiation, β is the full width at half maximum (FWHM) of the diffraction peak and θ is the diffraction angle.

2.3.1.2 ^1H NMR Titrations

The prepared oleic acid-capped NaYF_4 NPs (NaYF_4 @oleic acid) were dispersed in CDCl_3 for ^1H NMR analysis. The ^1H NMR spectrum of NaYF_4 @oleic acid shows a broad peak at 5.34 ppm, corresponding to the two alkene protons of oleic acid ligands bound to the surface of NaYF_4 NPs. The observed signal broadening is caused by a slow tumbling rate of the oleic acid ligands in the sample, due to their coordination to the NP surface. This, along with the low solubility of oleic acid capped NaYF_4 , meant that long 128 scan ^1H NMR measurements were necessary to achieve a sufficient signal-to-noise ratio for analysis.

A ferrocene standard was used to integrate the alkene signals against. By comparing the integration of the alkene protons in oleic acid to a known quantity of ferrocene standard, added to the NMR sample (0.02 mg), it was possible to determine the mass of oleic acid ligands bound to the surface of the NPs. The singlet at 4.16 ppm corresponds to the 10 protons in ferrocene. As can be seen in figure 2.9, the ratio of integration of the oleic acid to ferrocene peaks is 1:0.46. It can therefore be determined that there was a 1:8.9 molar ratio of ferrocene:oleic acid in the sample. The peak at 4.95 ppm is likely to be an artefact.

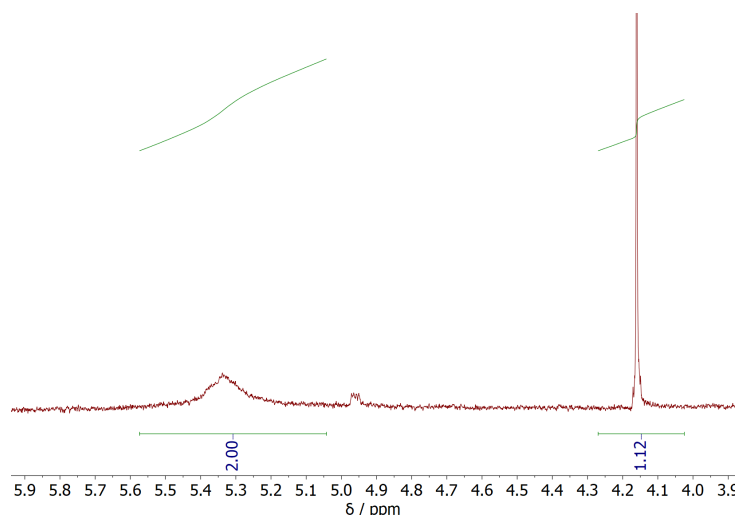


Figure 2.9: ^1H NMR spectrum of alkene region NaYF_4 NPs@oleic acid (CDCl_3 , 400 MHz, 295 K).

The number of oleic acid ligands in the sample was calculated as follows:

$$\text{Molar ratio of OA to ferrocene in sample} = 1 : 8.9$$

$$\begin{aligned} \text{Moles of ferrocene in sample} &= 0.02 \times 10^{-3} \times 186.04 \text{ mol} \\ &= 1.08 \times 10^{-7} \text{ mol} \end{aligned}$$

$$\begin{aligned} \text{Moles of OA in sample} &= 1.08 \times 10^{-7} \times 8.9 \text{ mol} \\ &= 9.57 \times 10^{-7} \text{ mol} \end{aligned}$$

$$\begin{aligned} \text{Number of OA ligands} &= 9.57 \times 10^{-7} \times N_A \\ &= 5.76 \times 10^{17} \end{aligned}$$

To calculate the number of NaYF₄ NPs in the sample, first it was necessary to determine the mass ratio of nanoparticle to ligand in the sample. For this, thermogravimetric analysis (TGA) was used. A dry sample of NaYF₄ NPs was heated at a controlled heating ramp rate and the mass loss was measured. This enabled the calculation of the amount of ligand compared to core nanomaterial in the sample. Figure 2.10 shows a plot of the TGA data obtained. The mass loss on heating to 350 °C was found to be 0.3 mg, of the total 2.3 mg sample. This means 13 % of the sample mass was oleic acid, the other 87 % was the material of the nanoparticle. Any mass loss above 350 °C is likely due to be due to decomposition of the nanomaterial.

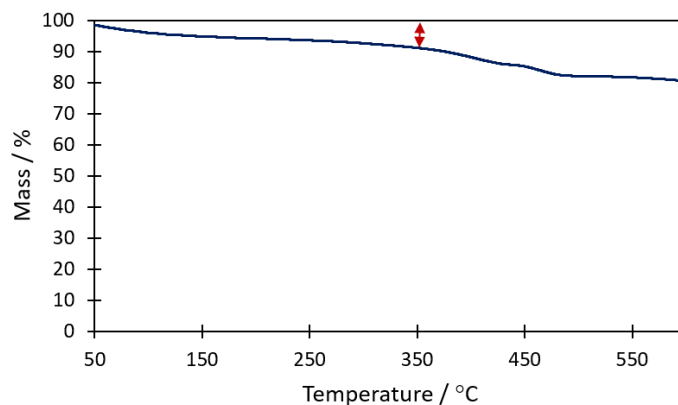


Figure 2.10: TGA NaYF₄ NPs with oleic acid surface ligand.

This could then be used to calculate the number of NaYF₄ NPs in the NMR sample. The mass of NaYF₄@oleic acid NPs used for ¹H NMR analysis was 10 mg and the density of NaYF₄ is 4.3 gcm⁻³.

The number of NaYF₄ NPs in the sample was calculated as follows:

$$\begin{aligned}
 \text{Volume of 1 NaYF}_4 \text{ NP} &= \frac{4}{3}\pi r^3 \\
 &= 1.91 \times 10^{-24} \text{ m}^3 \\
 \text{Mass of 1 NaYF}_4 \text{ NP} &= \rho V \\
 &= 8.22 \times 10^{-18} \text{ g} \\
 \text{Mass of NPs in sample} &= 0.87 \times 10 \text{ mg} \\
 &= 8.7 \times 10^{-3} \text{ g} \\
 \text{Number of NPs in sample} &= \frac{8.7 \times 10^{-3}}{8.22 \times 10^{-18}} = 1.06 \times 10^{15}
 \end{aligned}$$

Therefore, the number of oleic acid ligands per NaYF₄ NP was:

$$\frac{5.76 \times 10^{17}}{1.06 \times 10^{15}} = \mathbf{545} \text{ Oleic acid ligands per NaYF}_4 \text{ NP}$$

This value can be used to calculate the ligand density, which was found to be 2.4 ligands per nm^2 . This is close to the theoretical monolayer value calculated by De Roo et al. of 2.9 oleate ligands per nm^2 on the surface of CsPbBr_3 NPs.⁵² It is also very similar to the 2.4 - 3.0 ligands per nm^2 Smock et al. found experimentally for their system of oleate ligands bound to the surface of CsPbBr_3 NPs.³¹ The calculated ligand surface density and theoretical maximum value can be used to estimate the % surface coverage, at 83 %.

A stock solution of 10-undecenylphosphonic acid in deuterated chloroform was added to the dispersion of NaYF_4 NPs in CDCl_3 . The ^1H NMR spectra obtained during the titration are shown in figure 2.11. The spectra show that as the phosphonic acid ligand was added into solution, oleic acid was liberated from the surface of the NPs. This is shown by a sharpening of the alkene peaks at 5.34 ppm, with the better definition of the peak caused by free rotation of oleic acid in solution. As not all the phosphonic acid added binds to the surface of the NaYF_4 NPs, free phosphonic acid is visible in the ^1H NMR spectra. This obscures the ability to see the ^1H NMR signal corresponding to phosphonic acid bound to the NP surface.

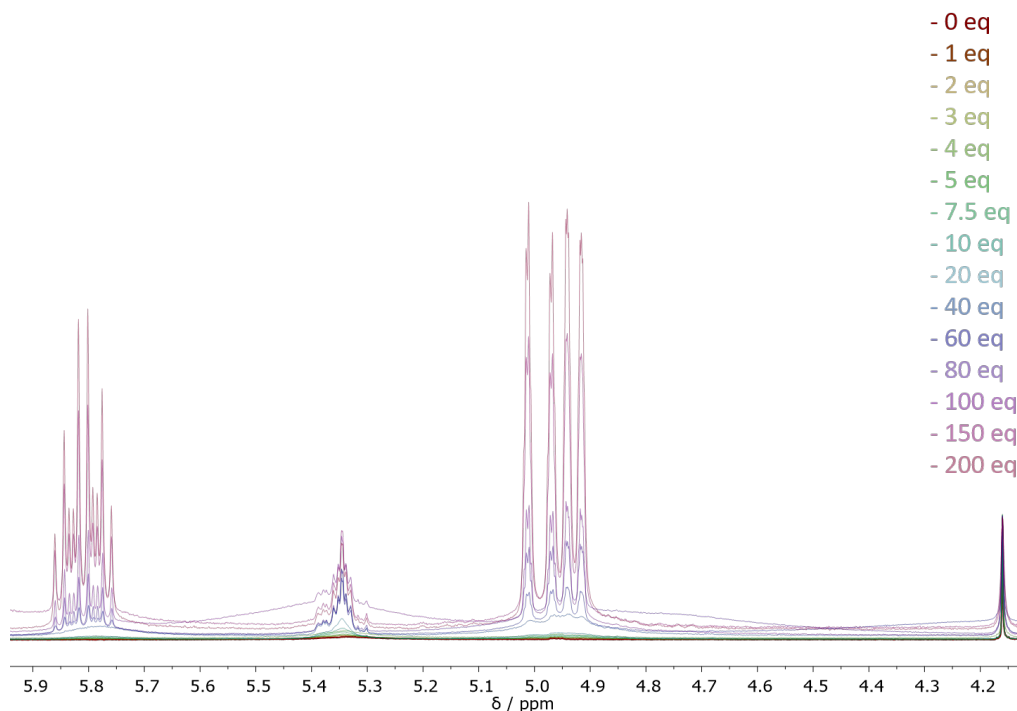


Figure 2.11: ^1H NMR spectra of NaYF_4 NPs@oleic acid, adding increasing amounts of 10-undecenylphosphonic acid. Legend shows equivalents of phosphonic acid ligand added, compared to original oleic acid on surface of NPs (CDCl_3 , 400 MHz, 295 K).

Figure 2.12 shows the 5.24–5.44 ppm region of the ^1H NMR spectrum shown in figure 2.11. After 100 equivalents of phosphonic acid were added, there was a negligible change in the integration of the oleic acid peak, with respect to the ferrocene peak. This suggests that at this point, all the oleic acid had been liberated from the surface of the NPs and replaced with the phosphonic acid ligand. The ligand exchange process was attempted to be followed by ^{31}P NMR

spectroscopy, however the signal corresponding to the phosphorus bound to the nanoparticle surface was broadened to the extent that it was not visible. This is due to the immobilisation of the phosphorus atom bound to the surface and therefore the reduction in tumbling rate of the ligand.

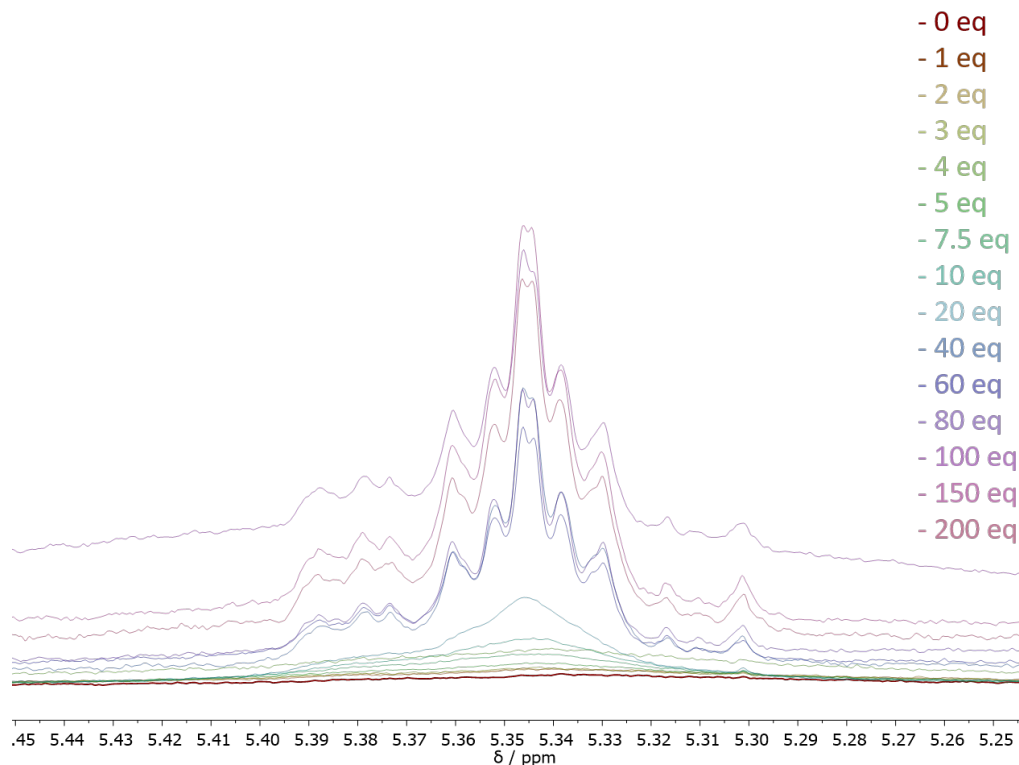


Figure 2.12: ^1H NMR spectra of 5.24–5.44 ppm region of NaYF_4 NPs@oleic acid, adding increasing amounts of 10-undecenylphosphonic acid. Legend shows equivalents of phosphonic acid ligand added, compared to original oleic acid on surface of NPs (CDCl_3 , 400 MHz, 295 K).

The free oleic acid was then washed out by sequential precipitation and centrifugation cycles. This enabled a dispersion of NaYF_4 NPs to be obtained, without oleic acid contamination. The ^1H NMR spectrum for the NaYF_4 @phosphonic acid dispersion in CDCl_3 is shown in figure 2.13. Using an analogous method to that described above, the surface coverage of the phosphonic acid was calculated. This was found to be 1.64 ligands per nm^2 .

An analogous process was then used to carry out a titration of oleic acid into the dispersion of NaYF_4 @phosphonic acid NPs. A solution of oleic acid in CDCl_3 was added to the NaYF_4 @phosphonic acid dispersion. The resulting ^1H NMR spectra are shown in figure 2.14. The data shows that the ligand exchange reaction of phosphonic acid, replacing oleic acid on the surface of NaYF_4 NPs is irreversible. There are no visible ^1H peaks corresponding to free phosphonic acid in solution. This means that the oleic acid was not able to displace the phosphonic acid on the nanoparticle surface, even when a large excess of the oleic acid ligand was added. This result demonstrates the strong binding and irreversible binding of phosphonic acid ligands to NaYF_4 NPs. The work agrees with previous literature, which showed strong binding

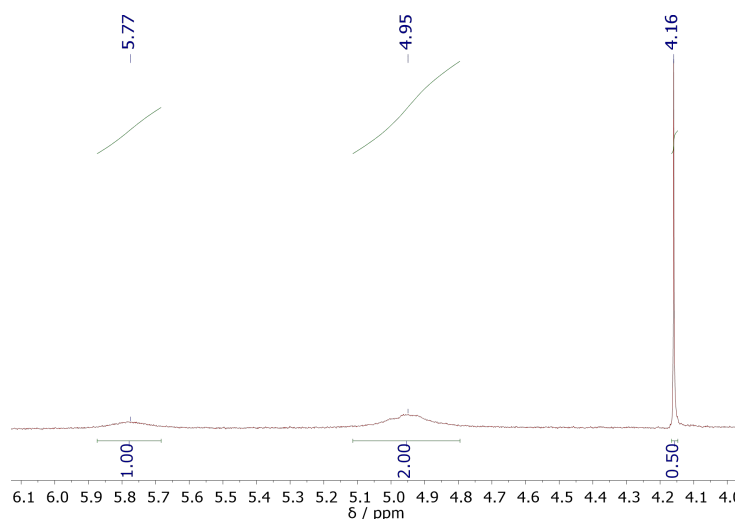


Figure 2.13: ^1H NMR spectrum of NaYF_4 NPs@phosphonic acid (CDCl_3 , 400 MHz, 295 K).

of phosphonic acids to QDs.^{25,31} The phosphonic acid ligands in these studies were shown to completely and irreversibly displace native oleic acid ligands.

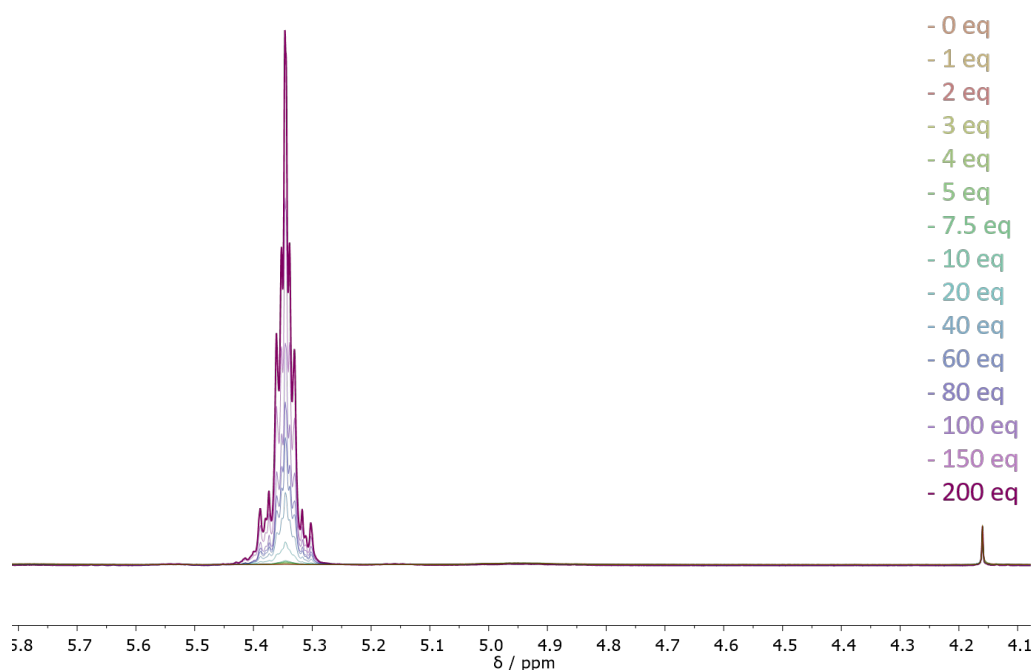


Figure 2.14: ^1H NMR spectrum of NaYF_4 NPs@phosphonic acid, adding increasing amounts of oleic acid. Legend shows equivalents of oleic acid ligand added, compared to original phosphonic acid on surface of NPs. Legend shows equivalents of oleic acid ligand added, compared to original phosphonic acid on surface of NPs (CDCl_3 , 400 MHz).

2.3.2 PEG-Phosphonate Ligands for Phase Transfer

Preparing UCNPs suitable for bioimaging applications requires replacing the native hydrophobic ligand shell with a hydrophilic coating, so that the nanomaterial can be dispersed in biological media. Ligand exchange arguably provides the most versatile technique for achieving this. Ligands can be specifically designed for their required use, synthesised and then exchanged with the native surface ligands.

For bioimaging there are a number of factors that need be considered when designing surface ligands:

- The hydrophilic ligands need to be robustly anchored to the nanoparticle surface to prevent desorption of ligands and aggregation of the nanoparticles.
- The ligands need to effectively repel proteins and biomolecules found in the blood and cytosol, which prevent the imaging probes reaching their target sites.
- There needs to be flexibility to functionalise the ligands with a variety of functional units which can allow sensing, targeting and/or therapy.

Figure 2.15 shows the structure of a ligand developed by the Torres group in 2013.⁵³ The ligand was shown to effectively coordinate to the surface of superparamagnetic iron oxide nanoparticles (SPIONs) and the resulting MR imaging probe showed slow reticuloendothelial system clearance. Earlier in this chapter, it was shown that phosphonate ligands coordinate preferentially to NaYF₄ NPs. It was therefore thought that the PEG-bisphosphonate ligand (PEG-BP) could be used to coordinate to NaYF₄:Yb,Er UCNPs in the synthesis of imaging nanoprobes. This was predicted to result in nanomaterials which would have high colloidal stability and resistance to aggregation.

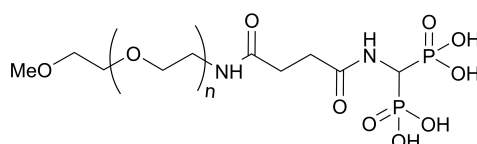


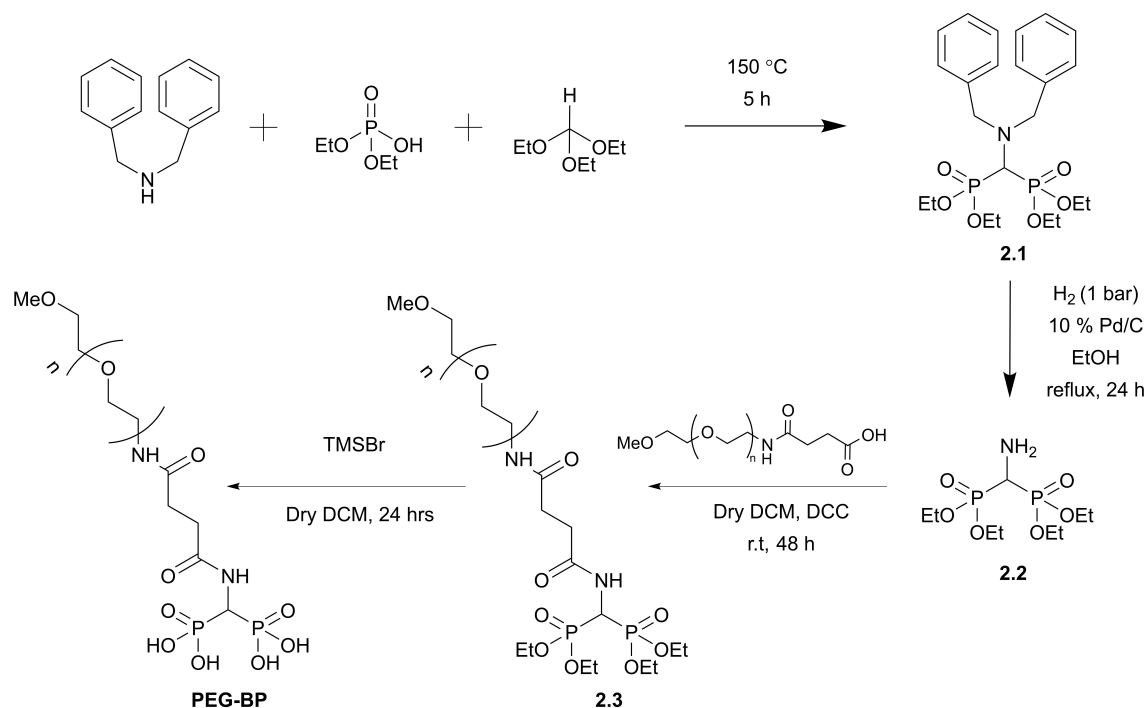
Figure 2.15: Structure of PEG-bisphosphonate ligand (**PEG-BP**).

The inclusion of polyethylene glycol (PEG) in bioimaging probes has been shown to increase biocompatibility of nanoparticles by prolonging their circulation time. PEG has been shown to repel protein adsorption and minimise the formation of a protein corona around the imaging probe.^{4,54,55} This slows down the particle uptake by the phagocytic system and increases the circulation time of the nanomaterial.

In the following sections the synthesis of PEG-bisphosphonate and PEG-monophosphonate ligands will be discussed. These ligands, along with the commercially available PEG-monocarboxylate ligands, were exchanged with the native oleate ligands on NaYF₄:Yb,Er UCNPs. The colloidal stability of these three systems could then be compared.

2.3.2.1 Synthesis of PEG-Bisphosphonate Ligand

The route used to synthesise **PEG-BP** is shown in scheme 2.2.⁵³ The first step towards formation of the target ligand was synthesis of the bisphosphonate ester (**2.1**). This was carried out in accordance to literature protocols as a one-pot reaction between dibenzylamine, diethylphosphite and triethylorthoformate.⁵⁶



Scheme 2.2: Four-step synthetic route to prepare **PEG-BP** ligand.

The synthetic route is based on perhaps the most common procedure for the preparation of aminomethylenebisphosphonic acids.^{57–59} The reaction is most commonly undertaken with a primary amine and this often results in a complex mixture of products, which are hydrolysed and then separated. The 1996 paper by Kantoci et al. reports that use of the primary amine, benzylamine, resulted in extremely low yields of the bisphosphonate ester, instead preferentially forming diethyl (bis(dibenzylamino)methyl)phosphonate. The authors found that the use of dibenzylamine allowed the bisphosphonate ester product to be produced in a 58 % yield but did not suggest a mechanistic reason for the improved yield. More recently, the mechanism of this reaction has been rigorously studied by Dabrowska et al.⁶⁰ The authors proposed a number of reaction intermediates which are able to interconvert, thereby resulting in a range of products from the synthesis. Many of these intermediates (such as ethyl N-(p-nitrophenyl)formimidate and N,N'-di(p-nitrophenyl)formamidine) were able to be isolated and characterised, supporting the proposed mechanism.

The mechanism Dabrowska et al. proposed was based upon the synthesis using a primary amine starting material. This can be modified to understand the use of secondary amines in the reaction and the suggested route for this is shown in scheme 2.3. The synthesis of the benzyl-

protected bisphosphonate was carried out on a multigram scale, with the product present along with three impurities. Silica column chromatography was used to purify the product, with it eluting in 100 % ethyl acetate from a hexane:ethyl acetate silica column.

Debenzylation was achieved by reaction of **2.1** under H_2 with a 10 % Pd/C catalyst. The benzylated starting material, Pd/C catalyst and ethanol was added to a thick-walled Schlenk charged with 1 bar H_2 . The reaction mixture was heated to reflux in the sealed Schlenk for 24 hours and this was sufficient time for the reaction to reach completion. A simple celite filtration removed the catalyst and this resulted in the isolation of pure product in quantitative yields. The hydrogenation deprotection step has been shown to be clean up to a 1 g scale.

1H NMR spectra for **2.1** and **2.2** are shown in figure 2.16. Upon hydrogenation of **2.1**, there is visible loss of the aromatic proton. The spectra have characteristic broad $^2J_{HP}$ coupling constants of ~ 20 Hz.

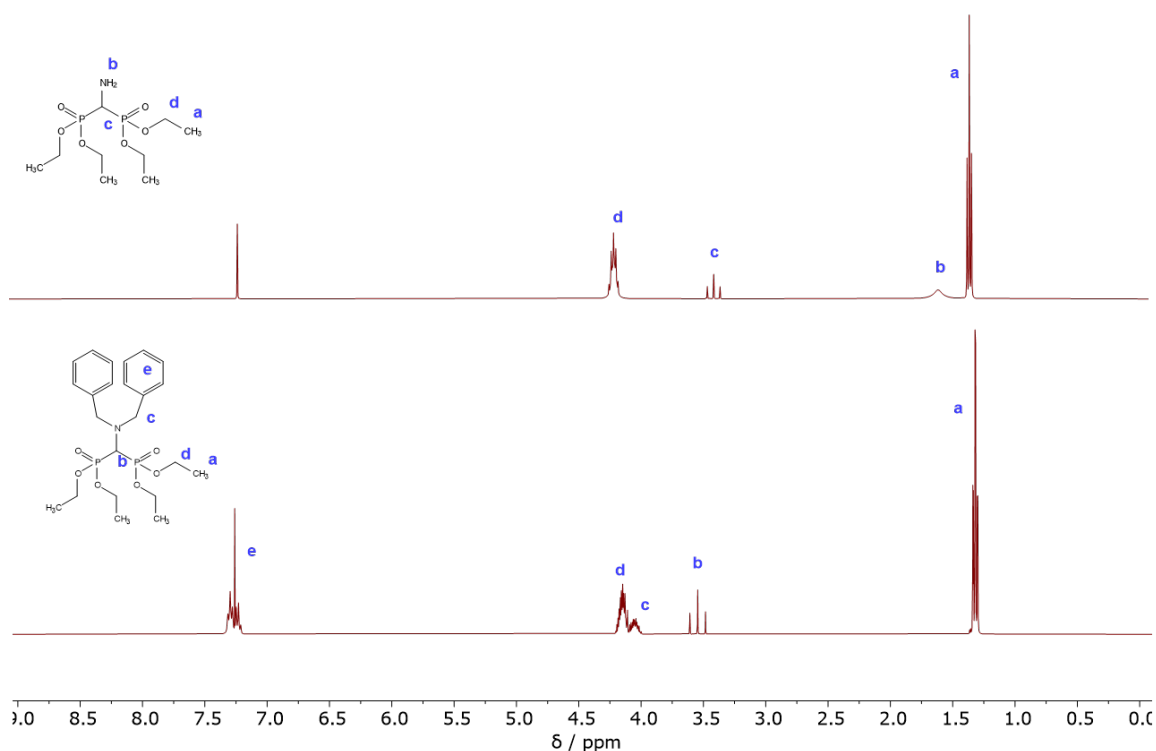
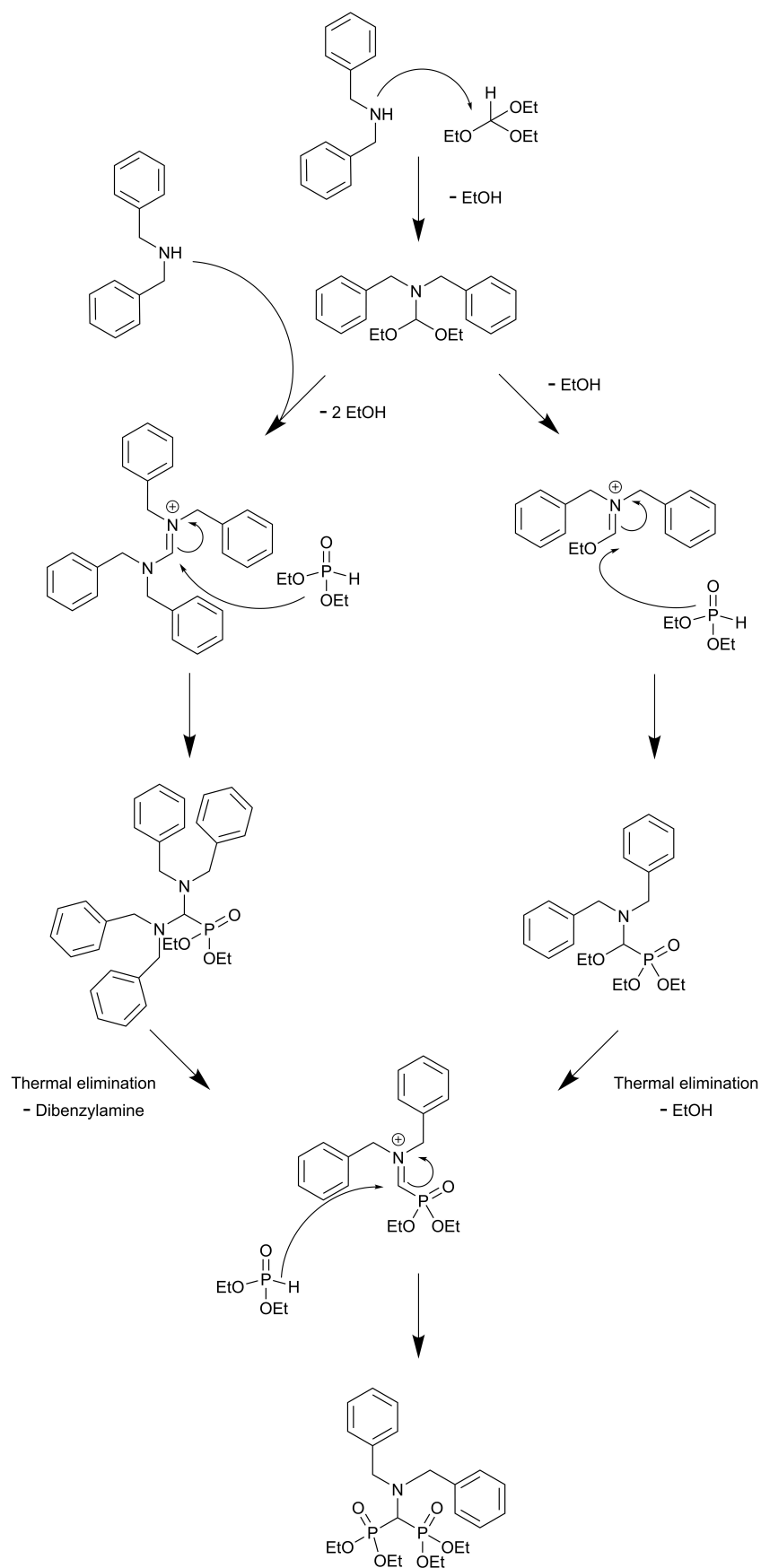


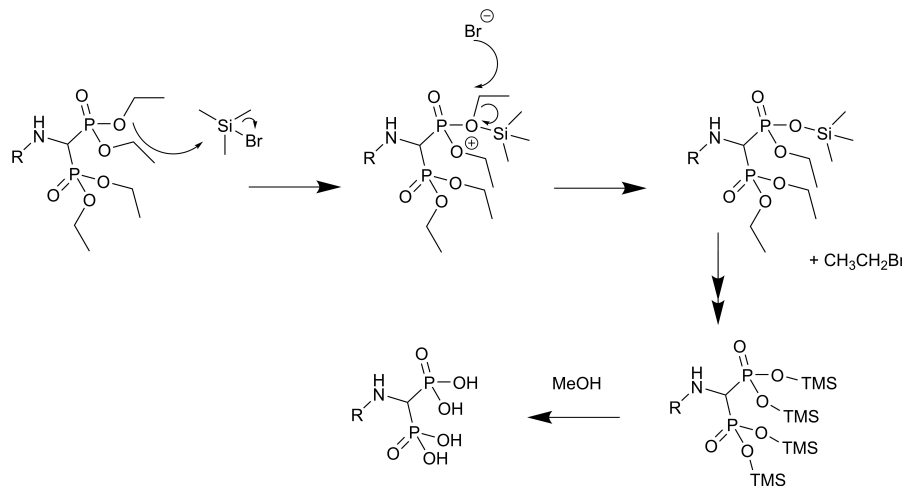
Figure 2.16: 1H NMR spectra of benzyl protected bisphosphonate ester (**2.1**) and the deprotected bisphosphonate ester (**2.2**) ($CDCl_3$, 400 MHz, 295 K).

An amide bond formation reaction was then carried out, using DCC as the coupling agent, between **2.2** and the terminal carboxylic acid of the polymer PEG(5000)-OMe ($M_w = 4450$ Da). Finally the bisphosphonate ester was deprotected with trimethylsilyl bromide (TMSBr), revealing the bisphosphonic acid product (**PEG-BP**). The **PEG-BP** ligand was purified by dialysis through a 3500 Da molecular weight cut-off membrane.



Scheme 2.3: Reaction mechanism for the multicomponent reaction forming tetraethyl (N,N-dibenzyl)aminomethyl-bis(phosphonate).

The mechanism for the phosphonate ester deprotection is shown in scheme 2.4. The mechanism proceeds via a trimethylsilyl ester formed by the reaction of the phosphonate ester with TMSBr, with the bromoethane side product removed under vacuum. A hydrolysis step in methanol liberates the bisphosphonic acid product in quantitative yields.^{61,62}



Scheme 2.4: Reaction mechanism showing bisphosphonate deprotection with TMSBr.

Characterisation of the **PEG-BP** ligand was not straightforward due to the presence of the large polymer chain. It dominates the ^1H NMR spectrum as a large singlet at 3.64 ppm and the low quantity of phosphorus in the sample, along with the slow tumbling rate of the polymer, made obtaining a $^{31}\text{P}\{^1\text{H}\}$ NMR spectrum challenging.

Figure 2.17 shows the ^1H and $^1\text{H}\{^{31}\text{P}\}$ NMR spectra. The triplet at 4.49 ppm has characteristic $^2J_{\text{HP}}$ coupling between the phosphorus centre and the proton on the carbon between the two phosphorus atoms (19.9 Hz). The $^1\text{H}\{^{31}\text{P}\}$ confirms this as it shows that upon running a ^{31}P decoupled ^1H NMR spectrum, the triplet collapses to a singlet.

At room temperature, the $^{31}\text{P}\{^1\text{H}\}$ NMR spectrum of **PEG-BP** shows a broad peak covering 0-20 ppm. It was thought that due to the large size of the polymer ligand chain, the tumbling rate of the ligand was likely to be restricted and therefore a broad signal observed in the $^{31}\text{P}\{^1\text{H}\}$ spectrum. Heating to 85 °C resulted in a sharpening of the signal and a chemical shift of 13.7 ppm.

A $^1\text{H}-^{31}\text{P}\{^1\text{H}\}$ HMBC experiment was used to help validate that **2.3** had successfully coupled with the PEG moiety. Figure 2.18 shows that the broad singlet in the $^{31}\text{P}\{^1\text{H}\}$ at 13.7 ppm coupled with the wide triplet at 4.49 ppm in the ^1H NMR spectrum. This suggested that the amide coupling reaction had been successful. This $^1\text{H}-^{31}\text{P}\{^1\text{H}\}$ HMBC NMR spectrum was run with an incomplete dialysis, so some of the deprotected **2.2** ligand was still in the sample. This can be seen as a sharp singlet at 9.40 ppm in the $^{31}\text{P}\{^1\text{H}\}$ NMR spectrum. There is no coupling between the ^1H NMR triplet and the sharp singlet, therefore it can be concluded that the triplet in the ^1H NMR spectrum and the broad singlet in the $^{31}\text{P}\{^1\text{H}\}$ NMR spectrum originate from successful coupling of **2.2** to the PEG ligand.

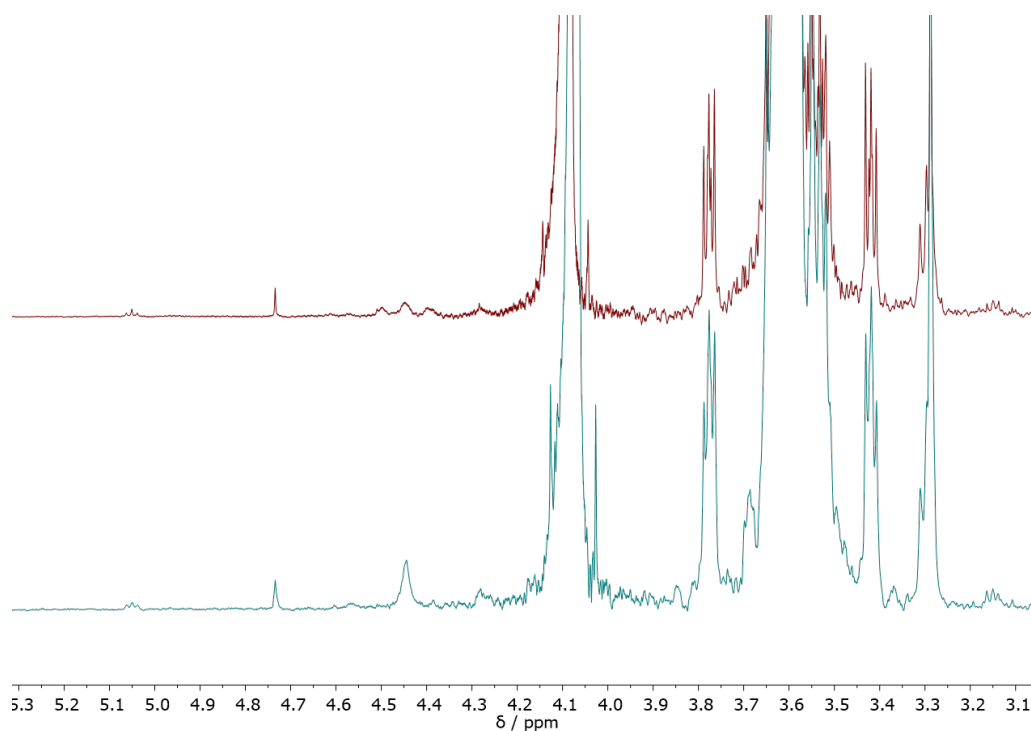


Figure 2.17: ^1H NMR spectra of **PEG-BP**. Top trace is ^1H NMR spectrum; bottom trace is $^1\text{H}\{^{31}\text{P}\}$ NMR spectrum. (D_2O , 400 MHz, 358 K).

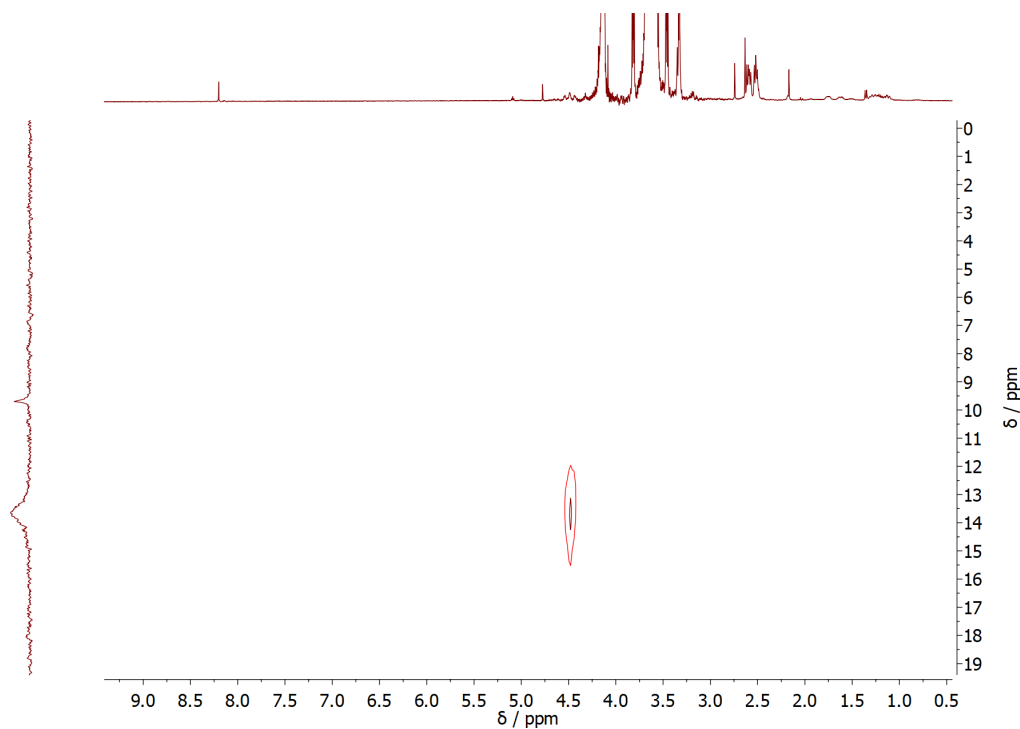
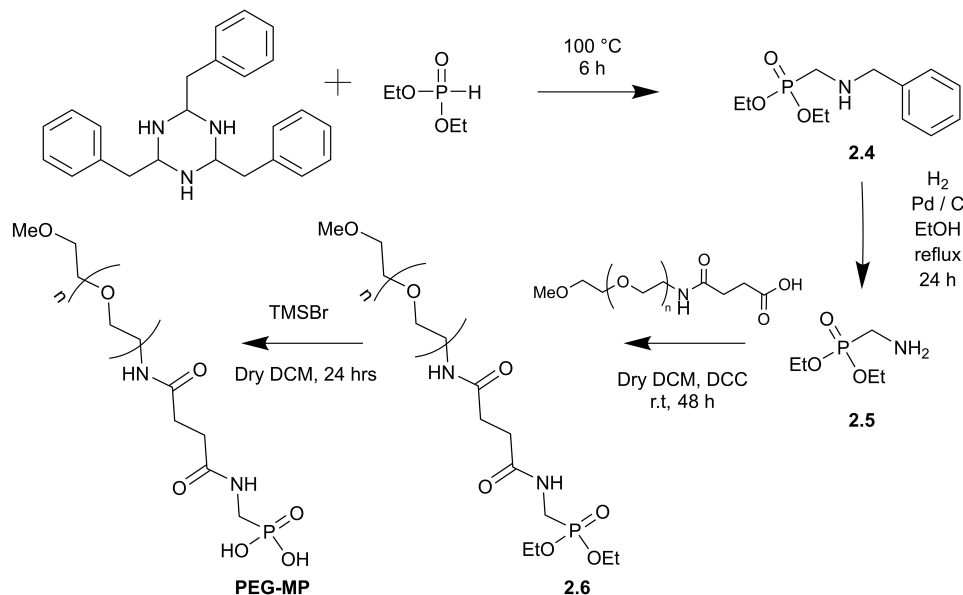


Figure 2.18: $^1\text{H}-^{31}\text{P}\{\text{H}\}$ HMBC NMR spectrum of incomplete dialysis of **PEG-BP** ligand. Some residual deprotected **2.2** can be seen as the sharp singlet in the $^{31}\text{P}\{\text{H}\}$ NMR spectrum. (D_2O , 400 MHz, 358 K).

It was thought that matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) could be used to validate successful coupling between the PEG polymer and the bisphosphonate ligand. If the coupling occurred, the m/z distribution would be expected to shift by 157 units. This corresponds to the mass of the bisphosphonic acid group. Unfortunately, the mass spectra obtained were not high enough resolution to be able to confidently confirm the successful coupling. It is thought that the long PEG chains are responsible for the large background noise in all the MS spectra obtained, with the PEG affecting the ability for the ligand to fly. Both positive and negative mode MALDI-MS was attempted, alongside ESI (electrospray ionisation), but none of these methods were successful. The highly noisy spectra do not have the resolution to distinguish the expected 44 unit m/z difference between the peaks of the polymer chain, corresponding to one ethylene glycol unit. The relatively small shift of 157 m/z difference, compared to the overall mass of the polymer (4500 Da) is therefore not visible from these spectra.

2.3.2.2 Synthesis of PEG-Monophosphonate Ligand

A similar synthetic route taken to prepare the **PEG-BP** ligand was used to prepare the equivalent monophosphonate-PEG ligand. The synthetic route to prepare the **PEG-MP** ligand is outlined in scheme 2.5. The first step is a variation of the Kabachnik—Fields reaction, where three equivalents of diethylphosphite were reacted with one equivalent of 1,3,5-tribenzylhexahydro-1,3,5-triazine.^{63,64}



Scheme 2.5: Four-step synthetic route to prepare **PEG-MP** ligand.

The reaction proceeds via the formation of an imine, which is attacked by the nucleophilic phosphorus species. Silica gel column chromatography (hexane:ethyl acetate) was used to purify the product. Debenzylation was achieved by reflux of **2.4** in ethanol, under one atmosphere of H_2 and with a Pd/C catalyst. A celite filtration, followed by a DCM:methanol silica gel column

yielded the pure product.

The assigned ^1H NMR spectra for **2.4** and **2.5** is shown in figure 2.19. There is a visible loss of the aromatic protons upon hydrogenation. Both **2.4** and **2.5** show characteristic $^2J_{\text{HP}}$ coupling constants of 10.2 Hz at ~ 3 ppm.

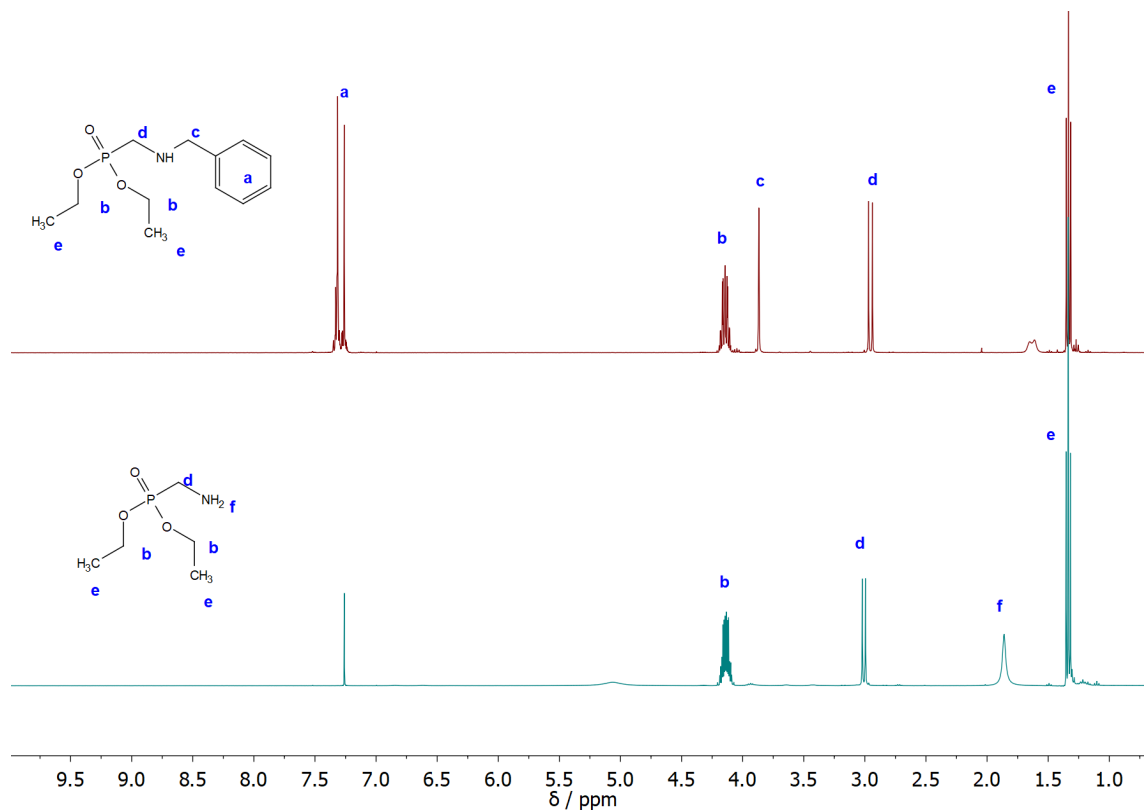


Figure 2.19: ^1H NMR spectra of benzyl protected monophosphonate ester (**2.4**) and the deprotected monophosphonate ester (**2.5**) (CDCl_3 , 400 MHz, 295 K).

2.5 was then coupled to PEG-COOH in an analogous method to that described for **PEG-BP**. **2.6** was deprotected using TMSBr, yielding the **PEG-MP** ligand. $^{31}\text{P}\{^1\text{H}\}$ NMR spectroscopy was used to verify the successful coupling of **2.5** with the PEG ligand. **2.5** showed a $^{31}\text{P}\{^1\text{H}\}$ chemical shift of 26.0 ppm and **2.6** a shift of 27.6 ppm. Upon coupling with PEG, the $^{31}\text{P}\{^1\text{H}\}$ NMR peak broadened and shifted to 20.3 ppm. This broadening suggests a slower tumbling rate in solution, consistent with the phosphorus being attached to a large polymer. The data supports the successful coupling of the monophosphonate **2.5** and the PEG ligand.

2.3.2.3 Synthesis of $\text{NaYF}_4\text{:Yb,Er}$ UCNPs

The synthesis of $\text{NaYF}_4\text{:Yb,Er}$ UCNPs was carried out using a similar method to that described in section 2.3.1.1. YbCl_3 and ErCl_3 were added in a ratio of 20 mol % ytterbium and 2 mol % erbium. There has been extensive research into the optimum lanthanide doping in upconversion nanoparticles.⁶⁵ As discussed in section 1.4.1, there is a careful balance to be struck with lanthanide ion dopant concentration. There needs to be a high enough concentration lanthanide

ions in the crystal structure to achieve the required sensitizer / activator effect, but not too high that the visible emission is decreased by non-radiative transitions.⁶⁶ The consensus in the literature is that the optimum concentration of ytterbium and erbium in a $\text{NaYF}_4:\text{Yb,Er}$ nanocrystal is 20 and 2 % respectively, hence the chosen concentration in this work.

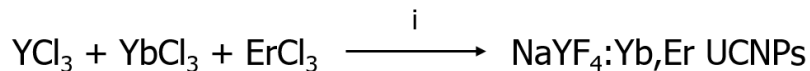
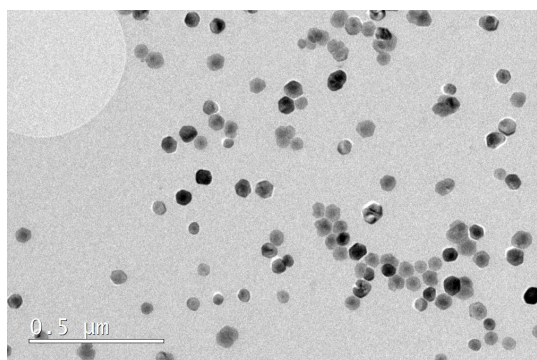
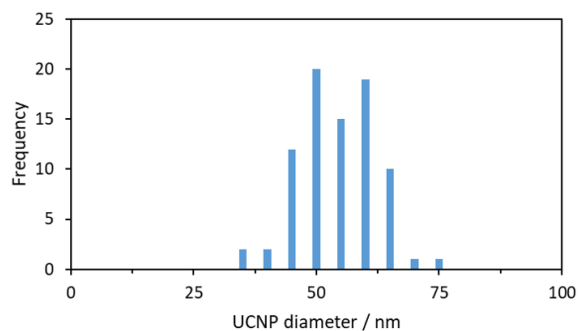


Figure 2.20: Synthesis of $\text{NaYF}_4:\text{Yb,Er}$ UCNPs. Reagents and reaction conditions: (i) 1-octadecene, oleic acid, sodium trifluoroacetate, 300 °C.

A representative TEM image of a sample of $\text{NaYF}_4:\text{Yb,Er}$ UCNPs is shown in figure 2.21, along with the corresponding size distribution data. The UCNPs synthesised had a size of 54.3 ± 8.2 nm and were hexagonal in shape, suggesting the material was $\beta\text{-NaYF}_4:\text{Yb,Er}$ UCNPs.



(a) Low resolution TEM.



(b) Size = 54.3 ± 8.2 nm ($n = 80$).

Figure 2.21: (a) Low resolution TEM and (b) Size distribution of $\text{NaYF}_4:\text{Yb,Er}$ UCNPs.

The crystal phase of the UCNPs synthesised was confirmed by XRD analysis, as shown in figure 2.22. As discussed in section 1.4.1, the $\beta\text{-NaYF}_4:\text{Yb,Er}$ has been shown to have a much higher upconversion efficiency than $\alpha\text{-NaYF}_4:\text{Yb,Er}$ nanomaterial.^{67,68} The XRD indeed shows the synthesis of the brighter hexagonal-phase $\text{NaYF}_4:\text{Yb,Er}$ UCNPs.

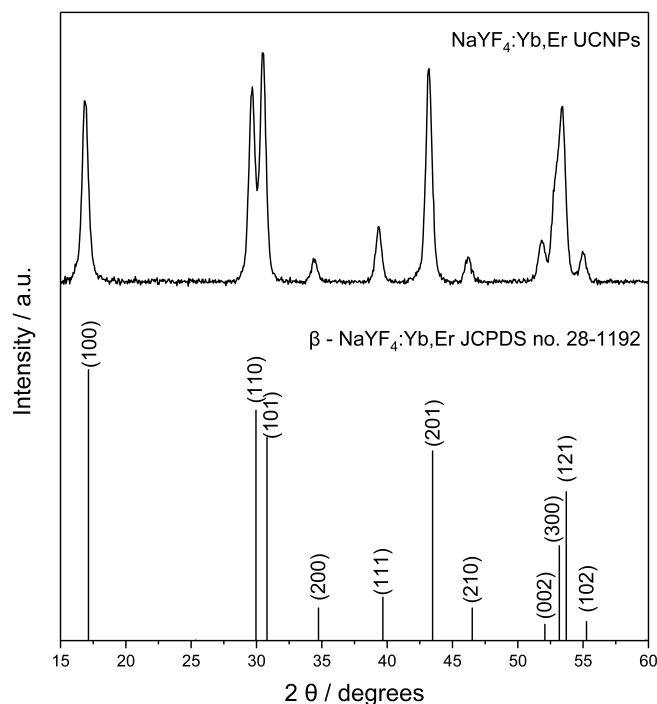
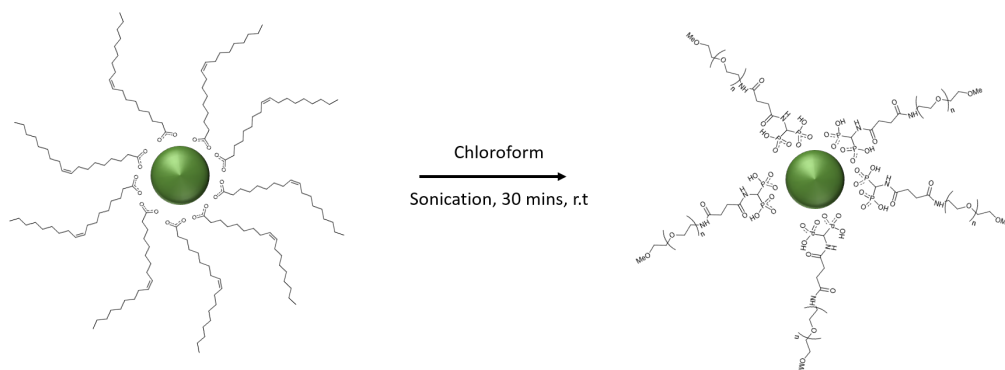


Figure 2.22: XRD pattern of $\text{NaYF}_4:\text{Yb,Er}$ UCNPs, compared to the ICDD database of $\beta\text{-NaYF}_4:\text{Yb,Er}$. The Miller indices are shown for each diffraction peak.

2.3.2.4 Ligand Exchange with $\text{NaYF}_4:\text{Yb,Er}$ @Oleic acid UCNPs

A method for ligand exchange on the surface of SPIONs was developed by Sandiford et al. in 2013.⁵³ In this technique, sonication was used to free the oleic acid from the surface of SPIONs, inducing **PEG-BP** to preferentially bind to the surface of the NPs, as shown in scheme 2.6. The oleic acid and any hydrophobic nanoparticles were then removed by a hexane wash, resulting in water soluble NPs. This same method was applied to ligand exchange on the synthesised $\text{NaYF}_4:\text{Yb,Er}$ UCNPs, exchanging oleic acid with PEG-COOH, **PEG-MP** and **PEG-BP** ligands. The process with the **PEG-BP** ligand is shown in scheme 2.6.



Scheme 2.6: Schematic showing phase transfer process of UCNPs; oleic acid ligands were replaced by **PEG-BP** ligands.

IR spectroscopy can be used to validate that surface of the UCNPs are capped by **PEG-BP** ligands after the phase transfer process. Oleic acid-capped UCNPs have a number of distinctive bands in their IR spectra. Medium strength bands at 2848 and 2915 cm^{-1} correspond to alkyl C–H stretches. The bands at 1547 and 1461 cm^{-1} are assigned to $-\text{COO}-(\delta_{\text{as}})$ and $-\text{COO}-(\delta_{\text{s}})$ stretching. The IR spectrum of $\text{NaYF}_4:\text{Yb,Er@PEG-BP}$ is more complex. Particularly of note is the strong band at 1096 cm^{-1} , which is assigned to the C–O stretch of the ether. The alkane C–H stretching absorption is visible at 2981 cm^{-1} . The P=O stretch can be found in the region 1140–1320 cm^{-1} .

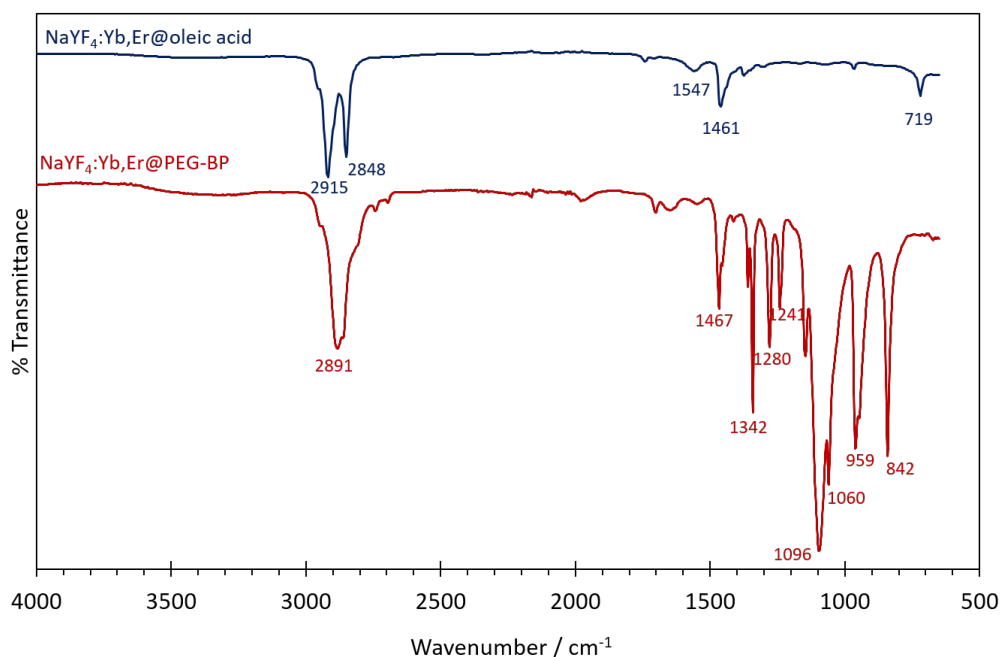


Figure 2.23: FT-IR spectra comparing $\text{NaYF}_4:\text{Yb,Er@oleic acid}$ and $\text{NaYF}_4:\text{Yb,Er@PEG-BP}$.

2.3.2.5 Colloidal Stability Studies of PEG-UCNP Dispersions by DLS

The colloidal stability of $\text{NaYF}_4:\text{Yb,Er@PEG-COOH}$, $\text{NaYF}_4:\text{Yb,Er@PEG-MP}$ and $\text{NaYF}_4:\text{Yb,Er@PEG-BP}$ nanomaterials was assessed by dynamic light scattering (DLS). The colloidal stability of the UCNPs dispersions were determined by assessing their resistance to aggregation. The pH of the dispersions were adjusted and the hydrodynamic diameter of the nanomaterial measured. The nanomaterial which did not show significant size increase over a broad pH range was concluded to have the greatest colloidal stability.

The UCNPs were added to water, at a concentration of 1 mg/mL . The size distribution of the dispersions were measured by DLS, with the pH adjusted by addition of 0.1 M HCl or 0.1 M NaOH. The size distribution data is shown in figure 2.24.

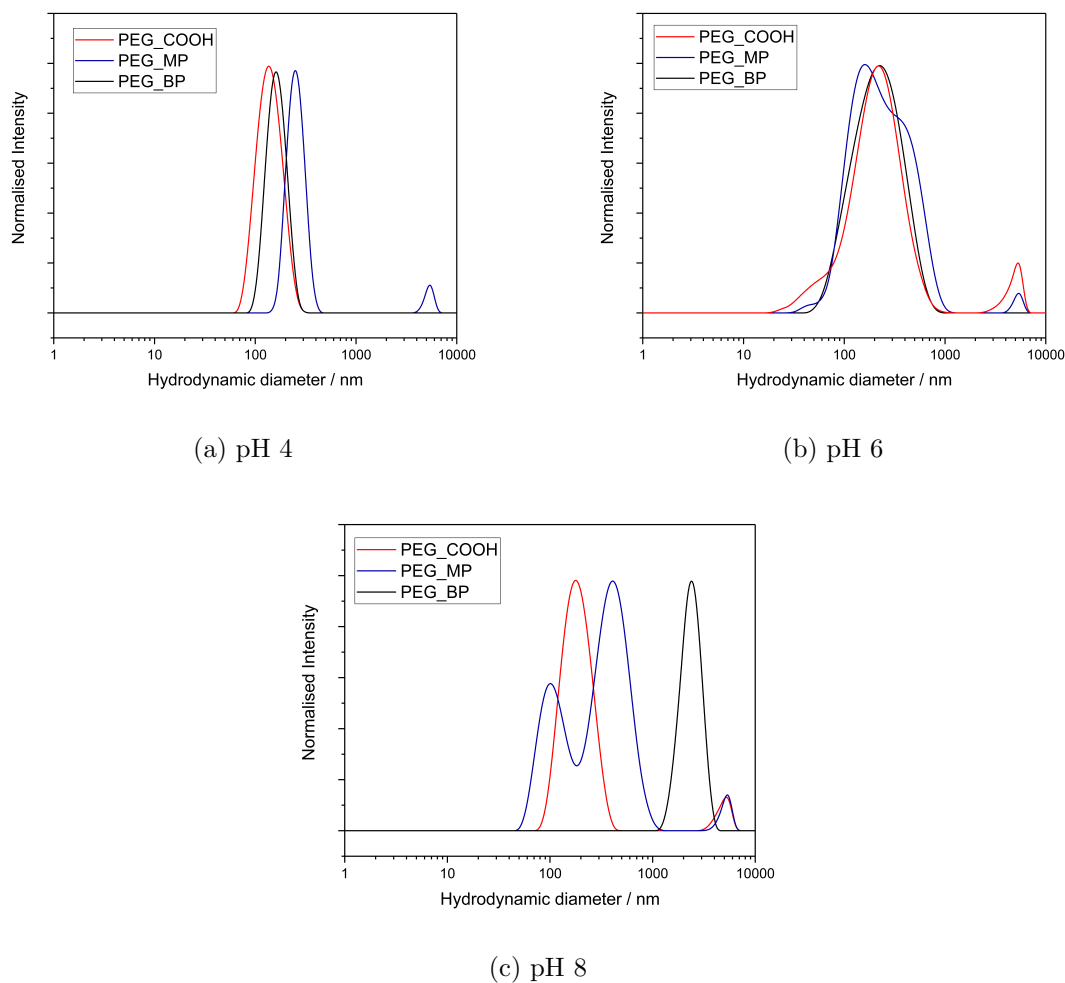


Figure 2.24: Size distribution data of NaYF₄:Yb,Er UCNPs in a range of pH conditions, measured by DLS. Samples at a concentration of 1 mg/mL; n=3 measurements.

At pH 4, the NaYF₄:Yb,Er@PEG-BP colloid appeared to be stable, with only one size distribution by DLS measurement. The hydrodynamic diameter was measured as 146.1 ± 8.2 nm for NaYF₄:Yb,Er@PEG-BP UCNPs. The NaYF₄:Yb,Er@PEG-MP dispersion showed aggregation at pH 4, with a visible peak above 5400 nm. The DLS data for NaYF₄:Yb,Er@PEG-COOH does not reflect the observation that there were visible solid aggregates at the bottom of the cuvettes. The data suggests that NaYF₄:Yb,Er@PEG-COOH had a size of 122.5 ± 2.5 nm; there is no peak suggesting presence of aggregates. It is likely that these aggregates were heavy enough to immediately fall to the bottom of the cuvette and so were not detected by the laser beam. The data shows that at pH 4, the nanomaterial with the greatest colloidal stability is NaYF₄:Yb,Er@PEG-BP.

At pH 7, only NaYF₄:Yb,Er@PEG-BP displayed a stable colloidal dispersion. This is shown by a single peak in the DLS data. Both NaYF₄:Yb,Er@PEG-MP and NaYF₄:Yb,Er@PEG-COOH were found to have large aggregates present in the samples. The data supports the theory that the chelate effect results in stronger binding of a ligand to the nanoparticle surface. The

bisphosphonate ligand has two phosphonate groups available for interaction with the nanoparticle, resulting in a stronger interaction. These ligands are less likely to undergo desorption than carboxylate or monophosphonate ligands. This results in bisphosphonate ligand-capped UCNPs forming stable colloids over a wider range of pH conditions.

At pH 8, there was visible aggregation in all three samples. In $\text{NaYF}_4\text{:Yb,Er@PEG-MP}$ and $\text{NaYF}_4\text{:Yb,Er@PEG-COOH}$, the aggregation can be seen in the DLS data as a peak around 5400 nm. In the $\text{NaYF}_4\text{:Yb,Er@PEG-BP}$ dispersion, there is just one peak. However, this peak is broadened and shifted to a larger size in comparison to the dispersion at pH 4 and pH 6. This suggests that at pH 8, there $\text{NaYF}_4\text{:Yb,Er@PEG-BP}$ dispersion begins to aggregate.

It was found that the $\text{NaYF}_4\text{:Yb,Er@PEG-BP}$ dispersion had good long term colloidal stability. Storage of the dispersion at pH 6 for up to 6 months resulted in negligible aggregation of the sample. After 6 months, the UCNPs did show some aggregation, but these aggregates were able to be removed by passing the dispersion through a 0.2 μm syringe filter. DLS analysis of the resulting dispersion showed the presence of UCNPs with a single size distribution. This shows that the aggregation only occurred for some of the UCNPs in the sample. The results are very promising, suggesting that **PEG-BP** could be an effective UCNP capping ligand *in vivo*. Further measurements need to be undertaken in biologically relevant media, for example PBS. Unfortunately due to time constraints in this project, this work was not able to be undertaken and will have to be carried out in the future.

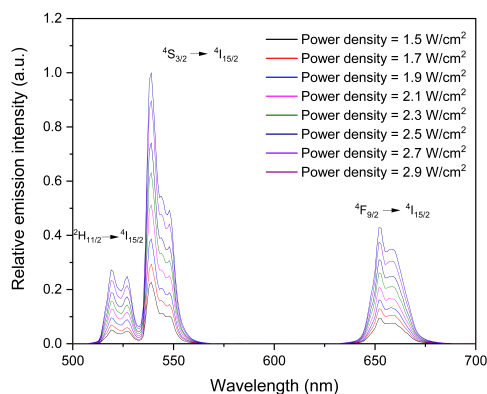
Zeta potential is a measure of the surface charge of a nanomaterial in a colloidal system. The term describes a measurement of the electrokinetic potential between the inner-most bound solvent layer and the non interacting surrounding solvent. A strong positive or negative ζ -potential gives an indication that the colloid is electrically stable, due to electrostatic repulsion between the individual nanoparticles. Nanomaterials with a large positive or negative ζ -potential are resistant to aggregation or agglomeration. *In vivo*, it is advantageous for nanoparticles to have a neutral or slight negative charge as this has shown to result in significantly prolonged circulation half-lives.⁶⁹ ζ -potential measurements were taken for the colloidally stable $\text{NaYF}_4\text{:Yb,Er@PEG-BP}$ UCNPs at pH 6 and found to be -41.5 ± 8.5 mV. This value lies within the widely accepted delineation of stability for colloids of $|\zeta\text{-potential}| > 30$ mV.⁷⁰ The result gives another indication that $\text{NaYF}_4\text{:Yb,Er@PEG-BP}$ UCNPs could be stable *in vivo*. The ζ -potential value is strongly dependent on pH and so ζ -potential measurements should be carried out in future studies of the UCNPs in PBS and other biologically relevant media.

2.3.2.6 Upconversion Luminescence Measurements of UCNPs@PEG-BP

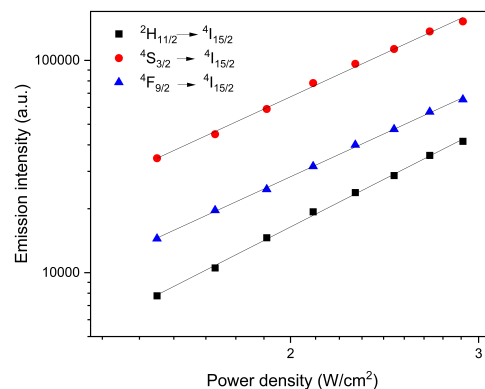
As the $\text{NaYF}_4\text{:Yb,Er@PEG-BP}$ UCNPs were shown to be the most resistant to aggregation, this nanomaterial was taken through to upconversion photoluminescence spectroscopy measurements. It has been widely reported in the literature that when UCNPs are transferred from organic solvents to water, the intensity of the upconversion fluorescence significantly decreases.⁷¹ To validate the claim, upconversion photoluminescence measurements were made of $\text{NaYF}_4\text{:Yb,Er@PEG-BP}$ in chloroform and compared to that of $\text{NaYF}_4\text{:Yb,Er@PEG-BP}$ in wa-

ter. (These measurements were kindly carried out by Dr Shuai Zha at Hong Kong Baptist University.)

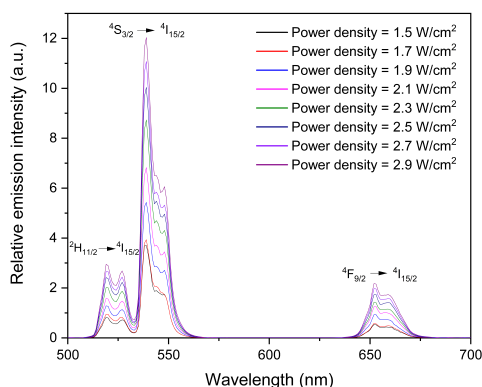
In order to adjust for the possible differences in concentration of the UCNPs in solution, ICP-MS measurements were carried out. This validated the concentration of yttrium in the two samples and then the upconversion emission data was scaled to reflect this. The upconversion emission spectra in figure 2.25 are plotted as relative emission intensity, relative to the highest emission intensity measured from the sample of NaYF₄:Yb,Er@PEG-BP UCNPs in water.



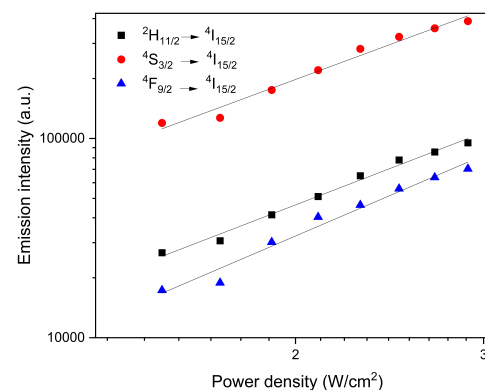
(a) Upconversion photoluminescence spectrum of NaYF₄:Yb,Er UCNPs in water.



(b) Power dependency study for NaYF₄:Yb,Er UCNPs in water.



(c) Upconversion photoluminescence spectrum of NaYF₄:Yb,Er UCNPs in chloroform.



(d) Power dependency study for NaYF₄:Yb,Er UCNPs in chloroform.

Figure 2.25: Comparing upconversion emission of NaYF₄:Yb,Er UCNPs in water and in chloroform; (a) and (c) upconversion photoluminescence spectra; (b) and (d) corresponding excitation power density dependence of $^4S_{3/2} \rightarrow ^4I_{15/2}$, $^4F_{9/2} \rightarrow ^4I_{15/2}$ and $^2H_{11/2} \rightarrow ^4I_{15/2}$ transition emission intensities. $\lambda_{\text{ex}} = 980 \text{ nm}$ with samples at a concentration of 2 mg/mL.

It can be seen in figure 2.25, that the intensity of the emission from the **PEG-BP** capped UCNPs was significantly lower in water than in chloroform. In chloroform, the UCNPs have an approximately 12 x stronger emission at 540 nm than the same concentration of UCNPs in water. Another noticeable difference is that the NaYF₄:Yb,Er@PEG-BP UCNPs in water have

a stronger red emission than the UCNPs in chloroform. The green:red (540:650 nm) ratio for NaYF₄:Yb,Er@PEG-BP UCNPs in chloroform was 5.6 and in water was 2.4.

This is likely to be due to surface quenching effects caused by surrounding water molecules. The water causes multiphonon relaxation, deactivating the $^2H_{11/2}$ and $^4S_{3/2}$ energy states thereby increasing the population of the $^4F_{9/2}$ state.⁷¹ This is the origin of the higher red:green emission ratio for NaYF₄:Yb,Er@PEG-BP in water compared to the UCNPs in NaYF₄:Yb,Er@PEG-BP in chloroform.

The gradient of the log-log plots gives the number of photons in the corresponding upconversion process (n), according to: $I_{UC} = (I_P)^n$, where I_{UC} is the intensity of the upconversion emission, I_P is the excitation power density and n is the number of NIR photons absorbed per photon emitted.

For NaYF₄:Yb,Er@PEG-BP in water:

$$^2H_{11/2} \rightarrow ^4I_{15/2} \text{ (525 nm) } n = 2.57 \pm 0.04 \text{ **2 / 3 photon process**}$$

$$^4S_{3/2} \rightarrow ^4I_{15/2} \text{ (540 nm) } n = 2.32 \pm 0.05 \text{ **2 photon process**}$$

$$^4F_{9/2} \rightarrow ^4I_{15/2} \text{ (650 nm) } n = 2.31 \pm 0.03 \text{ **2 photon process**}$$

For NaYF₄:Yb,Er@PEG-BP in chloroform:

$$^2H_{11/2} \rightarrow ^4I_{15/2} \text{ (525 nm) } n = 2.30 \pm 0.15 \text{ **2 photon process**}$$

$$^4S_{3/2} \rightarrow ^4I_{15/2} \text{ (540 nm) } n = 1.98 \pm 0.12 \text{ **2 photon process**}$$

$$^4F_{9/2} \rightarrow ^4I_{15/2} \text{ (650 nm) } n = 2.06 \pm 0.08 \text{ **2 photon process**}$$

If the upconversion mechanism was purely ESA (excited state absorption) or ETU (energy transfer upconversion), then it would be expected that $n \leq 2$, at low excitation densities. The $^2H_{11/2} \rightarrow ^4I_{15/2}$ transition of the UCNPs in water had a value of n of 2.57, considerably higher than the value of $n = 2$ expected. This is likely to be due to a three photon pathway contribution to the emission, with non-radiative transition contributions.⁷²

2.4 Conclusions

In this chapter, the upconversion nanoparticle–ligand interface was studied by two complementary techniques. NaYF_4 NPs were synthesised as a surrogate for $\text{NaYF}_4\text{:Yb,Er}$ UCNPs. This allowed ^1H NMR titrations to be carried out with the nanomaterial and enabled the relative binding strength of phosphonic acid and carboxylic acid ligands to be ascertained. It was found that 10-undecenylphosphonic acid binds preferentially and irreversibly to the surface of NaYF_4 NPs, in comparison to oleic acid. When 10-undecenylphosphonic acid was added to $\text{NaYF}_4\text{:oleic acid}$ NPs, the phosphonic acid completely displaced the carboxylic acid. In the reverse process, when oleic acid was added to $\text{NaYF}_4\text{:phosphonic acid}$ there was no ligand displacement. These results support the widely held view that phosphonic acids are suitable binding groups on ligands for UCNPs. The work suggests that the molecular coordination chemistry can be successfully applied to nanoparticle–ligand interaction to help predict strength of ligand-nanoparticle bonds.

The results from the ^1H NMR studies helped inform the design of ligands suitable for phase-transfer of hydrophobic UCNPs. PEG groups were incorporated in the ligand structure to improve the biocompatibility. PEG-COOH ligands were functionalised with mono- and bis-phosphonic acid groups, creating a set of three ligands which were used to phase transfer UCNPs. PEG-COOH, **PEG-MP** and **PEG-BP** ligands were synthesised and exchanged onto the surface of $\text{NaYF}_4\text{:Yb,Er}$ UCNPs. The colloidal stability of dispersions of these UCNPs in water were determined. It was found that $\text{NaYF}_4\text{:Yb,Er@PEG-BP}$ UCNPs had the best colloidal stability, over a range of pH values. The results suggest that **PEG-BP** is a suitable ligands for bioimaging probes. The colloidal stability of UCNPs@PEG-BP in physiological buffer conditions needs to be ascertained, in order to help further predict the biocompatibility of this nanomaterial. Unfortunately, due to time constraints these measurements were not able to be obtained during the time scale of this project.

Photoluminescence spectra for $\text{NaYF}_4\text{:Yb,Er@PEG-BP}$ UCNPs were obtained. Under excitation at 980 nm, the UCNPs displayed emission at 525, 540 and 650 nm. The UCNPs dispersed in water had a significantly lower emission than the chloroform dispersions, with UCNPs in water showing a higher red:green emission ratio. This emission quenching in water is an issue must be navigated in the application of UCNPs for bioimaging. Here, it is essential that the nanomaterials have a bright emission so that the signal from the UCNPs can be readily detected. This issue will be covered in greater detail in chapter 4.

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Chapter 3

Upconversion Nanoparticles as Nanosensors

3.1 Introduction

Upconversion nanoparticles are increasingly being investigated for use as reporter systems both *in vitro* and *in vivo*.^{1,2} The sharp emission bands of UCNPs make the materials suitable for use as nanosensors. This can be achieved by coupling the nanoparticles with chemical species which have an absorption band overlapping with selected UCNP emission bands. Figure 3.1 shows the spectral overlap necessary for energy transfer to occur in these sensing systems. This energy transfer causes a modulation of the emission of the nanomaterial, thereby allowing biodetection of selected analytes.

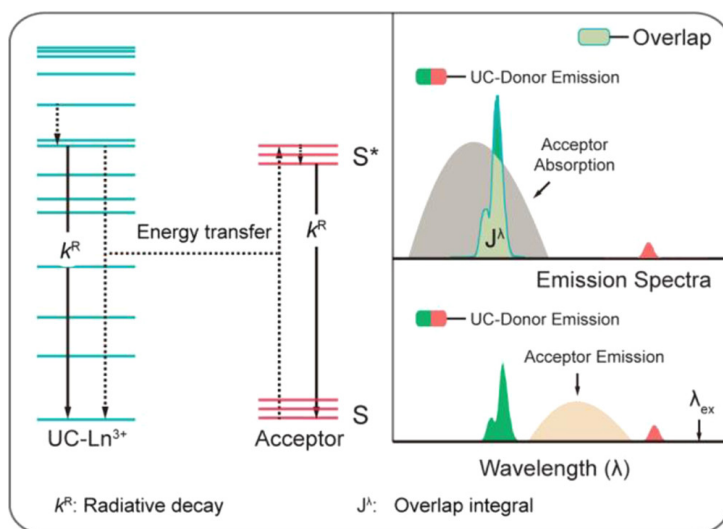


Figure 3.1: Illustration of UCNP donor paired with organic fluorophore acceptor. If there is an overlap between the UCNP emission and fluorophore absorption spectra then energy transfer can occur. Reprinted with permission from reference 1. Copyright 2017 American Chemical Society.

Resonance energy transfer (RET) describes the process in which energy is transferred between a donor species in an excited state and an acceptor species. This process is referred to as Förster resonance energy transfer or fluorescence resonance energy transfer (FRET) when the donor is a fluorophore and luminescence energy transfer (LRET) where the donor is a lanthanide.² RET is a non-radiative process which can occur over a distance of up to 10 nm under physiological conditions.¹

If the acceptor species is selected so that their absorption and/or emission properties change upon interaction with a target analyte, the nanomaterial can act as a chemical sensor. For imaging using UCNPs *in vivo*, this is particularly powerful as the excitation of the nanosensor requires NIR light, at a wavelength within the tissue optical window. In the following sections, examples of UCNP nanosensors will be discussed. In these examples, the emission of the nanoprobe is modulated when in the presence of specific analytes. The presence of the chemical species of interest can be quantified by either measuring the quenching of UCNP emission, emission from the bound fluorophore or increased emission from the UCNPs.

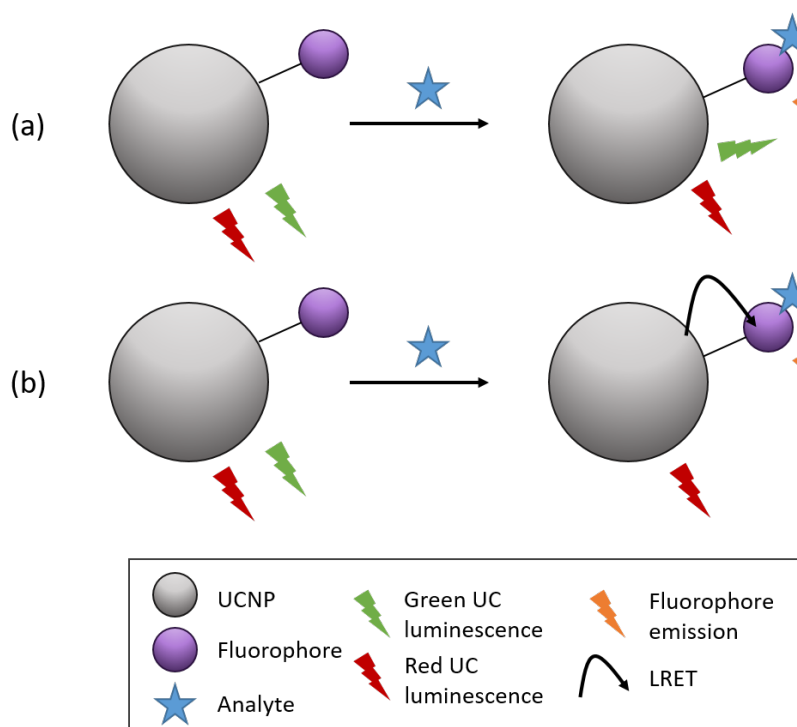


Figure 3.2: Energy transfer based analyte detection strategies with UCNPs. (a) Inner-filter mechanism. When the analyte is bound, light emitted by the UCNP can be reabsorbed by the fluorophore acceptor on the NP surface; (b) RET mechanism. When the analyte is bound to the fluorophore, energy transfer can take place between the UCNPs and fluorophore.

Figure 3.2 shows two mechanisms by which UCNPs can have their spectral properties modulated upon interacting with the specific analyte. (a) shows the inner-filter effect whereby excitation of the fluorophore occurs by reabsorption of light emitted by the UCNPs. The fluorophore in this case does not need to be bound to the surface of the nanoparticle.³⁻⁵

(b) shows RET, a distance dependent non-radiative process. When a specific analyte binds to the fluorophore, the photoluminescent properties of the dye are modulated. The change in spectral properties of the fluorophore energy transfer can take place between the UCNP and fluorophore. This process can also occur in the opposite direction i.e. energy transfer occurs between the UCNP and fluorophore without analyte binding. Upon binding, the fluorophore emission can be quenched. For *in vivo* imaging applications, RET is usually the more relevant process. This is because *in vivo*, the fluorophore and UCNP would need to be bound. This keeps the nanomaterial as one entity as it circulates in the body.

3.1.1 UCNP-RET Acceptor Systems

There were many examples published in the last 10 years of RET systems comprising UCNPs and various acceptors. These acceptors have included: Au NPs (for IgG detection⁶), CdSe QDs⁷, metal complexes (for Hg⁺ and cyanide anion detection⁸⁻¹⁰) and carbon NPs¹¹. This energy transfer can be used to induce photodynamic therapy (PDT) by loading the nanoparticle with photosensitizers.^{12,13} Examples of photosensitizers include zinc phthalocyanine¹⁴, TiO₂ NPs¹⁵ and rose bengal¹⁶. Reviews by Su et al., Andresen et al. and Zhang et al. provide detailed summaries of the recent progress made in the design and synthesis of UCNP-based nanosensors.^{1,2,17}

For efficient RET to take place between the UCNP donor and surface acceptor species, a number of requirements must be fulfilled:

- **Spectral overlap:** There must be significant overlap between the emission profile of the donor and the absorption spectrum of the acceptor to allow efficient RET to take place.
- **Distance:** Non-radiative energy transfer has a distance dependence of $\frac{1}{r^6}$, therefore the donor and acceptor need to be close enough together to allow RET to occur. Typically this is <10 nm.
- **Brightness:** The quantum yield of the UCNPs need to be sufficiently high so there is enough emission from the UCNP to transfer energy to the acceptor species, given that there will be competing energy loss processes such as surface quenching occurring.

The design of nanomaterial sensors requires taking a number of factors into consideration. These include:

- A reliable method for surface functionalisation
- Colloidal stability in biological media
- Stable attachment of the indicator molecules

The intensity of the upconversion emission is dependent on the concentration of the nanoprobe, which *in vivo* would be impossible to quantify. A ratiometric analysis is therefore a reliable way of assessing RET between the UCNP and absorbing species, as it is a concentration independent measurement. RET should only occur from the energy state which overlaps with the absorbing

species. This allows other UCNP emission peaks to be used as a reference and a ratiometric output can be calculated.

3.1.2 UCNP-Rhodamine RET Nanosensors

Organic fluorophores have been a popular choice as RET acceptors in UCNP-based nanosensors as there is a large volume of research into stimuli responsive organic fluorophores. Many of these can be grafted to the surface of UCNP, without complex synthetic modification.

N-substituted rhodamines are particularly versatile for use as nanosensors. They were briefly discussed in section 1.2.2.1. There are a range of examples of rhodamine derivatives where the intensity of emission from the fluorophore depends on the concentration of a specific analyte.^{18,19} This feature has made rhodamine-based compounds particularly interesting to be used as sensors *in vivo*.

There are many examples of rhodamine-based UCNP sensors in the literature, detecting: HOCl²⁰, nitric oxide²¹, Cu²⁺^{19,22,23}, Fe³⁺²⁴ and Hg²⁺.²⁵ These probes all show substantial quenching of the Er emission peak at 550 nm but only a very weak emission from the rhodamine species. This weak emission has been attributed to surface quenching due to interaction of erbium ions on the surface of the nanocrystal with the solvent and is a particular issue in aqueous solvent (containing O-H bonds).^{26–28}

Figure 3.3 shows work carried out by Xu et al. in 2016. They synthesised a probe capable of detecting Cu²⁺ ions and showed its selectivity for Cu²⁺ over other biologically relevant ions, such as zinc and iron. The probe comprised a NaYF₄:Yb,Er UCNP core, which was covered with a mesoporous silica shell. A Cu²⁺ sensitive rhodamine derivative was then grafted onto the silica shell, as shown in figure 3.3a. Upon direct rhodamine excitation, at 520 nm, the probe showed strong pink emission when in the presence of Cu²⁺ but no visible emission with various other metal ions. Figure 3.3b shows this colorimetric response. However, this response did not occur so starkly when the probe was excited at 980 nm as there was limited RET between the UCNP core and surface rhodamine species. Only a weak emission from rhodamine was visible in the presence of Cu²⁺, when the nanosensor was excited at 980 nm. Most of the signal observed was from the UCNP and this limits the *in vivo* applications of this probe.

There are a number of examples of nanosensors where the UC luminescence is recovered in the presence of the target analyte, in the reverse process to the reported cases described above.^{29–32} Peng et al. developed a nanosensor that upon exposure to Zn²⁺, the surface RET acceptor (a pyridyl chelating agent) inhibited RET and therefore ‘switched on’ emission from the UCNP.²⁹ However, these nanosensors experience the same issues of low analyte responsive emission from the probe.

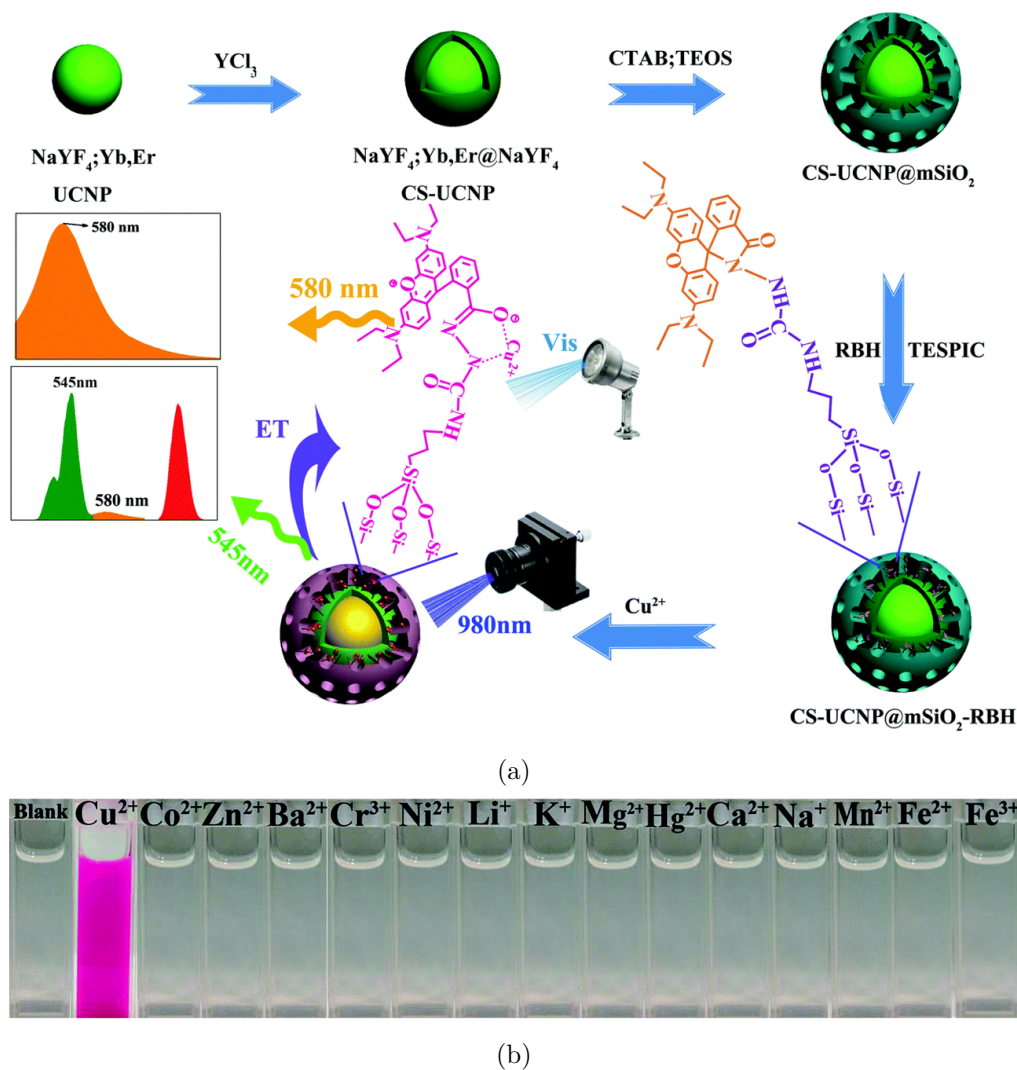


Figure 3.3: Structure and analyte sensing of Cu^{2+} selective UCNPs-based nanoprobe. (a) Method for synthesis of UCNPs-rhodamine nanoprobe; (b) Photographs showing the colorimetric performance of the nanoprobe in ethanol, upon the addition of metal ions. Reproduced from reference 23 with permission from the Centre National de la Recherche Scientifique (CNRS) and The Royal Society of Chemistry.

3.1.3 pH Sensing with UCNP-Rhodamine RET Nanosensors

pH can be used as a fundamental indicator of cell function and structure. The pH of the cell cytoplasm regulates ionic transport, cell migration and proliferation.^{33,34} An abnormal intracellular or extracellular pH can be an indicator of cell dysfunction and of diseases such as cancer.² Therefore, there is a desire to be able to image cellular pH, to both better understand cell function and to detect disease.

The functionalisation of UCNPs with pH responsive fluorophores provides a method to image cellular pH, circumventing the common issues of photobleaching and autofluorescence encountered with conventional molecular fluorophores.^{35,36} There have been a number of papers published in the last six years, which demonstrate the pH sensing ability of UCNPs functionalised with fluorophores.^{35,37–40}

Figure 3.4 shows work carried out by Nareoja et al. in 2017 and is a typical example of the construction of fluorophore functionalised UCNPs for pH sensing.³⁷ A NaYF₄:Yb,Er UCNP core was coated with a polyethylenimine (PEI) shell. The pH sensing dye, pHrodo red (a rhodamine-based fluorophore) was then coupled to the surface of the UCNPs. The authors showed that the UCNPs were internalised by HeLa cells and an increase in red to green ratio was observed for particles located in acidic cell compartments, such as endosomes (shown in figure 3.4). There was found to be limited RET efficiencies, leading to low ratios between the sensor signal and reference signal. This significantly limits the sensitivity of the system.

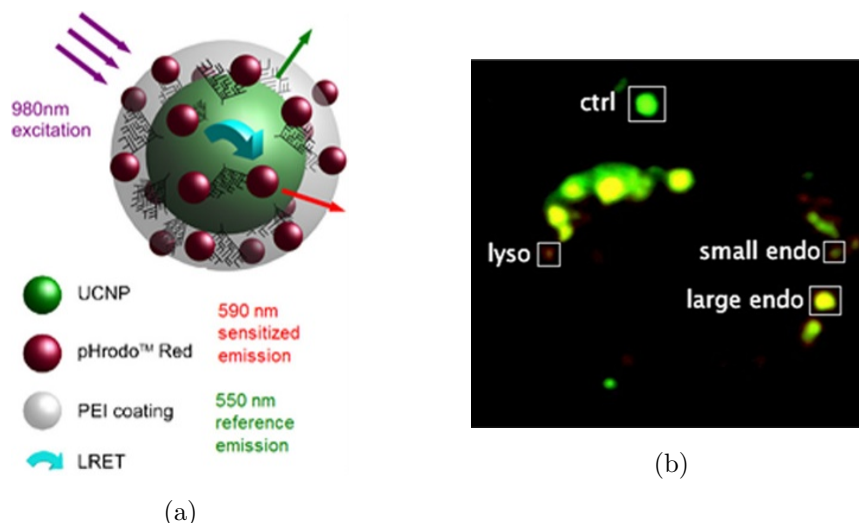


Figure 3.4: (a) Representation of structure of pH sensing nanoprobe, showing excitation at 980 nm and either emission of green (550 nm) or LRET and emission of red (590 nm) light (b)

An overlaid ratiometric image of a cell with the pH nanoprobes localising in the lysosome (predominately red emitting) and other cellular compartments such as endosomes (predominately green emitting). Reprinted with permission from reference 37. Copyright 2016 American Chemical Society.

3.2 Aims and Objectives

The aim of this chapter was to prepare UCNPs functionalised with rhodamine fluorophores, suitable for biological sensing. In the previous chapter, the stability of the bisphosphonate-UCNP interaction was discussed in detail. Based on this work, the bisphosphonate moiety has been chosen as the approach to anchor the fluorophore to the surface of UCNPs. The covalent assembly of the nanoprobe guarantees a short distance between the nanoparticle and fluorescent dye, maximising the energy transfer between the two. An aspect of this work was to prepare both an ‘pH-insensitive’ rhodamine (locked into its permanent fluorescent state) and a ‘pH-sensitive’ rhodamine (where the fluorescence intensity depends on the pH of surrounding media) bisphosphonate derivative.

These rhodamine-bisphosphonate derivatives would then be attached to the surface of $\text{NaYF}_4:\text{Yb,Er}$ UCNPs. In order to render these nanosensors biocompatible, the water-soluble **PEG-BP** ligand was incorporated into the design of the nanoprobe. The structure of which is shown in figure 3.5a. A further aim was to study the energy transfer and fluorescence of these systems. The donor and acceptor were chosen so that there would be spectral overlap, this can be seen in figure 3.5b. It was hoped that the ‘pH-sensitive’ rhodamine-based UCNP probe would be capable of sensing the pH of the surrounding media. This nanoprobe could have applications in various disease detection, including intra-operative cancer detection in image-guided surgery.

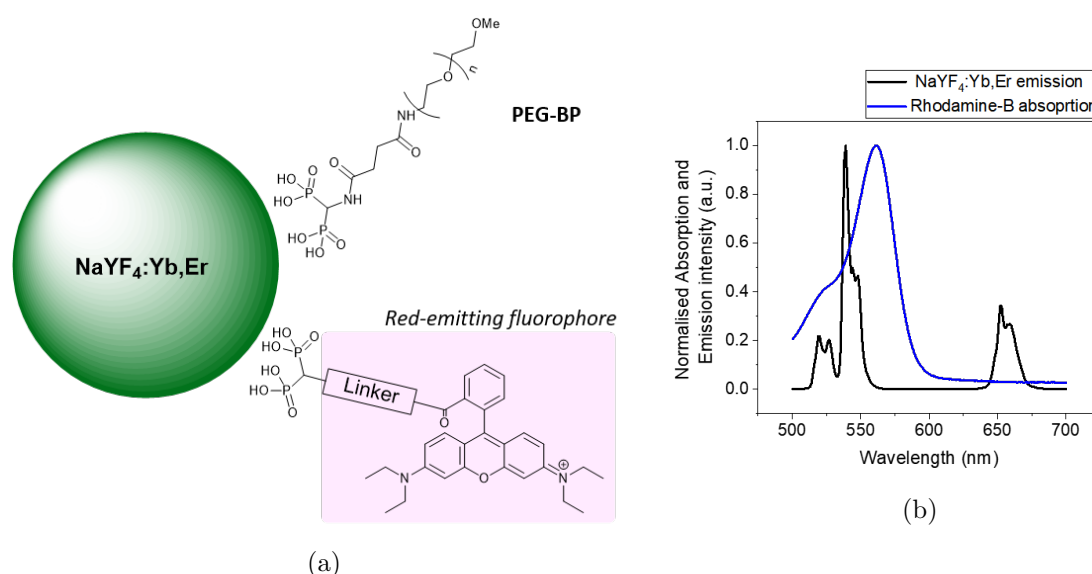


Figure 3.5: (a) Structural representation showing components of rhodamine-based probes; (b) Normalised absorption spectrum of rhodamine-B and emission spectra of $\text{NaYF}_4:\text{Yb,Er}$, showing spectra overlap.

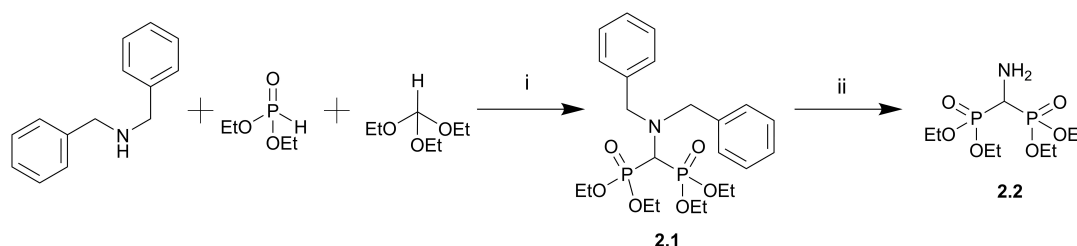
3.3 Results and Discussion

The general structure of the proposed rhodamine-containing probe is shown in figure 3.5a. The probe comprises: a $\text{NaYF}_4:\text{Yb,Er}$ UCNP core, **PEG-BP** ligands to induce water solubil-

ity and rhodamine-bisphosphonate ligands. The rhodamine ligands provide environmentally-responsive fluorescence. In the following sections, the synthesis and characterisation of two rhodamine-bisphosphonate species will be described. Firstly, a pH-sensitive derivative and then a pH-insensitive rhodamine. The assembly and testing of pH-sensitive nanoprobe will then be discussed.

3.3.1 Rhodamine-Bisphosphonate Ligand Synthesis

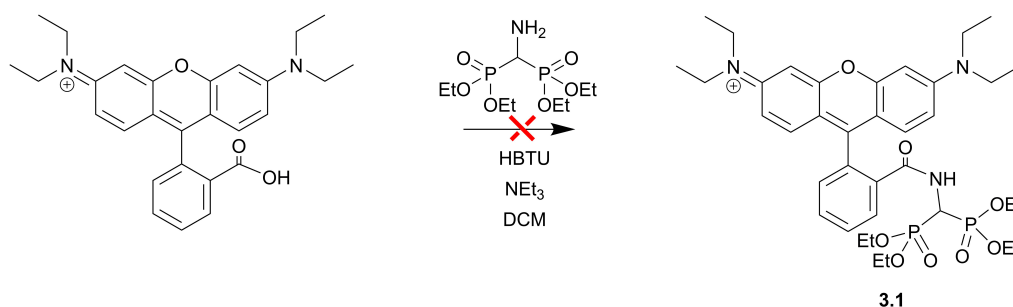
The first step in functionalising rhodamine-based fluorophores with a bisphosphonate moiety was preparation of an amine-containing bisphosphonate. This was carried out using the same method as was used to prepare **PEG-BP**, as shown in scheme 3.1. This was discussed in detail in section 2.3.2.



Scheme 3.1: Synthesis of amine-functionalised bisphosphonic ester, **2.2**. Reagents and reaction conditions: (i) 150 °C, 5 h; (ii) H₂ (1 bar), 10 % Pd/C, EtOH, reflux, 24 h.

3.3.1.1 pH-Sensitive Rhodamine Synthesis

Rhodamine-B is a commercially available rhodamine derivative which contains a free carboxylic acid. Initially, it was thought that the **2.2** could be directly coupled to the carboxylate on rhodamine-B (as shown in scheme 3.2), using a peptide coupling agent.

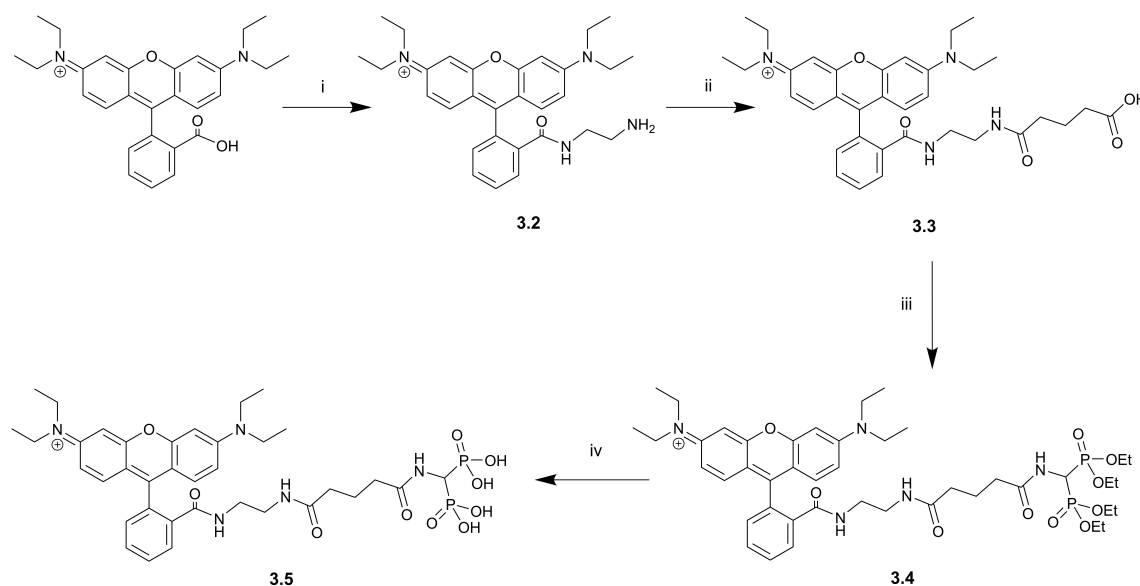


Scheme 3.2: Attempt at a direct rhodamine-B reaction with aminobisphosphonate.

Unfortunately this reaction was not successful. Even after attempting to force the reaction by increasing the reaction temperature (in acetonitrile, under reflux), there was no evidence of reaction between the two starting materials. The ³¹P{¹H} NMR spectra showed only the bisphosphonate starting material and no product peak was visible by ES-MS. The failure of this reaction is likely to be due to the sterics of the reactants. The bulky bisphosphonate must

be too large to attack the carboxylate on the rhodamine, which is sterically hindered by the surrounding dye structure. The primary amine on the bisphosphonate ligand is also buried, surrounded by the two bulky phosphonic ester groups. This prevented the HBTU-facilitated peptide coupling from occurring.

The solution to this lack of reactivity was to add a spacer unit. In this case, the chosen strategy was to add an ethylenediamine unit and then glutaric acid unit, leaving a free acid available for attack by **2.2**. The length of this spacer was important to consider. Increasing the distance between the Er emitters in the UCNP and the fluorophore would have a detrimental effect on the RET efficiency, as discussed in section 3.1.1.

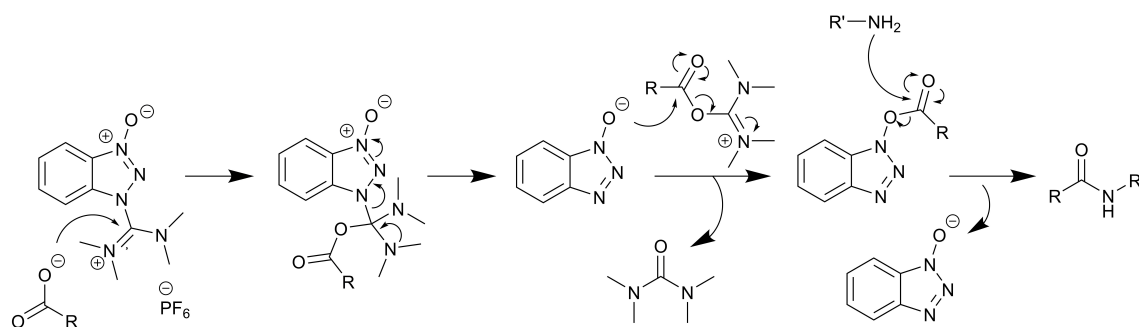


Scheme 3.3: Synthesis of pH-sensitive rhodamine-bisphosphonate ligand, **3.5**. Reagents and reaction conditions: (i) ethylenediamine, EtOH, reflux, 16 h; (ii) glutaric anhydride, MeCN, reflux, 16 h; (iii) **2.2**, HBTU, NEt₃, DCM, r.t., 16 h; (iv) TMSBr, dry DCM, N₂, r.t., 16 h followed by: MeOH, r.t., 1.5 h.

Scheme 3.3 shows the four reaction steps carried out to functionalise rhodamine with a bisphosphonate moiety. In the first reaction step, commercially available rhodamine-B was reacted with excess ethylenediamine. This is a previously published reaction which requires a simple water wash to remove excess starting material.⁴¹ In the second step, the free primary amine underwent reaction with excess glutaric anhydride and was purified by silica gel column chromatography, eluting with a DCM:methanol solvent system. The reaction produced **3.3**, a rhodamine species with a free and unhindered carboxylic acid which was available to couple to **2.2**.

In the third step, **3.3** was reacted with **2.2** in DCM, with the reaction facilitated by 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), a commercially available peptide coupling reagent. A general mechanism for HBTU-mediated peptide coupling is shown in scheme 3.4.^{42,43} The by-product tetramethylurea can be observed as a singlet in the ¹H NMR spectrum at 2.80 ppm. This can be removed by sequential washing with 10 % citric

acid, saturated sodium bicarbonate and then brine.



Scheme 3.4: Mechanism of HBTU-mediated amide coupling.

Finally, a deprotection step was undertaken. This removed the bisphosphonate esters, to reveal the free bisphosphonic acids. The reaction was shown to proceed cleanly, as was found in the TMSBr deprotection reactions discussed in chapter 2. Upon deprotection, there was a dramatic change in solubility, with **3.5** no longer soluble in CDCl_3 for ^1H NMR analysis. This change in solubility supports the conclusion that the bisphosphonate ester was successfully deprotected.

The $^{31}\text{P}\{^1\text{H}\}$ NMR spectrum of **3.5** showed a single ^{31}P shift at 14.6 ppm, this can be seen in figure 3.6. $^{31}\text{P}\{^1\text{H}\}$ NMR provided a useful handle to monitor these reactions. There was an observed δ from 20.3 ppm (**2.2**) to 16.9 ppm (**3.4**) and finally to 14.6 ppm (**3.5**).

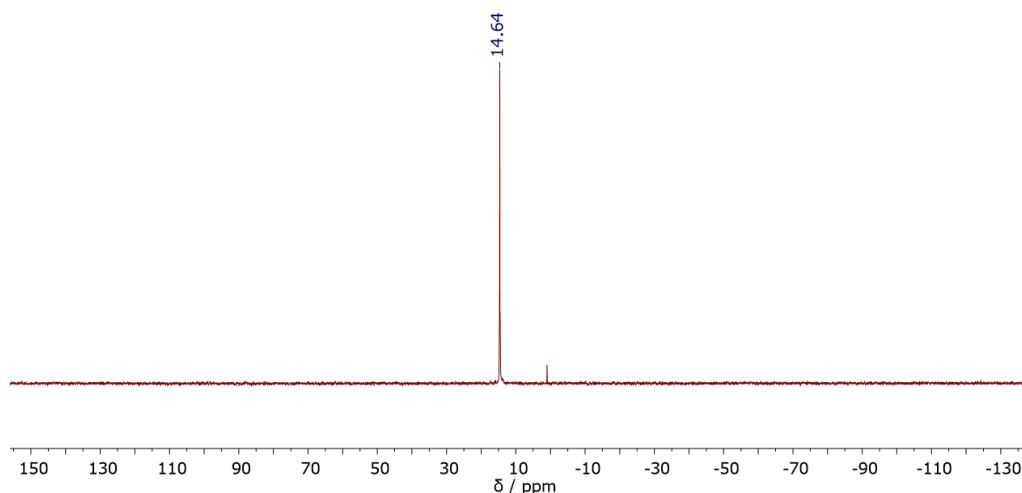


Figure 3.6: $^{31}\text{P}\{^1\text{H}\}$ NMR spectrum showing a single phosphorus environment in **3.5** (CDCl_3 , 161.84 MHz, 295 K).

3.3.1.2 pH-Sensitive Rhodamine Photophysical Properties

As discussed in section 1.2.2.1, the N-substituted rhodamine derivative **3.5** was expected to be pH-sensitive i.e. they should undergo an acid-catalysed ring opening. Figure 3.7 shows the ring-open and ring-closed conformers of the synthesised rhodamine-bisphosphonic acid ligand.

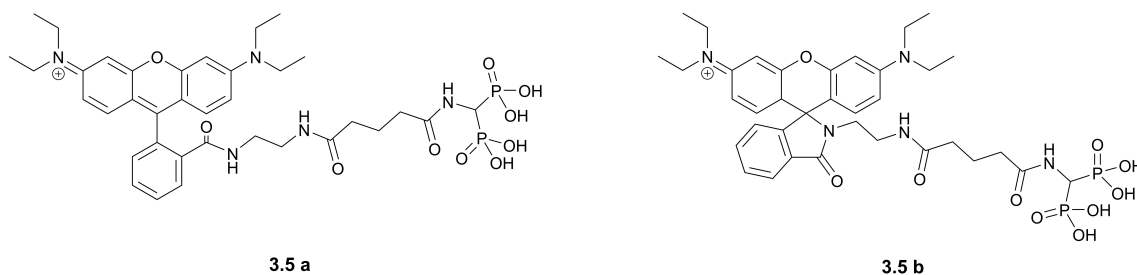


Figure 3.7: Highly fluorescent ring-opened **3.5a** and non-fluorescent ring-closed **3.5b** rhodamine derivatives.

The photophysical properties of the molecule were investigated by UV-Vis and fluorescence spectroscopy. 30 μM samples of **3.5** were prepared in 0.1 M KCl in distilled water. This provided a solvent with constant ionic strength. This was an important variable to control as it is known that pH indicators can be affected by the ionic strength of the media.⁴⁴ The pH of the solutions were adjusted by the addition of 0.1 M HCl and/or 0.1 M NaOH. Solutions were prepared in the pH range 3-8, allowing assessment of how the fluorophore behaved over a range of relevant physiological pH values. Figure 3.8 shows the absorbance and emission spectra of **3.5**, measured at pH values between 3 and 8. The spectra show that both the absorbance and emission of the rhodamine derivatives are dependent on the pH of the solution. Low pH values were found to result in **3.5** absorbing and emitting highly and high pH values showed both low absorption and emission.

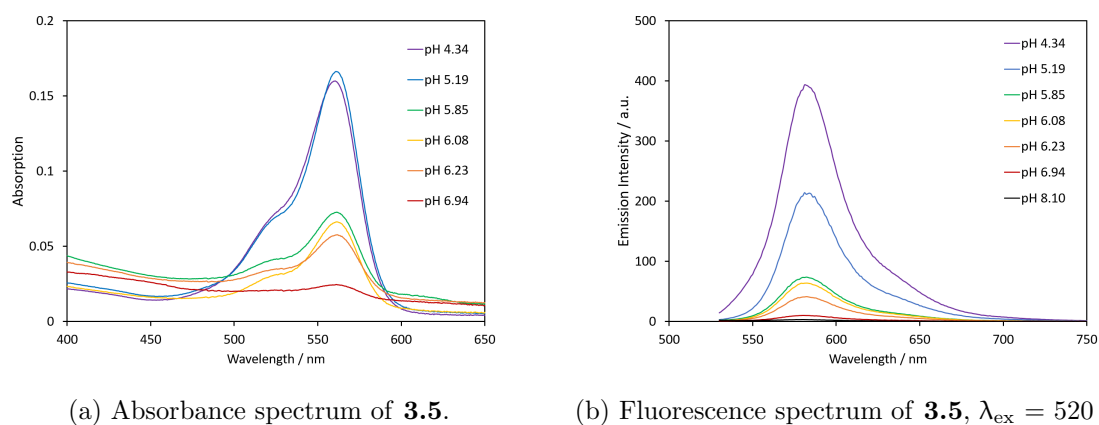


Figure 3.8: pH dependent absorbance and emission spectra of **3.5**. Samples at a concentration of 30 μM in aqueous 0.1 M KCl.

Figure 3.9 shows normalised absorption intensity at 560 nm plotted against measured pH. OriginPro 2017 was then used to fit the data, producing the sigmoidal fit shown in figure 3.9. The half-maximum absorbance value was then used to calculate the pKa of cyclisation, as described by Uno et al.⁴⁵. This was determined to be 5.85 for the pH-sensitive rhodamine fluorophore, **3.5**. This cyclisation pKa can widely differ between rhodamine derivatives, with values from 2.8 to 6.4 reported.^{46,47} A pKa value of 5.85 sits within a biologically relevant window, with a reduced intracellular pH of 6.0–7.2 being found in endosomal compartments and a pH of 4.0–6.0 for lysosomes.³⁷ It has been well reported that in cancer cells, both the intracellular and extracellular pH is dysregulated.^{48–51} The extracellular pH of a healthy differentiated cell is ~ 7.4 but in a cancer cell, the extracellular pH reduces to 6.7–7.1.⁴⁸

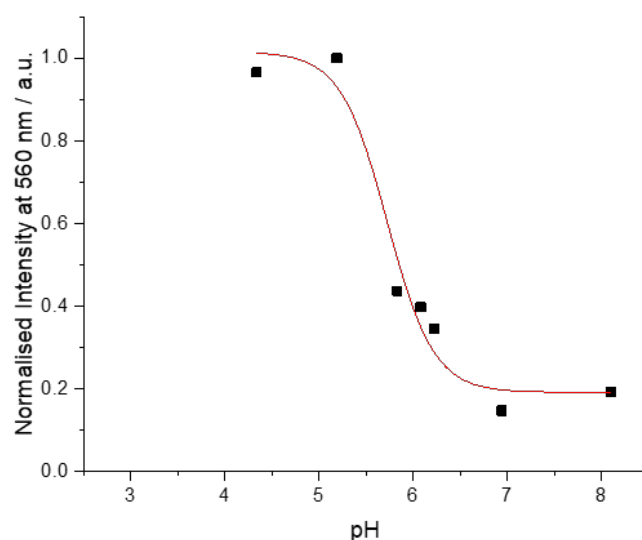


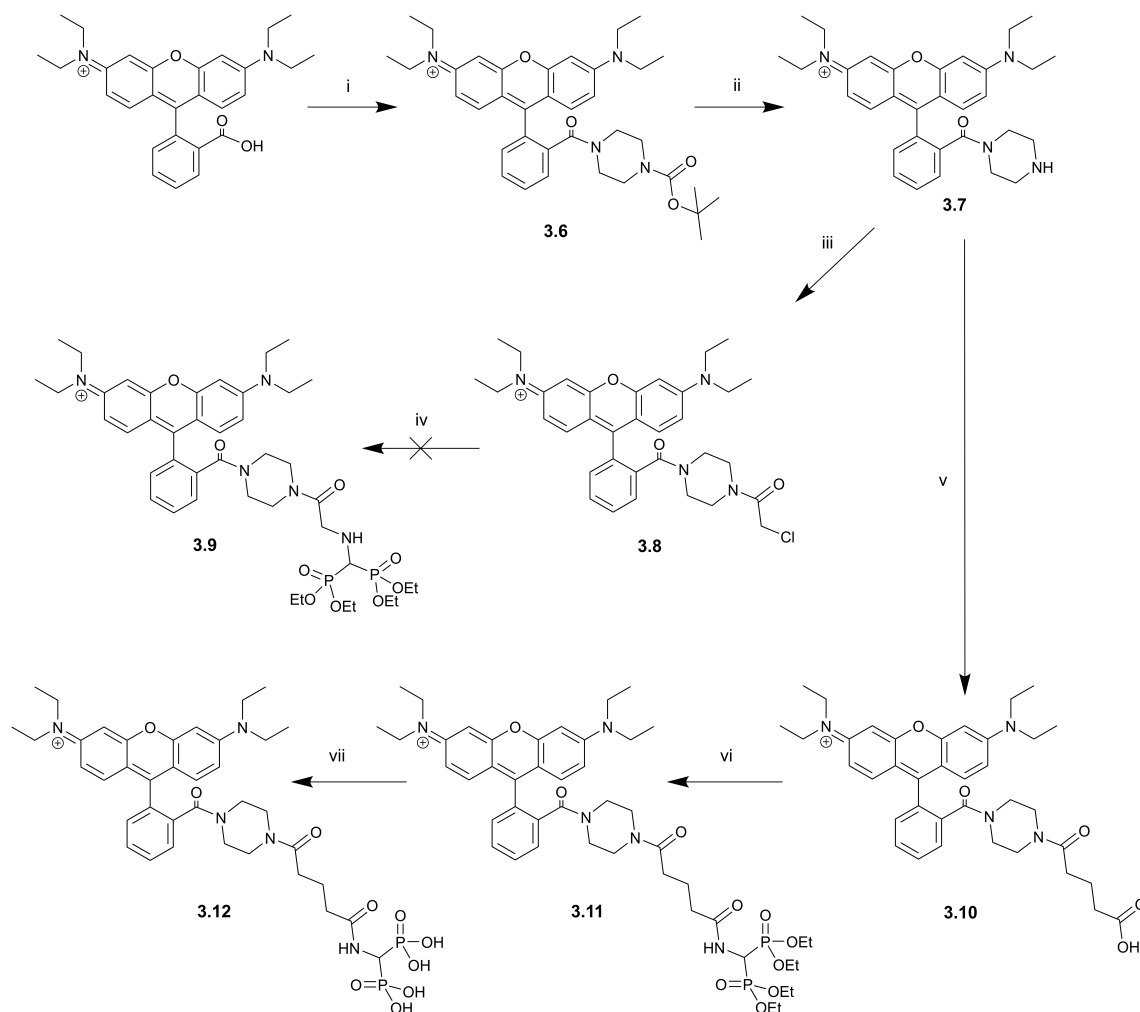
Figure 3.9: Plot showing normalised absorption intensity at 560 nm of **3.5** over a range of pH values, allowing the calculation of ring-opening pKa.

The rate of response to a target analyte is important to consider for bioimaging applications. The kinetics of the acid catalysed ring opening process of **2.2** was found to be slow. The samples needed to be left for 2 to 3 hours to equilibrate, with the acidic samples gradually becoming more strongly pink coloured over time. In their 2014 paper, Czaplyski et al. discuss the variation in ‘turn on’ behaviour of the studied rhodamine derivatives. The response time was found to vary from seconds for certain rhodamine derivatives to hours for other derivatives.⁴⁷ For *in vivo* applications, slow kinetics may prove to be beneficial. This would allow the imaging probe to localise at the target site before the ‘switch on’ of the fluorescence from the fluorophore. This may prove to be beneficial for target imaging as it would reduce background noise from probes not located at the site of interest. Due to time constraints, the reaction kinetics of the ring-opening process were not able to be studied. Further work on this may provide valuable insights into the suitability for the rhodamine derivative to be used *in vivo*.

3.3.1.3 pH-Insensitive Rhodamine Synthesis

To study the energy transfer between UCNP and rhodamine, it was thought that synthesis of a rhodamine derivative that was not sensitive to the pH of the surrounding media would be useful. This means that in all pH conditions, the fluorophore would be highly fluorescent and energy transfer between the UCNP core and fluorophore should occur independently of the environmental conditions.

Synthesis of an pH-insensitive rhodamine species has been achieved previously by inclusion of a piperazine unit.⁵² The piperazine group locks the rhodamine into its ring-opened form and prevents spirolactam ring closing from occurring, thereby resulting in a highly fluorescent rhodamine species which is fluorescent over a wide pH range.



Scheme 3.5: Synthesis of an pH-insensitive rhodamine bisphosphonate derivative. Reagents and reaction conditions: (i) 1-Boc piperazine, HBTU, DCM, NEt_3 , r.t, 2 h; (ii) TFA, DCM, r.t, 16 h; (iii) chloroacetyl chloride, DCM, NEt_3 , 0 °C, 3 h; (iv) **2.2**, MeCN, NEt_3 , 80 °C, 5 days; (v) glutaric anhydride, MeCN, reflux, 16 h; (vi) **2.2**, HBTU, NEt_3 , DCM, r.t, 16 h; (vii) TMSBr, dry DCM, N_2 , r.t, 16 h followed by MeOH, r.t, 1.5 h.

The first synthetic route attempted is shown in scheme 3.5, steps (i) to (iv). In the first step,

rhodamine-B was reacted with the mono-protected, 1-Boc-piperazine. This was achieved using HBTU as the coupling agent, with triethylamine as the base. It was found that the yield was much greater if this product was taken through to the deprotection step without purification. In the deprotection step, the Boc group was removed by TFA in DCM. The reaction proceeds via protonation of the *tert*-butyl carbamate, followed by loss of a *tert*-butyl cation. Decarboxylation of the carbamic acid results in the free amine. This product was purified by silica gel column chromatography, using a DCM:methanol solvent system. A dark pink solid resulted, the ^1H NMR spectrum of which is shown in figure 3.10.

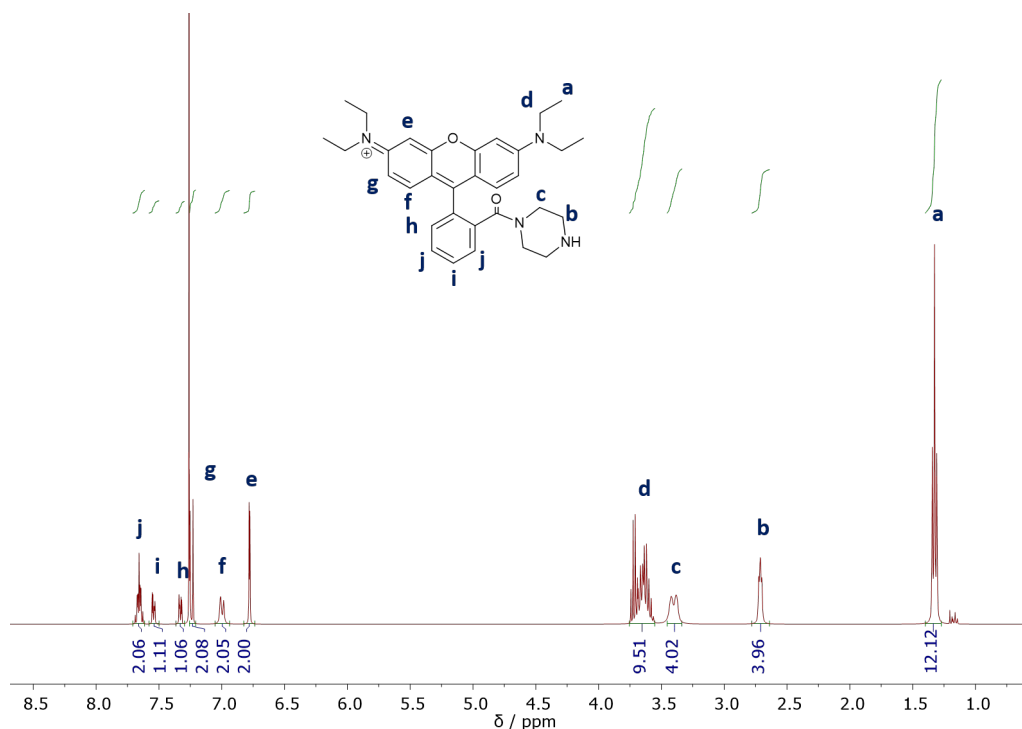


Figure 3.10: Assigned ^1H NMR spectrum of **3.7** (CDCl_3 , 400 MHz, 295 K).

Step (iii) was an acylation reaction of chloroacetyl chloride with **3.7**. The purpose was to prepare an pH-insensitive rhodamine derivative which could be functionalised with the bisphosphonate moiety **2.2** via an $\text{S}_{\text{N}}2$ reaction. **3.4** was purified by silica gel column chromatography, eluting with a DCM:methanol solvent system. Successful isolation of **3.4** was confirmed by ^1H NMR spectroscopy and by ESI-MS with a molecular ion m/z of 587. The following step proved to be problematic, with no reaction seen between **3.8** and **2.2**. After no reaction was seen in DCM at room temperature, harsher reaction conditions were trialled, heating the reaction at reflux in acetonitrile. The reaction was followed over 5 days by LCMS but the expected product m/z of 854 was never observed. The lack of reactivity is likely to be due to the bulky bisphosphonate ligand, hindering its approach to the alpha carbon. This is the same issue as was seen in the attempt at direct reaction of rhodamine-B with **2.2** (section 3.3.1.1). An alternative route was then developed, shown in scheme 3.5 steps (v) to (vii).

It was thought that a similar method to the synthesis of the pH-sensitive rhodamine (section

3.3.1.1) could be used, using a spacer group between the piperazine and bisphosphonate moieties. Reaction of **3.7** with glutaric anhydride led to the isolation of **3.10**, which was purified by silica gel column chromatography. In a similar method to that described in the synthesis of **3.5**, **3.10** was coupled to the bisphosphonate ligand **2.2** using HBTU as the coupling agent. The bisphosphonate ester was then deprotected with TMSBr to reveal the free bisphosphonic acid **3.12**.

3.3.1.4 pH-Insensitive Rhodamine Photophysical Properties

The photophysical properties of the pH-insensitive rhodamine derivative **3.12** were determined in an analogous method to that described in section 3.3.1.2. It was noted that the samples of the pH-insensitive rhodamine were much brighter than the pH-sensitive rhodamine and so samples were prepared at 6 μM , 5 times more dilute than the pH-sensitive rhodamine. As before, solutions of the rhodamine were prepared in aqueous 0.1 M KCl with the pH adjusted using 0.1 M HCl and 0.1 M NaOH. Solutions were prepared in the pH range 3 - 8. The absorption and emission spectra over the range of pH values are shown in figure 3.11. The spectra show consistent absorbance and emission across the pH range measured.

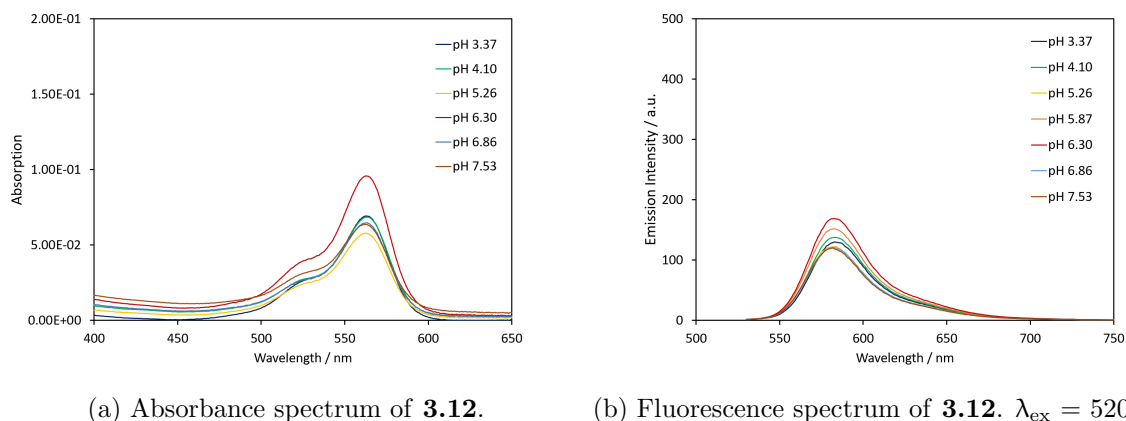


Figure 3.11: pH dependent absorption and emission spectra of **3.12**. Samples at a concentration of 6 μM in aqueous 0.1 M KCl.

The absorption values at 560 nm were normalised across the pH range and these values plotted against the pH of the solutions. Figure 3.12 shows that the pH does not affect the absorption of **3.12** and the compound fluoresces highly across biologically relevant pH values. It can be concluded that a pH-insensitive rhodamine bisphosphonate derivative was successfully synthesised.

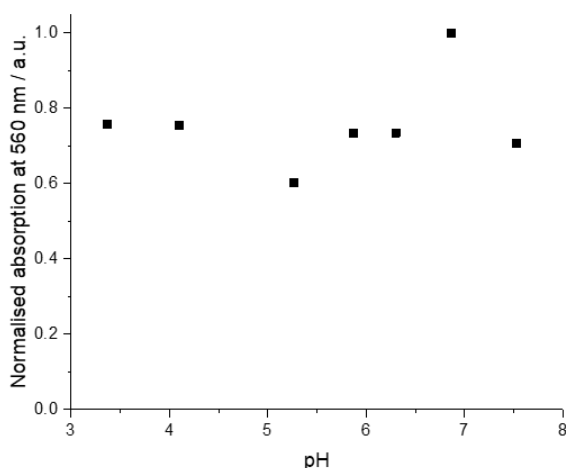


Figure 3.12: Plot showing absorption intensity at 560 nm at various pH solutions of **3.12**.

3.3.2 UCNP Functionalisation with Rhodamine-Bisphosphonates

After preparation of the pH-sensitive and pH-insensitive rhodamine bisphosphonic acid derivatives, the next step was to append these to the surface of $\text{NaYF}_4\text{:Yb,Er}$ UCNPs. The inclusion of **PEG-BP** ligands was required to ensure the nanoprobe were water soluble.

Multiple factors needed to be considered when deciding on the appropriate dye quantity to load on the nanoparticle surface. There needed to be a sufficient amount so that significant RET could occur between the UCNP and the dye. This would achieve modulation of the overall emission of the nanoprobe signal under varying environmental conditions. However, adding too much dye could compromise the solubility of the nanoprobe and could also lead to self-quenching between the fluorophore molecules.^{53–55} The dependence of RET on fluorophore density is not something which has been systematically investigated.⁵⁶ In order to optimise the output signal from the nanosensor, nanoparticles with a range of concentration of rhodamine on the surface of the UCNPs were prepared. These were: 1, 2, 5, 10, 20 & 50 mol %, relative to **PEG-BP** ligand concentration.

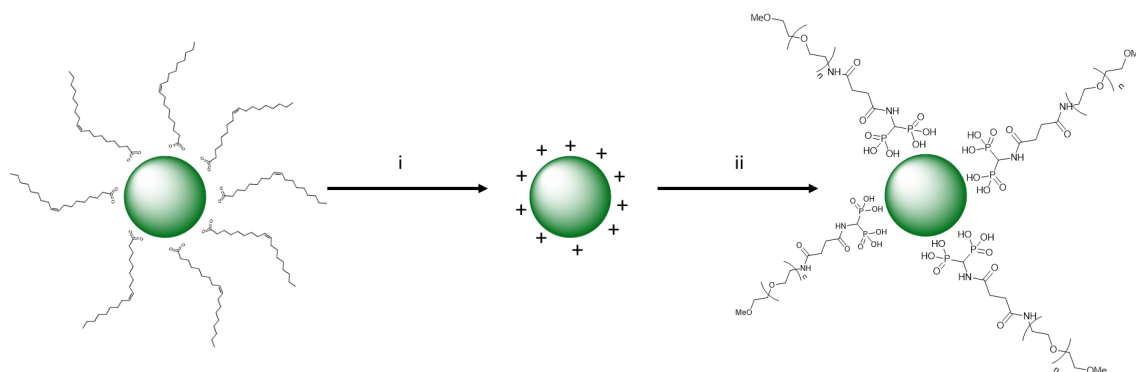
3.3.2.1 Ligand-Free Technique for Phase Transfer of UCNPs

A technique was required to exchange the native surface ligands for the synthesised **PEG-BP** and rhodamine-bisphosphonate ligands, **3.5** and **3.12**. In functionalising the UCNPs, it was necessary to be able to control the ratio of **PEG-BP** and rhodamine-BP ligands binding to the UCNP surface. The ligand-free technique is widely used as a method to functionalise the surface of UCNPs.⁵⁷ In this method, the surface oleate ligands are protonated by the addition of a weak acid and are stripped from the surface of the nanoparticles. At this stage, the UCNP surface is bare and this allows functionalisation with a controlled ratio of new ligands.

Scheme 3.6 shows the conditions required to achieve removal of oleic acid from the surface of

NaYF₄ NPs. The oleic acid capped NaYF₄:Yb,Er UCNPs were added to dilute HCl and the suspension was stirred at room temperature for four hours. The pH was monitored and adjusted to maintain pH 4. This pH value was shown by Bogdan et al. to result in nanoparticles with a positive ζ -potential of ~ 20 mV.^{57,58} This value is just below what would be considered a stable dispersion, suggesting the UCNPs may aggregate. It was found that if the ligand-free UCNPs were functionalised within 24 hours of preparation, no significant aggregation occurred.

The unbound oleic acid was then removed by washing with diethyl ether. A small amount of the sample was freeze dried and IR spectroscopy was used to verify the successful removal of the oleic acid ligand. The remaining sample was left as a dispersion in water, to help maintain the colloidal stability of the system.



Scheme 3.6: Two-step reaction to replace oleic acid ligands with **PEG** ligands on surface of NaYF₄:Yb,Er UCNPs, via ligand-free UCNPs. Reagents and reaction conditions: (i) 0.1 M HCl, pH 4, r.t, 4 h; (ii) **PEG-BP**, H₂O, pH 8, r.t, 2 h.

In the second step, **PEG-BP** was added to ligand-free UCNPs, in water. The pH was adjusted to 8 and then suspension was stirred at room temperature for 2 hours. Unbound **PEG-BP** was removed by precipitation of the UCNPs with addition of acetone, followed centrifugation. This process was carried out a total of three times. A small sample of the NaYF₄:Yb,Er@PEG-BP solid was retained for IR analysis; the remaining sample was resuspended in distilled water.

The IR spectra shown in figure 3.13 validate the successful removal of oleic acid ligands from the surface of the nanoparticles. This is most prominently observable as the lack of bands at 2800-2900 cm⁻¹ in the ligand-free UCNP samples, corresponding to the CH₂ and CH₃ stretches. The bands at 1557 and 1457 cm⁻¹ are also absent in the ligand-free UCNP sample, corresponding to -COO-(δ_{as}) and -COO-(δ_s). Water molecules directly coordinated to the nanoparticle surface are responsible for the band at 3200 cm⁻¹ in the ligand free samples. It is thought that the surface of the ligand-free nanoparticles are capped by -[OH₂]⁺, with a chloride counterion.⁵⁷

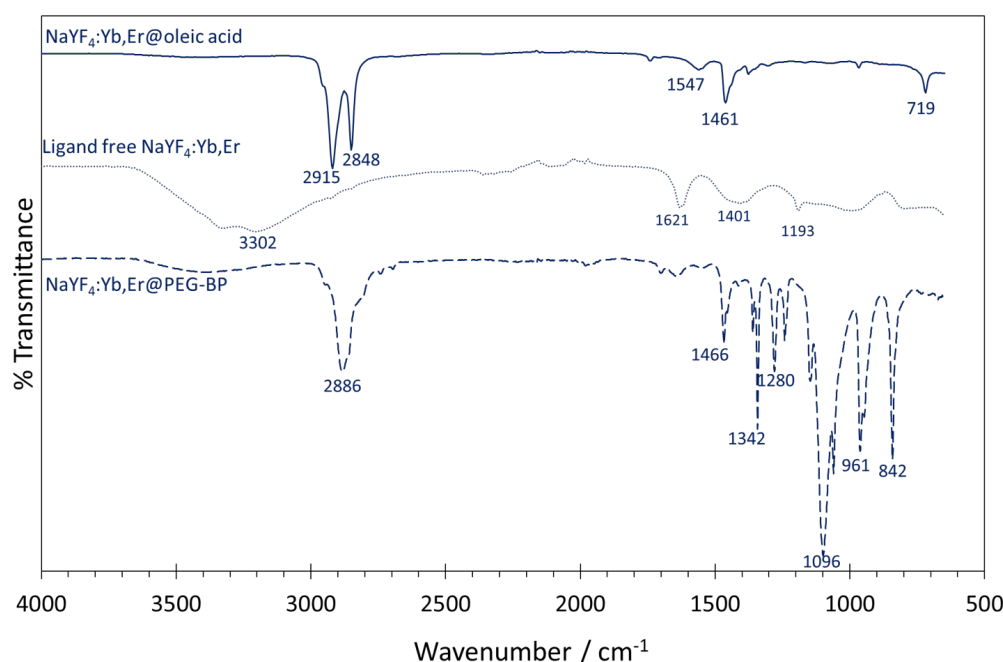


Figure 3.13: IR spectra of oleic acid-capped, ligand-free and **PEG-BP**-capped NaYF₄:Yb,Er UCNPs.

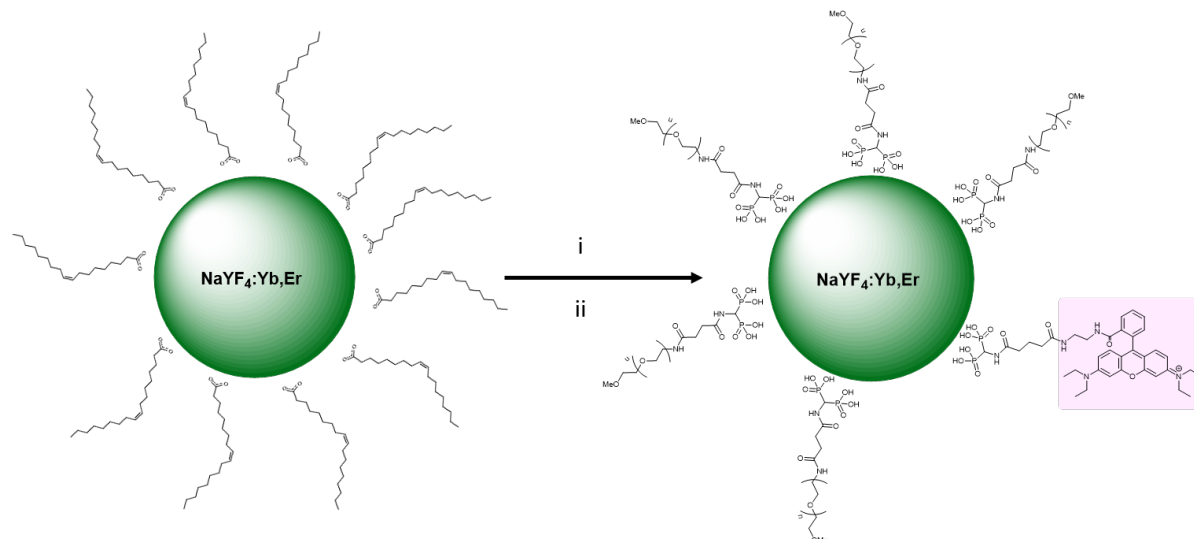
After the addition of **PEG-BP**, the IR spectrum resulting was almost identical to that of NaYF₄:Yb,Er@PEG-BP synthesised by direct ligand exchange. The IR of the direct ligand exchange by sonication, was shown previously in figure 2.23. The similarity between the spectra shows that ligand exchange via a ligand-free method is successful in functionalising the surface of NaYF₄:Yb,Er UCNPs.

3.3.2.2 One-Pot Functionalisation of NaYF₄:Yb,Er UCNPs

3.5 was the chosen rhodamine ligand for UCNP functionalisation due to its pH-dependent photoluminescent properties. It was thought that the functionalised NaYF₄:Yb,Er UCNPs could act as a nanosensor. At physiological pH 7.4, **3.5** is in the ring-closed form. Under excitation of the nanoprobe at 980 nm, the emission would be only from the UCNPs. At a pH below 5.8, **3.5** would be in the ring-opened conformer. Excitation of the nanoprobe under these conditions should result in quenching of the 650 nm emission from the UCNPs and a detectable rhodamine emission at 580 nm.

In the first functionalisation method attempted, **PEG-BP** and **3.5** were added to the UCNPs in one step. This is termed the ‘one-pot functionalisation’ method. In the one-pot UCNP functionalisation method, first the UCNPs were treated with dilute HCl to remove the native oleic acid ligands. Then a mixture of **PEG-BP** and **3.5** were added to the ligand-free NaYF₄:Yb,Er UCNPs in water, with the pH adjusted to 8. This is shown in scheme 3.7. The ligands were added in large excess; to a 2 mg sample of ligand-free UCNPs, 10 mg **PEG-BP** was added.

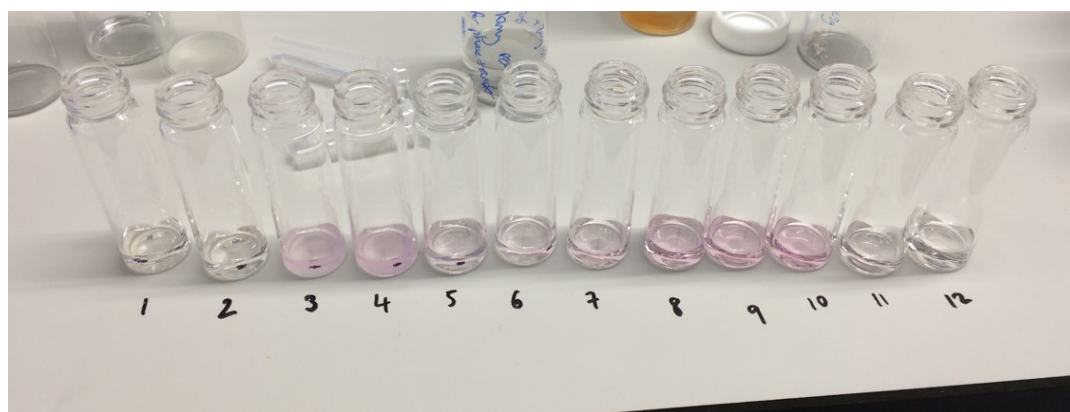
The mass of **3.5** was calculated as a molar ratio in comparison to 10 mg **PEG-BP**. Functionalisation of UCNPs with **3.5** was carried out in a range of ratios: 1, 2, 5, 10, 20 & 50 mol % (**3.5** : **PEG-BP**). A large excess of ligand was added to ensure the surfaces of the UCNPs were fully saturated with ligand.



Scheme 3.7: The one-pot method of functionalising NaYF₄:Yb,Er UCNPs with rhodamine and **PEG-BP** ligands. Reagents and reaction conditions: (i) 0.1 M HCl, r.t, 4 h; (ii) **PEG-BP**, **3.5**, H₂O, pH 8, r.t, 2 h.

Two different purification techniques were used to separate the fluorophore-functionalised UC-NPs and the free ligands. Initially an Amicon centrifugal filter unit was used with a molecular weight cut-off of 30,000 Da. In this method, the free ligand should pass through the filter and the nanoparticle sample should remain in the retentate. However it was found that **3.5** stuck to the filter membrane and the sample could not be recovered.

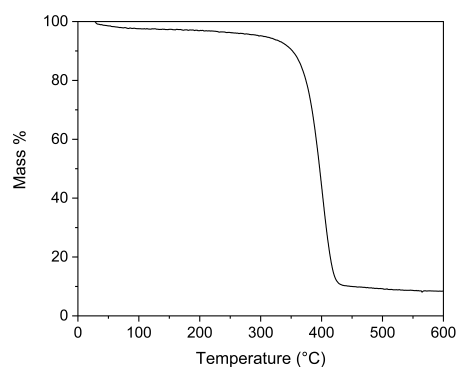
The second purification technique explored was the use of a size-exclusion column. A Sephadex PD10 column was chosen, where the nanoparticle sample should pass through the column first and then the free ligand should be eluted subsequently. The size exclusion column did result in separation of the sample into separate bands, as can be seen in figure 3.14 and 3.14b. However, thermogravimetric analysis (TGA) of the samples recovered from the column showed that the sample recovered was predominantly ligand. Decomposition of NaYF₄ NPs occurs above 450 °C, as was seen in figure 2.10. Figure 3.14c shows that no significant mass loss could be seen above that temperature and 90 % of the sample decomposed by 400 °C. This mass loss therefore must be due to presence of the ligand. The TGA results demonstrate that the UCNPs stuck to the size exclusion column and could not be recovered.



(a)



(b)



(c)

Figure 3.14: (a) Photograph of fractions obtained by size-exclusion chromatography of $\text{NaYF}_4:\text{Yb,Er}$ UCNPs functionalised with **3.5** and **PEG-BP**; (b) Photograph of size-exclusion column with remaining pink band which was unable to be eluted; (c) Representative TGA of sample recovered from column. Plot shows 90 % of sample decomposed before 400 °C, identifying the sample as predominately ligand.

Due to the issues of lack of recovery of the product from the centrifugal filter units and size exclusion columns, a simple purification technique where the nanomaterial was precipitated by addition of acetone was adopted. In this process, the free ligand remained in solution and could be easily removed by centrifugation. The drawback to this technique is that some of the nanoparticle product can be lost in the washing step; it is challenging to precipitate all of the UCNPs and some nanomaterial stays dispersed in the solvent.

Photophysical Characterisation:

The pH-sensitive rhodamine-functionalised nanoparticles were characterised by upconversion photoluminescence spectroscopy. The data is shown in figure 3.15. Initially, the emission properties of the nanoprobe were studied at either end of the pH responsive range, as determined in section 3.3.1.2. Solutions of pH-sensitive rhodamine-functionalised UCNPs (1, 2, 5, 10, 20 & 50 mol %) were prepared at pH 4 and pH 7 and excited at 980 nm. (These measurements were kindly carried out by Dr Shuai Zha at Hong Kong Baptist University.)

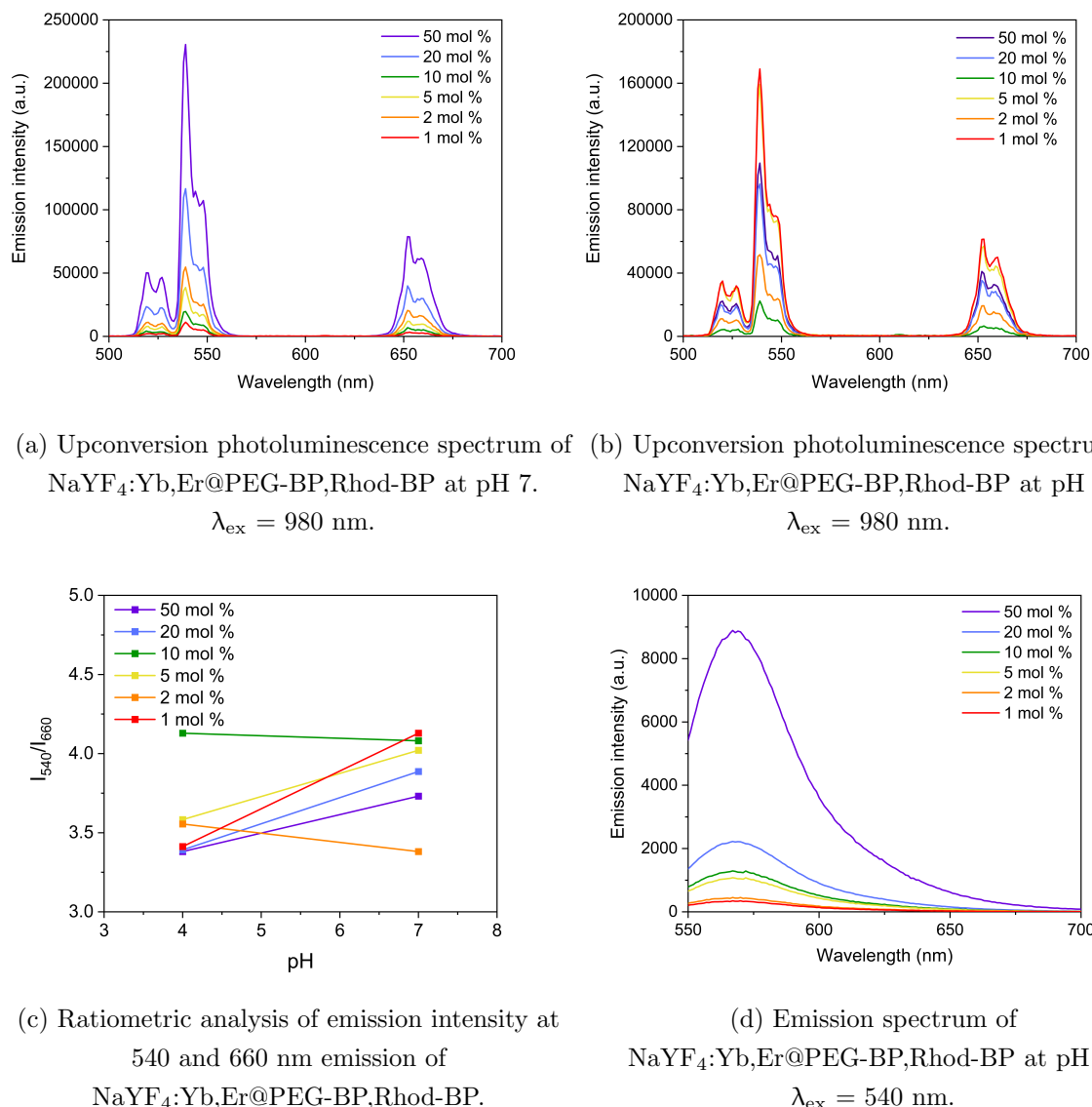


Figure 3.15: Photoluminescence spectra of NaYF₄:Yb,Er UCNPs functionalised with **3.5** and PEG-BP. Stated are the mol % of rhodamine (**3.5**) added in each sample. Samples at a concentration of 1 mg/mL.

At pH 7, it was expected that there would be no emission from rhodamine and only emission from the UCNPs should be visible. This is indeed what is seen in figure 3.15a. At pH 4, **3.5** should be in its ring-opened conformer and therefore highly fluorescent. Upon excitation at 980 nm, it was expected that the green UCNP emission peak at 540 nm would be quenched by RET to the surface fluorophore. Rhodamine emission should be visible at 580 nm. However, this is not what is seen in figure 3.15b. There is no rhodamine emission visible at 580 nm and the green UCNP emission peak at 540 nm is not visibly quenched.

A ratiometric analysis is shown in figure 3.15c, showing the green:red (540:660 nm) emission intensity. The data shows that there is no significant quenching of the erbium emission peak at 540 nm, suggesting energy transfer to rhodamine is not occurring. To check there was **3.5**

present in the nanomaterial, the sample was excited at 540 nm. This acted to directly excite the rhodamine fluorophore, rather than the indirect excitation at 980 nm. Figure 3.15d shows weak emission from rhodamine at 580 nm. The weak intensity of this emission suggested that the reason RET could not be detected may have been due to a low amount of rhodamine binding to the UCNP surface.

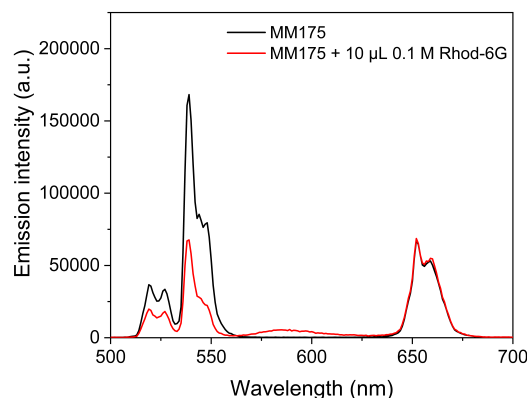


Figure 3.16: Upconversion photoluminescence spectrum of $\text{NaYF}_4:\text{Yb,Er@PEG-BP,Rhod-BP}$ at pH 4 after the addition of 10 μL 0.1 M Rhodamine-6G. $\lambda_{\text{ex}} = 980 \text{ nm}$.

To validate energy transfer could occur between $\text{NaYF}_4:\text{Yb,Er@PEG-BP,Rhod-BP}$ UCNPs in water, additional rhodamine was added. Rhodamine-6G was added in large excess, to a dispersion of $\text{NaYF}_4:\text{Yb,Er}$ UCNPs capped with **PEG-BP** and **3.5**. The sample was then excited at 980 nm. Figure 3.16 shows a quenching of the erbium emission peak at 540 nm, compared to the peak at 660 nm. There is also appearance of a rhodamine emission peak, centred at 580 nm. This result confirms that energy transfer can occur between $\text{NaYF}_4:\text{Yb,Er:PEG-BP,Rhod-BP}$ UCNPs and rhodamine, in this case by an inner-filter mechanism.

3.3.2.3 Stepwise Functionalisation of $\text{NaYF}_4:\text{Yb,Er}$ UCNPs

It was thought that the reason significant RET was not observed between the UCNP and the pH-sensitive rhodamine fluorophore on the surface, was that the density of fluorophores on the UCNP surface was low. The **PEG-BP** ligand is bulky so this may have prevented **3.5** from coordinating to the surface of the UCNPs, when both ligands were added in the same step. A 2-step process was proposed to facilitate the coordination of a greater amount of **3.5** to the UCNP surface. In this method, first **3.5** was added and stirred with ligand-free UCNPs, at pH 8. After 2 hours, **PEG-BP** was added and this dispersion was left stirring at room temperature overnight. The nanomaterial was purified by precipitation with acetone and centrifugation, as discussed in section 3.3.2.2.

It was impossible to calculate the amount of each ligand necessary to allow a specific molar ratio of **PEG-BP** to **3.5**. This is because it was not possible to know how much of the ligand **3.5** would bind to the ligand-free UCNPs in dispersion in the first functionalisation step. Therefore

the quantity of **3.5** used was in the same excess as in the one-pot method, referred to as 1 eq, 2 eq etc.

Photophysical Characterisation:

The NaYF₄:Yb,Er UCNPs, functionalised with **PEG-BP** and **3.5** were characterised in an analogous method to that described in the one-pot synthesis method. Figure 3.17 shows the upconversion photoluminescence spectra of the samples, excited at 980 nm. (These measurements were kindly carried out by Dr Shuai Zha at Hong Kong Baptist University.)

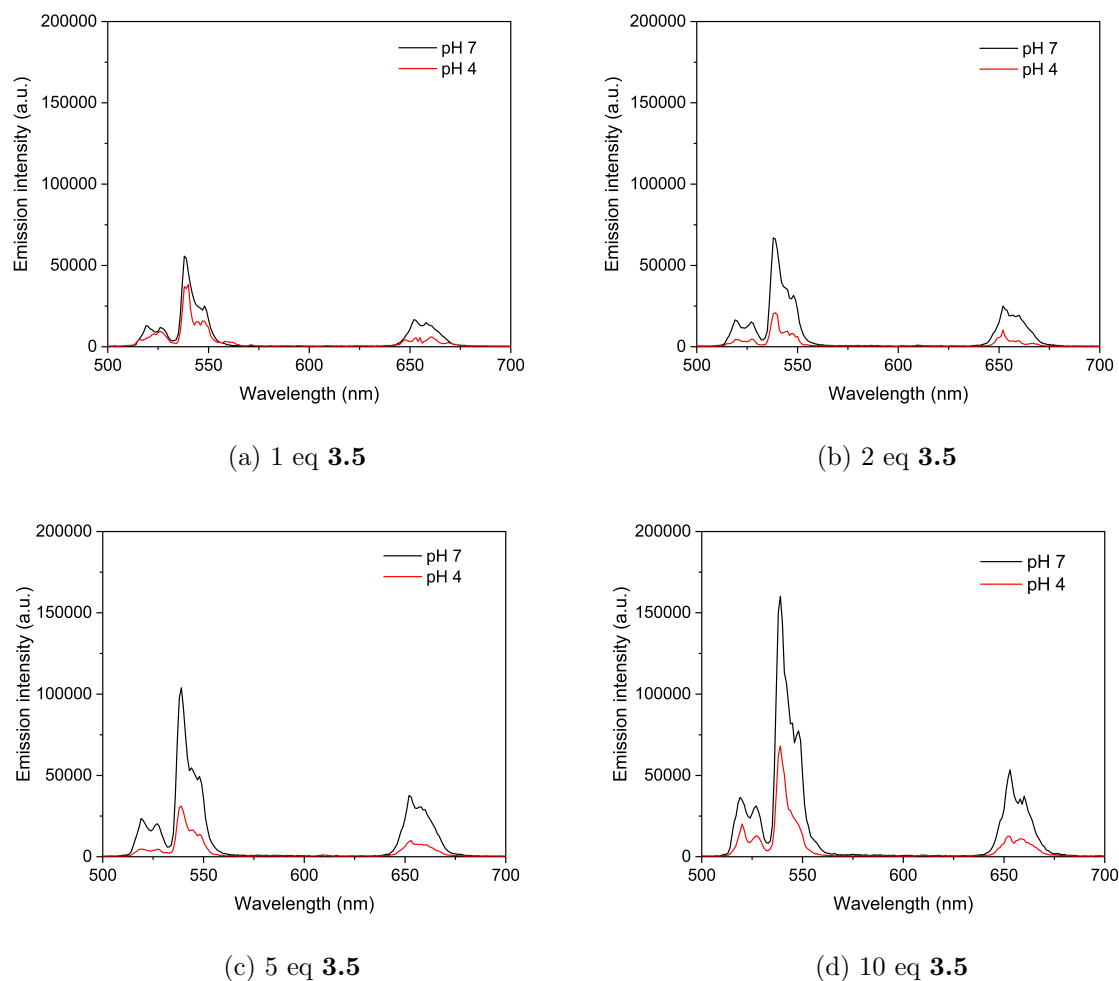


Figure 3.17: Photoluminescence spectra of NaYF₄:Yb,Er UCNPs functionalised with **3.5** and **PEG-BP**, using a stepwise method. $\lambda_{\text{ex}} = 980$ nm. Samples at a concentration of 2 mg/mL.

Unfortunately, the results do not show evidence of RET between the UCNP core and surface rhodamine ligands. There was no observable rhodamine emission at 580 nm. In comparison to literature results, the lack of rhodamine emission at 580 nm is not unexpected. There are examples of energy transfer from NaYF₄:Yb,Er to surface rhodamine, where only quenching of the emission at 540 nm was observed; there was no rhodamine emission visible at 580 nm.¹⁹

Analysis of the ratiometric data in figure 3.18 disappointingly shows that there is no correlation

between the ratio of the emission peaks at 540 and 660 nm and the pH. Unexpectedly, the results show a weaker emission of both the 540 and 660 nm peaks at pH 4, in comparison to the samples at pH 7. This was also seen in the one-pot synthesis method, shown in figure 3.15. In using the red band as an internal reference, it is assumed that the transition is not affected by quenching of other emissive states. There is some suggestion in the literature that this assumption may not be valid. Dukhno et al. showed that the lifetime of NaYF₄:Yb,Er emission at 660 nm decreased when RET occurred from the 540 nm emission in the system.⁵⁶

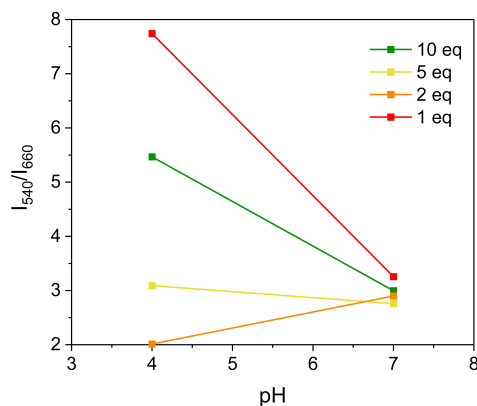


Figure 3.18: Ratiometric analysis of emission intensity at 540 and 660 nm emission of NaYF₄:Yb,Er@PEG-BP,Rhod-BP synthesised by a stepwise method.

3.4 Conclusions

The synthesis and characterisation of two novel rhodamine-bisphosphonate derivatives was described. **3.5** showed pH dependant emission, displaying strong fluorescence below pH 5.85. Above this pH value, the rhodamine ligand was in a ring-closed, non-fluorescent state. **3.12** was synthesised as a pH-insensitive rhodamine-bisphosphonate derivative and was highly fluorescent over all pH values tested.

The pH-sensitive rhodamine derivative **3.5** was used to functionalise NaYF₄:Yb,Er UCNPs. **PEG-BP** ligands were used to facilitate dispersibility of the nanoprobe in aqueous solvents. Two methods were trialled to functionalise the UCNP surface: a one-step method and a stepwise method. The one-step method resulted in very little **3.5** binding to the UCNP surface and this has been attributed to the large **PEG-BP** ligand blocking rhodamine from binding. It was thought that a stepwise method may be able to circumvent this issue. However, preliminary data using a stepwise method also did not result in RET between the UCNP and **3.5**.

Time constraints prevented this method being pursued any further but there is vast scope here to modify the system, promote RET and assemble a nanosensor. RET is distance dependent and therefore will only occur from surface lanthanide ions.^{55,59,60} The lanthanide ions in the centre of the nanocrystal will be too far from the fluorophore to contribute to energy transfer. This means for large UCNPs, most of the emission signal observed will be from lanthanide ions not undergoing RET. This saturates the observed signal and significant quenching in the system cannot be observed. By decreasing the size of the UCNPs, many more lanthanide ions proportionally will sit close to the UCNP surface. Further work should focus on using much smaller UCNPs, ~10 nm - compared to the 50 nm UCNPs used in this work.

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Chapter 4

Sensitizing UCNP Emission

4.1 Introduction

Upconversion nanoparticles absorb light poorly and often exhibit weak emission intensities.¹ This is most noticeable when the nanoparticles are phase-transferred into aqueous environments. The high energy vibrational modes of -OH groups in the surrounding solvents have been shown to increase the non-radiative relaxation of Er^{3+} emitters, thereby quenching the UC emission.^{2,3} Lanthanide f-f transitions are partially parity forbidden and so they exhibit long luminescence lifetimes, on the order of hundreds of microseconds.⁴ Non-radiative pathways, such as surface quenching, compete with the radiative emission and so a significant proportion of the energy input is lost via non-radiative pathways. Arppe et al. reported that up to 99.9 % of the luminescence intensity can be quenched by water.² Earlier in this thesis, it was shown $\text{NaYF}_4:\text{Yb},\text{Er}@ \text{PEG-BP}$ had a twelve times weaker emission in water than the same sample in chloroform. The weak emission intensity of upconversion nanoparticles has held back progress in developing UCNPs for applications in biomedical imaging. Improvements in both the upconversion efficiency and brightness of the systems is necessary for UCNPs to be used in real-world applications.^{1,5-8}

The issue of poor brightness of UCNPs can be at least partly attributed to the narrow absorption band of the sensitizer Yb^{3+} . Yb^{3+} has only one excited state, with the absorption at 980 nm corresponding to the $^2\text{F}_{7/2} \rightarrow ^2\text{F}_{5/2}$.⁴ Yb^{3+} has a small absorption cross section ($\sim 10^{-20} \text{ cm}^2$), much smaller than organic dyes ($\sim 10^{-17} - 10^{-16} \text{ cm}^2$).⁹ Another issue with direct excitation of Yb^{3+} at 980 nm, is that the wavelength overlaps with water absorption. As much of biological tissue is made up of water, irradiation with a 980 nm laser has been shown to lead to local temperature rises and significant overheating in *in vivo* studies.¹⁰ Wang et al. have shown, in both *in vitro* and *in vivo* investigations, that irradiation at 980 nm causes cell death. Thermal imaging of a mouse showed a significant hotspot caused by 980 nm irradiation. The images are shown in figure 4.1.

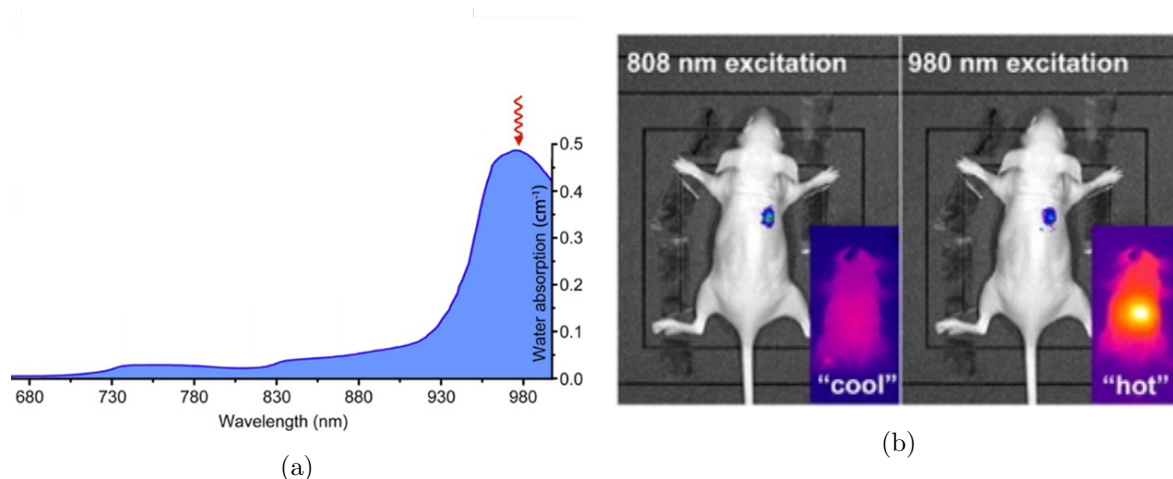


Figure 4.1: Images demonstrating issues with excitation at 980 nm. (a) Absorption spectrum of water, showing significant absorption at 980 nm; (b) thermal images of mice irradiated at 808 and 980 nm. 808 nm irradiation showed significantly less localised heating than 980 nm irradiation. Adapted with permission from reference 10. Copyright 2013 American Chemical Society.

Since 2012, there have been two main methods explored to overcome the issues of low upconversion efficiency, weak signal brightness and tissue heating effects. These strategies are: organic dye sensitization and Nd^{3+} sensitization. In the following sections, these two strategies will be discussed with a focus on how these methods can be used to improve the brightness of UCNPs for bioimaging applications.

4.1.1 Dye Sensitization

An approach to overcome the intrinsic issue with the small absorption cross section of Yb^{3+} ions is to introduce an organic fluorophore, as a light absorbing moiety. By exploiting the antenna effect, light can be harvested by an organic dye coordinated to the nanoparticle surface. If the emission band of the fluorophore overlaps with the absorption band of ytterbium, then energy transfer can take place. This can achieve sensitization of the system emission.

There are a number of requirements for an effective sensitizing dye:

- **Spectral overlap:** There must be an overlap of the emission band of the organic fluorophore and the absorption band of Yb^{3+} for efficient energy transfer to take place.
- **No reabsorption:** It is important that there is no absorption of the upconversion emission by the dye.
- **Suitable functionality for surface coordination:** The sensitizing dye must have functional groups which can coordinate to the surface of the nanoparticle with a binding strength sufficient to create a stable nanosystem.
- **Photostability:** NIR dyes are notoriously photosensitive but it is essential that the

surface dye is stable and will not decompose upon repeated exposure to radiation.

The first example of dye sensitization for UCNPs is shown in figure 4.2. In 2012, Zou et al. functionalised the surface of UCNPs with the NIR dye IR-806 (see figure 4.3).^{11,12} In this work, the authors achieved a 3,300 times emission enhancement compared to the non-sensitized nanoparticles, in the range 730 – 1,000 nm. Figure 4.2b shows the spectral overlap between the emission of IR-806 and the absorption of the UCNPs (predominately Yb^{3+} at 980 nm). The significantly larger absorption cross section of the organic dye (10^{-16} cm^2) compared to lanthanides (10^{-20} cm^2) allows broadband excitation, rather than the conventionally used narrow band NIR laser diode. The authors used a core-only UCNP system so that the distance between the organic surface molecules and surface ytterbium ions was minimised, thereby maximising the energy transfer efficiency.

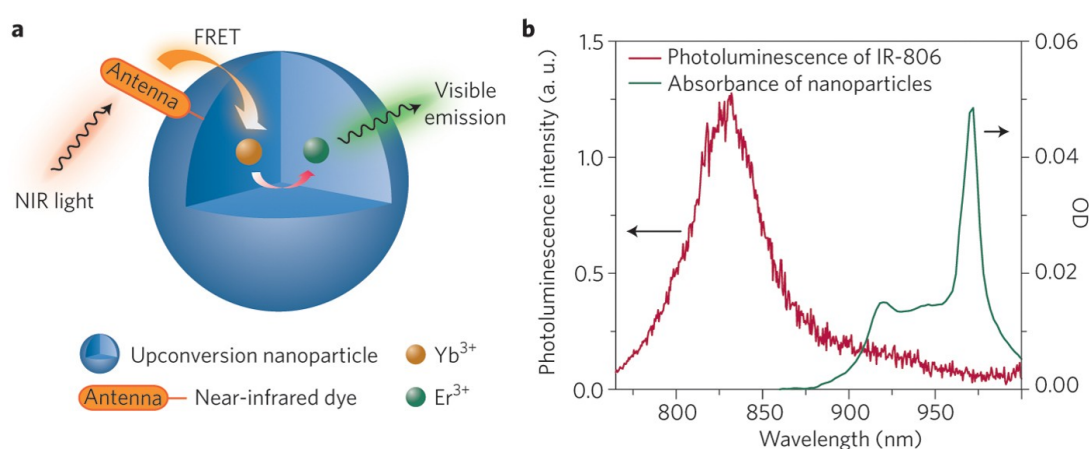


Figure 4.2: NIR sensitization of UCNPs by Zou et al. (a) schematic showing structure of IR-806 sensitized UCNPs; (b) spectral data showing overlap of absorbance band of UCNPs and photoluminescence of IR-806, suggesting ET could take place. Reprinted by permission from: Springer Nature, Nature Publishing Group, Nature Materials, reference 12. Copyright 2012.

Further studies have subsequently been carried out where a core-active shell structure is used.^{13–15} In these examples ytterbium is doped into the shell of the UCNPs. The use of a shell material reduces surface quenching of UCNPs by introducing a protecting layer between the lanthanide emitters and the surface quenchers. Doping ytterbium into the UCNP shell facilitates energy transfer from the surface organic dye, across the inorganic shell to the lanthanide ions in the core of the UCNP.

Work in 2018 by Garfield et al. uncovered a triplet state which formed when IR-806 was attached to the surface of $\text{NaYF}_4:\text{Yb},\text{Er}$ UCNPs.¹⁶ The inter-system crossing ($\text{S}_1 \rightarrow \text{T}_1$) to the triplet state is enhanced by the heavy atom effect, due to the presence of ytterbium ions close to the UCNP surface. At 30% Gd doping, the authors found there was an enhancement of emission intensity of 15,000 times in comparison to direct excitation of UCNPs at the same power density. The results demonstrate that by having a clearer understanding of the photophysics of these systems, this should allow the design of nanoprobes which display both enhanced emission intensity and

increased upconversion efficiency. There are however limitations with this particular system. The long-lived triplet state suffers from photooxidation by water and reactive oxygen species, making the system unsuitable for bioimaging based applications.¹⁷

Since 2012, a number of different NIR dyes have been used to increase the brightness of UCNPs. The structures of a selection of these are shown in figure 4.3.

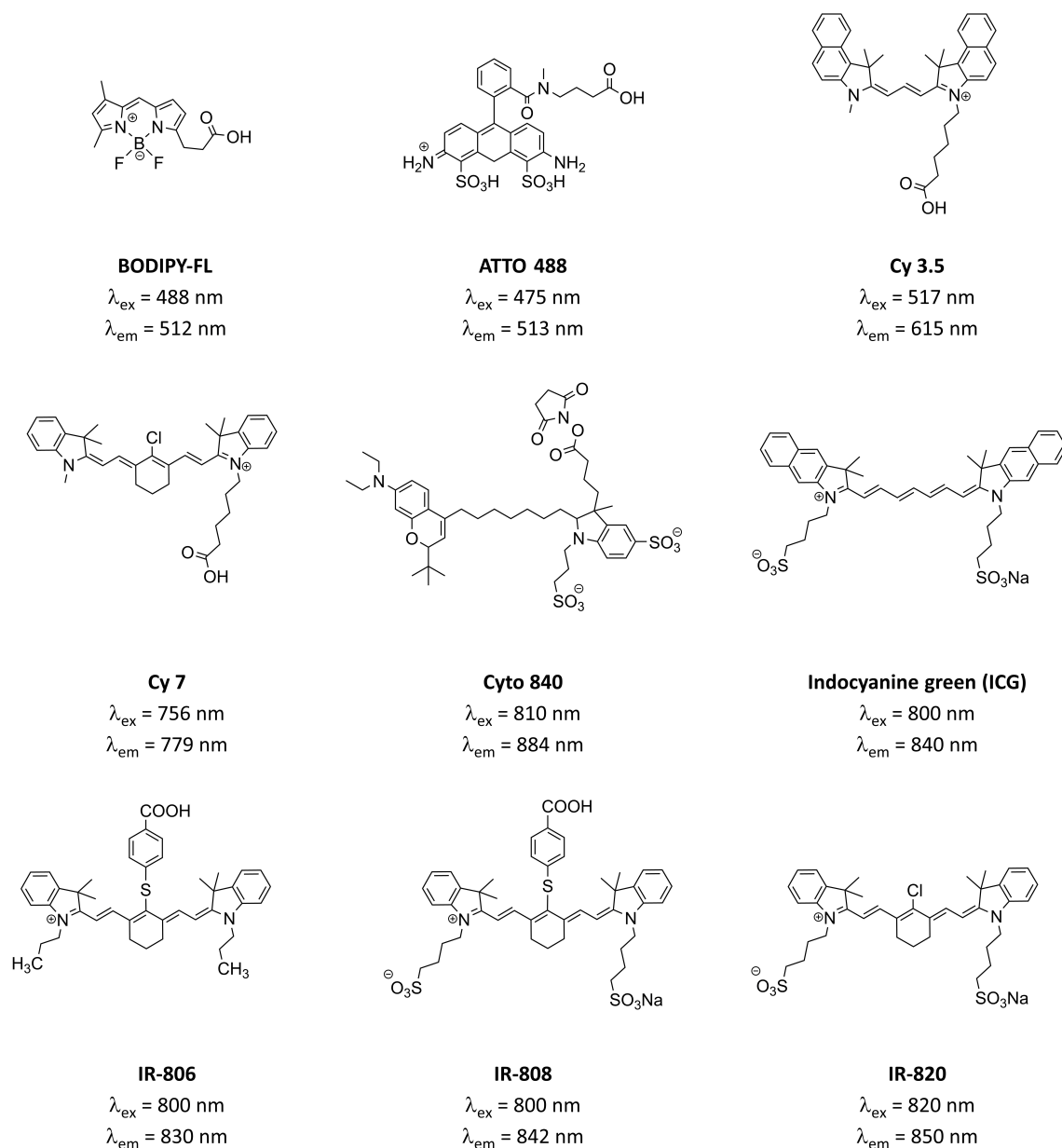


Figure 4.3: Examples of organic dyes which have been used to sensitize UCNP emission: ATTO¹⁸, BODIPY¹⁹, Cy3.5¹⁹, Cy7²⁰, Cyto840¹⁸, ICG²¹, IR-806¹⁹, IR-808⁹, IR-820⁹. The names of the NIR-dyes are often based on their absorption maxima; fluorophores with the same name may have slightly different structures in the literature.

The above examples all demonstrate absorption sensitization, where the absorption of the nanoparticle is enhanced by an organic dye on the surface of the nanoparticles. Wisser et al. demonstrated that surface ATTO 542 dyes can act as emission sensitizers, where the emission from the nanoparticle is enhanced by facilitating fast energy transfer from Er^{3+} to the surface dye.²² This was shown to enhance the radiative rates from the lanthanide emitters, out-competing the usual dominating non-radiative decay pathways. The emission from the system was shown to increase with the dye-decorated organically soluble nanoparticles twice as bright as the unmodified NPs and ten times as bright as the water soluble NPs. The relevant energy level diagram for the system is shown in figure 4.4.

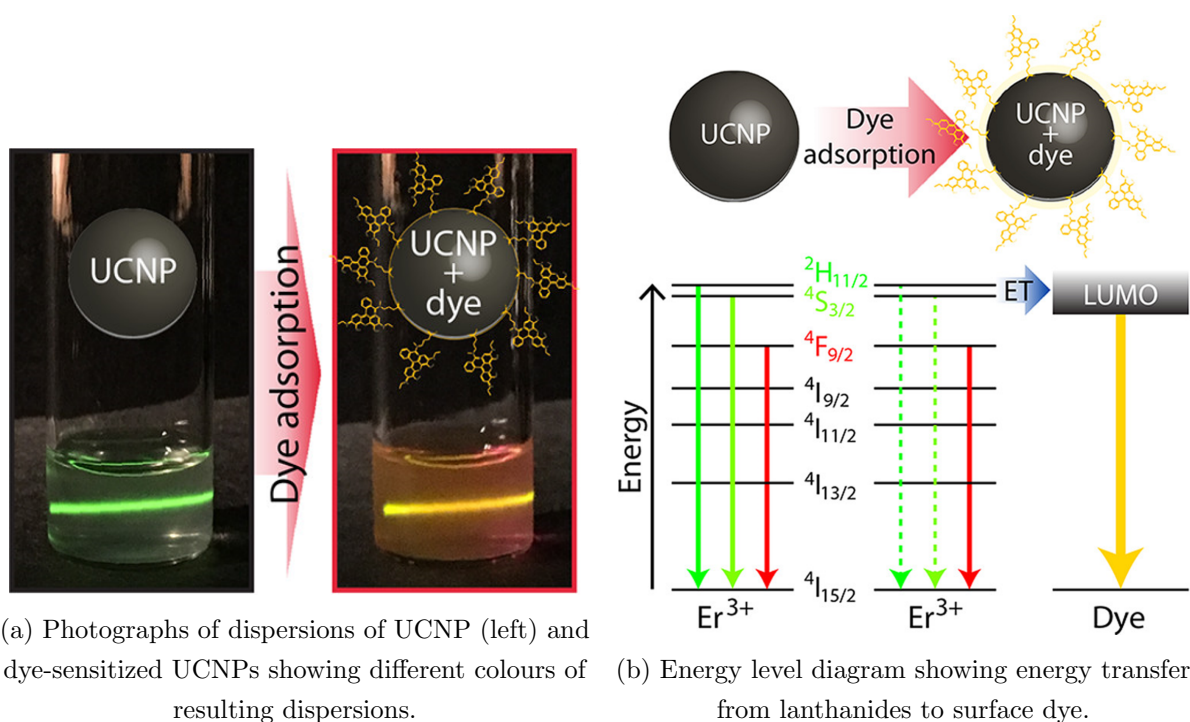


Figure 4.4: Work by Wisser et al. achieving emission sensitization with use of ATTO 542 surface dye. Reprinted with permission from reference 22. Copyright 2018 American Chemical Society.

4.1.2 Neodymium Sensitization

The absorption cross-section of neodymium(III) is $\sim 10^{-19} \text{ cm}^2$ at 808 nm. This value is ten times higher than the absorption cross-section of ytterbium(III) at 980 nm ($\sim 10^{-20} \text{ cm}^2$).^{23,24} The inclusion of Nd(III) in UCNPs allows excitation at 808 nm. This is particularly advantageous in bioimaging as at this wavelength, the absorption by water molecules in tissue is significantly lower than water absorption at 980 nm.⁸

In 2013 there were a flurry of papers published in which Nd^{3+} was used to sensitize UCNP emission.^{23–26} Initially, Shen et al. doped Nd^{3+} into the core of UCNPs, with the doping concentration limited to 1% due to deleterious back energy transfer from Er^{3+} .²³ Further work by other groups led to the adoption of a core-shell structure to spatially separate Nd^{3+} from

the activator ions, limiting this energy transfer.^{24,26} Xie et al. reported that doping Nd^{3+} at a concentration of 20 mol % resulted in an UC emission of the same intensity magnitude as the 980 nm excited UCNPs and with the additional benefit of reduced overheating in cells.²⁴ More recently there have been a number of papers published where a variety of core-shell structures with Nd^{3+} doping have been devised, in the attempt to increase the brightness of these systems.^{27–30} The increase in brightness achieved by inclusion of Nd^{3+} in the UCNP structure is not particularly significant but the real advantage for these systems is the reduction in tissue overheating. For this reason neodymium(III) doping is becoming more common in bioimaging UCNP probes developed in the past few years.

4.1.3 Combined NIR and Nd Sensitization

Recently, the strategies of dye sensitization and Nd^{3+} sensitization have been combined in a ‘energy cascade upconversion’ method.^{9,21,31–33} This strategy aims to overcome the issues with the mismatch in the emission band of NIR surface dyes and the absorption band of ytterbium. In this multistep energy transfer mechanism, the surface dye is excited and efficiently transfers energy to the Nd^{3+} species doped into the shell of the UCNP. This is possible due to the strong spectral overlap of the dye emission spectrum and the Nd^{3+} absorption spectrum. The energy is then transferred sequentially to the sensitizer (usually ytterbium) and then the emitting species (often Er^{3+} or Tm^{3+}). See figure 4.5 for a schematic showing the energy transfer pathway. Wei et al. reported that the ‘energy cascade upconversion’ method enabled high Nd^{3+} doping (up to 20 mol %) and resulted in a 10 times increase in brightness in comparison to the non dye-sensitized, Nd^{3+} doped UCNPs.³¹

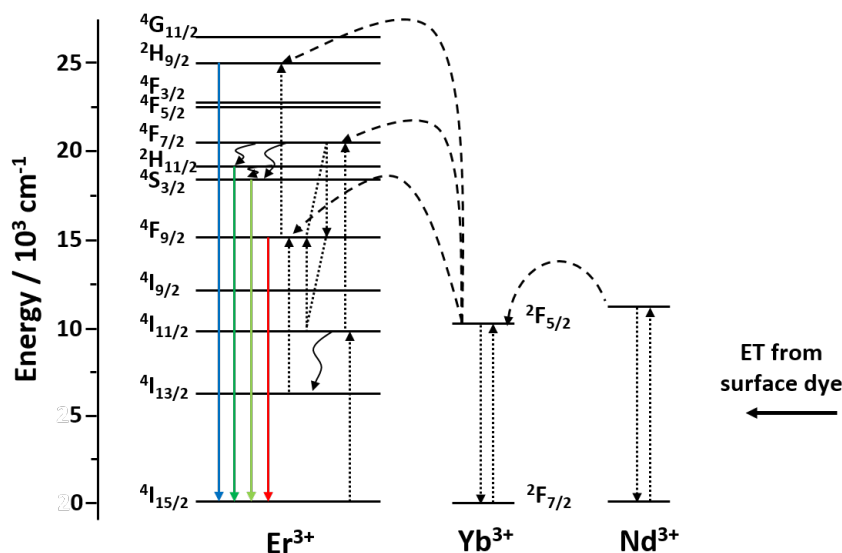


Figure 4.5: Energy transfer pathways in cyanine-sensitized UCNP nanosystems. Energy is transferred between cyanine surface dye, to neodymium in the nanoparticle shell, to the ytterbium sensitizers in the nanoparticle core and finally to the erbium emitter ions.

The energy cascade upconversion strategy has been employed for environmental sensing, with

examples of temperature sensing in the literature.^{34,35} More recently, there has been a focus on developing dye sensitized UCNPs suitable for *in vivo* applications.^{20,36–39} The first example of this was published by Zou et al. in 2016.³⁶ In this work, Nd^{3+} was doped in the shell of a core-shell UCNP. The NIR dye Cy7 was bound to the surface of the nanoparticle and the whole system was encapsulated in the amphiphilic phospholipid, phosphocholine. The nanocomposite was used for mouse lymph node imaging. There are more recent examples using dye sensitized UCNPs in blood vessel imaging and lymph node dissection²⁰, glutathione detection *in vivo*³⁸, brain imaging³⁹ and for *in vivo* PDT on mouse tumours³⁷.

4.2 Aims and Objectives

UCNP brightness has been found to be a limiting factor in the applicability of UCNP based nanoprobes in the field of bioimaging. In this chapter, an aim of the work was to improve the brightness of UCNP-based nanosystems. Cyanine dyes were chosen as the sensitizing species. Previous literature details the suitability of cyanines due to their excitation wavelength which is within the tissue optical window. There is also the overlap of their emission band with the absorption band of lanthanides such as ytterbium and neodymium.

In this chapter, the aim was to synthesise core-shell $\text{NaYF}_4:\text{Yb,Er}@ \text{NaYF}_4:\text{Nd}$ UCNPs, doping neodymium into the nanoparticle shell. It was thought that this should facilitate energy transfer from the sensitizer step-wise through to the activator species, erbium. The stability of the bisphosphonate bond to the UCNP surface was shown in chapter 2; the objective of the work in this chapter was to assemble a nanoprobe where the cyanine is bound to the UCNP surface through a bisphosphonate moiety. The structure of the proposed probe is shown in figure 4.6, where **PEG-BP** acts to facilitate water solubility of the nanoprobe.

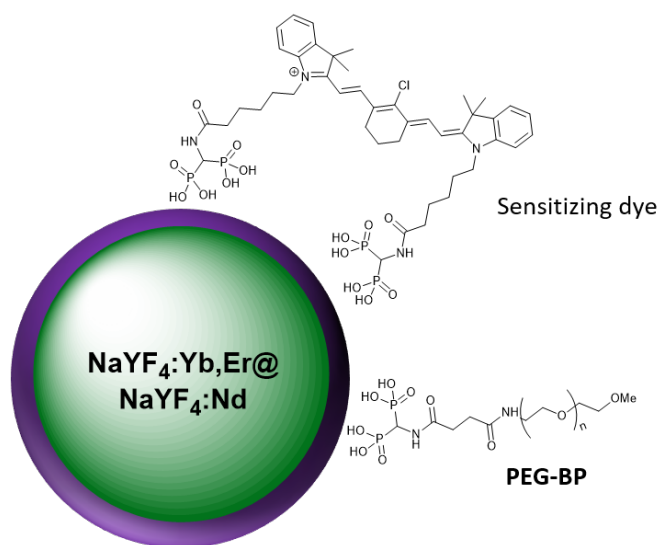


Figure 4.6: Structural representation of the proposed cyanine-sensitized $\text{NaYF}_4:\text{Yb,Er}@ \text{NaYF}_4:\text{Nd}$ UCNPs.

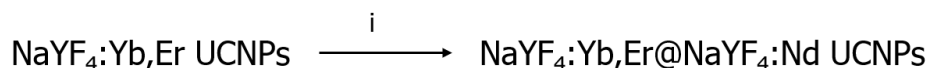
4.3 Results and Discussion

The first step towards dye-sensitized nanosystems was the synthesis of core-shell UCNPs, with neodymium doped into the shell. The synthesis and characterisation of these nanoprobes is discussed in section 4.3.1. A NIR dye which coordinates to the surface of neodymium doped core-shell UCNPs was developed. Heptamethiniocyanine dyes were chosen as the most suitable fluorophore and the synthesis of carboxylate-terminated and then bisphosphonic ester-terminated cyanine-808 are discussed in sections 4.3.2 and 4.3.3 respectively. Finally, the assembly of the dye-sensitized nanosystem is discussed in section 4.3.5.

4.3.1 Synthesis of Nd-doped NaYF₄:Yb,Er@NaYF₄:Nd UCNPs

In this work, Nd(III) was doped into the shell of UCNPs, allowing excitation of the nanomaterial at 803 nm. Core-shell UCNPs were synthesised in a method based on work by Wang et al.⁴⁰ In this method the authors used core NaGdF₄:Yb,Tm UCNPs as seeds for the epitaxial growth of a NaGdF₄:Tb shell. In an analogous technique, work in this project used NaYF₄:Yb,Er UCNPs synthesised in section 2.3.2.3 as the seed nanoparticles. A NaYF₄:Nd shell was grown on the surface, doping with a Nd(III) concentration of 20 mol %.

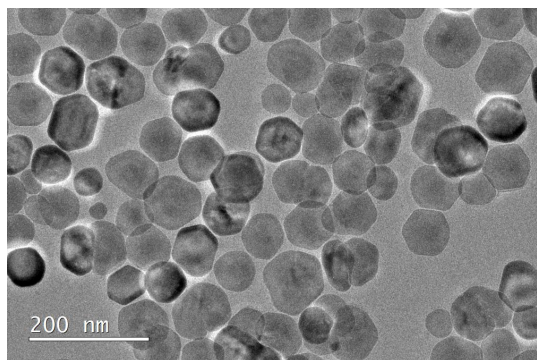
The method used for the shelling reaction is shown in scheme 4.1. The neodymium oleate precursor was prepared by heating neodymium chloride in oleic acid and 1-octadecene, at 120 °C and under vacuum for 1 hour. The reaction mixture was then cooled to room temperature and the core NaYF₄:Yb,Er UCNPs were added, along with sodium trifluoroacetate. This mixture was then heated at 300 °C, under N₂ for 1.5 hours. The resulting UCNPs were purified by precipitation with ethanol and centrifugation to collect the nanoparticle product.



Scheme 4.1: Synthesis of core-shell NaYF₄:Yb,Er@NaYF₄:Nd UCNPs. Reagents and reaction conditions: (i) NdCl₃, 1-octadecene, oleic acid, sodium trifluoroacetate, 300 °C.

The TEM data for synthesised NaYF₄:Yb,Er@NaYF₄:Nd UCNPs is shown in figure 4.7. There was a size increase from a diameter of 54.3 ± 8.2 nm for core only NaYF₄:Yb,Er, to a diameter of 63.6 ± 11.0 nm for core-shell NaYF₄:Yb,Er@NaYF₄:Nd UCNPs. The resulting UCNPs were monodisperse and hexagonal in shape, suggesting successful growth of a Nd(III)-doped β -NaYF₄ shell.

The XRD pattern for synthesised core-shell NaYF₄:Yb,Er@NaYF₄:Nd UCNPs is shown in figure 4.8a. It shows the expected pattern match with β -NaYF₄, the material in both the UCNP core and Nd-doped shell. Figure 4.8b shows the photoluminescence spectrum, with excitation at 803 nm. The characteristic erbium emission spectral shape is visible, with peaks at 525, 540 and 660 nm. Excitation at 803 nm cannot directly excite ytterbium, as Yb(III) has no absorption at this wavelength. The photoluminescence data therefore provides evidence of energy transfer



(a) Low resolution TEM.

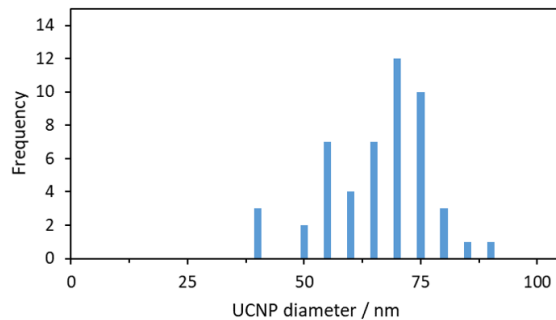
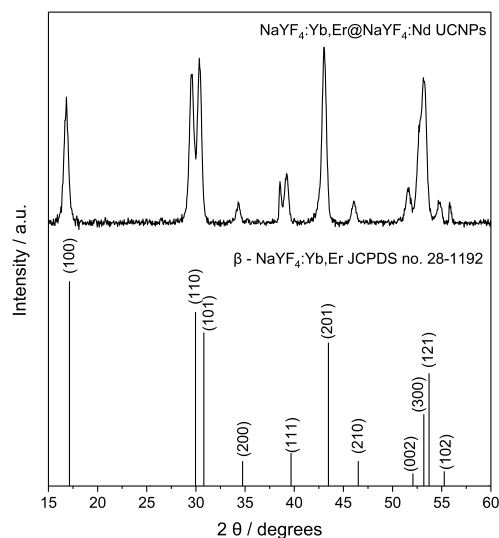
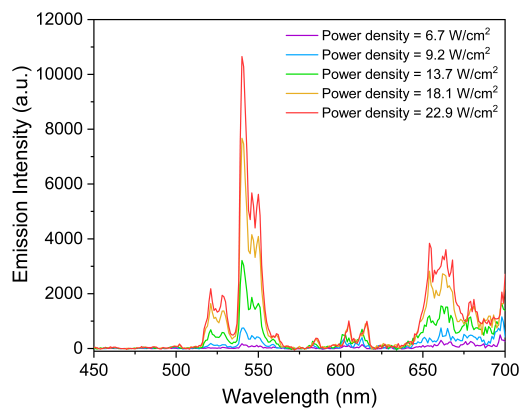
(b) Size = 63.6 ± 11.0 nm ($n = 50$).

Figure 4.7: (a) Low resolution TEM and (b) Size distribution of $\text{NaYF}_4:\text{Yb,Er}@\text{NaYF}_4:\text{Nd}$ UCNP.

in the system. Nd(III) ions in the UCNP shell were excited at 803 nm, followed by energy transfer to ytterbium sensitizers in the core of the nanomaterial. Further energy transfer to the erbium emitters in the system then resulted in emission from the erbium excited states. The upconversion photoluminescence spectrum is quite noisy; this is likely to be due to the low solubility of the $\text{NaYF}_4:\text{Yb,Er}@\text{NaYF}_4:\text{Nd}@\text{oleic acid}$ UCNP in chloroform.



(a)



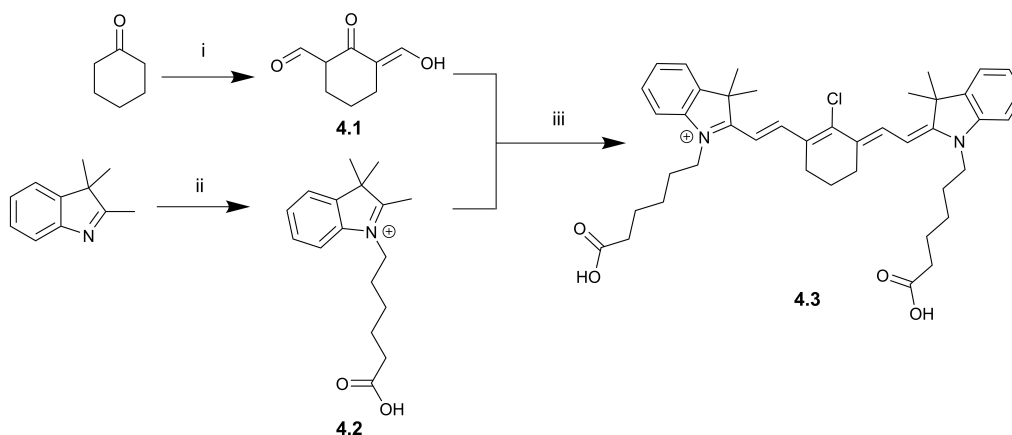
(b)

Figure 4.8: (a) XRD diffraction pattern of synthesised core-shell $\text{NaYF}_4:\text{Yb,Er}@\text{NaYF}_4:\text{Nd}$ UCNP, compared to the ICDD database sample for NaYF_4 . The Miller indices are shown for each diffraction peak; (b) Upconversion luminescence spectrum of $\text{NaYF}_4:\text{Yb,Er}@\text{NaYF}_4:\text{Nd}$ UCNP capped with oleic acid in chloroform. $\lambda_{\text{ex}} = 803$ nm.

4.3.2 Cyanine-808 Synthesis and Characterisation

Heptamethine cyanine dyes absorb in the wavelength range 730-820 nm. The NIR dyes comprise two aromatic or heterocyclic rings linked by seven adjacent methine units. The fluorophores have been used extensively in the field of optical imaging.^{41,42} The NIR dye **4.3**, shown in scheme 4.2, was first reported by Chen et al. in 2006. The dye was chosen as the basis of the sensitized nanoprobe as the free carboxylates make the fluorophore suitable for reaction with the bisphosphonic ester ligand, **2.2**. The photophysical properties of the dye make the moiety suitable as a sensitizing unit as the excitation wavelength is between 700 and 820 nm. The emission of the dye is at 808 nm in methanol, overlapping with the absorption of neodymium.

The synthetic route undertaken followed a slight modification of work published by Tan et al. in 2012.⁴³ The synthesis requires the preparation of two starting materials: the bisaldehyde **4.1** and the quaternary ammonium salt **4.2**. **4.1** was prepared by the reaction of phosphorus oxychloride and DMF to generate the Vilsmeier reagent *in-situ*. Cyclohexanone was then added to react with the intermediate Vilsmeier, thereby generating the desired product, 2-chloro-1-formyl-3-(hydroxymethylene) cyclohex-1-ene. This crude product was then recrystallised from DCM. The quaternary ammonium salt **4.2** was synthesised from the reaction 2,3,3-trimethyl-3H indole and 6-bromohexanoic acid in nitromethane, as reported by Kvach et al.⁴⁴ Addition of diethyl ether caused the product to precipitate and the purple solid was isolated by vacuum filtration. Reaction of **4.1** and **4.2** in ethanol and the weak base sodium acetate resulted in formation of the desired cyanine product **4.3**. This product was purified by silica gel column chromatography (DCM:methanol), yielding a dark green solid product.



Scheme 4.2: Synthesis of cyanine 808. Reagents and reaction conditions: (i) DMF, POCl₃, H₂O, r.t. 5 h; (ii) 6-bromohexanoic acid, nitromethane, 110 °C, 16 h; (iii) EtOH, NaOAc, 70 °C, 1.5hr.

4.3 was characterised by ¹H NMR, ¹³C NMR, COSY NMR and by high resolution mass spectrometry. ESI-MS confirmed the presence of the expected molecular ion at m/z = 683.3624 [M]⁺. COSY NMR was used to assist the assignment of the ¹H NMR spectrum and this assignment can be seen in figure 4.9. The positive charge, shown localised on the indole nitrogen in

figure 4.9, is delocalised over the conjugated π system and so the molecule is symmetric about the centre. The two sets of doublets at 8.33 ppm and 6.19 ppm in the ^1H NMR spectrum are particularly distinctive. These are assigned to the vinyl protons, with the electron-withdrawing nature of the chlorinated cyclohexene moiety in the centre of the molecule causing a significant downfield shift of the vinyl protons closest to the cyclohexene ring.

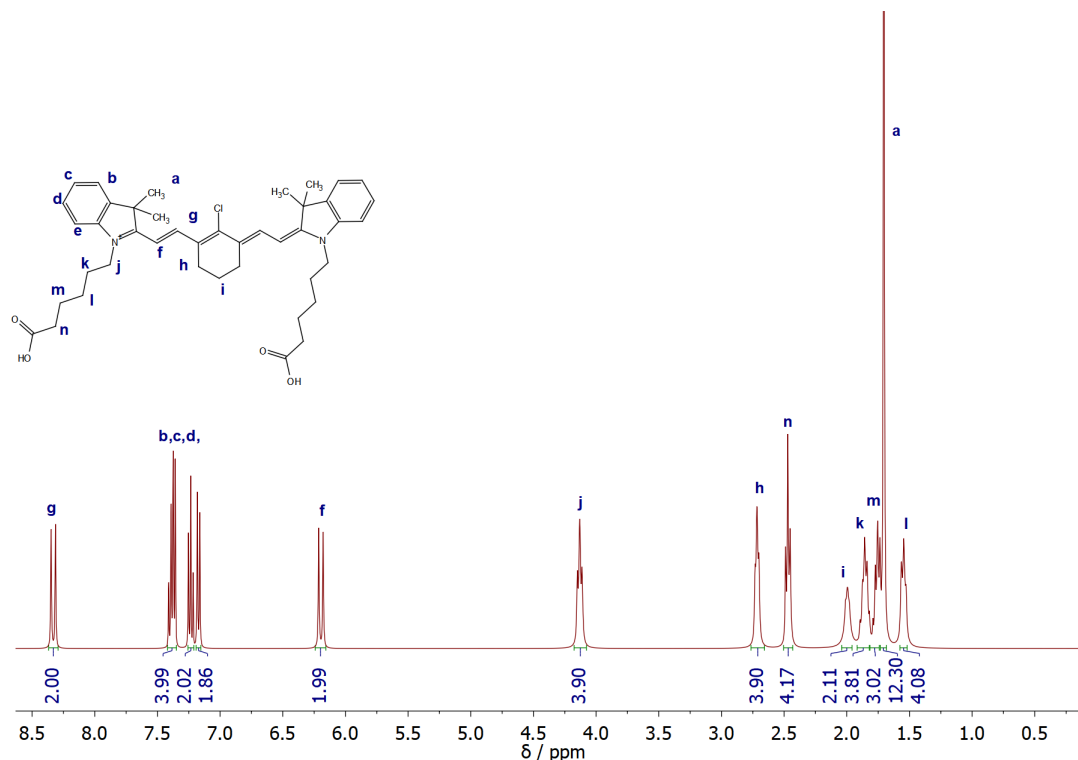


Figure 4.9: Assigned ^1H NMR spectrum of **4.3** (CDCl_3 , 400 MHz, 295 K).

The absorbance and emission spectra of **4.3** are shown in figure 4.10. The dye can be excited by broad-band excitation ~ 800 nm. There is a Stoke's shift of 21 nm and the emission of **4.3** overlaps with the absorption band of Nd(III). The photophysical properties make **4.3** a suitable sensitizer for $\text{NaYF}_4:\text{Yb,Er}@ \text{NaYF}_4:\text{Nd}$ UCNPs.

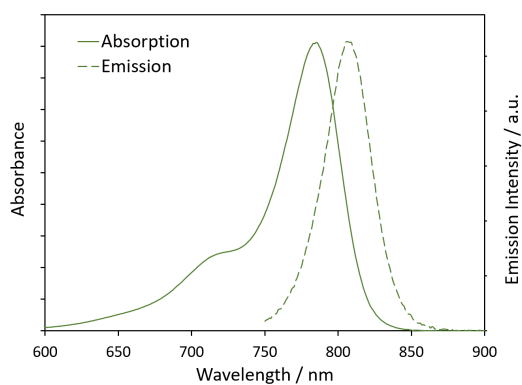
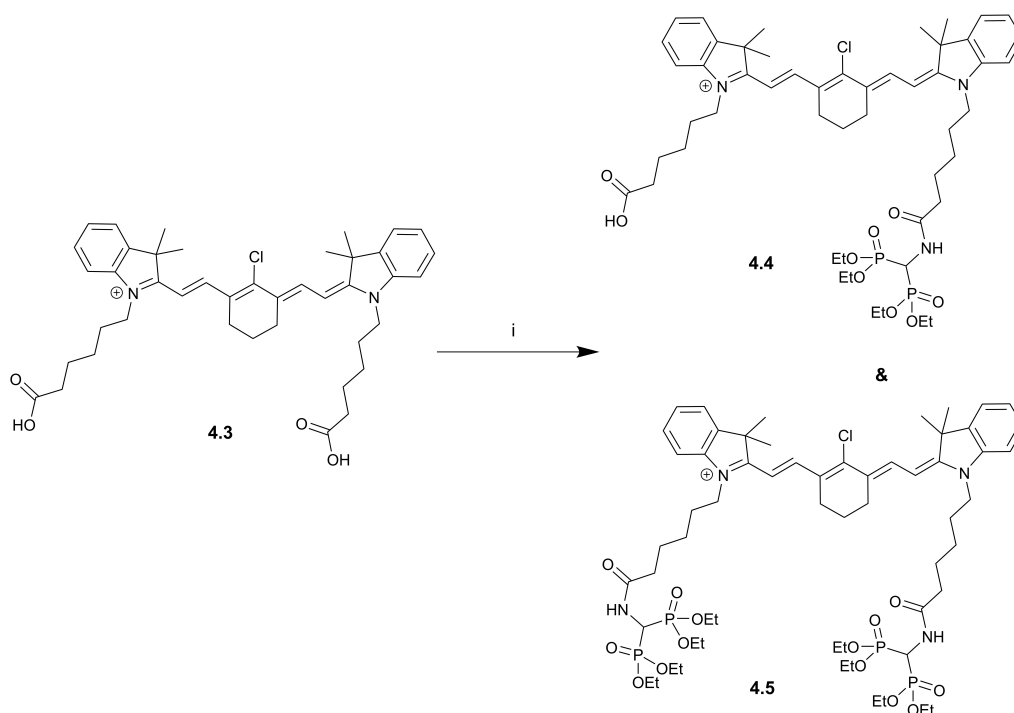


Figure 4.10: Absorbance and emission spectra of **4.3**. Samples at a concentration of $10\ \mu\text{M}$ in methanol.

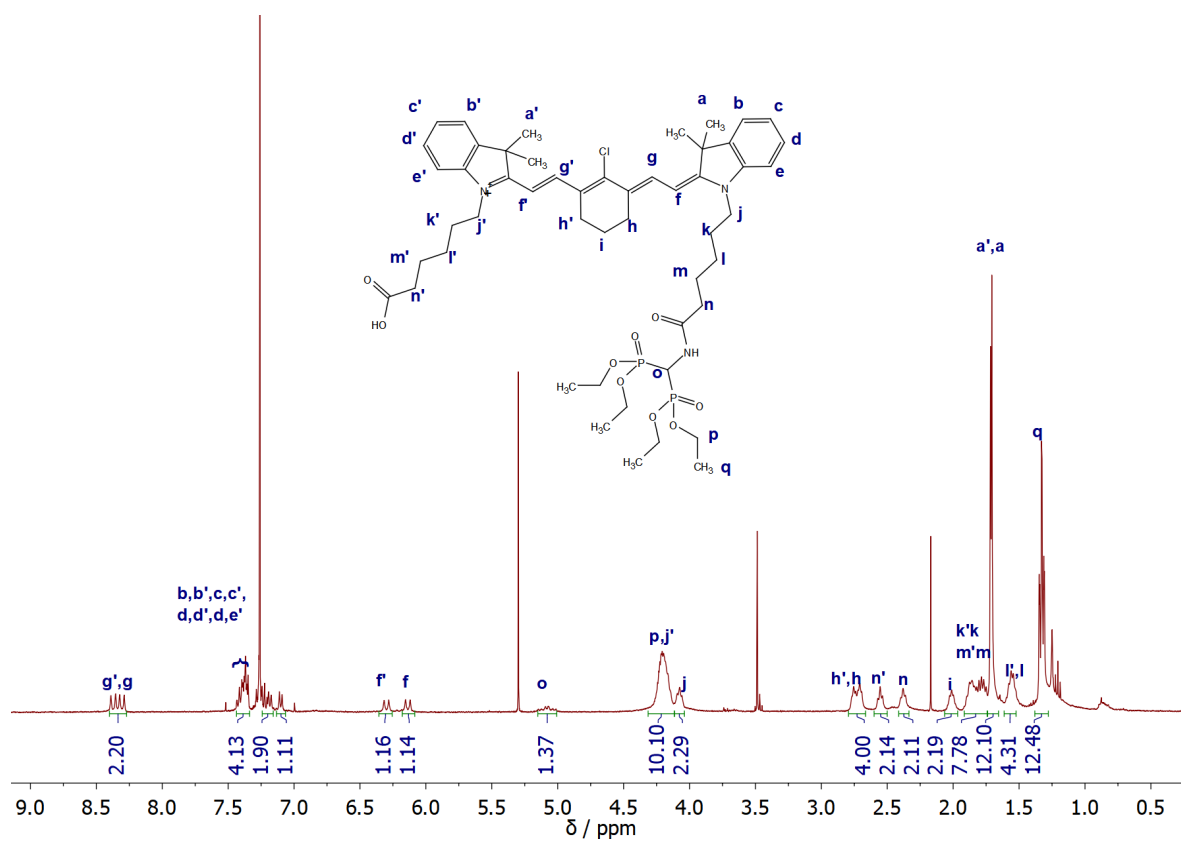
4.3.3 Cyanine-Bisphosphonate Synthesis

4.3 prepared in the previous section was coupled to the bisphosphonic ester ligand **2.2**, via an HBTU facilitated amide coupling reaction. The method used for the preparation of these fluorophores is shown in scheme 4.3. Both the phosphonate ester-protected mono- and di-bisphosphonic ester cyanine derivatives were prepared, purified and fully characterised. Depending on the length of the reaction time, it was straightforward to control the ratio of mono- to di-cyanine product. If the reaction was run at room temperature, overnight then mainly the mono- product was produced, with some starting material remaining. In contrast, running the reaction for 3 days produced near full conversion of starting material to the di-BP product, with around 10 % of the mono BP product isolated.

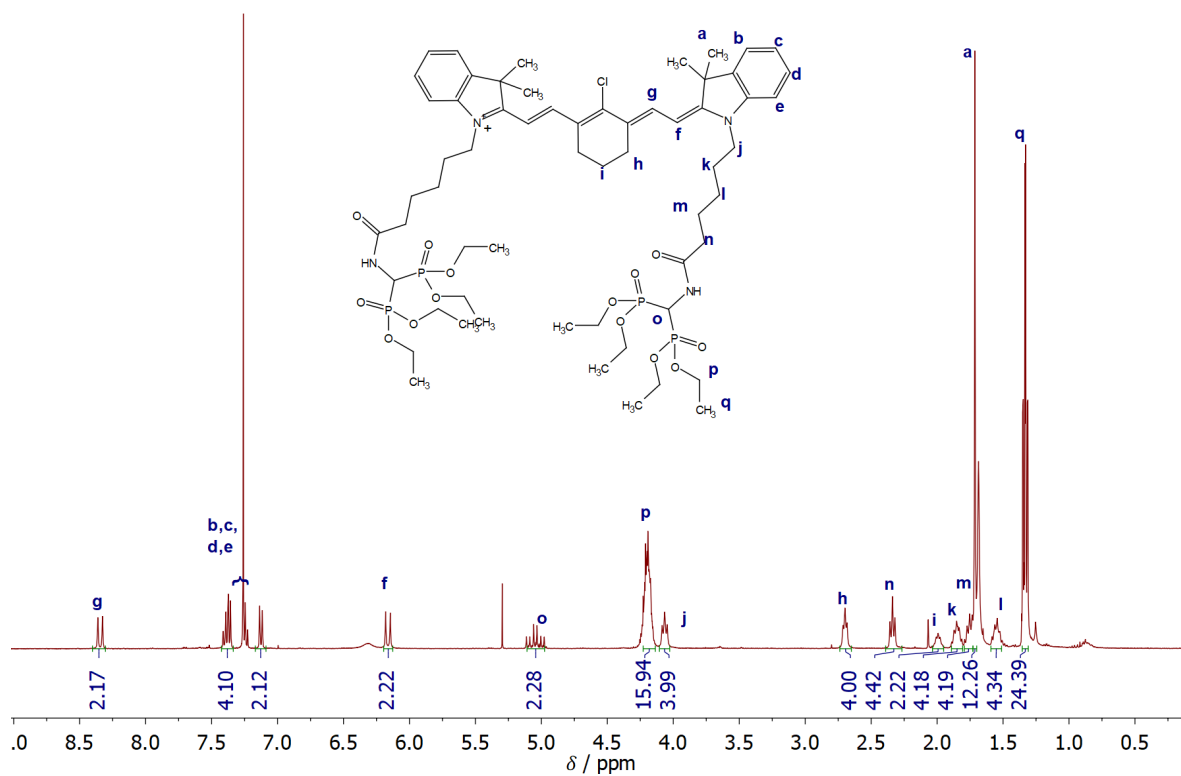


Scheme 4.3: Synthesis of bisphosphonate functionalised cyanines. Reagents and reaction conditions: (i) **2.2** (2 eq), DCM, HBTU, NEt₃, r.t., 16 h.

The ¹H NMR spectra of the two derivatives are shown in figure 4.11. The difference in symmetry between the two materials is apparent in the spectra. The signals corresponding to the vinyl protons of **4.5** are observed as two sets of doublets at 8.33 and 6.12 ppm, both integrating to 2 H. In **4.4** there is no symmetry in the molecule about the centre cyclohexene ring; the vinyl protons on either side of the cyclohexene ring have different chemical shifts. There are 4 sets of doublets, each integrating to 1 H. The triplet of doublets, displaying ~20 Hz ²J_{PH} coupling, is found at 5.04 ppm in **4.5** and 5.08 ppm in the **4.4**, with the triplet of doublets integrating as expected to 2 H in **4.5** and 1 H in **4.4**.



(a)



(b)

Figure 4.11: Assigned ^1H spectra of (a) **4.4** and (b) **4.5** (CDCl_3 , 400 MHz, 295 K).

Photophysical data for both mono- and di-bisphosphonic acid cyanine derivatives are shown in figure 4.12. The spectra are very similar to each other and to **4.3**. The fluorophores have a peak absorption wavelength of 784 nm and a peak emission wavelength of 803 nm, with a Stoke's shift of 20 nm.

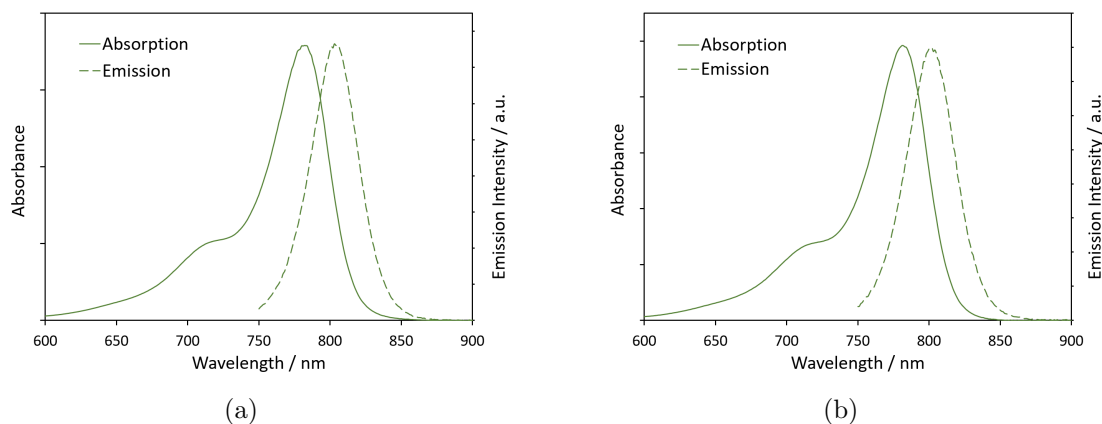
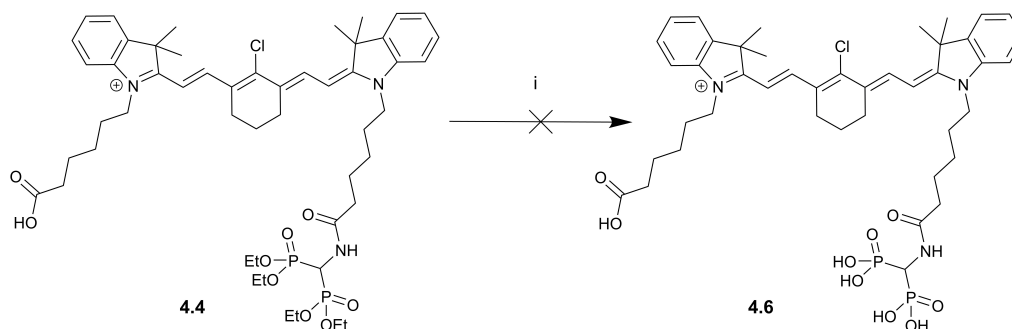


Figure 4.12: Absorbance and emission spectra of (a) **4.4** and (b) **4.5**. Samples at a concentration of 10 μ M in methanol.

The final step in preparation of cyanine bisphosphonate derivatives was the deprotection of the bisphosphonic esters to reveal bisphosphonic acids. This was attempted using the same method as discussed for the rhodamine-bisphosphonic ester derivatives as described in chapter 3, using TMSBr as the deprotecting agent (scheme 4.4).

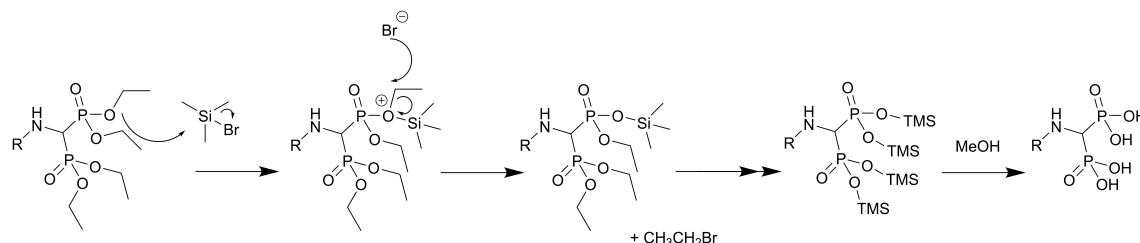


Scheme 4.4: Attempted deprotection of phosphonate esters. Reagents and reaction conditions: (i) TMSBr, dry DCM, r.t, 16 h, N₂.

There were numerous problems encountered during the phosphonate ester deprotection step. Unfortunately, it was found that the cyanine decomposed under the deprotection conditions, with the solution turning from green to brown overnight. The $^{31}\text{P}\{\text{H}\}$ NMR spectrum showed a large number of peaks in the 0-20 ppm region, contrary to the one peak that would be expected. The reaction conditions were adjusted to attempt to prevent decomposition. The reaction time was reduced to two hours and the reaction was monitored by LCMS to see whether the deprotected product could be observed at any point in the crude reaction mixture. Unfortunately,

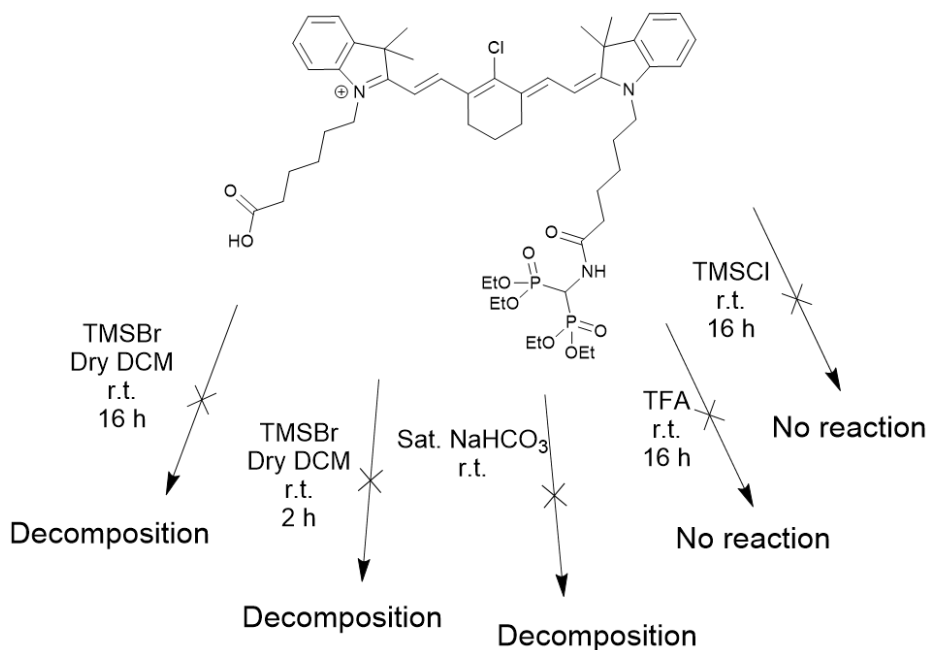
even with the much shorter reaction time of 2 h it was found that again the reaction mixture turned dark brown in colour. No product peak at $m/z = 798.3 [M]^+$ was visible in the LCMS and again the $^{31}\text{P}\{\text{H}\}$ NMR showed a eleven peaks between 0 and 20 ppm.

To understand why this decomposition was occurring, the mechanism of TMSBr deprotection of phosphonic esters was considered. The general TMSBr deprotection mechanism is shown in scheme 4.5.



Scheme 4.5: Mechanism showing general mechanism for TMSBr deprotection of phosphonate esters.

In the second step, a free bromide attacks the carbon adjacent to the phosphonate ester oxygen. This free bromide in the reaction mixture could also displace the cyanine *meso* chlorine in an $\text{S}_{\text{RN}}1$ mechanism.^{45–47} The resulting product would then have a *meso* bromide rather than a *meso* chloride. These bromo-cyanine derivatives have been reported to be much more reactive than the chloride derivatives.⁴⁵ Bromination inductively activates the C2 position (the vinyl carbon adjacent to the cyclohexene ring), making it more susceptible to nucleophilic attack. The free bromide available could further react here, creating a mixture of brominated products. This may explain the large number of peaks in the $^{31}\text{P}\{\text{H}\}$ NMR, because so many different phosphorus containing products were produced.

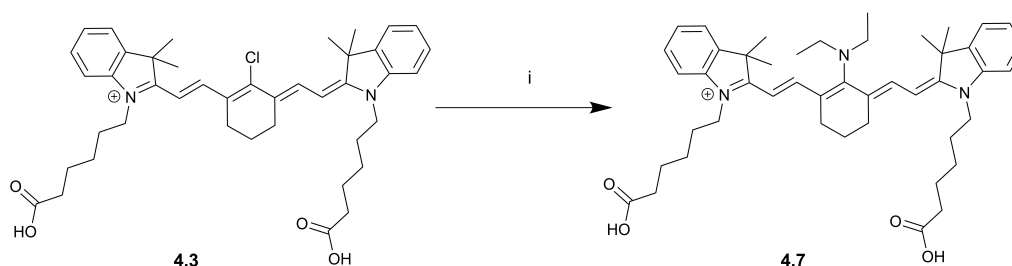


Scheme 4.6: Schematic showing various attempts at deprotection of **4.4**.

Unfortunately, other deprotection techniques attempted resulted in either decomposition of the cyanine or no reaction. A summary of those attempts is shown in scheme 4.6. As reported by Levitz *et al*, the basic conditions of saturated NaHCO_3 caused decomposition of the cyanine. Other methods, such as reaction with TFA or TMSCl resulted in no change to the cyanine. An attempt to deprotect the bisphosphonic ester ligand **2.2** first was undertaken, followed by reaction with the cyanine. The deprotection of the bisphosphonic ester was successful, with a $^{31}\text{P}\{\text{H}\}$ NMR shift of from 20.3 ppm to 10.3 ppm. However, reaction with the cyanine was then unsuccessful.

4.3.4 Functionalisation of the Cyanine *meso* Position

As a strategy to try to overcome the decomposition observed during the bisphosphonate ester deprotection step, the *meso* position was protected by the reaction with an amine. Introduction of alkylamino groups to the central position on heptamethine cyanine dyes have previously been shown to result in better photostability than the chlorine containing parent dye.⁴⁸ Figure 4.7 shows the strategy undertaken in order to achieve this. **4.3** was reacted with diethylamine in DMF. Optimisation of the reaction time was necessary; too little time led to a negligible amount of product formed, running the reaction for too long resulted in decomposition of the product. It was found that the optimum reaction conditions were to run the reaction for 4 hours at 40 °C, with DMF as the solvent.



Scheme 4.7: Reaction of **4.3** with diethylamine, protecting the *meso* chlorine position.

Reagents and reaction conditions: (i) diethylamine, DMF, 40 °C, 4 h.

The product of the reaction was purified by silica gel column chromatography; this resulted in a dark blue product of the amine-coupled cyanine product. The product **4.7** was characterised by ^1H NMR spectroscopy and ES-MS showed the expected product signal at $m/z = 720.4736$ $[\text{M}]^+$. The ^1H NMR spectrum is shown in figure 4.13. Substituting the electron withdrawing chlorine with the lone pair containing diethylamine led to an upfield shift of the vinyl protons, as can be seen in figure 4.13. The 2H doublet at 8.33 ppm of **4.3** shifted to 7.53 ppm in **4.7** and the 2H doublet at 6.19 ppm shifted to 5.88 ppm.

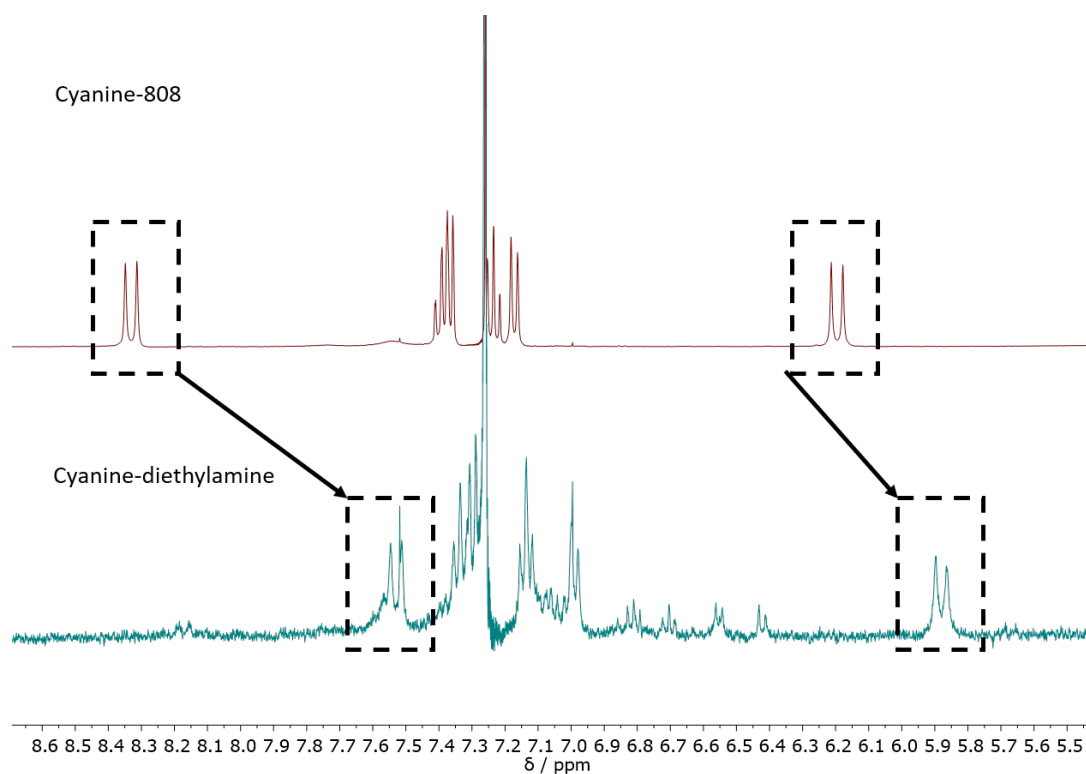
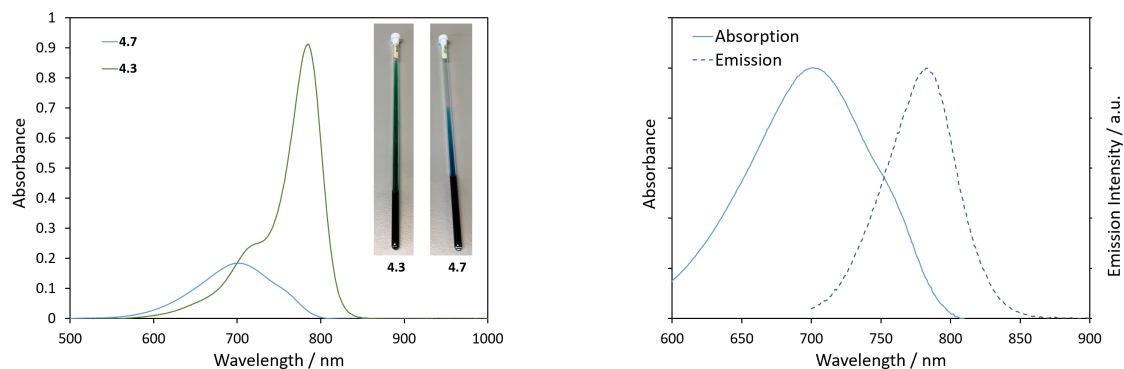


Figure 4.13: ^1H NMR spectra of **4.3** (top trace) and **4.7** (bottom trace) (CDCl_3 , 400 MHz, 295 K).

Figure 4.14a compares the absorption spectra of **4.3** and **4.7**. It is possible to see that there is a shift of peak absorption wavelength from 785 nm for **4.3** to 702 nm for **4.7** and a broadening of the line shape. This blue shift is typical of alkylamino-substituted heptamethine cyanine dyes.^{48,49} Figure 4.14b shows that **4.7** has a large Stoke's shift of 81 nm. These three features are thought to be due to either: intramolecular charge transfer or excited state proton transfer occurring in **4.7**.⁴⁸



(a) Absorption spectra comparing **4.3** and **4.7**.

Inset are photographs of the two fluorophores in solution.

(b) Absorption and emission spectra of **4.7**.

Figure 4.14: Photophysical characterisation of cyanine-diethylamine (**4.7**), with comparison to the absorption of cyanine-808 (**4.3**). All samples at a concentration of 10 μM , in methanol.

The stability of the *meso* functionalised cyanine was tested by addition of TMSBr to a solution of **4.7** in dry DCM. Milder conditions were used than standard bisphosphonic ester deprotection. 5 equivalents of TMSBr were added dropwise to the solution; this is less than the standard 20 equivalents used.⁵⁰

Figure 4.15 shows photographs of the reaction flask as TMSBr was added and then 5 minutes later. It was hoped that protection of the *meso* position of **4.3** by diethylamine would prevent decomposition of the dye. If successful, no visual change in the colour of the solution was expected. Unfortunately, this was not what was seen. There was a rapid colour change of the solution from blue to brown, suggesting dye decomposition. The ³¹P NMR spectrum supported this conclusion. There were a large number of peaks visible in the spectrum, showing that the free bromide in solution caused decomposition of **4.7**.

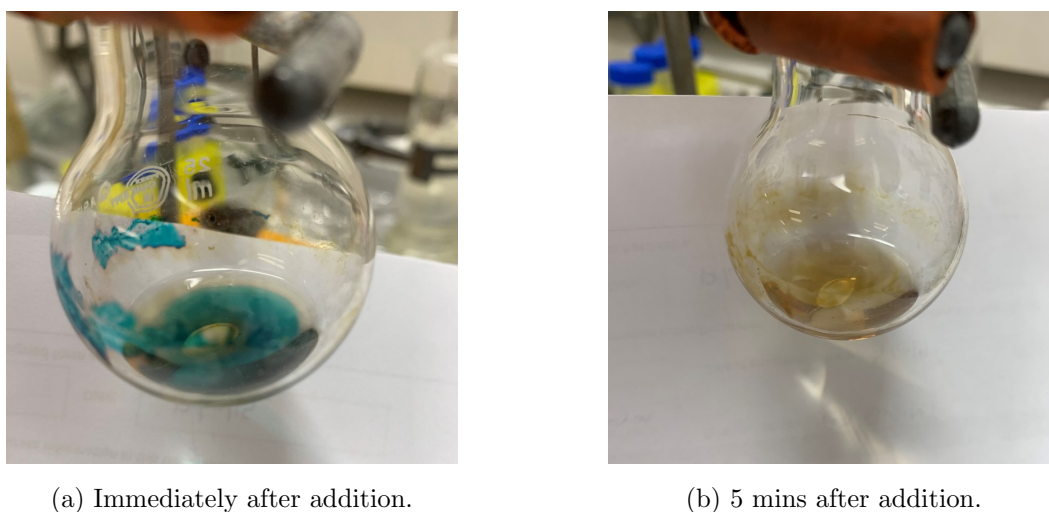


Figure 4.15: Photographs of the round bottom flask containing **4.7** in dry DCM, during the reactivity test with TMSBr.

4.3.5 Assembly of Cyanine-Capped NaYF₄:Yb,Er@NaYF₄:Nd Nanoprobes

Due to the issues with deprotection of the bisphosphonic ester groups on the cyanine fluorophores, it was decided that the dye-sensitization of NaYF₄:Yb,Er@NaYF₄:Nd would be carried out with cyanine-808, **4.3**. The free carboxylate groups on the fluorophore were predicted to be able to coordinate the UCNP surface. Two cyanine-functionalised UCNP nanosystems were synthesised and these will be discussed in the following sections. First, functionalisation of NaYF₄:Yb,Er@NaYF₄:Nd was achieved by replacing some of the oleic acid ligands with **4.3**. This led to a nanoprobe dispersible in chloroform and the work will be discussed in section 4.3.5.1. The second nanomaterial synthesised was water dispersible and used **PEG-BP** to phase-transfer the UCNP. This will be discussed in section 4.3.5.2. (All the photophysical characterisation measurements in this section were kindly carried out by Jingyue Fan at National University of Singapore.)

4.3.5.1 NaYF₄:Yb,Er@NaYF₄:Nd@Cyanine-808 in Chloroform

The first method used to functionalise NaYF₄:Yb,Er@NaYF₄:Nd UCNP with **4.3** was carried out in chloroform. The solubility of **4.3** allowed the direct functionalisation of oleic acid-capped UCNP with a fluorophore, without having to first strip the oleic acid ligands off the surface. This was not possible in the work discussed in chapter 3, as the rhodamine-bisphosphonate ligand (**3.5**) was not soluble in chloroform.

2 mg NaYF₄:Yb,Er@NaYF₄:Nd UCNP were dispersed in 2 mL chloroform. Varying concentrations of **4.3** were added to the dispersions and they were stirred at room temperature overnight. It was challenging to predict how much **4.3** should be added so that the fluorophore would bind to the UCNP surface. It was decided that concentrations of 0.01 mM (0.015 mg, 1 eq), 0.02 mM (0.03 mg, 2 eq), 0.05 mM (0.075 mg, 5 eq) and 0.1 mM (0.15 mg, 10 eq) **4.3** would be used as a starting point. The nanomaterials were purified by addition of ethanol to precipitate the product and centrifugation.

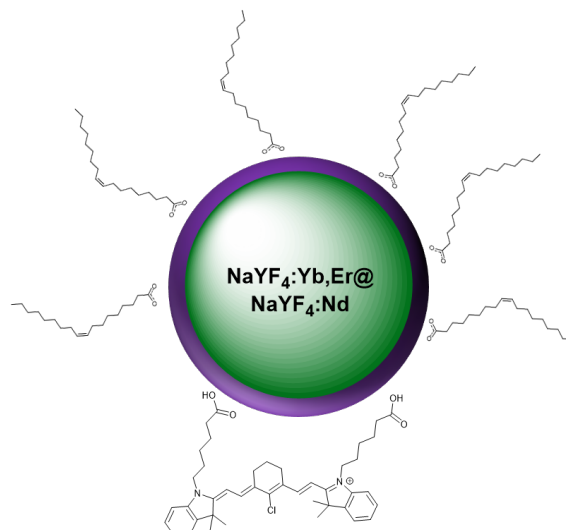


Figure 4.16: Structure of cyanine-808 functionalised NaYF₄:Yb,Er@NaYF₄:Nd@oleic acid UCNP.

Photophysical Characterisation:

Upconversion luminescence data for NaYF₄:Yb,Er@NaYF₄:Nd@Cyanine-808 UCNP in chloroform are shown in figure 4.17a. The corresponding absorption spectra for the samples are shown in 4.17b. ICP-MS measurements were carried out on all the samples and the photoluminescence data scaled to allow for differences in UCNP concentration. Any change in emission properties of the system could then be attributed to the difference in concentration of **4.3** on the surface and not any other factors.

The upconversion photoluminescence data shows that the optimum emission enhancement was achieved in the 5 eq **4.3** sample. This sample showed a 4.5 times emission enhancement in comparison to the sample with 1 eq **4.3**. It is likely that functionalisation at higher concentrations

than 5 eq caused concentration quenching, deactivating the excited states resulting in weaker visible emission from the system.

The data suggests that there was successful dye-sensitization of the UCNP as was achieved. Under excitation at 803 nm, the cyanine appended to the surface of the UCNP was excited. Energy transfer then occurred through Nd(III) in the UCNP shell, to Yb(III) in the UCNP core. Upconversion energy transfer could then take place to excite Er(III) in the UCNP core. The visible emission from erbium in the system was detected at 525, 550 and 660 nm, as shown in figure 4.17a.

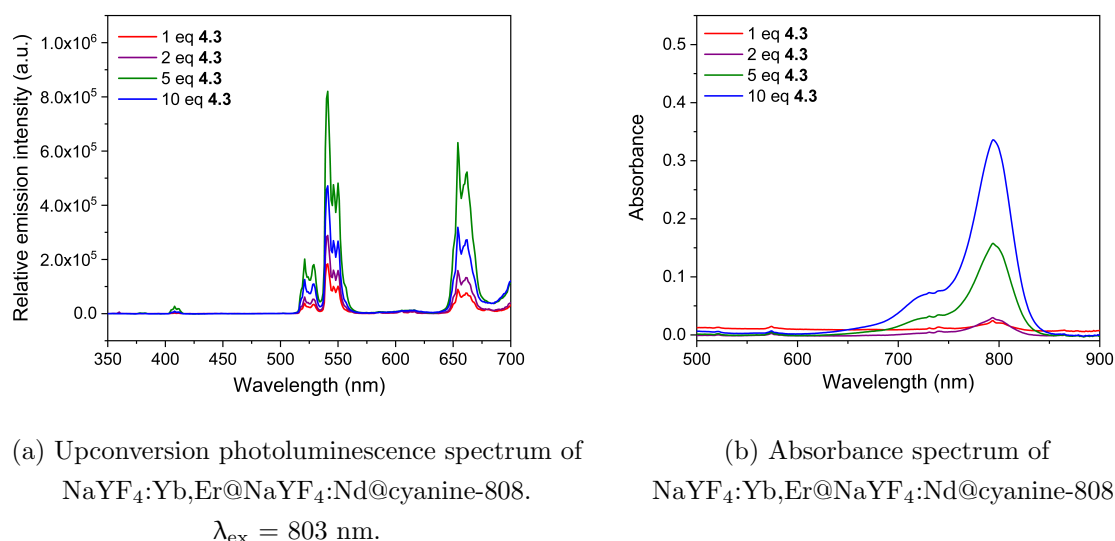


Figure 4.17: Photoluminescence spectra of NaYF₄:Yb,Er@NaYF₄:Nd UCNP functionalised with **4.3**. Samples at a concentration of 2 mg/mL in chloroform.

By using the extinction coefficient calculated for **4.3**, it was possible to determine the optimum number of molecules of the fluorophore per UCNP to achieve the brightest upconversion emission from the system. An analogous method to that described in section 2.3.1.2 was used to calculate the number of cyanine ligands per UCNP in the 5 eq **4.3** sample. ϵ for **4.3** = 91220 L mol⁻¹ cm⁻¹. The concentration of **4.3** in the sample was determined as 1.68 μM . This allowed the determination of the number of molecules of **4.3** in the sample, calculated to be 1.01×10^{15} .

Assuming a density of the NaYF₄:Yb,Er@NaYF₄:Nd UCNP equal to the density of NaYF₄, the number of UCNP (with a 31.8 nm radius) in the sample was determined to be 3.00×10^{12} .

The number of molecules of **4.3** per NaYF₄:Yb,Er@NaYF₄:Nd UCNP was calculated as 337. This gives a ligand density of 0.027 ligands per nm². As expected, this value is considerably lower than the 2.4 ligands per nm² oleic acid on the surface of NaYF₄ NPs calculated in section 2.3.1.2, as only some of the oleic acid ligand would have been displaced by **4.3**.

4.3.5.2 NaYF₄:Yb,Er@NaYF₄:Nd@Cyanine-808 in Water

The synthesis of water dispersible, cyanine-808 functionalised NaYF₄:Yb,Er@NaYF₄:Nd UC-NPs was achieved using a similar technique to the synthesis of rhodamine-functionalised UC-NPs, as described in section 3.3.2.3.

The oleic acid-capped NaYF₄:Yb,Er@NaYF₄:Nd UC-NPs were treated with dilute HCl to remove the oleic acid ligands. The ligand-free NaYF₄:Yb,Er@NaYF₄:Nd UC-NPs were then dispersed in water and varying concentrations of **4.3** were added. **4.3** was added dissolved in a minimum amount of methanol as the fluorophore was not water soluble. The same concentrations of **4.3** were used as in section 4.3.5.1: 0.01 mM (0.015 mg, 1 eq), 0.02 mM (0.03 mg, 2 eq), 0.05 mM (0.075 mg, 5 eq) and 0.1 mM (0.15 mg, 10 eq) **4.3**.

The dispersions were stirred at room temperature for 2 hours, before **PEG-BP** was added. These dispersion was stirred for a further 16 hours at room temperature. Excess ligand was removed by precipitation of the UC-NPs with acetone and centrifugation to collect the solid. The structure of the assembled nanoprobe is shown in 4.18.

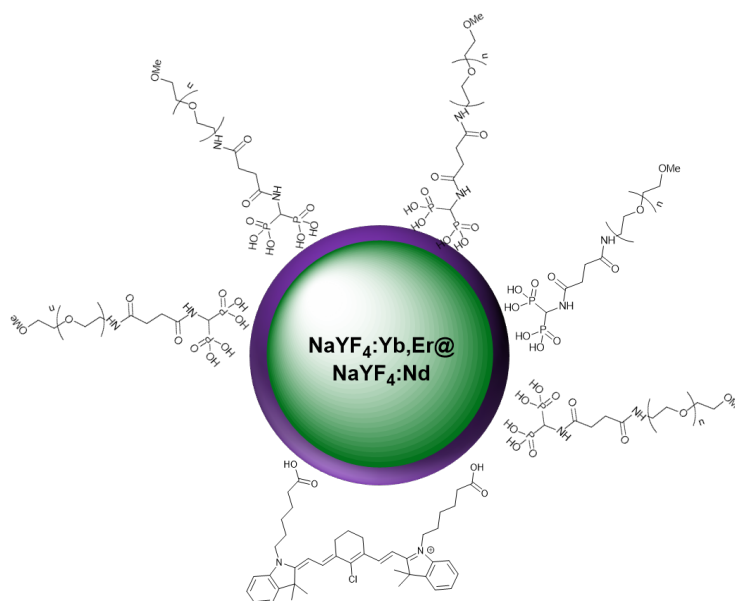


Figure 4.18: Structure of cyanine-808 and **PEG-BP** functionalised NaYF₄:Yb,Er@NaYF₄:Nd@oleic acid UC-NPs.

Photophysical Characterisation:

Upconversion photoluminescence measurements were undertaken for the samples and the data is shown in figure 4.19a. The spectra are all very noisy and the distinct erbium emission peaks are not visible. The absorbance spectra for the samples are shown in figure 4.19b. In these, the line shape corresponding to the absorption of **4.3** is not visible. There is a very weak absorption visible as a small deviation in the line shape ~ 800 nm. This corresponds to the neodymium(III) absorption in the sample. The lack of cyanine-808 absorption suggests that the dye did not successfully coordinate to the surface of the UC-NPs. This, along with

the quenching of UC emission which occurs when UCNPs are phase-transferred to water may explain the lack of erbium emission visible under excitation at 803 nm.

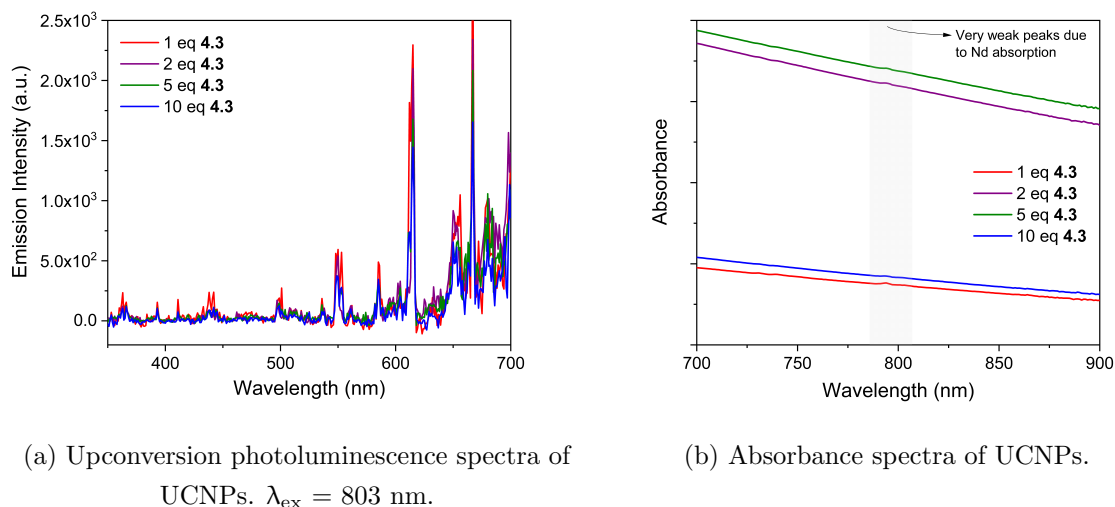


Figure 4.19: Photoluminescence spectra of NaYF₄:Yb,Er@NaYF₄:Nd UCNPs functionalised with **4.3**. Samples at a concentration of 2 mg/mL in water.

The samples were excited at 980 nm to validate there were UCNPs present in the samples. The data from the measurements are shown in figure 4.20. The distinctive erbium emission peaks are visible, showing that NaYF₄:Yb,Er@NaYF₄:Nd UCNPs are present in the samples.

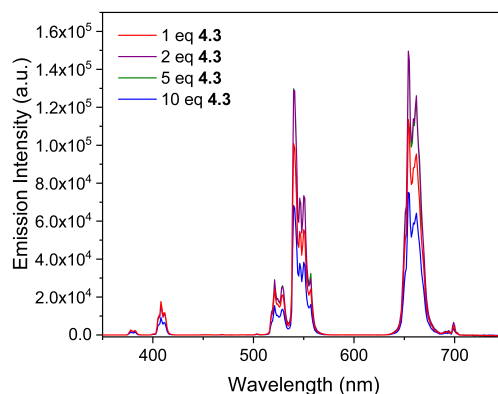


Figure 4.20: Photoluminescence spectra of NaYF₄:Yb,Er@NaYF₄:Nd UCNPs functionalised with **4.3**. Samples at a concentration of 2 mg/mL in water. $\lambda_{\text{ex}} = 980$ nm.

The lack of cyanine coordination to the surface NaYF₄:Yb,Er@NaYF₄:Nd UCNPs may be due to the synthesis method. It is likely that **4.3** did coordinate to the UCNP surface in the first step, but upon addition of **PEG-BP**, the fluorophore may have been displaced. This fits with results in chapter 2 which showed that phosphonate ligands completely displaced carboxylate ligands on the surface of NaYF₄ NPs.

4.4 Conclusions

In this chapter, work focused on improving the brightness of UCNPs by using sensitizing fluorophores and doping the nanoparticles with Nd(III), both of which have higher absorption cross-sections than ytterbium(III). Cyanine-808 was the chosen fluorophore, due to the emission overlap with the absorption band of Nd(III) and Yb(III).

Core-shell $\text{NaYF}_4\text{:Yb,Er@NaYF}_4\text{:Nd}$ UCNPs were synthesised and fully characterised. The Nd doping allowed the excitation wavelength to be shifted to 803 nm, with the UCNPs displaying characteristic erbium emission. The shift in excitation wavelength from 980 nm to 803 nm increases the biocompatibility of the nanoprobes. This is because of the reduced tissue heating issues which occur at 803 nm, in comparison to 980 nm.

Cyanine-808 was chosen as the sensitizing dye due to its photophysical properties and relatively good chemical stability, compared with other cyanine dyes. The aim was to synthesise cyanine-bisphosphonate ligands, providing a strong anchoring point for the cyanine ligand to bind to the surface of the UCNPs. Bisphosphonate esters were coupled to **4.3** via amide coupling and pure products of both the mono-bisphosphonate cyanine (**4.4**) and di-bisphosphonate cyanine (**4.5**) species were isolated. However issues were encountered with the chemical stability of the heptamethine dyes in the final bisphosphonate deprotecting step. TMSBr was used to convert the phosphonate esters to phosphonic acids but under these conditions, the cyanine dyes were found to undergo decomposition. A number of strategies were attempted to protect and stabilise the cyanines but unfortunately none proved successful.

Cyanine-808 was taken forward as the sensitizing dye and the surface of $\text{NaYF}_4\text{:Yb,Er@NaYF}_4\text{:Nd}$ UCNPs were functionalised with the fluorophore. First this was attempted with the system dispersed in chloroform; some oleic acid ligands were displaced by **4.3**. Excitation of the nanoprobes at 803 nm achieved emission enhancement, with the optimum dye doping found to be 337 dye molecules per 63.6 nm diameter $\text{NaYF}_4\text{:Yb,Er@NaYF}_4\text{:Nd}$ UCNP. This corresponded to a cyanine ligand density of 0.027 ligands per nm^2 . The dye-sensitized UCNPs showed a 4.5 times emission enhancement.

Attempts were made to assemble the nanosystems in water, however erbium emission from the system was not visible upon excitation at 803 nm. It is likely that this was due to the two-step synthesis technique used, where addition of **PEG-BP** ligands may have displaced the cyanine ligands already coordinated to the UCNP surface. This led to no detectable cyanine absorption in the system. Further work is necessary here, to investigate whether cyanine-sensitized UCNP systems are feasible in water. There are other factors which may explain the lack of cyanine absorption. The stability of the dye on the UCNP surface is unknown. It may be that the stability may not be sufficient and decomposition may have occurred. This warrants further investigation in future work in the project.

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Chapter 5

Dual-Modal Imaging

5.1 Introduction

The work in this thesis up to this point has focused on developing optical nanomaterials for imaging *in vivo*. However, there are intrinsic issues with optical bioimaging, most notably the poor depth penetration of visible light through tissue. In the last decade, there has been a noticeable increase in the number of articles published covering the synthesis of nanoparticle-based multimodal imaging probes.¹ Multimodal imaging allows the strengths of each imaging modality to be combined and can alleviate their intrinsic disadvantages.²

There are a number of points to consider when developing new multimodal imaging probes:

- Are the sensitivities of the imaging modalities compatible? For example combining highly sensitive PET probes with very insensitive MR probes will cause a large mismatch in the amount of each component of the probe needed for a scan.
- Does the material have specific clinical applications?
- What advantages are there from having a combined imaging agent, rather than carrying out two or more subsequent imaging scans?

Optical imaging and MRI is a particularly promising imaging combination. MRI has high spatial resolution and unlimited depth penetration through tissue, however it suffers from poor sensitivity.^{3,4} The synergy of combining MRI and optical imaging can produce imaging probes which are diagnostically more powerful than imaging with either modality alone. The combination of MRI and optical also allows for simultaneous acquisition of both anatomical and physiological information.² The MRI signal can build a detailed anatomical image and the fluorescence signal can be modulated with a dependence on the local chemical environment, giving physiological information.

Nanoparticles are a popular choice for multimodality imaging due to their functional versatility.⁵⁻⁷ It is synthetically straightforward to attach a variety of different ligands to the surface of nanoparticles and this can be used to introduce various functionality to the imaging probe.

There is also the opportunity to modify the material from which the core nanoparticle is made. By selecting a material with particular properties, it is possible to introduce further imaging functionality.

Superparamagnetic iron oxide nanoparticles (SPIONs) are a popular choice for MR imaging; some of these materials have made it into clinical use. Dextran-coated SPIONs, marketed as Feridex[®] and Resovist[®], were clinically approved for identifying liver lesions. These MRI contrast agents reduce the transverse relaxation time (T_2) of the water molecules in the vicinity of the contrast agent (shown in figure 5.1) by perturbation of the magnetic field of the surrounding protons. The transverse relaxivity (r_2) is inversely proportional to T_2 and r_2 is a measure of how effective the probe is as an MRI contrast agent. The greater the transverse relaxivity, the better the nanoparticle is as a T_2 contrast agent, thereby allowing visualisation of smaller tissue abnormalities. For T_2 -weighted MR imaging, a faster T_2 relaxation time leads to a darkening in the reconstructed MR image.⁸ There are a number of properties of SPIONs which can be tuned to influence the relaxivity of the nanoprobe including SPION: surface coverage, size and shape.

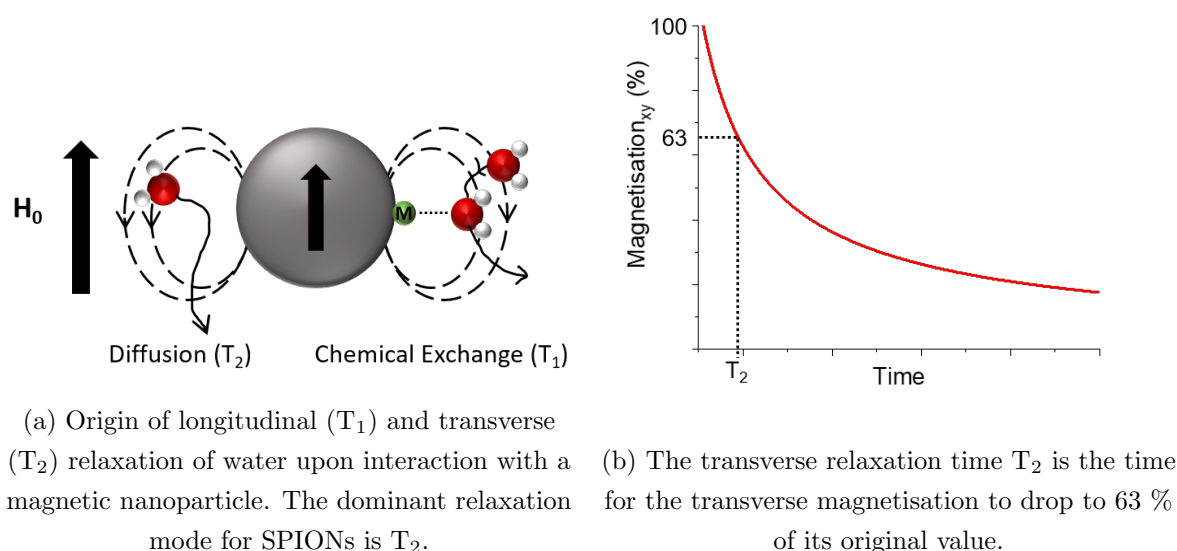


Figure 5.1: The origin and effect of transverse relaxation (T_2).

As MRI contrast agents, SPIONs have a number of advantages over clinically used Gd^{3+} chelates. Perhaps the most important of these is the lower toxicity risk of SPIONs in comparison to gadolinium chelates. This is specifically important when assessing MRI contrast agents, due to the issues which emerged around fifteen years ago in using gadolinium-based contrast agents. It was found that patients with renal impairment could suffer from nephrogenic system fibrosis, a devastating and irreversible condition caused by exposure to gadolinium contrast agents.^{9–11} The discovery led to the food and drugs administration (FDA) assigning a contraindication for the use of three gadolinium-based contrast agents in patients with severe renal failure or acute kidney injury.¹² In 2014, the issue of gadolinium toxicity re-emerged. In a

study of patients who had undergone six or more gadolinium-enhanced MR scans, gadolinium accumulation was found in the brain.¹³ A subsequent post-mortem study carried out elemental analysis of brains of patients who had received multiple gadolinium based contrast agents and also concluded gadolinium accumulated in the brains.¹⁴ As a result of these findings, regulatory bodies from around the world have issued warnings associated with using Gd-based contrast agents. This includes the European Medicines Agency suspending the marketing of three Gd-based contrast agents.¹² There is now an unmet clinical need for MR contrast agents suitable for use in patients with renal impairment.

Iron, on the other hand, is considered biologically innocuous. It is needed in a variety of biological processes and is mainly stored as ferritin in the blood.¹⁵ A very large quantity of iron is needed to cause iron overload syndrome (above 60 mg / kg), much larger than any quantity which would be used in imaging studies (typically below 10 mg / kg).¹⁶

5.1.1 MR/Optical Dual-Modal Imaging Agents

The first example of a dual-modal nanomaterial MRI/optical imaging probe was reported by the group of Weissleder in 1999. In this work, SPIONs were functionalised with the organic fluorophore rhodamine, thereby producing a dual-modal imaging probe.¹⁷ This method was further developed by the same group and in a 2002 paper they reported synthesis of a protease detection probe based on SPIONs and the NIR fluorescent dye Cy5.5.¹⁸ The ‘off’ form of the probe is shown in figure 5.2. In this state fluorescence from the Cy5.5 was quenched, due to the proximity to the nanoparticle surface. In the presence of the protease DTT, the linker was cleaved and Cy5.5 was liberated from the nanoparticle surface. The dual-modal probe demonstrated a non-reversible optical response in the presence of DTT and could be used to detect the presence of the protease.

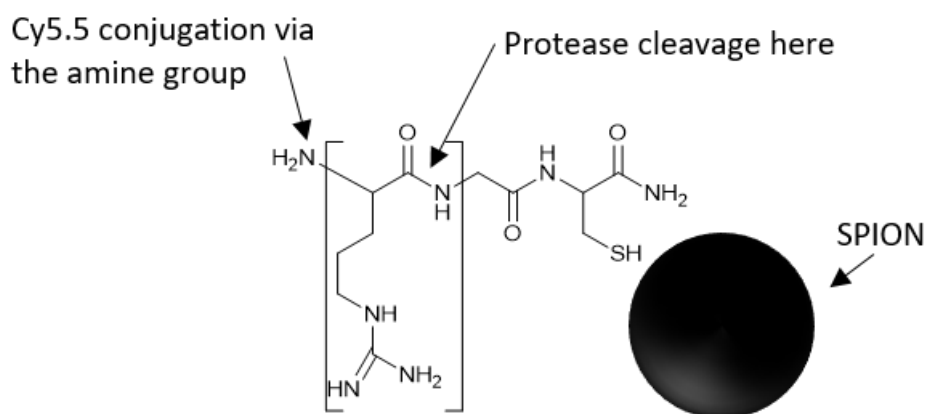


Figure 5.2: Dual-modal MRI/optical imaging probe developed by Weissleder et al. Cleavage by the protease DTT turned on a fluorescent signal from the fluorophore Cy5.5.¹⁸

In the last 15 years, there have been a significant number of papers published which report the synthesis of dual-modal SPION / optical imaging probes, where organic fluorophores are either conjugated directly to the surface of SPIONs or embedded in a silica coating. In conjugating the

fluorophore directly to the nanoparticle surface, there are often issues with quenching of the fluorescent signal. The other significant issue with using organic fluorophores as the optical probe, is the lack of tissue penetration of the UV or visible light required to excite the fluorophores. To navigate the issue of depth penetration of excitation light, UCNPs have been explored as a material which could be combined with MR imaging agents to create a dual-modal probe.

5.1.2 Dual-Modal UCNP-Based Nanomaterials

One method to synthesise a dual-modal NP imaging agent is to dope UCNPs with paramagnetic ions, such as Gd^{3+} . The first such probe was reported by Park et al. in 2009, where the authors synthesised upconverting $\text{NaGdF}_4:\text{Yb,Er}$ nanoparticles.¹⁹ The longitudinal relaxivity (r_1) value of the synthesised probe was found to be $1.4 \text{ mM}^{-1}\text{s}^{-1}$. This is significantly lower when than the value of $5\text{--}6 \text{ mM}^{-1}\text{s}^{-1}$ for the clinically used Gd-DTPA .²⁰

There have also been attempts to combine UCNPs with SPIONs to create a composite material, dual-modal MRI/optical imaging probe.^{21–26} There have been a number of different methods used to combine SPIONs and UCNPs in a dual-modal imaging probe. There are examples in the literature of anisotropic composites, where a spherical UCNP is bound to a SPION, either with a bridge or a Janus system.^{23,27} Core-satellite structures have also been explored, both with a SPION core and UCNP satellites and vice versa.^{28–35} An example of one such system is shown in figure 5.3. Fu et al. attached SPIONs to $\text{NaYF}_4:\text{Yb,Er}$ nanoparticles by three polyelectrolyte layers.

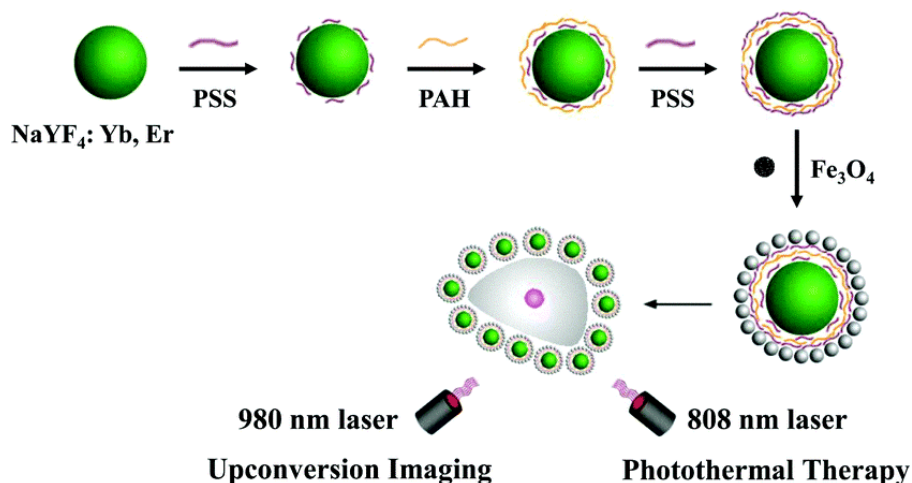


Figure 5.3: Preparation of multifunctional $\text{NaYF}_4:\text{Yb,Er}@ \text{Fe}_3\text{O}_4$ nanocomposites developed by Fu et al. PAH = poly(allylamine hydrochloride) ($\text{Mw} \approx 15,000 \text{ Da}$) and PSS = poly(sodium 4-styrenesulfonate) ($\text{Mw} \approx 7,000 \text{ Da}$). Republished with permission of Royal Society of Chemistry, from reference 35.

The issue with development of satellite composite materials for *in vivo* applications is their size and their lack of uniformity. The attachment of nanoparticles on the surface of other nanoparticles leads to the final imaging probe being large in size, often over 100 nm. Larger

sized nanomaterials *in vivo* can be an issue as larger nanoparticles have been shown to have shorter circulation times. They can also be too large to extravasate out of blood vessels into the interstitial space so they circulate in the blood stream rather than reaching the target imaging site. A lack of uniformity of composition of nanomaterials is an issue for *in vivo* applications due to the differences in biodistribution materials with different size / surface properties exhibit.

In order to overcome these issues, a number of groups have explored the possibility of synthesising core@shell SPION@UCNP nanomaterials. The technique was used by Cui et al. in a 2016 paper where a trimodal MRI/optical/SPECT probe was synthesised and tested *in vivo*.³⁶ Intravenous injection of $\text{Fe}_3\text{O}_4@\text{NaYF}_4:\text{Yb},\text{Tm}$ in a healthy mouse model showed the probe could be used to identify the lymph nodes in which the nanoparticles accumulated after intravenous injection. This is knowledge which could be vital in understanding tumour metastasis.

A number of papers have reported similar core-shell structures but the reported characterisation of these systems is often not sufficient to distinguish between a core-shell composite material and separate core nanoparticles.^{21,22,24–26,37,38} There is a gap in the literature, with SPION@UCNP core-shell nanomaterials yet to be comprehensively studied and characterised. There is still work to be done to optimise the imaging properties of these composite material imaging probes, for example: UCNP emission intensity and transverse relaxivity.

5.2 Aims and Objectives

In this chapter, two different routes to introduce MR functionality to UCNP-based imaging probes were explored. The first aim of this chapter was to synthesise SPIONs with high transverse relaxivity. These SPIONs would then be used as the core material of core-shell nanoparticles; the aim was to grow an upconversion nanomaterial shell on the surface of the SPIONs. The material chosen for the upconversion shell was $\text{NaScF}_4:\text{Yb},\text{Er}$, as this material has been shown to have a higher red:green emission ratio than $\text{NaYF}_4:\text{Yb},\text{Er}$ UCNPs.^{39–43} The higher red:green ratio has an advantage as red light has a deeper penetration than green light. The core-shell nanoparticles were designed to exhibit contrast in both MR and optical imaging modalities, thereby making them dual-modal MR / optical imaging probes. The second route explored was to introduce gadolinium(III) into the core UCNP nanomaterial with the objective of synthesising nanomaterials exhibiting longitudinal contrast (T_1). The aim was to compare the imaging properties of the composite SPION@UCNP nanomaterials to Gd-doped UCNPs.

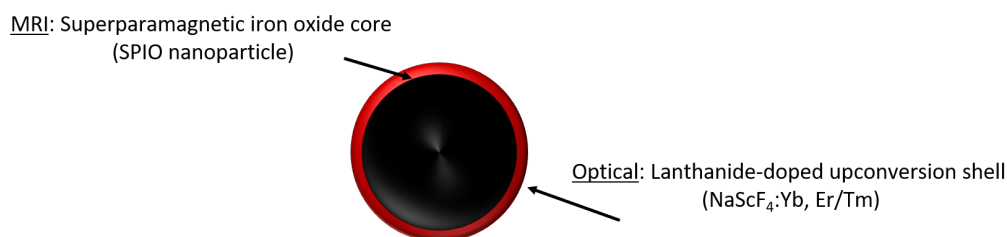


Figure 5.4: Structure of dual-modal $\text{Fe}_3\text{O}_4:\text{NaScF}_4:\text{Yb},\text{Er}$ SPION@UCNPs.

5.3 Results and Discussion

The results in this chapter are split into three sections. In the first, the synthesis of SPIONs is discussed. The second section details the attempts made at synthesising SPION@UCNP, core@shell nanoparticles. In the third section, the synthesis and characterisation of NaGdF₄:Yb,Er UCNPs is discussed.

5.3.1 Anisotropic SPIONs

The starting point for work in this chapter was the synthesis of SPIONs, which make up the core of the proposed core-shell SPION@UCNP imaging probes. The target here was synthesis of SPIONs with maximal transverse relaxivity (r_2). In this endeavour, the work of Zhao et al. is particularly notable.⁴⁴ In their 2013 paper, the group reported the synthesis of highly faceted octapod-shaped SPIONs with ultra-high transverse relaxivity.^{44–47} Nanoparticles with an edge length of 30 nm exhibited $r_2 = 679.3 \pm 30 \text{ mM}^{-1} \text{ s}^{-1}$. This can be compared to the same volume spherical nanoparticles ($r_2 = 125.9 \pm 9 \text{ mM}^{-1} \text{ s}^{-1}$) and the clinically approved liver contrast agent Resovist® ($r_2 = 151.0 \text{ mM}^{-1} \text{ s}^{-1}$). Replication of the synthesis of octapod-shaped SPIONs was the aim of this first section of work.

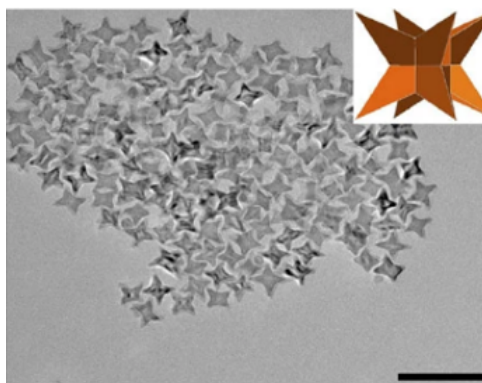


Figure 5.5: TEM images of octapod-shaped SPIONs, from work by Zhao et al. Scale bar = 100 nm. Reprinted by permission from Springer Nature: Nature Publishing Group, Nature Communications, reference 44. Copyright 2013.

5.3.1.1 Synthesis from Metal Oleate Precursor

The first step towards the synthesis of octapod SPIONs was the synthesis of iron(III) oleate, a common precursor for SPION synthesis. In oleate, iron(III) is bound to three oleic acid ligands.^{44,48–50} During the nanoparticle synthesis step, the iron(III) oleate is subjected to high temperatures above 300 °C. This achieves decomposition of the iron complex and nucleation of iron oxide, to form SPIONs. The resulting nanoparticles are capped with the hydrophobic ligand oleic acid. By controlling the ratio of iron oleate to oleic acid in the reaction mixture, it has been shown that the size and morphology of the SPIONs synthesised can be controlled and shapes such as spheres, cubes and stars can form.^{51–53}

Synthesis of Iron Oleate

Iron oleate was synthesised by the reaction of iron(III) chloride with sodium oleate in a 4:3:7 solvent mixture of ethanol:water:hexane. The iron oleate in the organic layer was then washed with water.⁴⁸ As iron(III) is paramagnetic, NMR is not helpful for characterisation. IR spectroscopy and TGA were used to characterise the synthesised iron(III) oleate and determine the ratio of free and unbound oleate species.

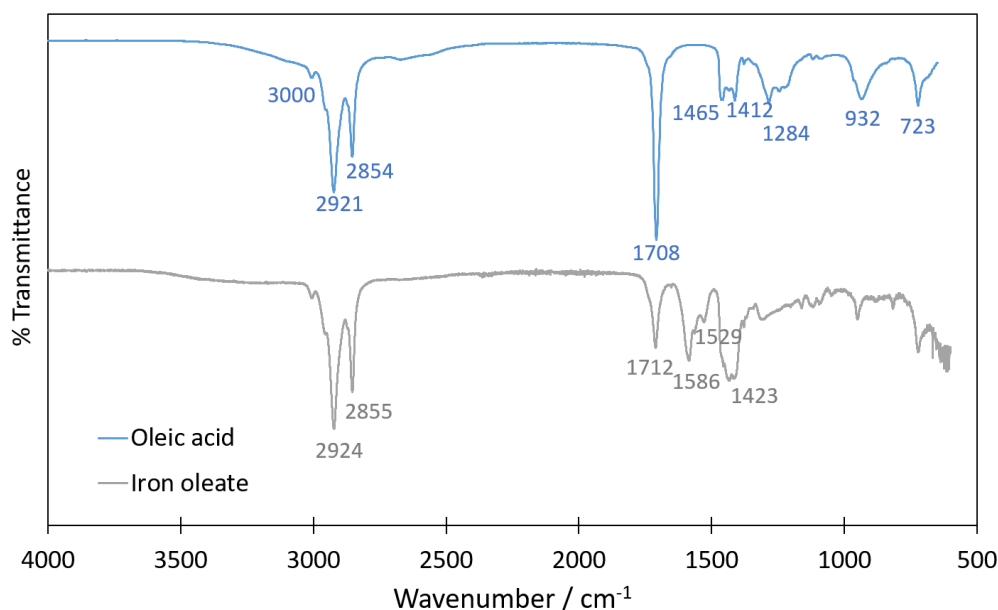


Figure 5.6: IR spectra comparing oleic acid and synthesised iron(III) oleate.

The IR spectrum for the synthesised iron oleate is shown in figure 5.6 and matches well with literature, with expected peaks at 1712, 1586, 1529, 1423 cm⁻¹.⁴⁸ The band at 1712 cm⁻¹ has been assigned to the carbonyl band of free oleic acid; the similarity between the band in the iron oleate spectrum and the spectrum of free oleic acid supports this assignment.⁵⁰ Bronstein et al. assigned the bands at 1586, 1529 and 1423 cm⁻¹ to the various binding modes of the carboxylate ligand to iron; monodentate, bidentate and bridging coordination modes are all possible.^{50,54,55} Representations of these binding modes are shown in figure 5.7.

In the literature, iron oleate is often described as a waxy orange solid.^{44,48} However when synthesised in this work, the resulting product was a black viscous oil. There is evidence that this black oil is also a typical product from the synthesis.⁵⁶ Excess oleic acid in the iron oleate product causes the product to form a viscous oil, rather than a solid. There was concern that a difference in the amount of free oleic acid in the iron oleate precursor may result in a different morphology of the resulting SPIONs. Work by Bronstein et al. demonstrated that treatment of iron(III) oleate with varying washing / drying methods resulted in nanoparticles with differing morphology.⁵⁰ A number of methods to remove some of the free oleic acid from the iron(III)

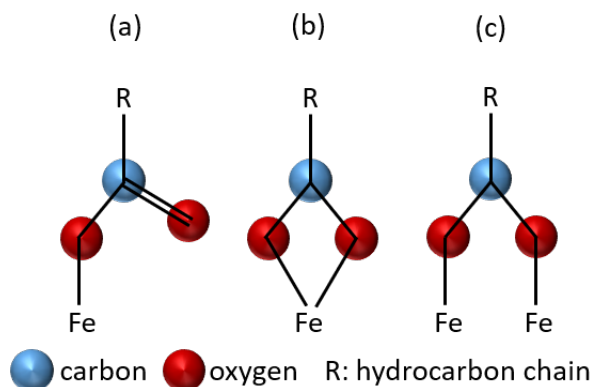


Figure 5.7: Coordination modes of the oleate ligand:
(a) monodentate; (b) bidentate; (c) bridging.

oleate product were undertaken but ultimately none were successful in transforming the viscous black oil to a waxy orange solid. These methods included combinations of: water, acetone and ethanol washing and drying under vacuum.

As the attempts at purifying the synthesised iron(III) oleate were unsuccessful, it was necessary to be able to compare the amount of free oleic in the iron(III) oleate synthesised in this work to the literature. Thermogravimetric analysis was undertaken to determine the ratio of free to bound oleic acid in the product. The results show a 30 % mass loss with an onset at 200 °C, followed by a 50 % mass loss with an onset at 280 °C. This corresponds well to the results in the paper by Park et al, who reported a 30 % mass loss, followed by a 55 % mass loss.⁴⁸ The initial decomposition of free oleic acid starts at 200 °C. This is followed by decomposition of the oleate coordinated to the iron, with decomposition of this starting at 280 °C.

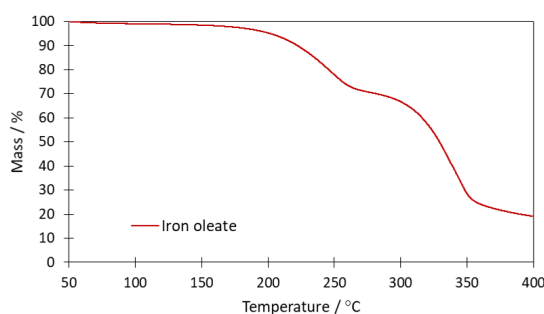
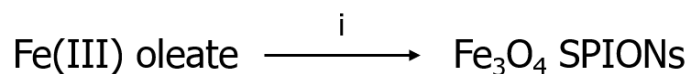


Figure 5.8: TGA spectrum of synthesised iron oleate.

Spherical SPIONs

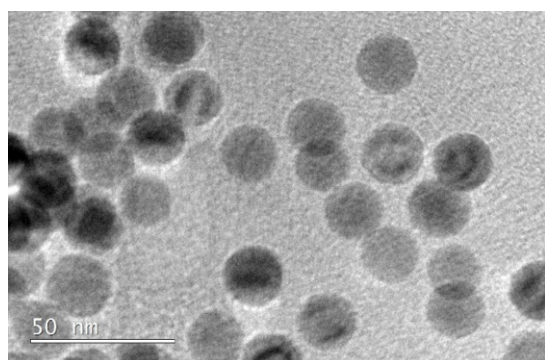
SPIONs were synthesised from iron(III) oleate by heating the iron precursor to 320 °C in the presence of oleic acid and the high boiling point solvent 1-octadecene. The heating rate was carefully controlled and undertaken at 3.3 °C min⁻¹, with a programmed heating control mantle. This has been shown to minimise the size distribution of the resulting nanoparticles.^{48,57} Thorough cleaning of the SPIONs was required to remove excess oleic acid. This was achieved

by adding ethanol to precipitate the SPIONs. The suspensions were centrifuged and the supernatant discarded. This process was repeated until the brown viscous oil containing the nanoparticles turned into a black solid.

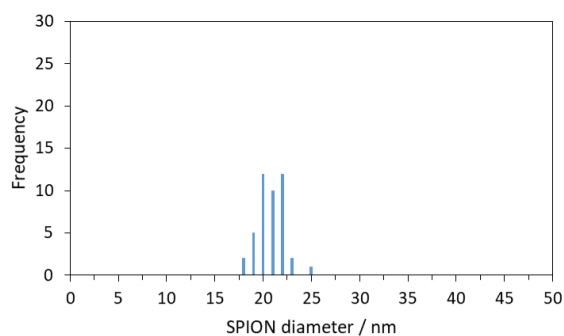


Scheme 5.1: Synthesis of Fe_3O_4 SPIONs from iron(III) oleate. Reagents and reaction conditions: (i) oleic acid, 1-octadecene, 320 °C, N_2 , 2 h.

TEM was used to characterise the size and shape of the synthesised SPIONs. A typical TEM image is shown in figure 5.9, with the size distribution data also shown. The method produced high quality SPIONs with a spherical shape and with a small size distribution, with the nanoparticles 18.4 ± 3.8 nm in size. If the samples were not sufficiently cleaned then excess oleic acid was observed during TEM characterisation as a build-up of organic contamination. This could be seen during TEM as a thick layer on the TEM sample grid, with no nanoparticles visible.



(a) Low resolution TEM.



(b) Size = 18.4 ± 3.8 nm ($n = 78$).

Figure 5.9: (a) Low resolution TEM and (b) Size distribution of SPIONs synthesised from iron oleate.

Anisotropic-Shaped SPIONs

In pursuit of synthesising SPIONs with high transverse relaxivity, the aim was to replicate the synthesis of octapod-shaped nanoparticles, reported by the group of Jinhao Gao.^{33,44} In their 2018 paper, they reported that by varying the quantity of NaCl added to the reaction, they were able to control the morphology of the nanoparticles synthesised.⁵⁸ Their results can be summarised as follows:

Spherical SPIONs $\rightarrow$ 0 mol % NaCl
 Cubic SPIONs $\rightarrow$ 17 mol % NaCl
 Octapod SPIONs $\rightarrow$ 19 mol % NaCl
 Nanoplate SPIONs $\rightarrow$ 39 mol % NaCl

Table 5.1 shows a summary of the attempts that were carried out towards replicating these results. Unfortunately, octapod-shaped SPIONs were never observed. It was very challenging to replicate any of the results. Addition of NaCl to the reaction resulted in a variety of shapes and sizes of SPIONs produced under the same reaction conditions.

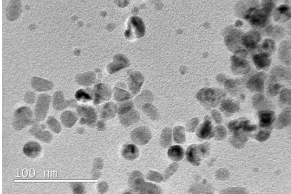
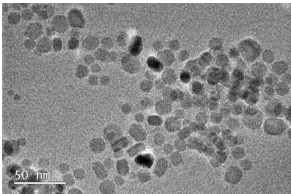
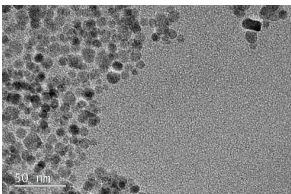
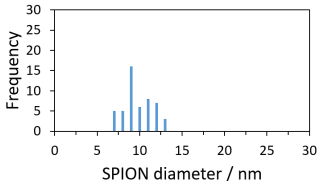
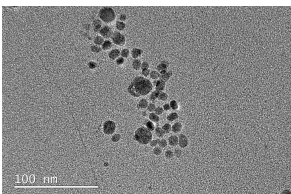
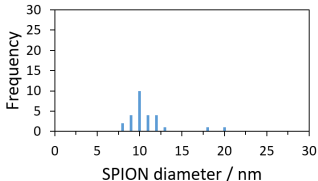
Amount of NaCl	TEM	Size
10 mol %		Approximately 25 x 15 nm
20 mol %		Approximately 20 x 10 nm
30 mol %		 Size = 9.3 ± 1.6 nm (n = 50)
40 mol %		 Size = 10.5 ± 2.6 nm (n = 27)

Table 5.1: TEM images of SPIONs synthesised with varying quantity of added NaCl.

The explanation for this irreproducibility is likely to lie with the purity of the iron oleate used in the reaction. In a 2018 paper by Situ-Loewenstein et al, the authors stated that the ‘dryness of the iron oleate plays a critical role in the resulting morphology’.⁵⁵ There are also examples in the literature referring to the ‘ageing time’ of iron oleate before use but attempts to characterise what this ageing time does to the structure of the complex is absent.^{59,60} Recent work in 2019 by Balakrishnan et al, studied the effect that the iron oleate drying time had on the binding modes of the oleate ligands to the iron centre and consequently, the size of the SPIONs synthesised from the precursor.⁶¹ The work moves towards a better understanding of the structure of the iron oleate nanoparticle precursor, allowing the reliable synthesis of large scale batches of spherical SPIONs. However, absent is any discussion of how the shape of nanoparticles can be modified

by adjusting the drying time of iron oleate.

Recently, a paper published by Plummer et al. discussed alternative methods for synthesising iron(II/III) oleate from iron(II) acetate or iron(III) acetylacetonate.⁵⁶ The authors argue that the current standard method for synthesising iron oleate, as discussed extensively above, results in iron(III) oleate with a variety of binding environments. This uncertainty and irreproducibility of the structure of iron oleate species present in nanoparticle precursors carries through to affect the reproducibility of the nanoparticle synthesis reactions.^{50,62} It makes it very challenging to reliably synthesise SPIONs with a consistent size and shape. Without having access to characterisation data of the iron oleate synthesised by the group of Jinhao Gao, it was not possible to reproduce the synthesis of octapod-shaped SPIONs. The lack of reproducibility of this synthesis is evidenced by the absence of literature published by other researchers. Even though the octapod paper has been cited over 240 times since its publication in 2013, no other research groups have been successful in reproducing the synthesis. In the context of bioimaging probes, this makes the technique unsuitable for preparing SPIONs for *in vivo* use. Here, reliable methods to reproduce consistent batches of SPIONs are necessary for the materials to be used in the clinic.

5.3.1.2 Synthesis from Iron Acetylacetonate Precursor

Due to difficulties with the reproducibility and purification of SPION synthesis from iron oleate, another thermal decomposition route was investigated. This alternative route is also well-established and uses iron(III) acetylacetonate ($\text{Fe}(\text{acac})_3$) as a precursor.^{63,64}

Spherical SPIONs

The protocol from Xu et al. was followed without modification to synthesise spherical-shaped nanoparticles.⁶⁴ $\text{Fe}(\text{acac})_3$ was heated at 300 °C in a mixture of benzyl ether and oleylamine, producing SPIONs capped with the ligand oleylamine. The nanoparticle product from the reaction was found to be straightforward to purify. Excess oleylamine was simply washed from the SPIONs with ethanol. No issues with organic contamination when undertaking TEM characterisation were observed.

Figure 5.10 shows the SPIONs synthesised were smaller than those produced by the iron oleate route, around 10 nm in size and broadly spherical in shape. The XRD pattern (shown in 5.10b) confirms the SPIONs were the expected Fe_3O_4 magnetite material. The high background for the SPION sample is due to iron fluorescence.⁶⁵

Attempt at Formation of Other SPION Shapes

As in the synthesis of anisotropic-shaped Fe_3O_4 nanoparticles from iron oleate, it was postulated that addition of NaCl to the reaction using $\text{Fe}(\text{acac})_3$ could produce nanoparticles with varying shapes. The mol % of NaCl added was chosen to match the mol % used in the iron oleate attempts and 20 mol % of NaCl to $\text{Fe}(\text{acac})_3$ was used in order to try to replicate the octapod

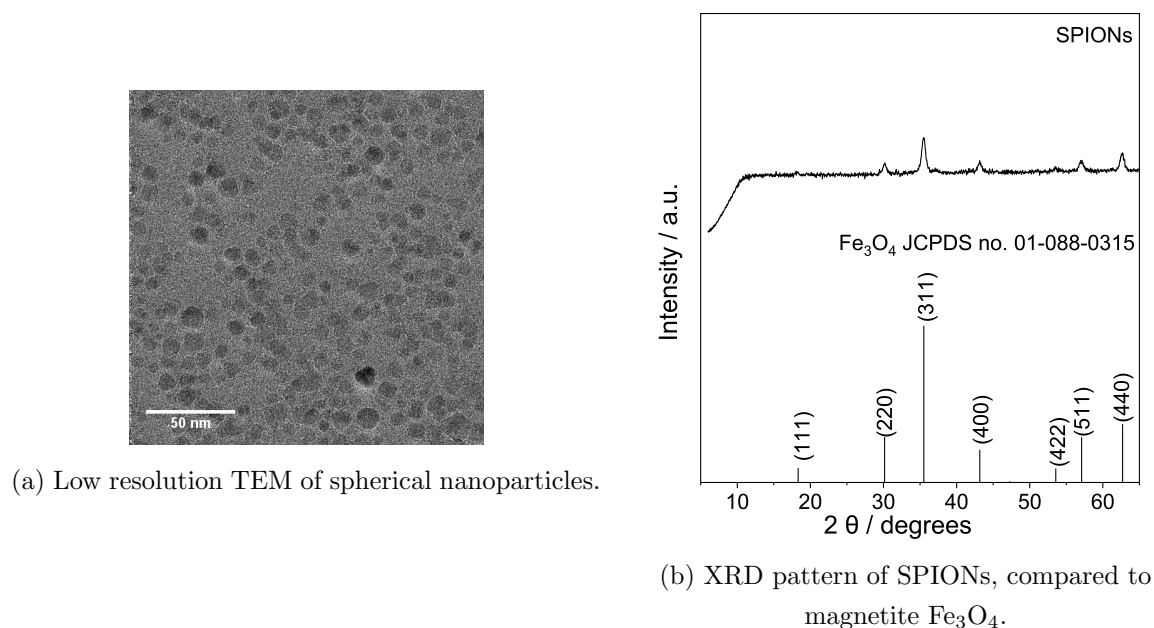


Figure 5.10: TEM image and XRD pattern of SPIONs produced via $\text{Fe}(\text{acac})_3$ synthesis.

synthesis. Unfortunately the synthesis resulted in a precipitate which was only barely soluble in hexane and toluene. This suggested a highly aggregated product was produced, likely to have occurred because a small quantity of water was introduced in the first step, required to dissolve the NaCl. The water is likely to have interfered with nanoparticle formation, creating the large aggregates observed. The TEM images shown in figure 5.11 come from some of the soluble precipitate. The images show that the synthesis produced nanoparticles of around 15 nm in size, with a variety of shapes and sizes. It can be concluded that adding NaCl to SPION synthesis reactions does not reliably produce anisotropic-shaped SPIONs.

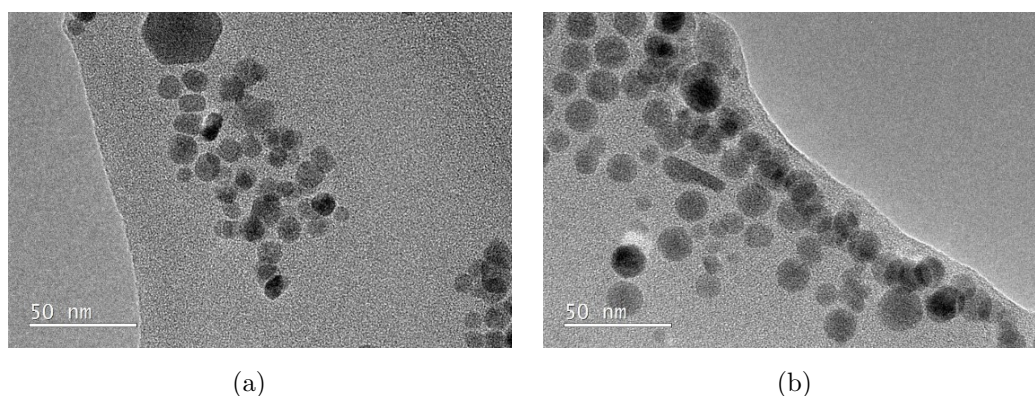


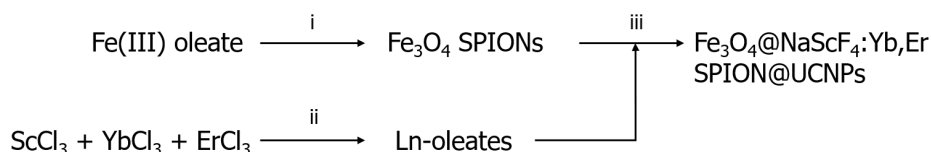
Figure 5.11: (a) and (b) are both low resolution TEM images of attempted synthesis of anisotropic shaped iron oxide nanoparticles from a $\text{Fe}(\text{acac})_3$ precursor.

5.3.2 Dual-Modal, Core-Shell SPION@UCNPs

After the issues with synthesising octapod shaped SPIONs, it was decided that the core-shell SPION@UCNP structure would be pursued using spherical SPIONs as the core nanomaterial. The chosen material for the upconversion shell was the red-emitting material NaScF₄:Yb,Er. The starting point for this work was the one-step core@shell SPION@UCNP reactions published by Cui et al.³⁶ Subsequently, a two-step strategy was employed and the results from this work will then be discussed.

5.3.2.1 One-Step Synthesis of Fe₃O₄@NaScF₄:Yb,Er SPION@UCNPs

The reported protocol for synthesis of core-shell nanoparticles was followed according to work by Cui et al.³⁶ Iron(III) oleate was heated to 320 °C, with oleic acid and 1-octadecene. Separately, lanthanide oleate complexes were prepared by heating the lanthanide chlorides at 180 °C in oleic acid and ODE. After 2 hours, the lanthanide precursors were hot injected (at 180 °C) into the SPION reaction and heating was continued at 320 °C for a further 15 mins. The reaction mixture was then cooled to room temperature over 30 mins and washed with ethanol. The molar ratio of iron precursor to lanthanide precursor was calculated to be the same as in the Cui et al. paper, i.e. 2:7 total lanthanide precursors:iron precursor.³⁶

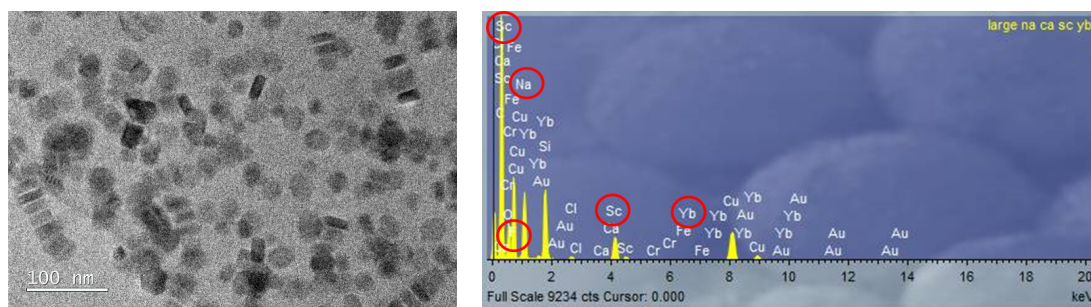


Scheme 5.2: One step synthesis of Fe₃O₄@NaScF₄:Yb,Er NPs. Reagents and reaction conditions: (i) oleic acid, 1-octadecene, 320 °C, N₂, 2 h; (ii) oleic acid, 1-octadecene, , NaF, 180 °C, N₂, 30 mins (iii) 320 °C, N₂, 15 mins.

TEM imaging and EDX spectroscopy data are shown in figure 5.12. The figures show that the nanoparticles isolated from the synthesis were scandium containing; there was no evidence of iron containing nanoparticles present.

The upconversion photoluminescence spectrum, shown in figure 5.13, confirms the synthesis of NaScF₄:Yb,Er UCNPs. (These measurements were kindly carried out by Dr Shuai Zha at Hong Kong Baptist University.) Upon excitation at 980 nm, the sample displayed characteristic erbium emission. The sample showed stronger red emission compared to yttrium containing NaScF₄:Yb,Er. This finding agreed with synthesis of NaScF₄:Yb,Er UCNPs by Teng et al. in 2012. As discussed in section 2.3.2.6, the green:red (550:660 nm) ratio for NaYF₄:Yb,Er@PEG-BP UCNPs in chloroform was 5.6 and in water was 2.4. This can be compared to the green:red ratio for solid NaScF₄:Yb,Er@oleic acid found to be 0.32; the scandium UCNPs have a much stronger red emission than the equivalent yttrium-containing NaYF₄:Yb,Er UCNPs.

Scandium is directly above yttrium in group 3 of the periodic table. The difference in ionic radii



(a) TEM showing NPs produced in one-step synthesis. (b) EDX showing presence of Sc, Yb, F and Na.

Figure 5.12: (a) TEM and (b) EDX showing failed synthesis of core-shell $\text{Fe}_3\text{O}_4@\text{NaScF}_4:\text{Yb,Er}$. Instead irregular-sized $\text{NaScF}_4:\text{Yb,Er}$ UCNPs formed.

of Sc^{3+} (0.83 Å) and Y^{3+} (1.06 Å) can explain the difference in emission properties. Scandium has a smaller ionic radius than yttrium so the lattice distance between Er^{3+} and Yb^{3+} is smaller in $\text{NaScF}_4:\text{Yb,Er}$ than $\text{NaYF}_4:\text{Yb,Er}$. This causes an enhancement in cross-relaxation between Er^{3+} and Yb^{3+} , decreasing the population of $^2\text{H}_{11/2}$ and $^4\text{S}_{3/2}$ energy states and increasing the population of $^4\text{F}_{9/2}$.³⁹ This results in the stronger red emission of $\text{NaScF}_4:\text{Yb,Er}$ than $\text{NaYF}_4:\text{Yb,Er}$.

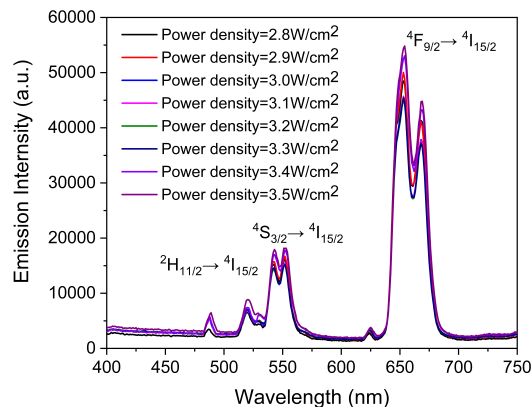


Figure 5.13: Upconversion photoluminescence spectrum of solid $\text{NaScF}_4:\text{Yb,Er}$ UCNPs.

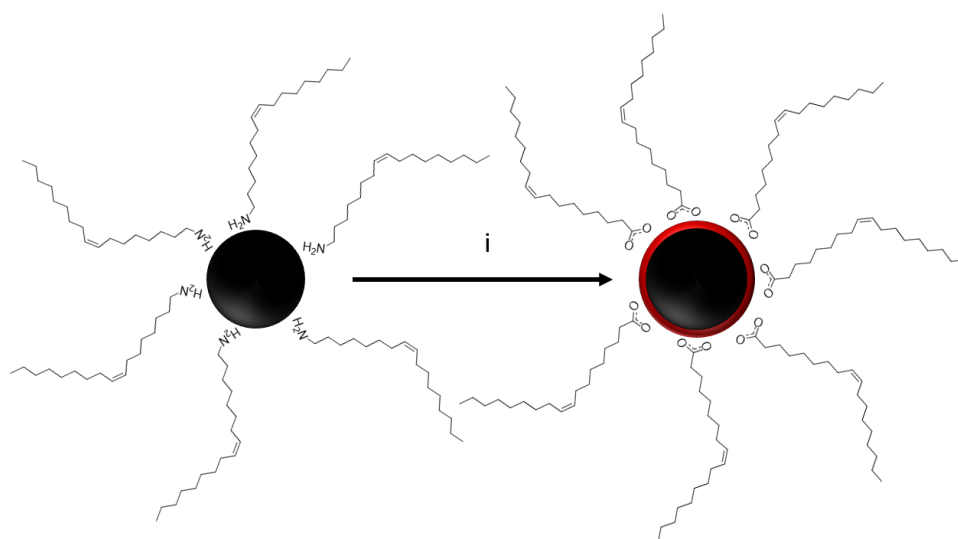
$$\lambda_{\text{ex}} = 980 \text{ nm.}$$

The issues which were encountered when synthesising and purifying SPIONs from an iron oleate precursor suggested that SPIONs may have formed during the one-step $\text{Fe}_3\text{O}_4@\text{NaScF}_4:\text{Yb,Er}$ synthesis attempt, but they may not have precipitated out of the reaction mixture in the ethanol washing stage. This was a typical issue encountered during SPION synthesis from iron oleate. The conclusion that could be drawn from the one-step core-shell SPION@UCNP data was that core-shell nanoparticles did not form.

5.3.2.2 Two-Step Synthesis of $\text{Fe}_3\text{O}_4@\text{NaScF}_4:\text{Yb,Er}$ SPION@UCNPs

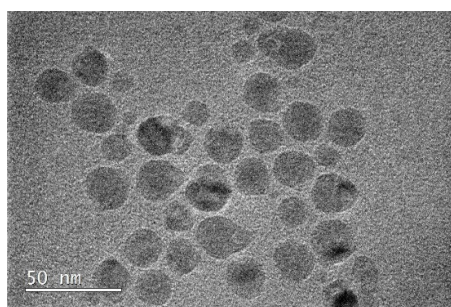
In the one-step synthesis method of synthesising $\text{Fe}_3\text{O}_4@\text{NaScF}_4:\text{Yb,Er}$ NPs, it was very challenging to understand what was happening in the reaction mixture. Therefore, it was decided that a more methodical strategy may be more successful. This would involve initially synthesising core SPIONs and characterising these nanoparticles. Then, in a second step, an upconversion shell would be added.

Due to the issues which had arisen with purification of SPIONs synthesised from an iron oleate precursor, it was decided that SPIONs synthesised from $\text{Fe}(\text{acac})_3$, as described in section 5.3.1.2, would be taken forward as the core of the core-shell SPION@UCNPs. The SPIONs were dispersed in hexane and added to the thermal decomposition of the lanthanide precursors with sodium hydroxide and ammonium fluoride in oleic acid and ODE.³⁹



Scheme 5.3: Second step of two-step synthesis of $\text{Fe}_3\text{O}_4@\text{NaScF}_4:\text{Yb,Er}$ NPs. Reagents and reaction conditions: (i) ScCl_3 , YbCl_3 , ErCl_3 , oleic acid, ODE, 180°C , N_2 followed by NaOH , NH_4F , 320°C , N_2 , 1 h.

Initial TEM imaging and EDX spectroscopy of the nanoparticle products are shown in figure 5.14. The results were promising but not conclusive. The nanoparticles did grow in size during the shelling reaction, from an average size of ~ 10 nm (figure 5.10a) to ~ 20 nm (figure 5.14a). This suggested either: growth of an upconversion shell on the surface of the iron oxide nanoparticle or Ostwald ripening. This is a phenomenon which describes the growth of nanoparticles in size over time.⁶⁶ It was not possible to distinguish between these two possibilities from the low resolution TEM imaging. EDX and upconversion photoluminescence data from the sample showed the presence of both Fe, Sc, Yb and Er but again was not conclusive evidence of a core-shell structure.



(a) Low resolution TEM.

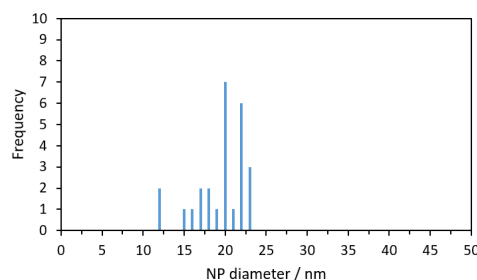
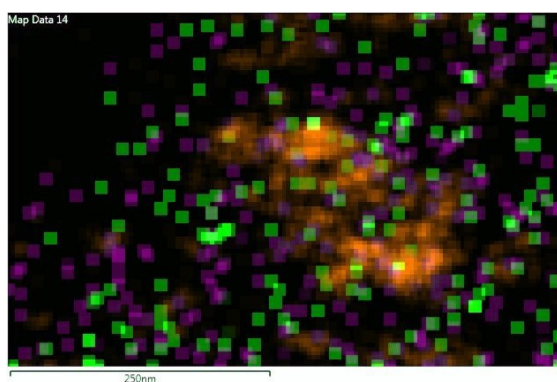
(b) Size = 18.9 ± 3.0 nm ($n = 26$ particles measured).

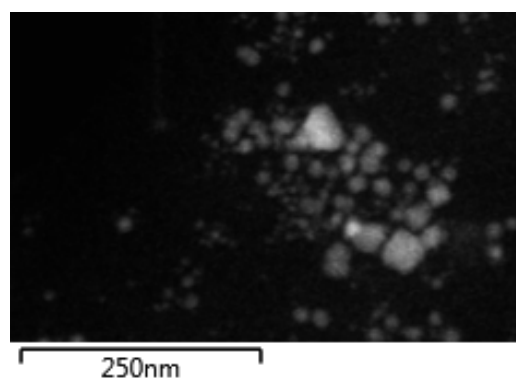
Figure 5.14: (a) Low resolution TEM and (b) Size distribution of the two-step core-shell SPION-UCNP reaction.

To definitively prove a core-shell structure, a technique which could probe the elemental composition of individual nanoparticles was necessary. Characterisation by scanning transmission electron microscopy energy dispersive X-ray spectroscopy (STEM EDX) was pursued. The technique allows elemental mapping across individual nanoparticles in a sample. Figures 5.15 and 5.16 show examples of sites on the sample grid which were chosen for analysis. Each coloured pixel represents a signal from a particular element, with the colour coding shown in the figure caption. The EDX image can be compared to the STEM image, enabling the identification of the elemental composition of particular areas of the STEM image.

Figure 5.15b shows a cluster of nanoparticles of varying size, between 10 and 40 nm. By comparing this to the elemental mapping image in 5.15a, it is possible to see that this cluster of nanoparticles are SPIONs, with scandium and ytterbium randomly distributed as noise across the image.

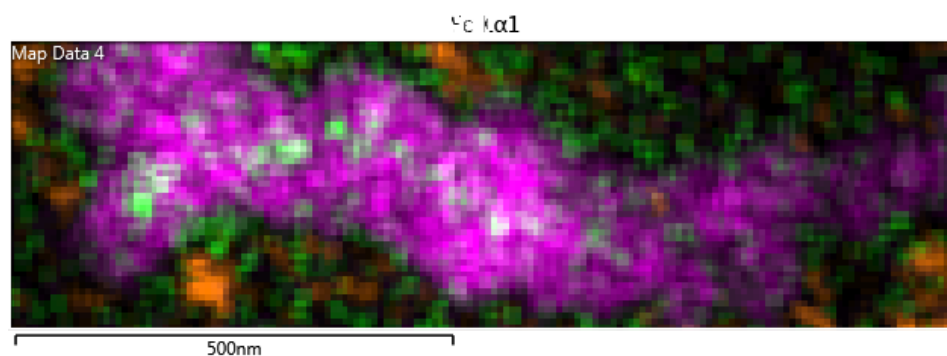


(a) Image merge of STEM EDX elemental mapping. Pink = scandium, green = ytterbium, orange = iron.

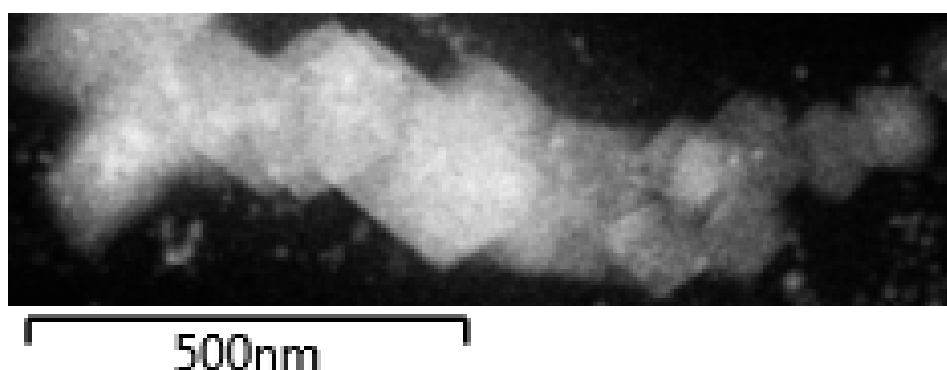


(b) Dark field STEM image.

Figure 5.15: STEM EDX elemental mapping of a cluster of smaller, mainly spherical shaped, nanoparticles.



(a) Image merge of STEM EDX elemental mapping. Pink = scandium, green = ytterbium, orange = iron.



(b) Dark field STEM image.

Figure 5.16: STEM EDX elemental mapping of large cubic nanoparticles.

Figure 5.16 shows another site on the same sample grid. Here, much larger cubic nanoparticles were visible, measuring 80 to 150 nm in size. The significant presence of scandium in figure 5.16a suggested that these larger nanoparticles were $\text{NaScF}_4\text{:Yb,Er}$. XRD analysis of the sample confirmed this assignment. $\text{NaScF}_4\text{:Yb,Er}$ UCNP of a similar size and shape nanoparticles were reported by Pei et al. when they used a ratio of fluoride: Ln^{3+} of 5:1.⁴² The ratio of fluoride: Ln^{3+} used in the two-step SPION shelling reaction was 4:1. According to results reported by Pei et al, this should have resulted in UCNP shell growth on the SPION surface, producing spherical nanoparticles around 50 nm in size.

The formation of separate SPIONs and UCNP, instead of the expected core-shell structure, led to a deeper consideration of the conditions needed to form a composite material. There has been some published literature studying the growth of active and passive shells on UCNP.⁶⁷ Work by Wang et al. showed that for $\text{NaGdF}_4\text{:Yb,Tm@NaGdF}_4\text{:Tb}$ core-shell UCNP, factors such as the ratio of core NPs to shell precursors affect the morphology of the product. Figure 5.17 shows that the volume of solvent in the reaction mixture is also fundamental in determining whether core-shell nanocrystals form or whether the reaction results in the formation of two types of core nanoparticles.

Another important factor to consider was the ratio of oleic acid to ODE in the reaction.

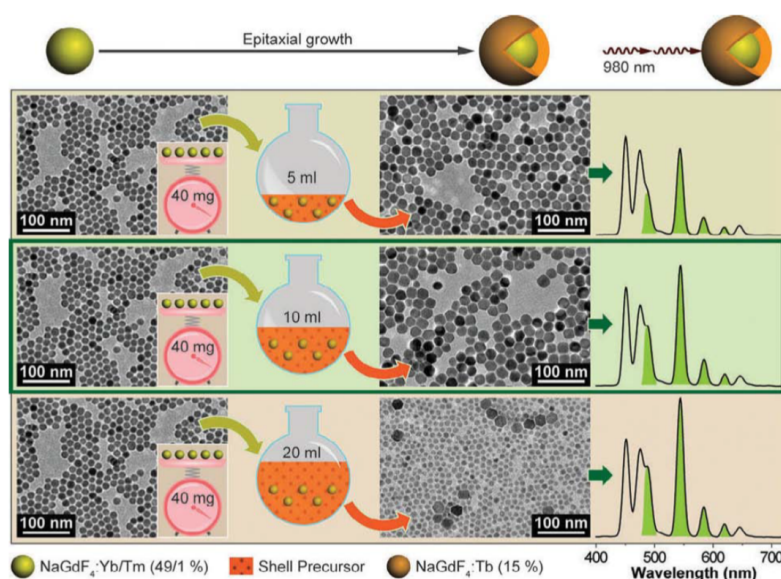


Figure 5.17: Experiments by Wang et al. investigating core-shell growth as a function of shell precursor function. Core-shell $\text{NaGdF}_4:\text{Yb,Tm}@\text{NaGdF}_4:\text{Tb}$ were synthesised. Reprinted by permission from Springer Nature: Nature Materials, reference 67.

Teng et al. reported that a ratio of oleic acid:ODE of 3:7 produced purely hexagonal phase $\text{NaScF}_4:\text{Yb,Er}$, which have been shown to have a brighter emission than the pure monoclinic phase Na_3ScF_6 .³⁹ The large number of factors determining the size, shape and crystal phase of the materials made planning and predicting reaction conditions to form core-shell reactions very challenging.

A series of two-step core-shell reactions were carried out, varying: SPION:lanthanide ratio, solvent volume and equivalents of fluoride precursor added. The samples were sent to the International Iberian Nanotechnology Laboratory in Braga, Portugal, where the analysis was carried out by Enrique Carbó-Argibay. STEM EDX was carried out using FEI Titan Themis microscope, which has much higher resolution than the JOEL JEM-F200 used in imaging in figures 5.15 and 5.16. The higher resolution microscope allowed individual nanoparticles to be imaged and mapped.

A significant issue arose when attempting to carry out STEM EDX analysis of the samples. Figure 5.18 demonstrates the issue encountered with organic contamination building up under the microscope beam. As was previously discussed in section 5.3.1.1, incomplete removal of excess oleic acid after nanoparticle synthesis created issues when carrying out TEM imaging of SPIONs. In STEM EDX, this contamination issue became much more prominent due to the way the sample was imaged. The electron beam in STEM moves across the sample in a raster-like pattern. The beam is focused at high intensity at each part of the sample and so the organic contamination quickly builds up on the sample grid. This contamination is faster still when high magnification is used and this prevents elemental mapping of single nanoparticles being obtained.



(a) STEM image after short beam exposure. (b) Visible contamination build-up after minutes of exposure to electron beam.

Figure 5.18: Organic contamination build-up on holey carbon grid after beam exposure. Scale bar represents 200 nm.

The issue of organic contamination was attempted to be circumvented by vacuum annealing and plasma cleaning the samples on the TEM grid before imaging. The vacuum annealing pre-treatment carried out on the core-shell samples was not successful. The contamination of the sample under the electron beam persisted, even after the sample was stored in a vacuum chamber for 24 h before imaging. The plasma cleaning pre-treatment technique consisted of exposing the sample grid to two cycles of plasma (Ar/O₂ - 75/25) for 3 + 5 seconds. The method did significantly reduce the contamination build-up on the sample but unfortunately the oxidative character of the plasma also damaged the sample. After imaging the sample for only a few seconds, holes appeared in the structure of the nanoparticles.

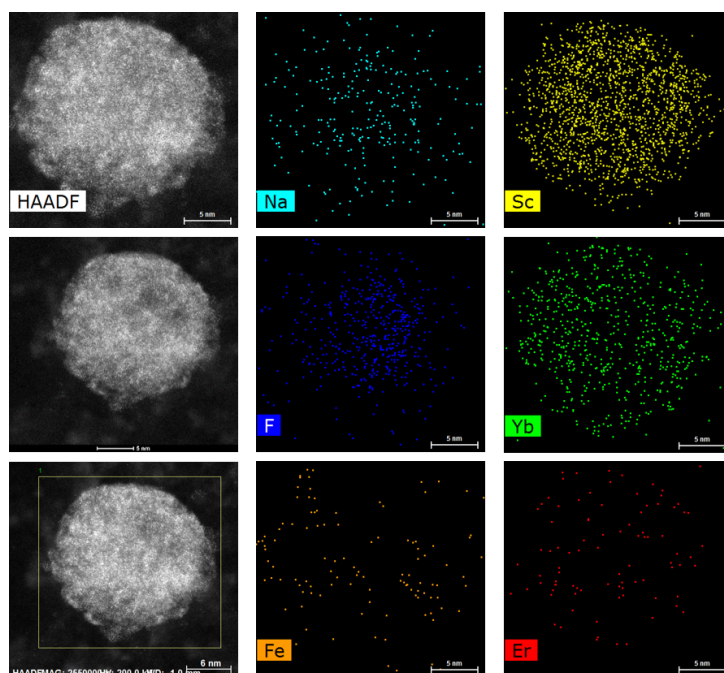
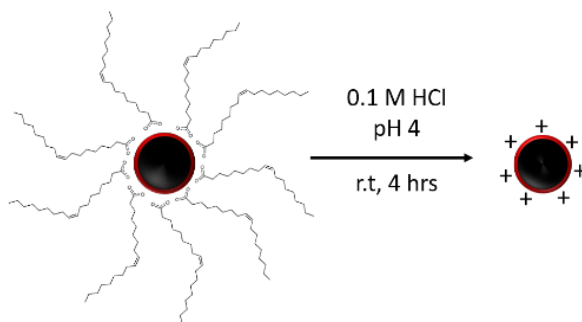


Figure 5.19: STEM EDX image of a damaged single nanoparticle after vacuum annealing to remove contamination. Scale bar represents 5 nm.

The images shown in figure 5.19 show elemental mapping of one nanoparticle, carried out for a few seconds before significant damage to the nanoparticle structure was caused. The elemental distribution of Na, Fe and Er mainly represent noise in the sample as the mapping could not be run for long enough to produce images with good resolution of these elements. The results demonstrate that elemental mapping of single nanocrystals is possible, but neither vacuum annealing nor plasma treatment work as methods to remove the organic contamination from the samples.

Ligand-Free Elemental Mapping

In chapter 3, section 3.3.2.1, a ligand-free method was carried out to exchange nanoparticle surface ligands. It was thought that this same ligand-free technique could be used to remove the oleic acid ligand from the surface of nanoparticles so the samples would be suitable for STEM EDX. The method used is shown in scheme 5.4.



Scheme 5.4: Dilute acid treatment of NPs from 2-step synthesis of $\text{Fe}_3\text{O}_4@\text{NaScF}_4:\text{Yb,Er}$, resulting in ligand-free NPs.

IR analysis was carried out to verify the oleic acid ligands had been successfully removed from the surface of the nanoparticles. TEM analysis was carried out to confirm the presence of nanoparticles in the sample, before the samples were sent to Dr Simon Fairclough at the University of Manchester. A Titan ChemiSTEM electron microscope was used for STEM EDX analysis.

Figure 5.20 shows the elemental maps obtained upon analysis of the samples by the Titan STEM in Manchester. Unfortunately the nanoparticle samples degraded in the time between TEM imaging and STEM EDX imaging, a month later. Individual nanoparticles could not be seen by the microscope, only the large aggregated structures as shown in figure 5.20.

More encouragingly, it was found that the sample did not contaminate under the electron beam. This shows that the ligand-free technique can provide a method to formally characterise core-shell nanoparticles, circumventing the issue of organic contamination. The technique will only be successful if the STEM EDX mapping is carried out soon after the ligand-free nanoparticles are prepared. In this case, the time between carrying out the ligand-free reactions and elemental mapping analysis was too long, resulting in degradation of the samples.

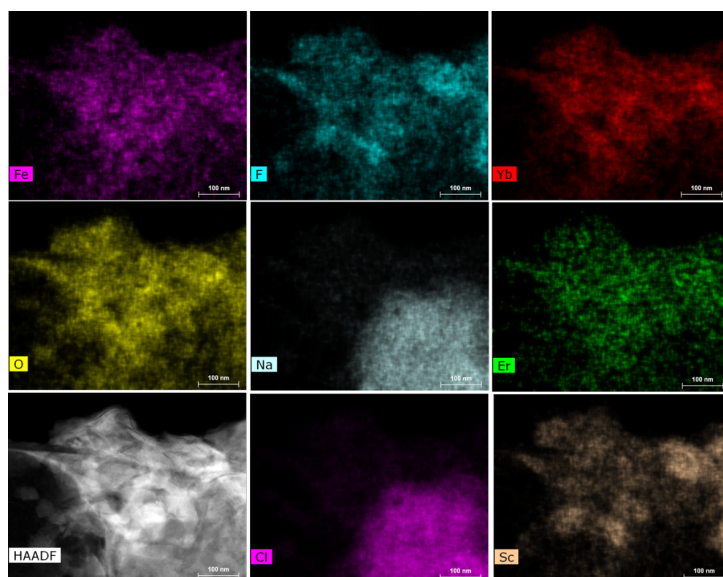


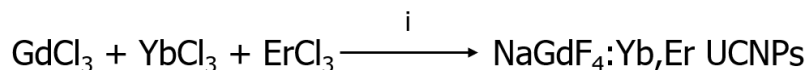
Figure 5.20: STEM EDX imaging of ligand-free core-shell UCNP-SPION sample.

The results show that the ligand-free technique can provide a way to prepare samples for STEM EDX. However, this is not an efficient method to screen core-shell synthesis attempts; the technique is costly, time consuming and was not available on site. On this basis, the work towards synthesising core-shell SPION-UCNPs was not pursued further. There is still a large unexplored field here. Characterising and optimising the synthesis of core-shell SPION-UCNPs has yet to be carried out in the field. It is hoped that this work could provide a springboard for further work in labs with reliable access to high resolution electron microscopy facilities.

5.3.3 Dual-Modal NaGdF₄:Yb,Er UCNPs

Due to the issues encountered in synthesising core-shell composite nanomaterials, Gd³⁺ doping of UCNPs was carried out. Gadolinium(III) has 7 unpaired electrons and as discussed in section 5.1.2, this can be used to decrease the T₁ relaxation time of surrounding water molecules.

NaGdF₄:Yb,Er NPs were synthesised according to work by Ryu et al.⁶⁸ The nanoparticles were synthesised in an analogous thermal decomposition technique to that discussed in chapter 2; a thermal decomposition process with lanthanide chlorides, oleic acid and ODE.



Scheme 5.5: Synthesis of NaGdF₄:Yb,Er UCNPs. Reagents and reaction conditions: (i) oleic acid, ODE, 120 °C, N₂ followed by sodium trifluoroacetate, 300 °C, N₂, 2 h.

TEM imaging of these nanoparticles was undertaken and a representative image for the sample is shown in figure 5.21. The images show monodisperse nanoparticles with a size of 30.6 ± 6.3 nm. The XRD pattern shown in figure 5.22b confirms the synthesis of a NaGdF₄ material.

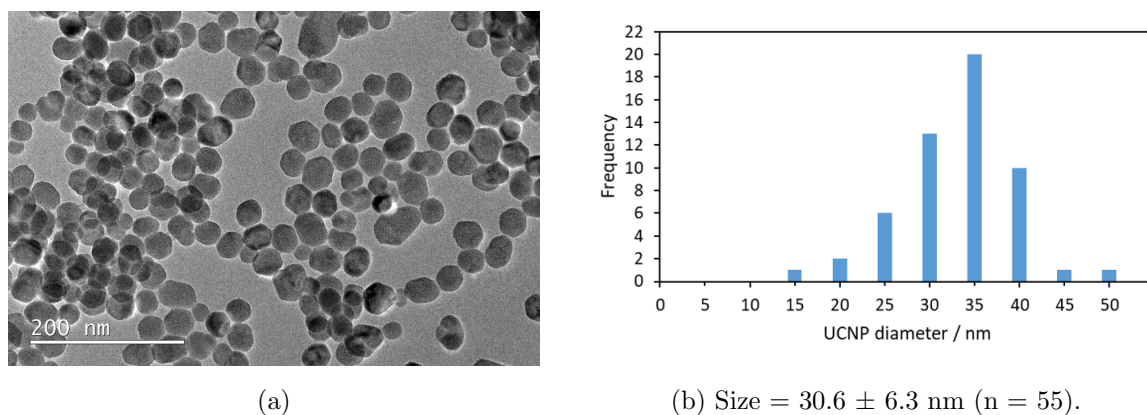


Figure 5.21: (a) Low resolution TEM and (b) Size distribution of NaGdF₄:Yb,Er UCNPs.

NaGdF₄:Yb,Er UCNPs were found to be less soluble than the yttrium containing NaYF₄:Yb,Er UCNPs. NaGdF₄:Yb,Er UCNPs were only soluble in toluene. Due to the low solubility and complications with sending the samples to Hong Kong for measurement, the upconversion photoluminescence measurements were carried out on solid samples of NaGdF₄:Yb,Er@oleic acid UCNPs. The results obtained are shown in figure 5.22a. (These measurements were kindly carried out by Dr Shuai Zha at Hong Kong Baptist University.) The spectrum shows the expected erbium emission peaks at 550 and 650 nm.

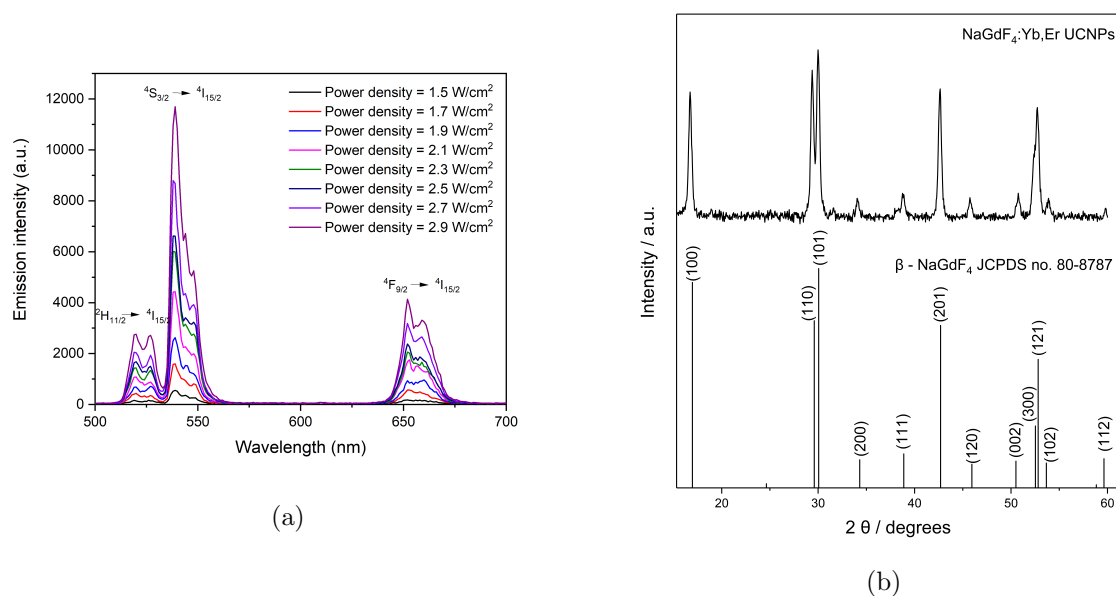


Figure 5.22: (a) Upconversion photoluminescence spectrum of solid sample of NaGdF₄:Yb,Er UCNPs capped with oleic acid. $\lambda_{\text{ex}} = 980$ nm; (b) XRD diffraction pattern of synthesised NaGdF₄:Yb,Er NPs, compared to the ICDD database sample for NaGdF₄. The Miller indices are shown for each diffraction peak.

The magnetic properties of the UCNPs were assessed by measurements of the longitudinal

relaxation time (T_1) of the probe. It was necessary to find out the Gd(III) concentration in the sample so that the longitudinal relaxivity (r_1) of the probe could be determined. Dispersions of approximately 2, 1 and 0.5 mM Gd(III) were prepared and the Gd(III) concentration was more accurately determined by ^1H NMR. ICP-MS was not readily available and for this reason, the Evans method to calculate gadolinium concentration was used.

The method uses the difference between the ^1H chemical shift of water with and without the Gd-containing probe to calculate Gd concentration. Water-soluble $\text{NaGdF}_4\text{:Yb,Er}$ nanoparticles were prepared by phase transferring the UCNPs with **PEG-BP**, in the same sonication method as that described in section 2.3.2.4. The $\text{NaGdF}_4\text{:Yb,Er@PEG-BP}$ UCNPs were dispersed in water and placed in a 1.7 mm diameter sealed capillary insert, within an NMR tube containing D_2O .⁶⁹

Equation 5.1 demonstrates how the difference in chemical shift can be used to calculate the concentration of gadolinium(III) in the sample.

$$\Delta_\chi = \frac{4\pi cS}{T} \left(\frac{\mu_{eff}}{2.84} \right)^2 \times 10^3 \quad (5.1)$$

where Δ_χ is the difference between the sample H_2O and reference H_2O chemical shifts (ppm) in the ^1H NMR, c is the concentration of Gd(III), $T = 298\text{ K}$, $\mu_{eff} = 7.94$ for Gd and $S = \frac{1}{3}$.

Inversion recovery experiments were conducted by Peter Haycock at Imperial College London, determining T_1 of each sample. The concentration of Gd(III) was plotted against $1/T_1$, as shown in figure 5.23. The gradient of this graph represents the longitudinal relaxivity of the system, calculated to be 0.300 mM s^{-1} .

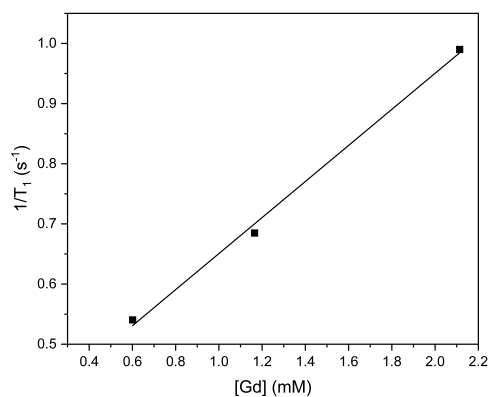


Figure 5.23: Gd concentration plotted against the respective $1/T_1$ value. The gradient of the graph represents the longitudinal relaxivity, R_1 value of the probe.

The calculated r_1 value of 0.300 mM s^{-1} is similar to that obtained by Ryu et al. for 40 nm $\text{NaGdF}_4\text{:Yb,Er}$ coated with amphiphilic polyacrylic acid derivatives (0.47 mM s^{-1}).⁶⁸ The r_1 value of a UCNP system has been shown to be size dependent, as well as dependent on the

surface ligands.⁶⁸ Larger particles have been demonstrated to have lower r_1 relaxivity values due to their proportionally smaller amount of surface Gd(III) ions. The relaxivity value of NaGdF₄:Yb,Er UCNP is significantly lower than that of the clinically approved Gd-DTPA (5.0 mM s⁻¹ at 1.5 Tesla). This, along with the toxicity considerations of Gd and the slow hepatic clearance of nanoparticles suggest that NaGdF₄:Yb,Er UCNP may not be suitable for *in vivo* imaging.

5.4 Conclusions

In summary, work described in this chapter moves towards the synthesis of UCNP-based multi-modal probes for imaging *in vivo*. Two dual-modal probe designs were explored: the synthesis of composite Fe₃O₄@NaScF₄:Yb,Er core-shell nanoparticles and the synthesis of Gd(III)-doped UCNP. The first route provides a probe which shortens the T₂ relaxation time of surrounding water protons, the second shortens the T₁ relaxation time.

In pursuit of synthesising core@shell Fe₃O₄@NaScF₄:Yb,Er NPs with high transverse relaxivity, attempts were made at synthesising anisotropic-shaped SPIONs. The first method used iron(III) oleate as the iron precursor, however issues were found with reproducibility of the reactions. Synthesis under the same reaction conditions resulted in nanoparticles of different sizes and shape. The lack of characterisation of iron(III) oleate is likely to be the cause of the reproducibility issues. The structure and controlled synthesis of iron oleate is not fully understood; different batches of iron oleate synthesised are likely to have different ratios of free to bound oleic acid. This irreproducibility makes the route unsuitable for clinical applications, where strict GMP (good manufacturing practice) controls the quality of pharmaceutical products. Synthesis of spherical SPIONs from iron acetylacetonate was found to be more straightforward and reproducible. This was the chosen route for further SPION synthesis.

Attempts were made to synthesise core-shell Fe₃O₄@NaScF₄:Yb,Er NPs. The initial one-step reaction, previously reported in the literature, was unsuccessful and so a two-step strategy was employed. It was found that the concentration of precursors during the shelling reaction is vital in determining the nature of the product produced. Initial reactions produced separate SPIONs and UCNP, rather than a composite material. Further attempts were made with the aim to optimise the synthesis, but there were issues encountered with the characterisation. High organic contamination of the nanoparticles during STEM EDX mapping was overcome by removing oleic acid from the surface of the nanoparticles by treatment with dilute HCl. Due to issues with access to high resolution electron microscopes and time constraints, it has not yet been possible to demonstrate the successful synthesis of core-shell SPION-UCNP materials.

Gd(III)-doping of UCNP was achieved and NaGdF₄:Yb,Er UCNP were synthesised. The dual-modal UCNP were shown to exhibit upconversion luminescence under irradiation at 980 nm. The longitudinal relaxivity of water-dispersible NaGdF₄:Yb,Er@PEG-BP UCNP was determined ($r_1 = 0.300$ mM s⁻¹). Optimisation of the size and surface-coverage of NaGdF₄:Yb,Er@PEG-BP UCNP has the potential to increase this value, resulting in a more sensitive contrast agent.

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Chapter 6

Conclusions & Future Work

Work in this project has focused on developing phosphonate-based ligands and using these to assemble UCNP-based nanoprobe. The predicted strong UCNP-phosphonate binding affinity was the starting point for research. The high phosphonate binding affinity discovered led to development of bisphosphonate functionalised fluorophores and assembly of fluorophore-functionalised UCNP nanosystems. Finally, work to introduce other imaging functionality to the nanoprobe was explored by combining MR contrast agents with UCNPs.

6.1 Studies of the Nanoparticle-Ligand Interface

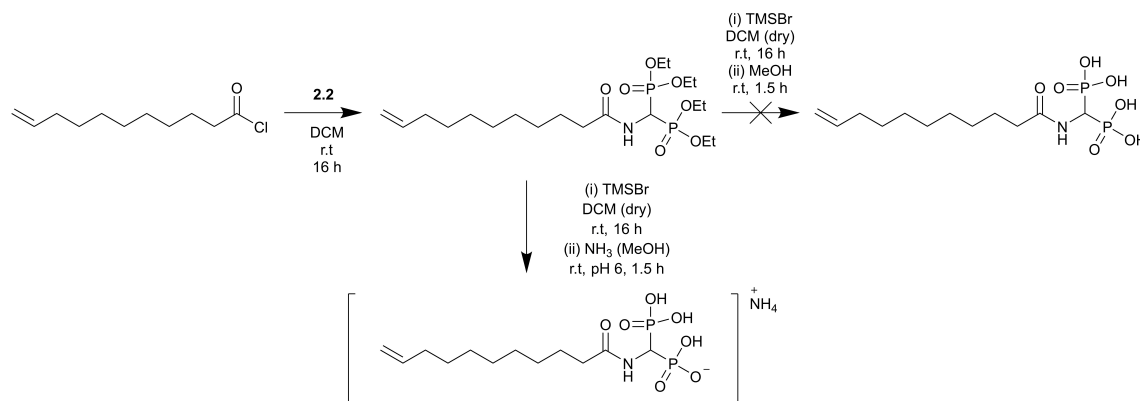
Ligand-UCNP binding affinity was studied using two methods: ^1H NMR titrations and DLS assessment of the colloidal stability of UCNP dispersions. Both of these methods demonstrated the strong binding of phosphonate ligands to the surface of UCNPs, in comparison to carboxylate ligands. The main conclusions from this work are summarised below, with suggestions of where future work may benefit the field.

6.1.1 ^1H NMR Studies:

^1H NMR studies were carried out, studying the relative ligand binding affinity of oleic acid and 10-undecenylphosphonic acid to NaYF_4 NPs. The alkene moiety in both ligands allowed the ligand exchange process to be followed. It was found that 10-undecenylphosphonic acid ligands irreversibly displaced oleic acid. This demonstrated the stronger binding affinity of phosphonates than carboxylates to NaYF_4 NPs.

It would be interesting to compare the binding affinity of a bisphosphonate ligand to a monophosphonate. For this, an alkene containing bisphosphonate ligand would need to be synthesised. Preliminary work (not discussed in this thesis) was attempted but unfortunately sufficient quantities of a alkene-bisphosphonate ligand was not able to be isolated. Scheme 6.1 shows the synthesis route attempted.

The alkene bisphosphonic ester was cleanly isolated, but then issues were encountered in the



Scheme 6.1: Synthesis of alkene-functionalised bisphosphonate ligand. It is envisioned a similar structure ligand could be used for ^1H NMR studies of bisphosphonate binding.

deprotection step. It was found that when methanol was added in part (ii), cleavage of the amide bond occurred. This was surprising but not unprecedented. When consulting the literature, it was found that Chougrani et al. reported ester cleavage during the deprotection of ester-containing bisphosphonic esters.¹ Addition of methanolic ammonia adjusted the pH to pH 6 and allowed isolation of the ammonium salt of the ligand. However, this was insoluble in chloroform. Due to time constraints in the project (re. the COVID-19 pandemic), unfortunately the ligand was not synthesised in high enough quantities to take through to a desalting step. Future work here would help elucidate the difference in binding strength between monophosphonic and bisphosphonic acids to UCNPs.

6.1.2 DLS Studies:

PEG-MP and **PEG-BP** ligands were synthesised and used, along with PEG-COOH, to phase transfer $\text{NaYF}_4:\text{Yb,Er}$ UCNPs. The colloidal stability of the resulting dispersions in water were assessed by DLS. The results from these studies suggest that the ligand with the strongest binding affinity to the UCNPs surface was **PEG-BP**. Further work needs to be undertaken with these water-dispersible nanoprobes. The colloidal stability of the UCNPs@PEG-BP dispersions in biologically relevant buffer should be assessed. For example, PBS contains phosphate groups which will have a strong binding affinity to the UCNPs surface. By conducting DLS studies in PBS, the affect of competing phosphate-based ligands on the colloidal stability of UCNPs could be assessed. If no large aggregates are observed when the UCNPs are dispersed in PBS, this would suggest the nanomaterial was colloidally stable. The **PEG-BP** ligand was designed to improve the biocompatibility of UCNPs and so the next logical steps for $\text{NaYF}_4@\text{PEG-BP}$ nanoprobes would be to take these forward into *in vitro* and if successful *in vivo* studies.

6.1.3 Photophysical Studies:

Another method to determine the long-term stability of UCNPs-bisphosphonate binding in aqueous environments could be to functionalise the surface of the UCNPs with a fluorophore. If the fluorophore is anchored to the UCNPs surface by a bisphosphonate moiety, then the fluorophore

should stay bound when the nanoprobe is stored as a dispersion. The fluorophore binding over time could be assessed by periodic precipitation of the UCNP and conducting photophysical measurements of the solvent. If the fluorophore remains bound to the UCNP surface, it would be expected that no UV-Vis absorption would be detected in the solvent; all fluorophore would be still bound to the UCNP. If an absorption or fluorescent signal was detected in the dispersing solvent then this would suggest desorption of the fluorophore from the UCNP surface. As discussed for DLS studies, these photophysical studies could be conducted in biologically relevant media.

Figure 6.1 shows the two novel rhodamine-bisphosphonate derivatives developed in this project. **3.5** is a pH-sensitive rhodamine derivative; the absorption and emission properties of the dye are dependent on the pH of the surrounding medium. The fluorophore is highly fluorescent at pH values below pH 5.85. **3.12** contains a piperazine ring, locking the rhodamine derivative into its permanently ring-open form. The fluorophore is highly fluorescent over all pH values measured. For the purpose of studying nanoparticle-fluorophore binding strength, **3.12** would be the more suitable derivative to use as then the pH of the media would not need to be controlled.

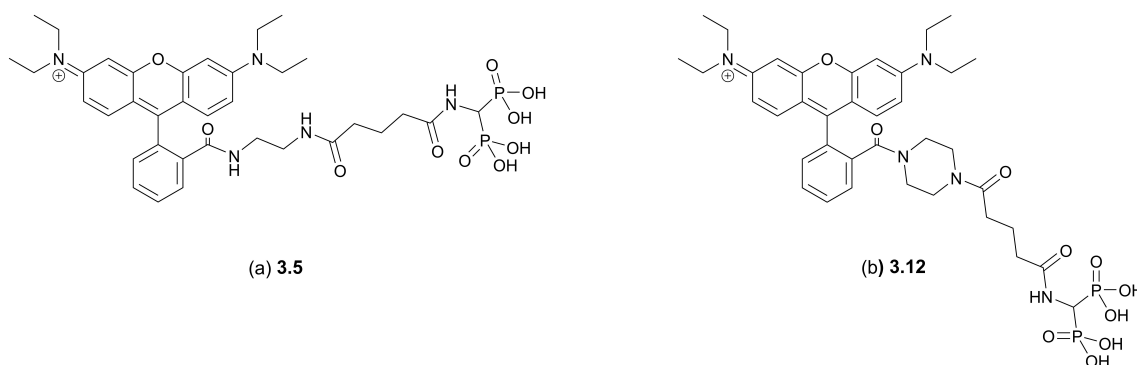


Figure 6.1: Two rhodamine-bisphosphonate derivatives synthesised in this thesis; (a) **3.5** is a pH-sensitive rhodamine bisphosphonate derivative with pka 5.85; (b) **3.12** is a pH-insensitive rhodamine bisphosphonate derivative, highly fluorescent between pH 3 and 8.

A literature search has been conducted and there were no examples found of studying UCNP-fluorophore binding affinity by this proposed method. It is envisioned that carrying out these ligand-binding studies would provide a valuable contribution to the field and help further understand the UCNP-ligand interface.

6.2 Energy Transfer Between UCNP and Surface Fluorophores

Two nanosystems were designed to promote energy transfer between UCNP and surface fluorophores. These are shown in figure 6.2. In the first, water dispersible UCNP, with pH-sensitive rhodamine-bisphosphonate were assembled. It was thought these nanoparticles could be used as environmental pH sensors. Unfortunately, there was no evidence of energy transfer occurring between the UCNP core and rhodamine ligands on the UCNP surface. In the second nanoprobe,

cyanine-808 ligands were exchanged with oleic acid on the surface of $\text{NaYF}_4:\text{Yb,Er}@ \text{NaYF}_4:\text{Nd}$ UCNP. This resulted in a nanomaterial which was dispersible in chloroform. Photophysical characterisation provided evidence of energy transfer, with the fluorophore acting as a sensitizer and enhancing UCNP emission.

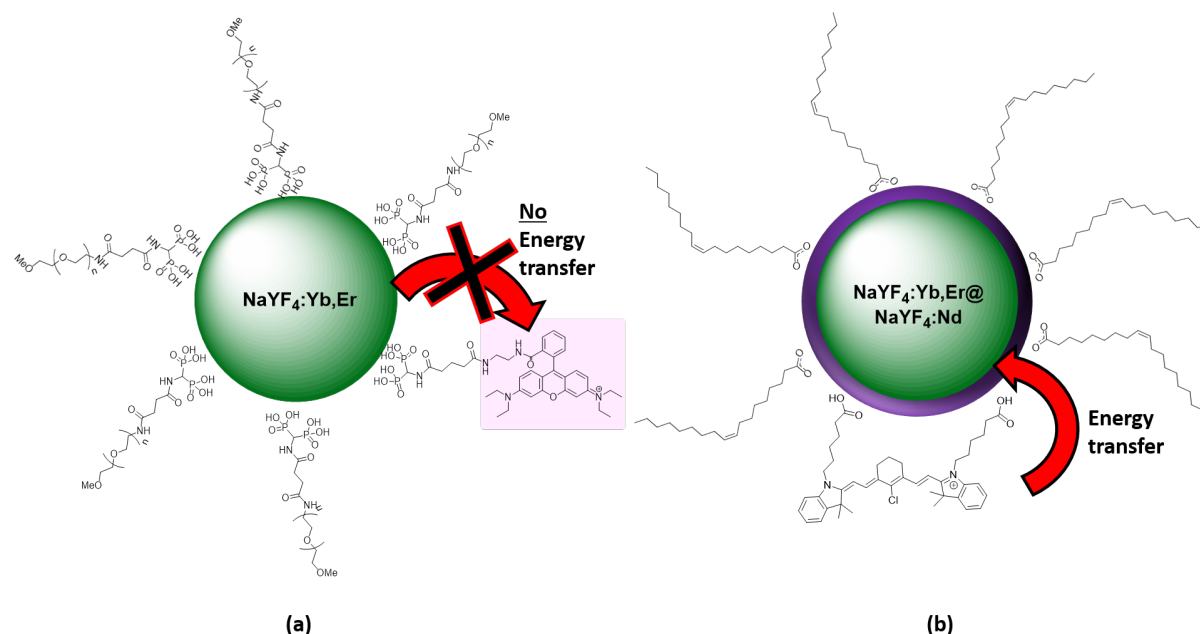


Figure 6.2: UCNP-based energy transfer nanoprobe designed in this thesis; (a) shows the design of the water dispersible, pH-sensitive nanoprobe discussed in chapter 3. Energy transfer was not detected between the UCNP core and surface rhodamine fluorophore; (b) shows the design of the chloroform dispersible, cyanine-sensitized nanoprobe discussed in chapter 4. Evidence of energy transfer was detected between surface cyanine fluorophore and the core UCNP.

6.2.1 Rhodamine-Based UCNP as Nanosensors:

The pH-sensitive rhodamine bisphosphonate derivative **3.5** was chosen as the fluorophore to functionalise the surface of $\text{NaYF}_4:\text{Yb,Er}$ UCNP. Upon excitation at 980 nm, it was thought that a ratiometric measurement of the 540:660 nm erbium emission would allow determination of the environmental pH. Unfortunately energy transfer was not detected in the system and no expected quenching of the UCNP emission at 540 nm was detected at pH 4. Two synthesis methods were attempted, as it was thought that **3.5** may have been prevented binding to the UCNP surface due to the bulky **PEG-BP** ligands. Ultimately this two-step strategy was also unsuccessful.

It was disappointing that energy transfer was not observed, but the system does warrant further investigation. It is important to understand what is preventing energy transfer in the system: water quenching, **PEG-BP** induced quenching, lack of rhodamine-BP or another factor. Logistical issues in this project prevented the synthesis of large numbers of samples, as these needed to be transported to Hong Kong for measurement. The next step for this work would be synthe-

sis of ligand-free $\text{NaYF}_4\text{:Yb,Er}$ UCNP and functionalisation with **3.5**, without the addition of **PEG-BP**. These samples should be measured in water to see whether energy transfer occurs.

There are also a number of other factors which could be optimised in these systems. Energy transfer will only occur from surface lanthanide ions and so energy transfer in smaller UCNP would be more apparent. The use of inert UCNP shells may prevent quenching of excited states by water, increase the lanthanide emission from the system and increase the excited states available for energy transfer to the surface fluorophores. There is a fine balance to be struck here: resonance energy transfer is distance dependent and so significantly increasing the distance between the UCNP emitting species and fluorophores will dramatically decrease the energy transfer efficiency.²⁻⁵

$\text{NaYF}_4\text{:Yb,Er}$ was chosen as the core nanomaterial for the rhodamine-functionalised nanoprobe as preliminary qualitative studies suggested that the material was brighter and more soluble than $\text{NaGdF}_4\text{:Yb,Er}$ UCNP. However, for energy transfer nanomaterials, $\text{NaGdF}_4\text{:Yb,Er}$ UCNP may be more useful. Deng et al. in 2016 reported that the use of $\text{NaGdF}_4\text{:Yb,Er}$ enabled a resonant energy migration process, where energy was transferred through Gd ions in the crystal lattice.⁶ This allowed emission to occur from fluorophores on the surface of large 30 nm UCNP.

6.2.2 Cyanine-Sensitized UCNP:

Cyanine-808 fluorophores were taken forward to functionalise the surface of synthesised $\text{NaYF}_4\text{:Yb,Er@NaYF}_4\text{:Nd}$ UCNP. Cyanine ligands functionalised with bisphosphonic esters were successfully synthesised, however all deprotection attempts resulted in decomposition of the ligands. The carboxylate terminated cyanine-808 was therefore used for UCNP dye-sensitization. Cyanine-functionalised $\text{NaYF}_4\text{:Yb,Er@NaYF}_4\text{:Nd}$ UCNP in chloroform showed successful sensitization. Energy transfer took place: cyanine-808 $\rightarrow$ Nd(III) in UCNP shell $\rightarrow$ Yb(III) in UCNP core $\rightarrow$ Er(III) in UCNP core.

An attempt was made to study the dye-sensitization process in water, phase transferring the $\text{NaYF}_4\text{:Yb,Er@NaYF}_4\text{:Nd}$ UCNP using **PEG-BP**. In these systems, Er emission was not visible. This needs further work. In developing UCNP for bioimaging, the sensitization must occur in aqueous conditions. Recent work by Han et al. and Garfield et al. suggest that a triplet state is involved in the energy transfer from surface fluorophore to lanthanides.^{7,8} If this is the case, the triplet state is likely to be quenched by water and this will prevent effective dye-sensitization of the UCNP. The instability of cyanine-808 may also be an issue; decomposition may be occurring in the aqueous conditions. This warrants further investigation to help understand the dye-sensitization mechanism and establish the applicability of this method to aqueous environments.

6.3 Dual-Modal Imaging with UCNP

MR functionality was attempted to be introduced to UCNP-based nanoprobe by two methods. In the first, SPIONs were synthesised as the core nanomaterial and it was attempted to grow

UCNP shells on the surface of the nanocrystals. The conditions for this shelling step were challenging to optimise and unfortunately no evidence of core-shell SPIONS@UCNPs was found. In the second method, Gd(III) was doped into the core of the UCNPs and NaGdF₄:Yb,Er UCNPs were synthesised.

6.3.1 SPION@UCNP Synthesis Attempts

SPIONs were synthesised as the core material of proposed core-shell Fe₃O₄@NaScF₄:Yb,Er NPs. Attempts were made at replication of the synthesis of octapod-shaped SPIONs but the published synthesis could not be reproduced. Instead, spherical SPIONs were taken forward as the core nanomaterial. The aim was to grow an upconversion shell (NaScF₄:Yb,Er) on the surface of SPIONs, but ultimately this was not successful. Progress was in characterising these nanomaterials. It was found that the most promising characterisation strategy was high resolution STEM-EDX imaging, allowing mapping of the elemental composition of a single nanoparticle. The issue of organic contamination was circumvented by treating the nanomaterial with dilute acid to strip the organic ligands off the surface. Unfortunately access to high resolution microscopy equipment was not readily available. It was found that there were many variables in the synthesis and optimisation of these to form core-shell nanomaterials was very challenging. It was not possible to readily analyse the large quantities of samples necessary for optimisation with the equipment available during this project. There is a gap in the literature for the synthesis and characterisation of these core-shell SPION@UCNP nanomaterials. It is hoped that this thesis can provide a stepping stone for future work,

6.3.2 Gd-Doping for T₁ Relaxivity Enhancement

Gd-doping of UCNPs was achieved by synthesis of NaGdF₄:Yb,Er UCNPs. These UCNPs were phase-transferred to water and a relaxivity (r_1) value of 0.300 mM⁻¹s was measured. This is comparable to other NaGdF₄:Yb,Er UCNPs synthesised, but considerably lower than 5.0 mM⁻¹s for the clinically used Gd-DTPA.

The r_1 value of UCNPs could be increased by exploring a different Gd-doping strategy. The chelate DO3A contains a free amine in the structure, which is available for functionalisation. It is envisaged that the BP ligand **2.2** could be coupled to Gd-DO3A and this ligand could be bound to the surface of UCNPs. The inner-sphere contribution is dominant in determining the T₁ relaxation rate and this occurs from water molecules directly coordinating to the paramagnetic ion. Therefore, containing the Gd(III) ions on the surface of UCNPs, rather than in the core, is likely to lead to higher r_1 relaxivity of the probe. A design for this nanosystem is shown in figure 6.3.

It is important to consider the specific applications for the nanoprobe to assess whether a dual-modal probe would be diagnostically useful. As discussed in chapter 5, Gd toxicity is becoming a more prominent issue especially after recent studies reported Gd deposits in the brains of patients who had undergone multiple Gd-enhanced MRI scans. Introduction of a Gd-chelate is likely to increase the relaxivity of the nanoprobe, in comparison to core Gd-doping

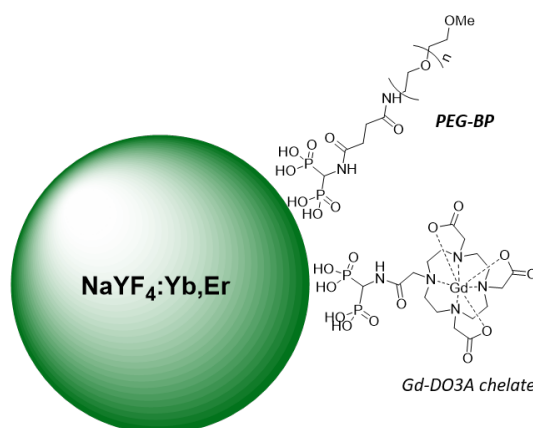


Figure 6.3: Speculative design of a dual-modal UCNP-based nanoprobe, with MR functionality achieved by introduction of Gd chelators on the UCNP surface, bound by a bisphosphonate moiety.

but importantly moving forward questions should be asked about whether this is necessary. Are we adding anything to the diagnostic power of these nanoprobe by introducing further functionality?

6.4 Final Remarks

In the introduction to this thesis, a number of factors were identified which are essential for UCNPs to make the step into clinical use. These are: reproducible control over the core nanomaterial size and shape, detectable signal and biocompatibility. Work presented in this thesis has shown how challenging meeting some of these points are.

In chapter 5, the challenges faced in developing reproducible nanoparticle syntheses were encountered. It is essential that nanomaterials used in *in vivo* have consistent properties. This therefore will favour simple materials, rather than complex core-shell structures.

Signal detectability is an issue especially encountered when UCNPs are dispersed in water. Work in this thesis has shown that dye-sensitization is possible in organic solvents. The challenge still remains to replicate this in aqueous conditions.

Finally, the UCNP nanoprobe must be biocompatible. In this thesis, work has focused on using phosphonate anchoring groups to robustly attach ligands to the surface of the UCNPs and the results are promising. The stability of these systems in biological conditions is yet to be determined and ultimately, the only way to assess biocompatibility of new imaging agents would be to test them *in vivo*.

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Chapter 7

Experimental

7.1 General Procedures

Materials and Experimental Methods: All chemicals were purchased from Sigma-Aldrich Chemical Co. unless otherwise stated and all were used without further purification. MeO-PEG-COOH (Mw = 5000 Da) was purchased from Iris Biotech GMBH. Column chromatography was performed using silica gel under mild pressure or using a Biotage automated column with Biotage SNAP KP-Sil cartridges. Nanoparticles were synthesised using a Horst HG - 450 °C, 100 mL Glass Yarn Heating Mantle, connected to a Horst HT MC11 - Microprocessor Temperature Regulator, with a Fe-CuNi thermocouple. Dialysis was performed using a Float-A-Lyser system with a 3500 Da molecular weight cut-off cellulose membrane, against distilled water. Samples were lyophilised using an Alpha 1-2LD plus -55 °C freeze dryer overnight.

Instrumentation: ^1H , ^{13}C , $^{31}\text{P}\{^1\text{H}\}$ NMR spectra were obtained using a Bruker AV-400 MHz spectrometer at 295 K and internally referenced to the residual solvent peaks of: CDCl_3 at 7.26 ppm (^1H) and 77.16 ppm (^{13}C), MeOH at 3.31 ppm (^1H) and 49.15 ppm (^{13}C) and H_2O at 4.79 ppm (^1H). Chemical shifts are reported in parts per million (ppm) and coupling constants in Hertz (Hz). Peak multiplicities are abbreviated as; s = singlet, d = doublet, t = triplet, q = quartet, P=pentet, m = multiple, dd = doublet of doublet and br = broad. Spectra were analysed using MestReNova software. High temperature NMR experiments were conducted by Peter Haycock of the NMR Service Facility of Imperial College London, Department of Chemistry.

Electrospray Time-of-Flight and high-resolution mass spectra were obtained using a Waters LCT PremierMass spectrometry by Dr Lisa Haigh of the Mass Spectrometry Service, Imperial College London. Thermogravimetric analysis was carried out by Dr Lisa Haigh using a Mettler Toledo TGA. ICP-OES measurements were carried out at the National University of Singapore using a Perkin Elmer Avio 500 Inductively Coupled Plasma-Optical Emission Spectrometer.

Transmission electron microscopy (TEM) images were taken on a JEOL 2100Plus at 200 keV, fitted with an Oxford Instruments INCA EDS 80 mm X-Max detector system for energy dis-

persive spectroscopy (EDS). Image analysis was performed using ImageJ. DLS and ζ -potential measurements were made using a Malvern Zetasizer Ultra. XRD measurements were carried out on solid samples with a Bruker D2 PHASER and the X-ray diffraction patterns were compared to the Cambridge Crystallographic Data Centre (CCDC) database of standard samples.

UV-Visible absorption spectra were measured using an Agilent Technologies Cary 60 Spectrophotometer operating with WinUV software. The samples were held in a quartz cuvette with a path length of 1 cm. Emission spectra were acquired using an Agilent Technologies Carry Eclipse Fluorescence Spectrophotometer. pH measurements were carried out using a Jenway model 3510 pH/mV/temperature meter with a Jenway 924 005 pH electrode, calibrated against 'NIST' pH, 10.00, pH 7.00 and pH 4.00 buffers. Visible emission spectra of UCNPs were measured using a SpectroFluorometer System (Horiba/Fluoromax-4) equipped with a 980 nm laser as the excitation source. Infrared spectra were collected in the solid state using a Perkin Elmer Spectrum 100 FTIR Spectrometer.

7.2 Chapter 2 Synthetic Protocols

NaYF₄ NPs:

YCl₃ (195.2 mg, 1.00 mmol) was added to 1-octadecene (15 mL) and oleic acid (6 mL). The mixture was heated to 120 °C under vacuum for 30 mins. The reaction mixture was cooled to room temperature and sodium trifluoroacetate (340 mg, 2.50 mmol) was added. The solution was then rapidly heated to 300 °C under N₂. After 2 hours the reaction mixture was cooled to room temperature. Ethanol (300 mL) was added to precipitate the nanoparticles and the solid pellet was collected by centrifugation at 4000 rpm for 10 mins. The nanoparticles were redispersed in hexane (10 mL) and ethanol (25 mL) was added to again precipitate the nanoparticles. This washing step was repeated once more resulting in an off-white solid of NaYF₄@oleic acid NPs (105.0 mg). The NPs were dispersed in hexane for storage at 4 °C.

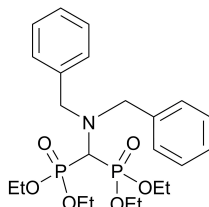
Sample preparation for ¹H titrations:

NaYF₄@oleic acid NPs were prepared for ¹H titrations by dispersing the dry solid (10 mg) in CDCl₃ (500 μ L). Ferrocene (0.02 mg, 0.1 μ mol) was added to the NP dispersion to act as an internal standard, allowing the determination of the concentration of oleic acid and phosphonic acid. Separately, a stock solution of 10-undecenylphosphonic acid was prepared (20 mg in 1 mL CDCl₃, 0.09 mM). 10-undecenylphosphonic acid was titrated into the NP sample (1 eq = 10 μ L stock solution). After each addition, a 128 scan ¹H NMR spectrum was recorded. Addition of aliquots of 10-undecenylphosphonic acid continued up to 200 equivalents of the ligand.

Isolation of NaYF₄@phosphonic acid NPs was achieved by precipitation of the final sample by the addition of ethanol (3 mL). The solid pellet was collected by centrifugation at 4000 rpm for 10 mins and redispersed in hexane (1 mL). The ethanol addition and resuspension steps were repeated twice more to wash out any free oleic acid, 10-undecenylphosphonic acid and ferrocene. The dry solid NaYF₄@phosphonic acid NPs was dispersed in CDCl₃ (500 μ L). Ferrocene (0.02

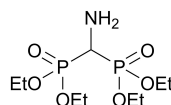
mg, 0.1 μmol) was again added to the NP dispersion to act as an internal standard. Separately, a stock solution of oleic acid was prepared (10 mg in 1 mL CDCl_3 , 0.07 mM). Oleic acid was titrated into the NP sample (1 eq = 10 μL stock solution). After each addition, a 128 scan ^1H NMR spectrum was recorded. Addition of aliquots of oleic acid continued up to 200 equivalents of the ligand.

Tetraethyl (N,N-dibenzyl)aminomethyl-bisphosphonate (2.1):

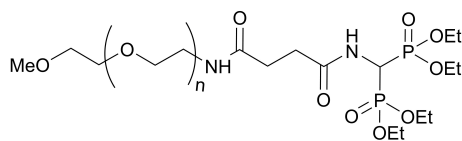


The synthesis of **2.1** was carried out according to literature procedures.^{1,2} Triethyl orthoformate (11.9 mL, 71.5 mmol), diethyl phosphite (23.9 mL, 185.5 mmol) and dibenzylamine (12.0 mL, 62.4 mmol) were refluxed under an N_2 atmosphere at 150 $^\circ\text{C}$ for 5 hours. The temperature was increased to 160 $^\circ\text{C}$ for a further 24 hours to remove the volatile impurities. Chloroform (300 mL) was added and the solution was washed with 2 M NaOH (3 x 60 mL) and brine (2 x 75 mL). The chloroform was then removed *in vacuo* resulting in a yellow coloured oil. The oil was purified by silica gel column chromatography (hexane:ethyl acetate), with the product eluting at 100 % ethyl acetate. A colourless oil of **2.1** was isolated (10.4 g, 21.5 mmol, 35 % yield). ^1H NMR (400 MHz, CDCl_3): δ 7.20–7.36 (m, 10H), 4.14 (m, 12H), 3.57 (t, $^2J_{\text{PH}} = 25.1$ Hz, 1H), 1.34 (t, $^3J_{\text{HH}} = 7.1$ Hz, $^4J_{\text{PH}} = 2.0$ Hz, 12H). ^{13}C NMR (100 MHz, CDCl_3): δ 129.4, 128.1, 127.2, 62.4, 56.3, 54.7, 16.5. $^{31}\text{P}\{^1\text{H}\}$ NMR (161.84 MHz, CDCl_3): δ 20.1. MS(ES $^+$): calcd. for $\text{C}_{23}\text{H}_{36}\text{NO}_6\text{P}_2$ 484.2018, found 484.2028 $[\text{M}]^+$.

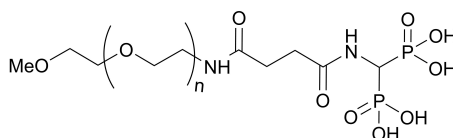
Tetraethyl aminomethyl-bisphosphonate (2.2):



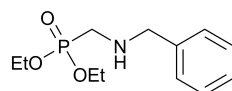
The synthesis of **2.2** was carried out according to literature procedures with minor modification.³ A solution of (**2.1**) (1.00 g, 2.07 mmol) in absolute ethanol (30 mL) was added to a Schlenk charged with 10 % Pd/C (400 mg). The solution was degassed and the Schlenk was filled with H_2 (1 bar). The black suspension was refluxed at 80 $^\circ\text{C}$ for 24 hours. The suspension was then cooled to room temperature and filtered through celite, resulting in a colourless oil. (605 mg, 2.00 mmol, 96 % yield). ^1H NMR (400 MHz, CDCl_3): δ 4.25 (m, 8H), 3.44 (t, $^2J_{\text{PH}} = 20.6$ Hz, 1H), 1.70 (br s), 1.39 (t, $^3J_{\text{HH}} = 7.1$ Hz, 12H). ^{13}C NMR (100 MHz, CDCl_3): δ 63.3, 31.1, 16.6. $^{31}\text{P}\{^1\text{H}\}$ NMR (161.84 MHz, CDCl_3): δ 20.3. MS(ES $^+$): calcd. for $\text{C}_9\text{H}_{24}\text{NO}_6\text{P}_2$ 304.1079, found 304.1088 $[\text{M}]^+$.

PEG-coupled bisphosphonic ester (2.3):

The synthesis of **2.3** was carried out according to literature procedures.³ MeO-PEG-COOH (400 mg, 0.08 mmol, 1 eq) and dicyclohexylcarbodiimide (DCC) (24 mg, 0.17 mmol, 2.1 eq) were dissolved in dry DCM (1.5 mL) and cooled to 0 °C, under N₂. The solution was stirred at 0 °C for 5 mins, and then a solution of **2.2** (24 mg, 0.08 mmol, 1 eq) in dry DCM (0.5 mL) was added dropwise. The solution was stirred under N₂ at room temperature for 16 hrs, in which time a white precipitate formed. The reaction mixture was cooled in the freezer for 4 hours and the precipitate was removed by gravity filtration. The solution was dried *in vacuo* resulting in an off-white solid of **2.3** (344.3 mg, 0.06 mmol, 75 % yield). ¹H NMR (400 MHz, CDCl₃): δ 3.67 (s, -(CH₂-CH₂-O)-). This peak dominates the ¹H NMR spectrum.

Deprotected PEG-coupled bisphosphonate (PEG-BP):

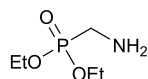
The synthesis of **PEG-BP** was carried out according to literature procedure.³ Briefly, **2.3** (330 mg, 0.06 mmol) was dissolved in dry DCM (2 mL). At 0 °C and under N₂, bromotrimethylsilane (TMSBr) (200 μL, 1.5 mmol, 25 eq) was added dropwise. The solution was left stirring at room temperature for 16 hrs and then dried *in vacuo*. The off-white solid was then dissolved in methanol (3 mL) and stirred for 1.5 hrs, before the methanol was removed *in vacuo*. The white solid was dissolved in distilled water (5 mL) and purified by dialysis (3,500 Mw cutoff). The resulting cloudy solution was lyophilised, resulting in a white solid of **PEG-BP** (239 mg, 0.05 mmol, 83 % yield). ¹H NMR (400 MHz, CDCl₃): δ 3.67 (s, -(CH₂-CH₂-O)-). This peak dominates the ¹H NMR spectrum. ³¹P{¹H} NMR (161.84 MHz, D₂O, 358 K): δ 13.7 (br).

Diethyl (N,N-dibenzyl)aminomethylphosphonate (2.4):

The synthesis of **2.4** was carried out by adaptation of a literature procedure.⁴ 1,3,5-tribenzylhexahydro-1,3,5-triazine (1.38 g, 3.86 mmol) and diethyl phosphite (1.5 mL, 10.13 mmol) were heated at 100 °C for 16 hours. The solution was cooled to room temperature and volatile impurities were removed *in vacuo*. Chloroform (30 mL) was added and the solution was washed with 2 M NaOH (3 x 10 mL) and brine (2 x 10 mL). The chloroform was removed

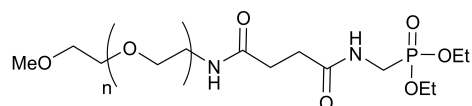
in vacuo and the yellow oil was purified by silica gel column chromatography (hexane:ethyl acetate), with the product eluting at 100 % ethyl acetate. A colourless oil of **2.4** was isolated (1.20 g, 4.65 mmol, 46 % yield). ^1H NMR (400 MHz, CDCl_3): δ 7.36–7.28 (m, 4H), 7.28–7.22 (m, 2H), 4.20–4.09 (m, 4H), 3.87 (s, 2H), 2.95 (d, $^3J_{\text{HH}} = 12.6$ Hz, 2H), 1.33 (t, $^3J_{\text{HH}} = 7.1$ Hz, 6H). ^{13}C NMR (100 MHz, CDCl_3): δ 128.6, 128.5, 127.5, 62.3, 62.3, 60.5, 54.9, 54.7, 44.9, 43.3, 16.7, 16.6. $^{31}\text{P}\{^1\text{H}\}$ NMR (161.84 MHz, CDCl_3): δ 26.0. MS(ES+): calcd. for $\text{C}_{12}\text{H}_{21}\text{NO}_3\text{P}$ 258.1259, found 258.1259 $[\text{M}]^+$.

Diethyl aminomethylphosphonate (**2.5**):

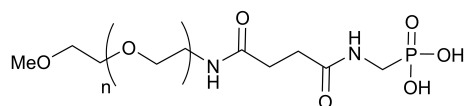


The synthesis of **2.5** was carried out by adaptation of a literature procedure.⁴ A solution of (**2.4**) (1.00 g, 3.88 mmol) in absolute ethanol (30 mL) was added to a Schlenk charged with 10 % Pd/C (400 mg). The solution was degassed and the Schlenk was filled with H_2 (1 bar). The black suspension was refluxed at 80 °C for 24 hours. The suspension was then cooled to room temperature and filtered through celite, resulting in a yellow oil. (605 mg, 2.00 mmol, 96 % yield). The yellow oil was purified by silica gel column chromatography twice (hexane:ethyl acetate) then (DCM:methanol). A colourless oil **2.5** was isolated (0.252 g, 1.50 mmol, 39 % yield). ^1H NMR (400 MHz, CDCl_3): δ 4.19 – 4.08 (m, 4H), 3.01 (d, $^3J_{\text{HH}} = 10.3$ Hz, 2H), 1.34 (t, $^3J_{\text{HH}} = 7.1$ Hz, 6H). ^{13}C NMR (100 MHz, CDCl_3): δ 62.1, 38.5, 37.1, 16.5. $^{31}\text{P}\{^1\text{H}\}$ NMR (161.84 MHz, CDCl_3): δ 27.3. MS(ES+): calcd. for $\text{C}_5\text{H}_{15}\text{NO}_3\text{P}$ 168.0784, found 168.0780 $[\text{M}]^+$.

PEG-coupled monophosphonic ester (**2.6**):* Novel compound



MeO-PEG-COOH (200 mg, 0.04 mmol) and dicyclohexylcarbodiimide (DCC) (12 mg, 0.085 mmol) were dissolved in dry DCM (2.0 mL) and cooled to 0 °C, under N_2 . The solution was stirred at 0 °C for 5 mins, and then a solution of **2.5** (10 mg, 0.06 mmol) in dry DCM (0.5 mL) was added dropwise. The light yellow solution was stirred under N_2 at room temperature for 16 hrs, in which time a white precipitate formed. The reaction mixture was cooled in the freezer for 4 hours and the precipitate was removed by gravity filtration. The solution was dried *in vacuo* resulting in a white solid of **2.6** (156.7 mg, 0.03 mmol, 76 % yield). ^1H NMR (400 MHz, CDCl_3): δ 3.67 (s, $-(\text{CH}_2\text{-CH}_2\text{-O})-$). This peak dominates the ^1H NMR spectrum.

Deprotected PEG-coupled monophosphonate (PEG-MP):* Novel compound

2.6 (150 mg, 0.03 mmol) was dissolved in dry DCM (2 mL). At 0 °C and under N₂, bromotrimethylsilane (TMSBr) (100 µL, 0.75 mmol) was added dropwise. The solution was left stirring at room temperature for 16 hrs and then dried *in vacuo*. The off-white solid was then dissolved in methanol (3 mL) and stirred for 1.5 hrs, before the methanol was removed *in vacuo*. The white solid was dissolved in distilled water (5 mL) and purified by dialysis (3,500 Mw cut-off). The resulting cloudy solution was lyophilised, resulting in an off-white solid of **PEG-MP** (101 mg, 0.02 mmol, 68 % yield). ¹H NMR (400 MHz, CDCl₃): δ 3.67 (s, -(CH₂-CH₂-O)-). This peak dominates the ¹H NMR spectrum. ³¹P{¹H} NMR (161.84 MHz, D₂O): δ 20.5 (br).

NaYF₄:Yb,Er UCNPs:

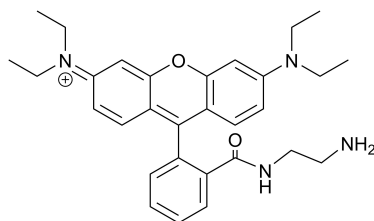
YCl₃ (121.0 mg, 0.800 mmol), YbCl₃ (50.3 mg, 0.180 mmol) and ErCl₃ (5.5 mg, 0.020 mmol) in methanol (5 mL) were added to oleic acid (6 mL) and 1-octadecene (15 mL). The solution was heated under vacuum at 120 °C for 1 h to remove the solvent and any residual water. The solution was then cooled to room temperature and sodium trifluoroacetate (340.0 mg, 2.500 mmol) was added under a flow of N₂. The solution was heated rapidly to 300 °C, under N₂ and this temperature was maintained for 2 h. The reaction mixture was then cooled to room temperature and ethanol (60 mL) was added to precipitate the nanoparticles. The nanoparticles were washed with ethanol (3 x 20 mL) resulting in a white solid of NaYF₄:Yb,Er UCNPs (142.8 mg). NaYF₄:Yb,Er UCNPs were dispersed in hexane (20 mL) at stored at 4 °C.

NaYF₄:Yb,Er@PEG-BP UCNPs:

NaYF₄:Yb,Er@oleic acid UCNPs (2 mg) and **PEG-BP** (10 mg) were added to chloroform (2 mL) in an open glass vial. The dispersion was sonicated until the solvent had evaporated. Water (2 mL) was added, dispersing the UCNPs. Hexane (1 mL) was added to the cloudy colourless dispersion, the vial was shaken and the hexane layer carefully removed. This acted to remove the free oleic acid and the process was repeated 3 times in total. Acetone (5 mL) was added to the resulting water suspensions of NaYF₄:Yb,Er@PEG-BP UCNPs to precipitate the UCNPs and remove any free textbfPEG-BP. The NaYF₄:Yb,Er@oleic acid UCNPs were collected by centrifugation at 4000 rpm for 10 mins (~ 2 mg).

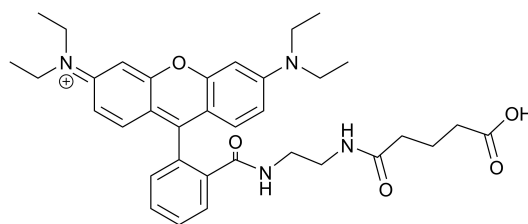
7.3 Chapter 3 Synthetic Protocols

Rhod-EA (3.2):



Preparation of **3.2** was carried out according to literature procedures.⁵ Ethylenediamine (2.5 mL, 37.4 mmol) was added dropwise to a stirring suspension of rhodamine B (2.49 g, 5.1 mmol) in ethanol (20 mL). The orange suspension was heated to reflux, upon which the solid dissolved. After 16 hours, the orange solution was cooled to room temperature. The solvent was removed *in vacuo* and then water (20 mL) was added. The product was extracted with DCM (3 x 10 mL), dried over MgSO₄ and the solvent was removed *in vacuo*. The product was isolated as a orange crystalline solid (1.80 g, 3.7 mmol, 73 % yield). ¹H NMR (400 MHz, CDCl₃): δ 7.88 (dd, ³J_{HH} = 5.8, 3.0 Hz, 1H), 7.41 (dt, ³J_{HH} = 7.4, 3.7 Hz, 2H), 7.11 – 7.01 (m, 1H), 6.41 (d, ³J_{HH} = 8.8 Hz, 2H), 6.35 (d, ³J_{HH} = 2.6 Hz, 2H), 6.25 (dd, ³J_{HH} = 8.8, 2.6 Hz, 2H), 5.26 (br s), 3.30 (q, ³J_{HH} = 7.1 Hz, 9H), 3.16 (t, ³J_{HH} = 6.7 Hz, 2H), 2.38 (t, ³J_{HH} = 6.7 Hz, 2H), 1.13 (t, ³J_{HH} = 7.0 Hz, 12H). ¹³C NMR (100 MHz, CDCl₃): δ 169.2, 153.7, 153.4, 149.0, 132.7, 131.0, 128.7, 128.3, 124.0, 123.0, 108.4, 105.3, 97.8, 77.5, 77.2, 76.8, 65.5, 44.5, 43.1, 41.1, 12.73. MS(ES⁺): calcd. for C₃₀H₃₇N₄O₂ 485.2917, found 485.2901 [M]⁺.

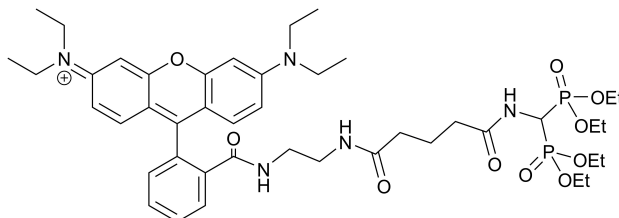
Rhod-EA-Glut (3.3): * Novel compound



Glutaric anhydride (1.17 g, 10.30 mmol) and **3.2** (1.00 g, 2.00 mmol) were added to acetonitrile (20 mL). The orange suspension was refluxed overnight, in which time the solid dissolved to give a bright pink solution. The solution was cooled to room temperature and the solvent removed *in vacuo*. The pink oil was redissolved in DCM (20 mL) and washed with water (3 x 10 mL) and then dried over MgSO₄. The crude product was purified by column chromatography (DCM:methanol) yielding a pink solid (0.56 g, 0.94 mmol, 47 % yield). ¹H NMR (400 MHz, CDCl₃): δ 7.94–7.87 (m, 1H), 7.51–7.43 (m, 2H), 7.13–7.07 (m, 1H), 6.96 (t, ³J_{HH} = 4.4 Hz, 1H), 6.41 (d, ³J_{HH} = 8.9 Hz, 2H), 6.37 (d, ³J_{HH} = 2.6 Hz, 2H), 6.28 (dd, ³J_{HH} = 8.9, 2.6 Hz, 2H), 5.30 (s, 1H), 3.33 (q, ³J_{HH} = 7.2 Hz, 10H), 3.05–2.97 (m, 2H), 2.41 (t, ³J_{HH} = 7.1 Hz, 2H), 2.23 (t, ³J_{HH} = 7.1 Hz, 2H), 1.94 (p, ³J_{HH} = 7.1 Hz, 2H), 1.16 (t, ³J_{HH} = 7.0 Hz, 12H). ¹³C

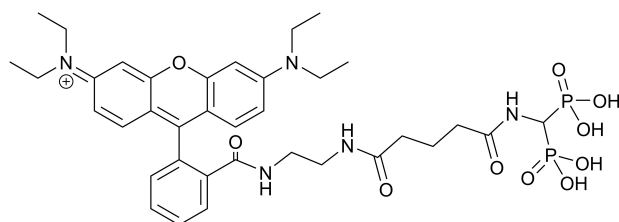
NMR (100 MHz, CDCl_3) δ 176.3, 172.9, 170.2, 153.8, 153.4, 150.0, 133.1, 130.4, 130.3, 128.5, 128.4, 124.0, 123.0, 110.6, 108.5, 98.4, 66.0, 44.6, 40.4, 40.3, 35.3, 33.5, 21.0, 12.6. MS(ES⁺): calcd. for $\text{C}_{35}\text{H}_{43}\text{N}_4\text{O}_5$ 599.3233, found 599.3257 $[\text{M}]^+$.

Protected Rhod-EA-Glut-BP (3.4): * Novel compound



A solution of **3.3** (396 mg, 0.660 mmol), **2.2** (200 mg, 0.660 mmol), HBTU (750 mg, 1.980 mmol) and triethylamine (145 μL , 1.980 mmol) in DCM (10 mL) was stirred for 16 hr at room temperature. After 16 hr a DCM (10 mL) was added to the light pink solution. The solution was washed sequentially with 10 % citric acid (5 mL), saturated sodium bicarbonate (5 mL) and then brine (5 mL). The solution was dried over MgSO_4 and the solvent was removed *in vacuo*. The purple solid product was purified by column chromatography (DCM:methanol) resulting in the dark pink solid of **3.4** (83 mg, 0.094 mmol, 14 % yield). ^1H NMR (400 MHz, CDCl_3): δ 8.01 – 7.92 (m, 1H), 7.48 – 7.43 (m, 2H), 7.14 – 7.05 (m, 1H), 7.03 (t, $^3J_{\text{HH}} = 4.9$ Hz, 1H), 6.41 (t, $^3J_{\text{HH}} = 8.6$ Hz, 2H), 6.38 – 6.35 (m, 2H), 6.30 – 6.24 (m, 2H), 5.19 – 5.03 (t, $^3J_{\text{HH}} = 22.2$ Hz, 1H), 4.27 – 4.09 (m, 8H), 3.35 – 3.27 (m, 10H), 3.03 – 2.97 (m, 2H), 2.39 – 2.31 (m, 2H), 1.96 – 1.90 (m, 2H), 1.31 (td, $^3J_{\text{HH}} = 7.1$, $^4J_{\text{PH}} = 1.4$ Hz, 12H), 1.16 (t, $^3J_{\text{HH}} = 7.0$ Hz, 12H). $^{31}\text{P}\{^1\text{H}\}$ NMR (161.84 MHz, CDCl_3): δ 16.9. MS(ES⁺): calcd. for $\text{C}_{44}\text{H}_{64}\text{N}_5\text{O}_{10}\text{P}_2$ 884.4128, found 884.4097 $[\text{M}]^+$.

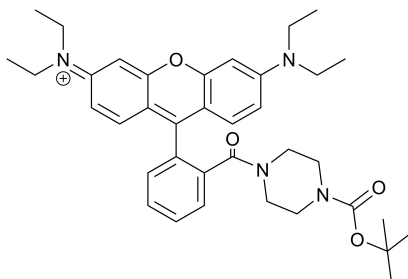
Deprotected Rhod-EA-Glut-BP (3.5): * Novel compound



Under N_2 , a solution of **3.4** (83.0 mg, 0.094 mmol) in dry DCM (5 mL) was prepared. Bromotrimethylsilane (100 μL , 0.758 mmol) was added dropwise and a colour change from light pink to dark pink was observed. The reaction mixture was stirred at room for 16 h and the solvent was subsequently removed *in vacuo*. Methanol was added to the pink solid and the solution was stirred at room temperature for 90 mins. Methanol and other volatiles were then removed *in vacuo* resulting in a bright pink solid (50.0 mg, 0.065 mmol, 69 % yield). ^1H NMR (400 MHz, Methanol - d_4) δ 8.03 – 7.99 (m, 1H), 7.85 – 7.81 (m, 2H), 7.70 – 7.61 (m, 3H), 7.44 – 7.37 (m, 2H), 7.24 – 7.18 (m, 1H), 7.08 – 7.02 (m, 2H), 4.85 (t, $^2J_{\text{PH}} = 22.1$ Hz, 1H), 3.74 (q, $^3J_{\text{HH}} = 7.1$ Hz, 10H), 3.00 – 2.87 (m, 2H), 2.30 (t, $^3J_{\text{HH}} = 7.2$ Hz, 2H), 2.18 – 2.10 (m, 2H), 1.84

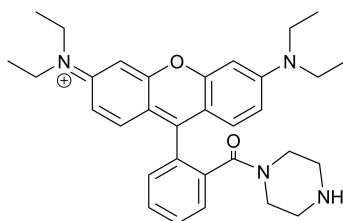
(t, $^3J_{\text{HH}} = 7.6$ Hz, 3H), 1.21 (t, $^3J_{\text{HH}} = 7.1$ Hz, 12H). ^{13}C NMR (100 MHz, CDCl_3) δ 175.7, 174.6, 170.5, 153.5, 153.4, 140.3, 135.2, 132.1, 131.2, 131.0, 125.1, 124.6, 122.4, 119.9, 113.6, 79.5, 65.3, 55.0, 49.9, 41.0, 38.8, 35.8, 35.7, 23.1, 10.8. $^{31}\text{P}\{^1\text{H}\}$ NMR (161.84 MHz, Methanol - d_4): δ 14.6. MS(ES+): calcd. for $\text{C}_{36}\text{H}_{48}\text{N}_5\text{O}_{10}\text{P}_2$ 772.2720, found 772.2704 $[\text{M}]^+$.

Rhod-Pip-Boc (3.6):

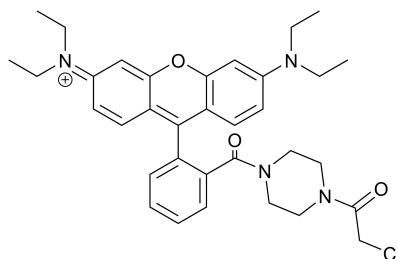


3.6 was prepared with a modification to literature procedures.⁶ Rhodamine B (2.00 g, 4.17 mmol), 1-Boc-piperazine (0.78 g, 4.18 mmol), HBTU (1.90 g, 5.02 mmol) and triethylamine (1.40 mL, 10.00 mmol) were added to DCM (50 mL). The purple solution was stirred for 2 h and then the DCM was removed *in vacuo*. The purple solid was redissolved in ethyl acetate (100 mL) and washed sequentially with 10 % citric acid (25 mL), saturated sodium bicarbonate (25 mL) and brine (25 mL). The purple solution was dried over MgSO_4 and then the solvent was removed *in vacuo*. The crude purple solid was taken through to the next step without purification (1.97 g, 3.31 mmol, 79 % crude yield).

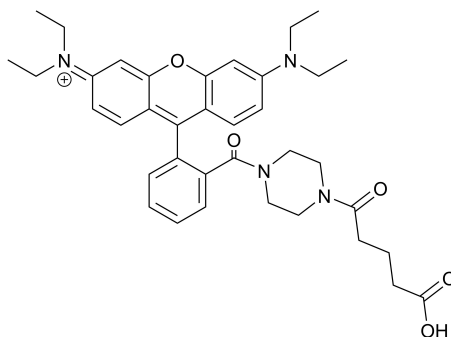
Rhod-Pip (3.7):



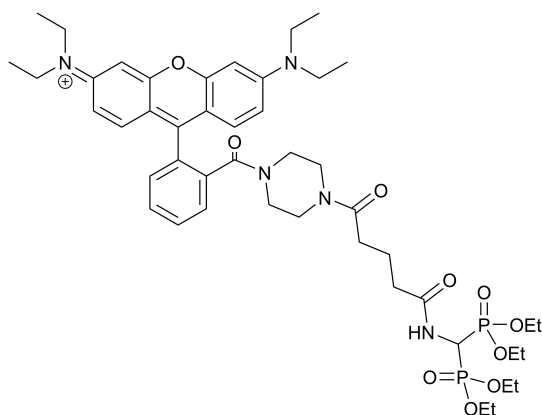
3.7 was prepared with a modification to literature procedures.⁶ **3.6** (1.97 g, 3.31 mmol) was added to DCM (5 mL) and TFA (5 mL) was added dropwise. An immediate colour change from purple to red was observed. The solution was stirred at room temperature for 16 h. Saturated sodium bicarbonate (50 mL) was added, to neutralise the TFA. The aqueous phase was discarded and the organic phase was dried over MgSO_4 . DCM was removed *in vacuo*, resulting in a dark pink solid of the crude product. The crude product was purified by silica gel column chromatography (DCM:methanol) resulting in a dark pink solid (567 mg, 1.11 mmol, 34 % yield). ^1H NMR (400 MHz, CDCl_3) δ 7.71 – 7.61 (m, 2H), 7.58 – 7.50 (m, 1H), 7.36 – 7.30 (m, 1H), 7.24 (d, $^3J_{\text{HH}} = 9.5$ Hz, 2H), 7.00 (d, $^3J_{\text{HH}} = 10.0$ Hz, 2H), 6.78 (d, $^3J_{\text{HH}} = 2.5$ Hz, 2H), 3.76 – 3.55 (m, 10H), 3.40 (d, $^3J_{\text{HH}} = 16.4$ Hz, 4H), 2.71 (t, $^3J_{\text{HH}} = 5.0$ Hz, 4H), 1.33 (t, $^3J_{\text{HH}} = 7.1$ Hz, 12H). MS(ES+): calcd. for $\text{C}_{32}\text{H}_{39}\text{N}_4\text{O}_2$ 511.3073, found 511.3077 $[\text{M}]^+$.

Rhod-Pip-Acetyl-Cl (3.8):

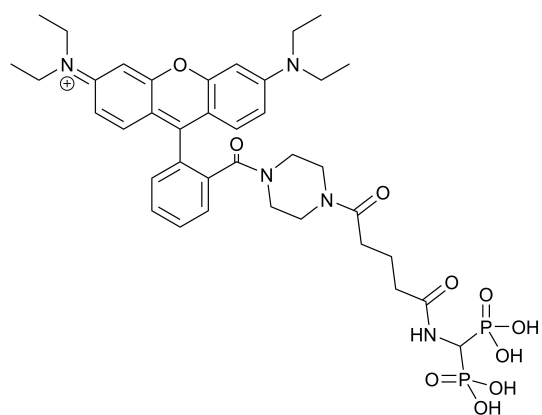
3.10 was prepared with a modification to literature procedures.⁷ **3.7** (560 mg, 1.10 mmol) and triethylamine (353 μ L) were added to DCM (20 mL) and the solution was cooled to 0 °C. A solution of chloroacetyl chloride (97 μ L, 1.57 mmol) in DCM (0.5 mL) was added dropwise and then the solution was stirred at room temperature for 3 h. The solvent was removed *in vacuo* and the crude purple solid was purified by silica gel column chromatography (DCM:methanol) to yield a dark pink solid (129 mg, 0.220 mmol, 20.0 % yield). ¹H NMR (400 MHz, CDCl₃) δ 7.64 (t, ³J_{HH} = 6.1 Hz, 2H), 7.51 (d, ³J_{HH} = 6.4 Hz, 1H), 7.30 (d, ³J_{HH} = 6.6 Hz, 1H), 7.25 – 7.13 (m, 2H), 7.03 (d, ³J_{HH} = 9.6 Hz, 1H), 6.89 – 6.65 (m, 3H), 4.26 (s, 2H), 3.71 – 3.52 (m, 8H), 3.48 – 3.04 (m, 8H), 1.28 (t, ³J_{HH} = 7.0 Hz, 13H). MS(ES⁺): calcd. for C₃₄H₄₀N₄O₃Cl 587.2789, found 587.2783 [M]⁺.

Rhod-Pip-Glut (3.10):

3.10 was prepared with a modification to literature procedures.⁸ **3.7** (383 g, 0.749 mmol) and glutaric anhydride (427 mg, 3.75 mmol) were added to acetonitrile (10 mL). The orange suspension was refluxed overnight, in which time the solid dissolved to give a purple solution. The solution was cooled to room temperature and the solvent removed *in vacuo*. The pink oil was redissolved in DCM (50 mL) and washed with water (3 x 10 mL) and then dried over MgSO₄. The crude product was purified by repeated column chromatography (DCM:methanol) yielding a pink solid (28.0 mg, 0.031 mmol, 4.1 % yield). ¹H NMR (400 MHz, CDCl₃) δ 7.66 (m, 2H), 7.52 (m, 1H), 7.30 (m, 1H), 7.27 (m, 2H), 7.11 (m, 2H), 6.75 (m, 2H), 3.64 (m, 10H), 3.28 (m, 12H), 2.40 (d, ³J_{HH} = 7.5 Hz, 2H), 2.20 (m, 2H), 1.31 (t, ³J_{HH} = 7.1 Hz, 12H). MS(ES⁺): calcd. for C₃₇H₄₅N₄O₅ 625.3384, found 625.3384 [M]⁺.

Protected Rhod-Pip-Glut-BP (3.11): * Novel compound

A solution of **3.10** (28.0 mg, 0.031 mmol), **2.2** (30.0 mg, 0.099 mmol), HBTU (55.0 mg, 0.145 mmol) and triethylamine (20.0 μ L, 0.143 mmol) in DCM (5 mL) was stirred for 16 hr at room temperature. After 16 h, the bright pink solution was diluted with DCM (10 mL) and washed sequentially with 10 % citric acid (5 mL), saturated sodium bicarbonate (5 mL) and then brine (5 mL). The organic phase was dried over MgSO_4 and the solvent was removed *in vacuo*. The pink crude product was purified by column chromatography (DCM:methanol), resulting in a pink solid (15.2 mg, 0.017 mmol, 54 % yield). ^1H NMR (400 MHz, Methanol- d_4) δ 7.21 – 7.12 (m, 2H), 7.09 (d, $^3J_{\text{HH}} = 4.0$ Hz, 1H), 6.92 (d, $^3J_{\text{HH}} = 11.3$ Hz, 1H), 6.67 (d, $^3J_{\text{HH}} = 9.4$ Hz, 2H), 6.45 (dd, $^3J_{\text{HH}} = 9.5, 2.4$ Hz, 2H), 6.35 (d, $^3J_{\text{HH}} = 2.5$ Hz, 2H), 4.45 (s, 2H), 3.67 – 3.51 (m, 8H), 3.18 – 2.98 (m, 8H), 2.88 – 2.73 (m, 8H), 0.70 – 0.66 (m, 24H). $^{31}\text{P}\{^1\text{H}\}$ NMR (161.84 MHz, CDCl_3): δ 16.5. MS(ES $^+$): calcd. for $\text{C}_{46}\text{H}_{66}\text{N}_5\text{O}_{10}\text{P}_2$ 910.4285, found 910.4326 $[\text{M}]^+$.

Deprotected Rhod-Pip-Glut-BP (3.12): * Novel compound

Under N_2 , a solution of **3.11** (15.2 mg, 0.017 mmol) in dry DCM (5 mL) was prepared. Bromotrimethylsilane (50 μ L, 0.379 mmol) was added dropwise and a colour change from pink to dark red was observed. The reaction mixture was stirred at room for 16 h and the solvent was then removed *in vacuo*. Methanol (5 mL) was added to the pink solid and the solution was stirred at room temperature for 90 mins. Methanol and other volatiles were then removed *in*

vacuo resulting in a bright pink solid (5.3 mg, 0.007 mmol, 41 % yield). ^1H NMR (400 MHz, Methanol - d_4): δ 7.28 (d, $^3J_{\text{HH}} = 9.5$ Hz, 1H), 7.23 – 7.17 (m, 3H), 7.16 – 7.07 (m, 5H), 6.96 (d, $^3J_{\text{HH}} = 2.5$ Hz, 1H), 3.69 (m, 5H), 3.40 (s, 4H), 2.44 (d, $^3J_{\text{HH}} = 23.2$ Hz, 2H), 2.31 (d, $^3J_{\text{HH}} = 0.7$ Hz, 4H), 1.92 – 1.79 (m, 2H), 1.66 – 1.54 (m, 1H), 1.33 – 1.26 (m, 12H). $^{31}\text{P}\{^1\text{H}\}$ NMR (161.84 MHz, Methanol - d_4): δ 14.7. MS(ES+): calcd. for $\text{C}_{38}\text{H}_{50}\text{N}_5\text{O}_{10}\text{P}_2$ 798.3033, found 798.3012. $[\text{M}]^+$.

Ligand-free $\text{NaYF}_4\text{:Yb,Er}$ UCNPs:

Ligand-free $\text{NaYF}_4\text{:Yb,Er}$ UCNPs were prepared according to literature procedures.⁹ $\text{NaYF}_4\text{:Yb,Er@oleic acid}$ UCNPs (100 mg) were added to distilled water (5 mL) and the pH was adjusted to pH 4 by the addition of 0.1 M HCl. The mixture was stirred at room temperature for 3 hours, where the pH was periodically tested and adjusted to maintain pH 4. The reaction mixture was extracted with diethyl ether (3 x 10.0 mL) to remove the free oleic acid. The organic layers were combined and further extracted with distilled water (30 mL). Finally the two aqueous layers were combined and extracted with diethyl ether (30.0 mL). The cloudy aqueous dispersion of ligand-free $\text{NaYF}_4\text{:Yb,Er}$ UCNPs was stored at 4 °C (~ 40.0 mg).

One-pot preparation of $\text{NaYF}_4\text{:Yb,Er@PEG-BP,Rhod-BP}$ UCNPs:

Ligand-free $\text{NaYF}_4\text{:Yb,Er}$ UCNPs (2 mg) and **PEG-BP** (10 mg) were added to distilled water (2 mL). Depending on the mol % of rhodamine-bisphosphonate functionalisation desired, a specific quantity of **3.5** was added to the dispersion (for example 1 mol % **3.5** = 0.0173 mg). The pH was adjusted to pH 8 by addition of 0.1 M NaOH and the dispersion was stirred at room temperature for 16 hours. Acetone (5 mL) was added to precipitate the nanoparticles and then the solid was collected by centrifugation at 4000 rpm for 10 mins. The washing step was repeated twice more, each time adding acetone (5 mL). This removed any free **PEG-BP** or **3.5** from the dispersion. The functionalised UCNPs were lyophilised, to allow transportation, yielding a pink solid (~ 2 mg).

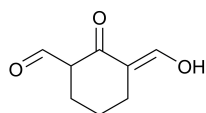
Stepwise preparation of $\text{NaYF}_4\text{:Yb,Er@PEG-BP,Rhod-BP}$ UCNPs:

Ligand-free $\text{NaYF}_4\text{:Yb,Er}$ UCNPs (2 mg) were added to distilled water (2 mL). Depending on the quantity of rhodamine-bisphosphonate functionalisation desired, a specific quantity of **3.5** was added to the dispersion (for example 1 equivalent **3.5** = 0.0173 mg). The pH was adjusted to pH 8 by addition of 0.1 M NaOH and the dispersion was stirred at room temperature for 2 hours. To the pink dispersion, **PEG-BP** (10 mg) was added. The dispersion was stirred at room temperature for another 16 hours. Acetone (5 mL) was added to precipitate the nanoparticles and then the solid was collected by centrifugation at 4000 rpm for 10 mins. The washing step was repeated twice more, each time adding acetone (5 mL). This removed any free **PEG-BP** or **3.5** from the dispersion. The functionalised UCNPs (~ 2 mg) were redispersed in distilled water (1 mL) and stored at 4 °C.

7.4 Chapter 4 Synthetic Protocols

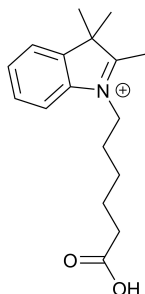
NaYF₄:Yb,Er@NaYF₄:Nd UCNPs: YCl₃ (132.6 mg, 0.680 mmol) and NdCl₃ (34.1 mg, 0.136 mmol) were added to oleic acid (3 mL) and 1-octadecene (7.5 mL). The solution was heated under vacuum at 120 °C for 1 h to remove any residual water. The solution was then cooled to room temperature and NaYF₄:Yb,Er UCNPs (71.4 mg) in hexane (10 mL) were added. The hexane was then removed *in vacuo* and sodium trifluoroacetate (170.0 mg, 1.250 mmol) was added under a flow of N₂. The solution was heated rapidly to 300 °C, under N₂ and this temperature was maintained for 2 h. The reaction mixture was then cooled to room temperature and ethanol (60 mL) was added to precipitate the nanoparticles. The nanoparticles were washed with ethanol (3 x 20 mL) resulting in a light purple solid of NaYF₄:Yb,Er@NaYF₄:Nd UCNPs (180.2 mg). The NaYF₄:Yb,Er@NaYF₄:Nd UCNPs were redispersed in distilled water (20 mL) and stored at 4 °C.

2-chloro-3-(hydroxymethylene)cyclohex-1-ene-1-carbaldehyde (4.1):



4.1 was prepared according to literature procedures.¹⁰ DMF (25.0 mL, 323 mmol) was cooled to 0 °C and phosphorus (V) oxychloride (7.0 mL, 74 mmol) was added dropwise. The solution was stirred at room temperature for 30 mins. Cyclohexanone (2.5 mL, 24 mmol) was added dropwise and the dark red solution was stirred at 50 °C for 5 h. The red reaction mixture was poured over a beaker of crushed ice (70 g) and left stirring at room temperature for 16 h. The yellow precipitate was isolated by vacuum filtration and recrystallised from DCM (350 mL). This resulted in a yellow crystalline solid (1.59 g, 9.2 mmol, 38 % yield). ¹H NMR (400 MHz, DMSO - d₆): δ 8.80 (br s, 1H), 2.36 (t, ³J_{HH} = 6.1 Hz, 4H), 1.58 (p, ³J_{HH} = 6.3 Hz, 2H). MS(ES⁺): calcd. for C₈H₉ClO₂ 173.0369, found 173.0374 [M]⁺.

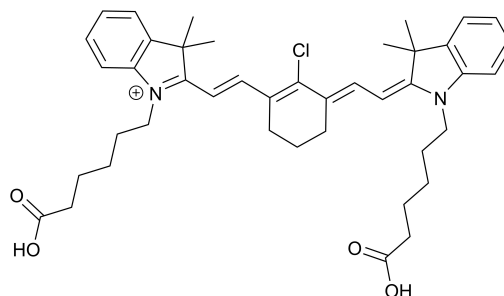
1-(5-carboxypentyl)-2,3,3-trimethyl-3H-indol-1-ium (4.2):



4.1 was prepared according to literature procedures.¹⁰ Under N₂, 2,3,3-trimethylindolenine (8.0 mL, 50 mmol) was added dropwise to a solution of 6-bromohexanoic acid (9.8 g, 49 mmol) in nitromethane (30 mL). The dark red solution was heated to 80 °C under N₂ and stirred at this

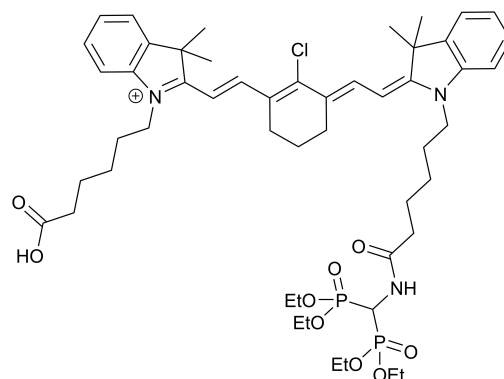
temperature for 16 h. The purple solution was then cooled to room temperature and diethyl ether (300 mL) was added to precipitate the purple product. The reaction mixture was filtered under vacuum, resulting in a purple solid (6.23 g, 22.7 mmol, 46 % yield). ^1H NMR (400 MHz, DMSO - d_6): δ 8.01 – 7.93 (m, 1H), 7.87 – 7.81 (m, 1H), 7.68 – 7.59 (m, 2H), 4.45 (t, $^3J_{\text{HH}} = 7.7$ Hz, 2H), 2.83 (s, 3H), 2.23 (t, $^3J_{\text{HH}} = 7.2$ Hz, 2H), 1.90 – 1.77 (m, 2H), 1.61 – 1.54 (m, 1H), 1.53 (s, 6H), 1.47 – 1.36 (m, 2H). MS(ES $^+$): calcd. for $\text{C}_{17}\text{H}_{24}\text{NO}_2$ 274.1807, found 274.1796 $[\text{M}]^+$.

Cyanine-808 (4.3):



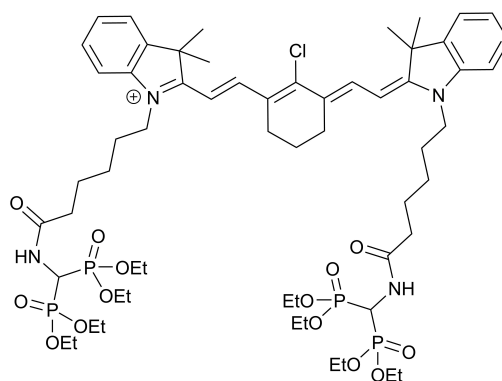
4.3 was prepared according to literature procedures.¹⁰ Sodium acetate (1.60 g, 19.5 mmol) was added to a solution of **4.1** (1.12 g, 6.49 mmol) and **4.2** (4.66 g, 17.0 mmol) in ethanol (65 mL). The mixture was heated at 70 °C and stirred at this temperature for 90 mins. The solvent was removed *in vacuo* and the purple coloured crude solid purified by silica gel column chromatography (DCM:methanol), resulting in the dark green solid of **4.3** (2.30 g, 3.36 mmol, 26 % yield). ^1H NMR (400 MHz, CDCl_3): δ 8.33 (d, $^3J_{\text{HH}} = 14.0$ Hz, 2H), 7.44 – 7.33 (m, 4H), 7.22 (t, $^3J_{\text{HH}} = 7.5$ Hz, 2H), 7.16 (d, $^3J_{\text{HH}} = 8.0$ Hz, 2H), 6.19 (d, $^3J_{\text{HH}} = 14.0$ Hz, 2H), 4.12 (t, $^3J_{\text{HH}} = 7.6$ Hz, 4H), 2.71 (t, $^3J_{\text{HH}} = 6.3$ Hz, 4H), 2.42 (t, $^3J_{\text{HH}} = 7.2$ Hz, 4H), 2.01 – 1.96 (m, 2H), 1.84 (q, $^3J_{\text{HH}} = 7.5$ Hz, 4H), 1.78 – 1.72 (m, 4H), 1.70 (s, 12H), 1.57 – 1.51 (m, 4H). ^{13}C NMR (100 MHz, CDCl_3): δ 177.0, 172.4, 150.8, 144.5, 142.2, 141.1, 129.0, 127.9, 125.4, 122.4, 111.1, 101.4, 49.4, 44.6, 34.4, 28.3, 27.1, 26.7, 26.3, 24.6, 20.8. MS(ES $^+$): calcd. for $\text{C}_{42}\text{H}_{52}\text{N}_2\text{O}_4\text{Cl}$ 683.3616, found 683.3624 $[\text{M}]^+$. λ_{max} (MeOH)/nm 785 (ϵ / $\text{dm}^3 \text{mol}^{-1} \text{cm}^{-1}$ 91220).

Protected Cyanine mono-BP (4.4): * Novel compound

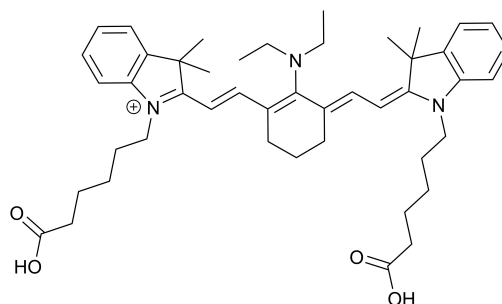


A solution of **4.3** (113 mg, 0.165 mmol), **2.2** (100 mg, 0.330 mmol), HBTU (375 mg, 0.475 mmol) and triethylamine (36.0 μ L, 0.475 mmol) in DCM (5 mL) was stirred for 48 hr at room temperature. The dark green solution was washed sequentially with 10 % citric acid (5 mL), saturated sodium bicarbonate (5 mL) and then brine (5 mL). The organic solution was then dried over MgSO_4 . The green crude product was purified by column chromatography (DCM:methanol) resulting in the dark green solid of **4.3** (38.0 mg, 0.039 mmol, 24 % yield). ^1H NMR (400 MHz, CDCl_3): δ 8.37 (d, $^3J_{\text{HH}} = 14.1$ Hz, 1H), 8.31 (d, $^3J_{\text{HH}} = 13.8$ Hz, 1H), 7.46 – 7.32 (m, 4H), 7.24 – 7.16 (m, 2H), 7.10 (d, $^3J_{\text{HH}} = 7.9$ Hz, 1H), 6.30 (d, $^3J_{\text{HH}} = 14.2$ Hz, 1H), 6.14 (d, $^3J_{\text{HH}} = 14.1$ Hz, 1H), 5.18 – 4.98 (m, 1H), 4.29 – 4.12 (m, 10H), 4.08 (t, $^3J_{\text{HH}} = 7.6$ Hz, 2H), 2.81 – 2.65 (m, 4H), 2.55 (t, $^3J_{\text{HH}} = 7.3$ Hz, 2H), 2.38 (t, $^3J_{\text{HH}} = 6.3$ Hz, 2H), 2.01 (t, $^3J_{\text{HH}} = 6.3$, 5.5 Hz, 2H), 1.92 – 1.74 (m, 8H), 1.71 (s, 12H), 1.61 – 1.49 (m, 4H), 1.33 (t, $^3J_{\text{HH}} = 7.1$ Hz, 12H). $^{31}\text{P}\{^1\text{H}\}$ NMR (161.84 MHz, CDCl_3): δ 16.6. MS(ES⁺): calcd. for $\text{C}_{51}\text{H}_{73}\text{N}_3\text{O}_9\text{ClP}_2$ 968.4511, found 968.4573 $[\text{M}]^+$. $\lambda_{\text{max}}(\text{MeOH})/\text{nm}$ 782 (ϵ / $\text{dm}^3 \text{mol}^{-1} \text{cm}^{-1}$ 152065).

Cyanine di-BP (4.5): * Novel compound



A solution of **4.3** (113 mg, 0.165 mmol), **2.2** (250 mg, 0.824 mmol), HBTU (180 mg, 0.475 mmol), triethylamine (36.0 μ L, 0.475 mmol) in DCM (5 mL) was stirred at room temperature for 60 h. 20 mL of DCM was then added to the green solution and was washed sequentially with 10 % citric acid (5 mL), saturated sodium bicarbonate (5 mL) and then brine (5 mL). The solvent was removed *in vacuo* and the brown / green solid was purified by silica gel column chromatography (DCM : methanol) resulting in a green solid (13.0 mg, 0.010 mmol, 6 % yield). ^1H NMR (400 MHz, CDCl_3): δ 8.34 (d, $^3J_{\text{HH}} = 14.0$ Hz, 2H), 7.38 (m, 4H), 7.13 (d, $^3J_{\text{HH}} = 8.0$ Hz, 2H), 6.16 (d, $^3J_{\text{HH}} = 14.1$ Hz, 2H), 5.11 – 4.98 (m, 2H), 4.23 – 4.14 (m, 16H), 4.07 (t, $^3J_{\text{HH}} = 7.7$ Hz, 4H), 2.70 (t, $^3J_{\text{HH}} = 6.2$ Hz, 4H), 2.34 (t, $^3J_{\text{HH}} = 7.4$ Hz, 4H), 2.03 – 1.95 (m, 2H), 1.85 (m, 4H), 1.79 – 1.73 (m, 4H), 1.70 (m, 12H), 1.55 (m, 4H), 1.33 (t, $^3J_{\text{HH}} = 7.1$ Hz, 24H). $^{31}\text{P}\{^1\text{H}\}$ NMR (161.84 MHz, CDCl_3): δ 16.5. MS(ES⁺): calcd. for $\text{C}_{60}\text{H}_{94}\text{N}_4\text{O}_{14}\text{ClP}_4$ 1253.5406, found 1253.5426 $[\text{M}]^+$. $\lambda_{\text{max}}(\text{MeOH})/\text{nm}$ 781 (ϵ / $\text{dm}^3 \text{mol}^{-1} \text{cm}^{-1}$ 98443).

Cyanine-diethylamine (4.7): * Novel compound

4.3 (100 mg, 0.146 mmol) and diethylamine (40 μ L, 0.387 mmol) were dissolved in DMF (5 mL). The solution was heated at 40 $^{\circ}$ C for 4 hours. The solvent was removed *in vacuo* and the blue/brown solid was purified by silica gel column chromatography (DCM:methanol) resulting in a blue solid of **4.3** (10.2 mg, 0.014 mmol, 10 % yield). ^1H NMR (400 MHz, CDCl_3): δ 7.53 (d, $^3J_{\text{HH}} = 13.7$ Hz, 2H), 7.39 – 7.26 (m, 4H), 7.14 (t, $^3J_{\text{HH}} = 7.5$ Hz, 2H), 7.04 – 6.92 (m, 2H), 5.88 (d, $^3J_{\text{HH}} = 13.5$ Hz, 2H), 4.02 – 3.77 (m, 4H), 3.77 – 3.53 (m, 4H), 3.14 – 2.98 (m, 2H), 2.58 – 2.25 (m, 8H), 1.70 (m, 8H), 1.66 – 1.55 (m, 10H), 1.54 – 1.41 (m, 6H). MS(ES $^{+}$): calcd. for $\text{C}_{46}\text{H}_{62}\text{N}_3\text{O}_4$ 720.4740, found 720.4736 $[\text{M}]^{+}$. $\lambda_{\text{max}}(\text{MeOH})/\text{nm}$ 702 (ϵ / $\text{dm}^3 \text{mol}^{-1} \text{cm}^{-1}$ 18378).

Functionalisation of $\text{NaYF}_4\text{:Yb,Er@NaYF}_4\text{:Nd}$ UCNP s with Cy-808 in chloroform:

$\text{NaYF}_4\text{:Yb,Er@NaYF}_4\text{:Nd}$ UCNP s (2 mg) were added to chloroform (2 mL). Depending on the quantity of cyanine-808 functionalisation desired, a specific quantity of **4.3** was added to the dispersion (for example 1 equivalent **4.3** = 0.0150 mg). The dispersion was stirred at room temperature for 16 hours. Ethanol (5 mL) was added to precipitate the nanoparticles and then the green solid was collected by centrifugation at 4000 rpm for 10 mins. The washing step was repeated twice more, each time adding acetone (5 mL); this removed any free **4.3** from the dispersion. The cyanine-functionalised $\text{NaYF}_4\text{:Yb,Er@NaYF}_4\text{:Nd}$ UCNP s ($\sim$ 2 mg) were redispersed in chloroform (1 mL) and stored at 4 $^{\circ}$ C.

Functionalisation of $\text{NaYF}_4\text{:Yb,Er@NaYF}_4\text{:Nd}$ UCNP s with Cy-808 in water:

Ligand-free $\text{NaYF}_4\text{:Yb,Er@NaYF}_4\text{:Nd}$ UCNP s (2 mg) were added to distilled water (2 mL). **4.3** was dissolved in methanol at a concentration of 1 mg/mL. Depending on the quantity of cyanine-808 functionalisation desired, a specific quantity of **4.3** was added to the UCNP dispersion (for example 1 equivalent **4.3** = 15 μ L). The dispersion was stirred at room temperature for 2 hours and then **PEG-BP** (10 mg) was added. The dispersion was stirred at room temperature for a further 16 hours. Acetone (5 mL) was added to precipitate the nanoparticles and the green solid was collected by centrifugation at 4000 rpm for 10 mins. The washing step was repeated twice more, each time adding acetone (5 mL). This removed any free **PEG-BP** or **4.3** from the dispersion. The functionalised UCNP s ($\sim$ 2 mg) were redispersed in distilled water (1 mL) and stored at 4 $^{\circ}$ C.

7.5 Chapter 5 Synthetic Protocols

Iron(III) oleate:

Synthesis of iron oleate was carried out according to a published procedure.¹¹ Iron chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 5.40 g, 20.0 mmol, 1 eq) and sodium oleate (18.30 g, 60.0 mmol, 3 eq) were dissolved in a 7:3:4 solvent mixture of hexane:water:ethanol (140 mL). Under N_2 , the orange solution was heated to reflux at 70 °C and maintained at that temperature for 4 hours. After cooling to room temperature, the solution was washed with distilled water (3 x 20 mL). The organic layer was decanted into an evaporating dish and left at room temperature for the hexane to volatilise naturally. This resulted in an orange waxy solid (12.0 g, 13.3 mmol, 66 % yield). IR (ν cm^{-1}): 2921 (CH), 2854 (CH), 1711 (COO^-), 1587 (COO^-), 1521 (COO^-), 1441 (COO^-), 724 (FeO).

Spherical SPIONs from iron (III) oleate:

The synthesis of SPIONs from iron(III) oleate was carried out according to a published procedure.¹¹ Iron oleate (800 mg, 0.89 mmol) and oleic acid (110 μL , 0.35 mmol) were added to 1-octadecene (10 mL). The reaction mixture was degassed under vacuum for 30 mins and the glassware was backfilled with N_2 . The solution was then heated to 320 °C at a constant heating rate of 3.3 °C min^{-1} . The solution underwent a visible colour change from orange/red to black and white effervescence was visible above 300 °C. After 2 hours at 320 °C, the solution was cooled to room temperature and isopropanol (60 mL) was added to precipitate the nanoparticles. The nanoparticles were collected by centrifugation at 4000 rpm for 10 mins and washed three times with ethanol (3 x 20 mL). The resulting black solid was dispersed in hexane (10 mL).

Octapod SPIONs attempt:

The attempted synthesis of octapod Fe_3O_4 nanoparticles was carried out according a published procedure, with minor modification.¹² Iron oleate (800 mg, 0.89 mmol) and oleic acid (110 μL , 0.35 mmol) were added to 1-octadecene (10 mL). Sodium chloride (10 mg, 0.17 mmol) was dissolved in distilled water (100 μL) and added to the iron oleate suspension. The reaction mixture was degassed under vacuum for 30 mins and the glassware was backfilled with N_2 . Once degassed, the temperature was increased to 320 °C at a constant heating rate of 3.3 °C min^{-1} . The solution underwent a visible colour change from orange/red to black and white effervescence was visible above 300 °C. After 2 hours at 320 °C, the solution was cooled to room temperature and isopropanol (60 mL) was added to precipitate the nanoparticles. The nanoparticles were separated by centrifugation at 4000 rpm for 10 mins and washed three times with ethanol (3 x 20 mL). The resulting black solid was dispersed in hexane (10 mL).

Spherical SPIONs from iron acetylacetonate:

The synthesis of spherical SPIONs from iron acetylacetonate was carried out according to a published procedure.¹³ Iron acetylacetonate (1.06 g, 3 mmol) was dissolved in oleylamine (15

mL) and benzyl ether (15 mL). The red solution was dehydrated at 110 °C under nitrogen for 1 hour. The temperature was then increased at a rate of 20 °C min⁻¹ to 300 °C and stirred under nitrogen at this temperature for 1 hour. The black reaction mixture was then cooled to room temperature and ethanol (60 mL) was added to precipitate the nanoparticles. The nanoparticles were separated by centrifugation at 4000 rpm for 10 mins and washed with ethanol (3 x 20 mL). The final black nanoparticle product was dispersed in hexane for storage (10 mL).

Attempted one-step synthesis of Fe₃O₄@NaScF₄:Yb,Er NPs:

Iron(III) oleate (800 mg, 0.89 mmol) and oleic acid (110 µL, 0.35 mmol) were added to 1-octadecene (10 mL). The reaction mixture was degassed under vacuum for 30 mins and the glassware was backfilled with N₂ to remove any volatile impurities. Once degassed, the temperature was increased to 320 °C at a constant heating rate of 3.3 °C min⁻¹. The reaction mixture was held at 320 for 2 hours. Separately, ScCl₃ (23.3 mg, 0.153 mmol), YbCl₃ (11.0 mg, 0.039 mmol) and ErCl₃ (1.1 mg, 0.004 mmol) were dissolved in methanol (1 mL) and added to a mixture of oleic acid (2.5 mL, 7.92 mmol) and 1-octadecene (2.5 mL). The solution was degassed under vacuum for 30 mins, removing the methanol and then the glassware was back-filled with N₂. The solution was heated to 160 °C and NaF (33.2 mg, 0.791 mmol) was added under a positive flow of N₂. The temperature was held at 160 °C for 30 mins, in which time the solution turned from yellow to orange. After the iron oleate solution had been heated for 2 hours, the lanthanide reaction mixture was added by a hot injection method. The reaction mixture was then heated at 320 °C for a further 15 mins. The reaction mixture was cooled to room temperature and isopropanol (60 mL) was added to precipitate the nanoparticles. The precipitate was collected by centrifugation at 4000 rpm for 10 mins and the nanoparticles were washed with ethanol (3 x 20 mL). The resulting off-white solid was dispersed in toluene (10 mL).

Attempted two-step synthesis of Fe₃O₄@NaScF₄:Yb,Er NPs:

ScCl₃ (23.3 mg, 0.153 mmol), YbCl₃ (11.0 mg, 0.039 mmol) and ErCl₃ (1.1 mg, 0.004 mmol) were added to a mixture of oleic acid (2.5 mL, 7.92 mmol) and 1-octadecene (2.5 mL). Fe₃O₄ nanoparticles (30 mg), produced via the Fe(acac)₃ route, in 2 mL hexane was added to the suspension. The solution was degassed under vacuum for 30 mins, removing the hexane, and then the glassware was backfilled with N₂. The solution was heated to 180 °C and NaF (33.2 mg, 0.791 mmol) was added under a positive flow of N₂. The temperature was increased to 340 °C and this was maintained for 1 hour. The heat was then removed and the solution cooled to room temperature. Isopropanol (60 mL) was added to precipitate the nanoparticles and the nanoparticles were separated by centrifugation at 4000 rpm for 10 mins. They were washed three times with ethanol (3 x 20 mL) and the resulting off light brown solid was dispersed in toluene (10 mL).

NaGdF₄:Yb,Er UCNPs:

NaGdF₄:Yb,Er UCNPs were synthesised according to literature protocols.¹⁴ GdCl₃ (211.0 mg, 0.800 mmol), YbCl₃ (50.3 mg, 0.180 mmol) and ErCl₃ (5.5 mg, 0.020 mmol) in methanol (5

mL) were added to oleic acid (6 mL) and 1-octadecene (15 mL). The solution was heated under vacuum at 120 °C for 1 h to remove the solvent and any residual water. The solution was then cooled to room temperature and sodium trifluoroacetate (340.0 mg, 2.500 mmol) was added under a flow of N₂. The solution was heated rapidly to 300 °C, under N₂ and this temperature was maintained for 2 h. The reaction mixture was then cooled to room temperature and ethanol (60 mL) was added to precipitate the nanoparticles. The nanoparticles were washed with ethanol (3 x 20 mL) resulting in a white solid of NaGdF₄:Yb,Er nanoparticles (176.5 mg).

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