Near-Infrared Triggered Anti-Cancer Drug Release from Upconverting Nanoparticles

By

Laura Lee Fedoryshin

A thesis submitted in conformity with the requirements

For the degree of Masters in Science

Graduate Department of Chemistry

University of Toronto

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1. Abstract

Externally-triggered drug delivery using functional nanoparticles has provided new strategies to improve therapeutic efficacy while concurrently minimizing toxicity. This thesis describes an investigation of photocleavage at the surface of UCNPs to release the chemotherapeutic 5-Fluorouracil (5-FU). Core-shell UCNPs composed of β -NaYF₄: 4.95%Yb, 0.08%Tm / β -NaYF₄ were decorated with o-phosphorylethanolamine ligands that were coupled to an o-nitrobenzyl (ONB) derivative of 5-FU. The UV photoluminescence (PL) was in resonance with the absorption band of the ONB-FU derivative and energy transfer resulted in photocleavage and subsequent release of 5-FU from the surfaces of UCNPs for these *in vitro* studies. Release of 5-FU was complete within minutes using a 980 nm NIR laser source that operated below 100 mW in power. The efficiency of triggered release was as high as 80% of the total conjugated ONB-FU. This work provides an important first step toward the development of a UCNP platform capable of targeted chemotherapy.

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7. Abbreviations

Abbreviation	Definition	Page Introduced
5-FU	5-fluorouracil	12
AES	Atomic Emission Spectroscopy	34
APTE	Addition de photon par transferts d'énergie	25
CNT	Carbon Nanotube	20
CUC	Cooperative Upconversion	25
DCM	Dichloromethane	41
DIC	N,N'-diisopropylcarbodiimide	54
DMSO	Dimethyl Sulfoxide	41
DOX	Doxorubicin	19
DPD	Dihydropyrimidine Dehydrogenase	40
ESA	Excited State Absorption	25
ESI	Electrospray Ionization	34
ETU	Energy Transfer Upconversion	25
FA	Folic acid	23
GSA	Ground State Absorption	26
HPLC	High Performance Liquid Chromatography	12
ICP	Inductively Coupled Plasma	34
MS	Mass Spectra	34
NHS	N-hydroxysuccinimide	54
NIR	Near-Infrared	2
NMR	Nuclear Magnetic Resonance	41
ONB	o-nitrobenzyl	12
PA	Photon Avalanche	25
PAA	Polyacrylic Acid	32
PAH	Poly(Allylamine Hydrochloride)	33

PDT	Photodynamic Therapy	2
PL	Photoluminescence	2
QD	Quantum Dots	20
ROS	Reactive Oxygen Species	15
TEM	Transmission electron microscopy	33
TFA	Trifluoroacetic Acid	41
TS	Thymidylate Synthase	40
UC	Upconversion	24
UCNP	Upconverting Nanoparticles	2

8. Overview

8.1. Overview

This thesis describes the use of UCNPs for the photo controlled release of a caged anticancer compound. (Figure 8-1) The drug release method utilized the o-nitrobenzyl (ONB) photolabile tether reported by the Rotello group [1] to uncage the well-known chemotherapeutic 5-fluorouracil (5-FU). UCNPs composed of β -NaYF₄: 4.95% Yb, 0.08% Tm were coated with o-phosphorylethanolamine. The amino functional group from the ligand was coupled to the carboxylic group of the ONB-FU molecule (Figure 8-1). Upon excitation with NIR at 980 nm, UV PL from the UCNPs caused photocleavage of the ONB group. The ONB group is a popular photolabile protecting group and was originally introduced by Kaplan *et al.* [2]. The ONB group is advantageous because it undergoes photolytic cleavage at 365 nm allowing for the controlled release of the covalently attached therapeutic. Moreover the ONB group can covalently bind to a large range of therapeutic compounds and therefore this strategy is not limited to photosensitizing agents. The extent of release of 5-FU was determined using high performance liquid chromatography (HPLC). Given the higher tissue penetration depth of NIR compared to UV wavelengths, this drug release mechanism should offer an attractive approach for targeted delivery *in vivo*. The work described herein is an important first step toward the development of a theranostic UCNP for *in vivo* imaging and effective delivery of chemotherapeutics.

Figure 8-1. Stepwise synthesis of water soluble $\beta\text{-NaYF}_4$: 4.95% Yb, 0.08% Tm / $\beta\text{-NaYF}_4$ doped core-shell UCNP (1-3). Doped cores were synthesized and then were subsequently coated with an additional layer of $\beta\text{-NaYF}_4$. Native oleic acid ligands were exchanged with *o*-phosphorylethanolamine to impart water solubility and enable further conjugation with ONB-FU molecules through amide coupling (4). Excitation of the UCNP with NIR photons resulted in upconverted PL emission at 365 nm that was in resonance with the absorption spectrum of the

ONB-FU. Energy transfer to the ONB-FU molecules resulted in photocleavage of the ONB-FU bond and subsequent release of 5-FU from the UCNP surface (5). Note that the dimensions of the nanoparticles and the ligands are not to scale.

8.2. Specific Objectives

The work of this thesis explored the development of a specific externally triggered photochemical drug release strategy toward the development of a UCNP platform capable of targeted chemotherapy. The specific objectives of this thesis were:

1. To synthesize and characterize water soluble UCNPs
2. To synthesize and characterize a ONB caging group for the chemotherapeutic 5-FU
3. To quantify the photolysis of the ONB caging group caused by the upconversion emission from UCNP for externally triggered drug delivery

9. Introduction

9.1. Remotely Triggered Delivery of Therapeutics

An ideal drug delivery method would provide for spatial and dosage control after the drug had been administered to a patient. This would avoid toxicity due to dosage beyond the therapeutic concentration, selectivity in targeting a tissue, and reduction of potential for ineffective treatment from under-dosing. The development of passive and remotely triggered drug delivery techniques evolved from the need to overcome the limitations of traditional drug administration. Passive drug delivery techniques allow for the prolonged release of therapeutics from a single dose. Typical examples include injectable liposomes for release of anesthetics [3] and contact lenses for the release of antifungal agents [4]. Although this method does not provide tight spatial control and only limited dosage control, it does allow for decreased patient inconvenience and

avoids tethering of patients to external devices [5]. Remotely triggered drug delivery techniques provide the administrator with control over one or all of the following parameters: time, duration, dosage, and location of drug delivery. There are 2 main approaches to remotely triggered drug delivery techniques: 1) Electrically triggered external or implantable devices [6], which are advantageous because they are able to control the time, duration and dosage of a therapeutic but lack the spatial control that is associated with; 2) irradiation triggered techniques, which fall into three categories: photodynamic therapy, photothermally triggered drug release and photochemically triggered drug release.

9.2. Photodynamic Therapy

An increasingly popular approach for drug delivery is photodynamic therapy (PDT) in which light is used to generate product for delivery as a therapeutic. PDT has 3 main components: the photosensitizer, light/radiation and tissue oxygen. In traditional PDT, the photosensitizing agent is administered to the cell/patient and becomes excited by Ultraviolet – visible (UV-vis) radiation. The photosensitizing agent is then able to transfer the absorbed energy to oxygen molecules in its surroundings and generate cytotoxic reactive oxygen species (ROS) that disrupt cell functions which ultimately leads to cell death [7, 8, 9]. Since light can be selected in terms of wavelength, power, and volume/area of illumination, this drug delivery approach is advantageous because it allows for external spatiotemporal control. Although this approach is effective it can be limited in practical application due to the poor tissue penetration of UV-vis radiation, the limited library of photosensitizing agents that are available, and the hydrophobic nature of many of these compounds.

When using light to trigger drug release it is important to consider the “optical window” associated with penetration into biological tissue. The optimal wavelengths for stimulating drug

release occur in the spectral regions where light has the least absorbance and scattering by skin, blood, water and lipids. This region covers approximately 650 to 950 nm [10]. The challenge with this optical window range is that the majority of photosensitizers utilized in PDT, photocleaveable compounds and compounds that undergo a conformational change in response to light, do so at wavelengths less than 450 nm. Additionally, use of high energy light for excitation has other disadvantages including induction of chemical degradation and side-reactions of organic molecules upon extended irradiation. The widespread application of PDT is also limited by the number of photosensitizing agents available. An ideal system for triggered drug delivery should be applicable for the treatment of a wide range of illnesses. Although many existing compounds can behave as photosensitizers with more being developed and discovered, very few meet the necessary requirements for commercial viability. Allison *et al.* described clinically relevant guidelines for photosensitizers including toxicity, elimination, activity, sunlight precautions, and other factors [11]. The development of photosensitizers is an active area of research, but currently the range of applications is small and struggles with unreliable dosimetry calculations resulting from inconsistencies in the selectivity and photoactivation efficiencies of photosensitizers [11]. A final important drawback for the use of photosensitizers is their hydrophobic nature. The broad majority of photosensitizers are hydrophobic and therefore require a liposomal coating, attachment to a metal surface or an additional modification to allow for water solubility [11]. These modifications can affect photoactivation efficiencies and therefore overall drug efficacy.

9.3. Caging and Irradiation Triggered Drug Release

Caging is the chemical modification of a molecule using a photo-removable protecting group. In the case of the caging of therapeutics the chemical modification should also prevent drug

activity. Photothermal drug release relies on irradiation-induced changes of local temperature. Photochemical drug release results from direct irradiation of the photo-removable protecting group following UV-vis irradiation or from a secondary emission from a nanoparticle platform.

9.3.1. Photothermal Drug Release

Photothermal drug release was developed to take advantage of using NIR irradiation for triggering drug release in biological applications. The majority of work in photothermal drug release has used NIR irradiation as an excitation source in combination with gold nanostructures, which due to their strong NIR absorbance can create localized heating. Other sources of external excitation that have been used include ultrasound, UV-vis light and magnetic fields [12]. Radt *et al.* were the first to demonstrate the photothermal technique. They used layer-by-layer colloid templating to prepare gold nanoparticles (NPs) that were incorporated into capsules that released the enzyme lysozyme upon dissociation of the capsule [13]. Photoexcitation of gold NPs resulted in the formation of a heated electron gas that exchanged energy with the NP lattice. The subsequent energy exchange with the surrounding medium resulted in localized heating (600 to 800 °C) on a 100 picosecond time scale, making these NPs an attractive platform for photothermal drug release [12]. Mechanical and thermal stress ultimately caused the dissociation of polymer capsules and liposomes that incorporated gold NPs. [14, 15, 16]. Further examples of applications with other NP platforms can be found in Table 9-1. The primary drawback of photothermal methods is the stability of the therapeutic under conditions of high temperature. To overcome this drawback the therapeutic can be coated in a lipid membrane [12] however, this results in increased overall size with the majority of such NP constructs being greater than 200 nm in diameter [17]. This can present a challenge because size influences both the clearance and biodistribution of NPs. To avoid clearance by the renal system, NPs should be greater than 20 –

30 nm in diameter [18, 19]. The range of fenestration sizes is between 1 nm and 1.2 μm [18, 19], but the size of a nanoparticle drug delivery system should be as small as possible while still being larger than 30 nm. Recent studies by Tang *et al.* suggest that nanoparticles less than 50 nm in diameter are ideal [20], but it is important to consider that this varies by material, coating and shape.

9.3.2. Photochemical Drug Release

Photochemical drug release can be divided into 2 groups: the first is large polymeric or liposomal capsules, and the second is direct caging of a therapeutic onto a nanoparticle.

The four categories of photochemical drug release from polymeric or liposomal capsules are: 1) Photoisomerization, 2) Photocrosslinking, 3) Photodegradation and 4) Photodecrosslinking. The majority of these strategies have the drawback of larger size (>150 nm) and potential leakiness due to degradation of an external shell by biological processes as well as imperfect fabrication procedures. Each of these four strategies will be presented briefly for comparison.

Photoisomerization describes a conformational change; a popular example of this is associated with azobenzene which undergoes *cis* to *trans* isomerization following UV irradiation. Kano *et al.* were the first to describe the use of azobenzene in liposomes, where the liposomes became leaky following UV irradiation as observed by the release of bromothymol blue dye [21].

Azobenzene has subsequently been incorporated into many liposome and micellar assemblies, and this topic has been reviewed in detail by Fomina *et al.* [12].

The strategy of photocrosslinking is used in polymeric or liposomal assemblies. Upon irradiation the polymerization results in shrinking of the polymeric structure or the hydrophobic domain in the case of liposomes, which disrupts the uniform packing and produces pores for drug release

[12]. Drawbacks to this strategy include spontaneous crosslinking, which may occur in the presence of radical initiators that occur naturally in patient tissues [12].

Photodegradation results in the disintegration of the nanocarrier and polymer fragmentation.

Fomina *et al.* were the first to demonstrate this technique, and reported a polymeric nanoparticle (170 nm diameter) to deliver Nile Red, a model dye. Excitation using UV or NIR irradiation to trigger drug release was not very efficient, and the polymeric carriers were additionally susceptible to pH degradation [22]. A number of different polymers have been developed for photodegradation. Johnson *et al.* developed 10 nm nanoparticles for doxorubicin (DOX) delivery following UV irradiation [23]. The advantage associated with this approach is that the disintegration of the nanocarrier allows for it to be cleared by the body. The challenge, however, still remains to effectively combine small size, minimal leakiness and NIR excitation.

Photo decrosslinking is designed so that when irradiated, the light sensitive crosslinks break to increase porosity, but still remain attached to the polymer preventing any potential toxicity from photocleavage byproducts. Yu *et al.* demonstrated this technique using micelles of 66 – 95 nm diameter which contained the photosensitive 2-nitrobenzyl group. Disruption occurred following UV irradiation and resulted in the released Nile Red as a model of therapeutic release [24].

Direct caging to a nanoparticle carrier combines the advantages of phototriggered drug release, small size and the potential diagnostic properties of the nanoparticle carrier. An example of this was demonstrated by Agasti *et al.* using gold NPs of 2 – 10 nm diameter that were functionalized with tetra(ethylene glycol) for water solubility and a o-nitrobenzyl group for UV triggered release of the chemotherapeutic 5-FU [1]. The limitation of this approach was the requirement of excitation using UV radiation, which is impractical for biological applications. The nanoparticle carrier is a crucial component to this approach, influencing overall size, diagnostic potential and

toxicity. Further examples of this approach with various nanoparticle carriers can be found in Table 9-1. The drawbacks of this approach tend to be specific to the nanoparticle carrier, immobilization strategy and selection of photochemical cage.

9.4. Nanoparticle Carriers

Many types of nanoplatforms including iron oxide nanoparticles, carbon nanotubes, gold nanoparticles, silica nanoparticles, quantum dots (QDs) and upconverting nanoparticles have been studied for drug delivery. Nanoparticles are appealing because they have large surface area allowing for multiple functionalities to be added to the surface in addition to a caged therapeutic, allowing for an increased range of therapeutic and diagnostic applications. In addition, some nanoparticles have advantageous intrinsic functionalities for therapeutics. For example, carbon nanotubes (CNTs) have the intrinsic ability to penetrate cell membranes [25], and UCNPs can convert NIR irradiation to UV-vis emission. Table 9-1 summarizes the advantages and disadvantages of each nanoparticle and includes some examples of therapeutic applications. UCNPs provide a competitive platform from which to build a drug delivery system since these are biocompatible and are able to convert NIR excitation to UV-vis emission, overcoming a major challenge associated with phototriggered drug delivery.

	Diameter (nm)	Advantages	Disadvantages	Externally triggered Drug Delivery		
				Photodynamic Drug Release	Photo - Thermal Drug Release	Photo - Chemical Drug Release
Magnetic	3 – 100 [26] [27]	MRI Imaging NIRF PET [28]	Non - Biodegradable [29] Limited to magnetically inert surface coatings [27]	[30] [31] [32] [33]		[34]
Gold	1 – 150 [35]	Photothermal Therapy	Non - Biodegradable [29]	[36] [37]	[14] [15] [16]	[1]
Carbon Nanotubes	0.5 – 20 [25]	Intrinsic ability to penetrate cell membranes [25] Photothermal Therapy [38]	Hydrophobic Cytotoxic No intrinsic imaging capabilities [25] Non-specific protein binding [39]	[40] [41]	[42] [43]	
Silica	>4nm [44] [45]	X-ray/ CT imaging Silica is often used to coat other nanoparticles.	Non - Biodegradable [29]	[31] [36] [46] [47]		[48] [49] [50]
Quantum Dots	2 – 10 nm [51]	Sharp, tunable emission peaks [25] Excitation: UV Emission: Vis	Toxic [51] Requires UV excitation for imaging [25]	Intrinsically [29]	[52]	
UCNP	>5 [53] [54]	Non Toxic [55] Fluorescence Excitation: IR Emission: UV, Vis	No standard method for measuring UC efficiency Poor understanding of surface chemistry and long term stability [53]	[33] [56] [57] [58] [59] [60] [61] [62] [63]		Mesoporous Shell [64] [65]

Table 9-1 Summary of the size, advantages and disadvantages of each nanoparticle as relevant to therapeutic applications. Please note that only externally triggered drug delivery is considered herein, and therefore applications that make use of passive drug release (desorption, pH triggered, enzymatic cleavage) are not described.

9.5. Upconverting nanoparticles

UCNPs provide potential for the development of platforms from which to build photochemical drug delivery systems. Upconversion is a process that can convert NIR radiation into UV-vis wavelengths, as would be required for activation and release of a toxic reagent. Core-shell UCNPs composed of NaYF₄ nanocrystals (host matrix) doped with lanthanides such as Tm³⁺ (activator) and Yb³⁺ (sensitizer) can convert continuous 980 nm laser radiation into a few emissions of higher energy and narrow bandwidth in UV-vis range. In upconversion, two or more photons are absorbed and combined through excited state absorption, energy transfer or photon avalanche mechanisms [66]. The upconversion luminescence originates from intra-configurational 4f transitions of lanthanide dopants. By varying the dopant concentration the emission spectra can be tuned. Upconversion takes place with two or more photon absorptions occurring in sequence rather than simultaneously, eliminating the need for high power density pulsed lasers as used in two-photon excitation. UCNPs are also advantageous due to the good biocompatibility of the materials that are used, and usefulness in optical diagnostic applications because of the low background signal that results from high photo stability and long luminescence lifetimes (milliseconds) [7, 67].

9.6. Contributions of this thesis

The unique optical properties of UCNP have the potential to provide a platform for externally triggered photochemical drug delivery. The advantages of UCNP-mediated PDT have been described [68], and the adsorption of a therapeutic to NP surfaces [69, 70, 71], use of a

mesoporous shell or micelle for drug release [72, 65, 73], and the activation of photosensitizing agents [74, 75, 59, 58] have been reported. The former two approaches are compromised by inherent instability associated with physical adsorption or encapsulation of therapeutic agents that result in loss over time, while the latter has shown improved control over on-demand delivery and localized therapy. Active PDT was reported in a study by Cui *et al.* that utilized UCNPs decorated with the photosensitizer zinc (II) phthalocyanine [59]. Upconversion PL bands at 540 nm and 660 nm were used for *in vivo* tumor imaging and generation of ROS through energy transfer to the interfacial zinc photosensitizer, respectively. Folic acid (FA) was co-immobilized for targeted therapy. The UCNP triggered ROS generation displayed 50% tumor inhibition compared to 18% when visible light was used as the excitation source. [59]

UCNPs tagged with FA and DOX were used in a study by Chien *et al.* for targeted chemotherapy [7]. FA ligands were caged using the photolabile ONB group through covalent attachment to the carbohydrate moiety of the FA molecule. Laser excitation at 980 nm was used to produce UCNP PL at 360 nm, and this short wavelength resulted in uncaging of FA by excitation of the NBz group. The system was used for targeted tumor binding to cell surface FA receptors. DOX was coupled to the UCNP surface via a labile disulfide bond and released intracellularly through cleavage by lysosomal enzymes after internalization of NPs and inhibition of tumor growth was noted [7]. Photoactivation of platinum pro-drugs have also been reported using the upconverted PL emission from UCNPs. UV PL from UCNPs was utilized to activate the dicarboxyl *trans*-platinum (IV) pro-drug to the highly toxic Pt (II) derivative to destroy cancerous cells and track efficacy *in vivo* using tri-modal imaging with PL, computer tomography, and magnetic resonance [74].

This thesis describes a drug release method that utilizes the ONB photolabile tether reported by the Rotello group [1] to allow caging of the well-known chemotherapeutic 5-FU. UCNPs composed of β -NaYF₄: 4.95% Yb, 0.08% Tm were coated with o-phosphorylethanolamine. The amino functional group from the ligand was coupled to the carboxylic group of the ONB-5FU molecule (Figure 8-1). Upon excitation with NIR at 980 nm, UV PL from the UCNPs caused photocleavage of the ONB group. The ONB group is a popular photolabile protecting group and was originally introduced by Kaplan *et al.* [2]. The ONB group is advantageous because it undergoes photolytic cleavage at 365 nm allowing for the controlled release of the covalently attached therapeutic. Moreover the ONB group can covalently bind to a large range of therapeutics and therefore this strategy is not limited to photosensitizing agents. The release of 5-FU was followed using HPLC. Given the higher tissue penetration depth of NIR compared to UV wavelengths, this drug release mechanism should offer an attractive approach for targeted delivery *in vivo*. The work described herein is an important first step toward the development of a theranostic UCNP for *in vivo* imaging and effective delivery of chemotherapeutics.

10. Upconverting Nanoparticle Synthesis and Characterization

10.1. Abstract

UCNPs show great potential for externally triggered drug-delivery applications due to their small size, biocompatibility and UC emission in the UV-vis range. Herein, the synthesis of β -NaYF₄ nanocrystals (host matrix) doped with lanthanides Tm³⁺ (activator) and Yb³⁺ (sensitizer) is described. The ICP-AES analysis of the nanoparticles revealed that they were 16.34% Na, 0.07% Tm, 13.25 Y, 4.96% Yb and 65.37% F percent by mass. Using these % ratios and 20.1 nm radius

the MW of the nanoparticles was calculated to be 5066.27 g/mol. The nanoparticles were characterized using fluorescence emission spectroscopy and TEM. The average size and homogeneity of the UCNPs were determined through electron microscopy. The UCNPs adopted a hexagonal structure characteristic of β -UCNP which was expected *a priori* due to the synthesis method chosen and the temperature of the reaction. The average size of the UCNPs was determined to be 20.1 nm by physical measurement of 100 particles. The spread of sizes of this ensemble 3.0 nm as given by the standard deviation, and indicated good monodispersity that was consistent with similar synthetic reports. [76, 77, 78, 79]

10.2. Introduction

UCNPs provide an attractive platform from which to build remotely triggered drug delivery systems. Upconversion (UC) is a process that can convert NIR radiation into UV-vis wavelengths, as would be required for drug delivery and activation of a toxic reagent. Core-shell UCNPs composed of NaYF₄ nanocrystals (host matrix) doped with lanthanides such as Tm³⁺ (activator) and Yb³⁺ (sensitizer) convert continuous 980 nm laser light into a few sharp emissions of higher energy in UV-vis range. In UC, two or more photons are absorbed and combined through excited state absorption, energy transfer or photon avalanche mechanisms [66]. The upconversion luminescence originates from intra-configurational 4f transitions of lanthanide dopants and by varying the dopant concentration the emission spectra can be tuned. A primary advantage of UCNPs when considering multiphoton absorption is that energy levels are real and multiple photon absorptions occur sequentially rather than simultaneously, eliminating the necessity for high power density pulsed lasers. UCNPs are also advantageous due to their biocompatibility and usefulness in optical diagnostic applications because of the low background

signal that results from high photostability and long luminescence lifetimes (milliseconds) [54, 67, 7].

The UC process or anti-Stokes emission is the photo physical process where the absorption of lower energy, longer wavelength radiation results in the emission of photons with higher energy and shorter wavelengths. The UC process was independently discovered by Azel, Ovsyankin and Feofilov in the mid-1960s [80]. There are four mechanisms for photon UC: Multistep excitation from an excited state absorption (ESA); Addition de photon par transferts d'énergie (APTE) effect or energy transfer UC (ETU); Cooperative UC (CUC); and the photon avalanche (PA) effect.

10.2.1. Upconversion Mechanisms

10.2.1.1. Multistep excitation from an excited state absorption (ESA)

The UC process by multistep excitation from an ESA is the result of the successive absorption of two photons by a single ion. If resonance conditions are met then the absorption of a single photon can occur and an electron is promoted to the first metastable excited state E1. This transition is referred to as ground state absorption (GSA). The absorption of a second photon can occur due to the long-lived nature of this metastable excited state allowing for promotion of the higher excited state E2. Radiative relaxation of this excited electron to G results in the emission of a photon that is of higher energy (shorter wavelength) than either of the two absorbed photons.

Figure 10-1. Excited-state absorption. G, E₁, and E₂ represent ground state, intermediate, and excited state, respectively [81].

10.2.1.2. Addition de photon par transferts d'énergie (APTE) effect or energy transfer upconversion (ETU)

Addition de photon par transferts d'énergie (APTE) effect or energy transfer UC (ETU) is the result of successive energy transfers between ions. The ETU can occur by 3 mechanisms: radiative, non-radiative and photon assisted (Figure 10-2) [82]. Radiative ETU is the process by which the sensitizer emits energy that is absorbed by the activator. Non-radiative energy transfer occurs between the two neighboring ions, increasing the population of the E₂ state and allowing for subsequent emission with higher energy than either of the two incoming photons.

Figure 10-2. Addition de photon par transferts d'énergie (APTE) effect or energy transfer UC (ETU). G, E1, and E2 represent ground state, intermediate, and excited state, respectively.

10.2.1.3. Cooperative UC between two ions or a pair of ions and a third ion

Often confused with ETU-type UC processes, the CUC mechanism is distinguished by second order electronic transitions. ETU and ESA processes are both one-center transitions, and involve, at most, the transfer of energy between two ions. CUC processes, however, can occur between a pair of ions and a third, single ion in the UC material, shown in Figure 10-3. While these types of transitions, from both a mathematical and practical context, are far less likely to occur, the CUC mechanism has been observed and reported in samples of significant dimensions (mm to cm scale) of Yb^{+3} doped glass [83]. Recently, it has been shown that at high dopant ion concentrations of approximately 75%, efficient CUC is observed in nanoparticles [39]. The low probabilities of these types of transitions make emission efficiencies and luminescence intensities very weak [40, 41]. As a consequence, materials exploiting CUC processes have not been used for any bio-analytical applications.

Figure 10-3. Cooperative UC between two ions or a pair of ions and a third ion. G, E₁, and E₂ represent ground state, intermediate, and excited state, respectively.

10.2.1.4. Photon Avalanche (PA) effect

The photon avalanche effect mechanism incorporates both excited state absorption and energy transfer UC. The photon avalanche process begins with weak non-resonant ground state absorption. Relaxation of this electron occurs to the first metastable state. Excited state absorption occurs resulting in promotion to the third excited state. Non-radiative relaxation from the third excited state to the first excited state results in ion-pair relaxation energy transfer, promoting two ground state electrons, either in the same ion or neighboring ions. Resonant ESA of incident radiation brings these excited E₁ electrons back up to the E₃ state. This process repeats over and over, resulting in an exponential population growth of the E₃ state. Radiative relaxation from the E₃ to the G state results in strong UC emission.

Figure 10-4. Photon Avalanche Effect. G, E1, E2 and E3 represent ground state, intermediate, and excited states, respectively.

10.2.2. Upconverting Nanoparticle Composition

10.2.2.1. Host Lattice Choice

UCNPs are generally composed of a host matrix/lattice, a sensitizer and an activator [84]. An ideal host matrix or host lattice, which are terms that can be used interchangeably, should have low lattice photon energies [84]. Low lattice photon energies result in the minimization of non-radiative energy losses and maximized radiative emission [84]. In addition, the host lattice should have close lattice matches to the dopant ions. The high chemical stability and low lattice photon energies of fluorides such as NaYF_4 , was selected for synthesis in this thesis work because it is most commonly used and studied. NaGdF_4 , NaLuF_4 , KYF_4 , NaYbF_4 , LaF_3 , CaF_2 , KMnF_3 , YF_3 , and KGdF_4 have also been demonstrated to be good host candidates [84].

10.2.2.2. Sensitizer

The sensitizer ion is the energy donor and is selected to have a large absorption cross-section in the range of the desired excitation energy to be used, and Yb^{3+} is used almost exclusively for this reason.

10.2.2.3. Activator

The activator is the ion that emits the upconverted radiation. It is required to have more than one excited state energy level. The energy differences between the excited states and ground state should be close enough to facilitate photon absorption and are selected based on the desired emission wavelengths. Common activator choices are Tm^{3+} , Er^{3+} and Ho^{3+} ions.

10.2.3. Emission Wavelength Tuning

The ability to select emission wavelength allows for spectral matching to the absorbance of the photocleavable compound. It is also worth mentioning that this characteristic of UCNPs is important for applications in multiplexed biological labeling [85]. Emission tuning results from the distinguishable spectroscopic fingerprints of each lanthanide ion [54]. Generally colour tuning is done by adjusting dopant concentrations of Tm^{3+} , Er^{3+} and Ho^{3+} ions allowing for emission wavelengths that range from UV to NIR [54, 86]. It has also been demonstrated that the relative intensity of the multiple emission peaks can be tuned by adjusting the dopant concentrations, and thereby the distances between the activator and sensitizer ions [87, 88]. For the purpose of designing a photochemically triggered drug delivery strategy, Tm^{3+} was selected as the sole activator ion. Resonant energy transfer from Yb^{3+} ions to Tm^{3+} ions enabled emission bands ranging from 348-490 nm [89, 90, 91].

10.2.4. UC Efficiencies and Luminescence Stability

To date there is no generally accepted definition of UC efficiency. Generally UC efficiency is determined by considering radiative and non-radiative energy transfer rates, photon energies, spectral overlap, temperature, surface defects and excitation power [54]. For UCNP cores consisting of Tm^{3+} and Yb^{3+} dopants, UC efficiencies of 0.005 – 0.3% have been reported for the aforementioned PL emissions [92]. An important characteristic of UCNPs for both drug delivery and diagnostic applications is luminescence stability. In comparison with QDs, UCNPs display no “blinking” behavior and no intensity loss following over six hours of continuous irradiation with NIR excitation [93, 94].

10.2.5. Synthesis Routes

The synthesis should produce UCNPs with strong emission intensity, uniform size, shape, and water solubility using ligands that allow for further bioconjugation. A number of synthesis methods have been developed including sol-gel, hydrothermal, co-precipitation, thermal decomposition and one-step methods.

The sol-gel method begins with a liquid molecular precursor and the formation of an oxide network following a series of polycondensation reactions. This process is not widely used because it requires a high temperature calcination procedure and results in significant particle aggregation [95]. The method of co-precipitation for UCNPs was pioneered by van Veggel. This method produces NPs with narrow although larger size distributions, with sizes from 37 – 166 nm [96, 97]. The thermal decomposition method is the most common method for UCNP synthesis and was selected for the experiments herein, using a well established procedure to consistently produce monodisperse NPs [76, 77, 78, 79]. The method of thermal decomposition is defined by the formation of nucleates from decomposition of precursors under high

temperature conditions. This method is typically done in oleic acid, which acts not only as a solvent but also as a capping agent to prevent agglomeration and to control the size of NPs. The quality of NPs synthesized by this method is highly dependent on the solvent composition, reaction temperature, and time [95]. In comparison the hydrothermal method relies on the pH in addition to the reaction temperature and time to determine NP quality. The one-step method follows the same procedure as the hydrothermal method or co-precipitation method but uses hydrophilic or binary cooperative ligands to produce water soluble nanoparticles. This method provides a simplified approach to the generation of hydrophilic UCNPs but generally produces nanoparticles that are large and lack uniformity [95].

10.2.6. Surface Modification

Surface modification is required in order to add functionalization to UCNPs. There are five main approaches to surface modification: 1) ligand exchange; 2) ligand oxidation; 3) ligand attraction; 4) layer-by-layer assembly; and 5) silanization.

In the method of ligand exchange, the original, generally hydrophobic oleic acid ligands are displaced by other ligands. The coordination ability of the incoming ligand must be stronger than that of the displaced ligands [95]. For example, the -COOH functional group of oleic acid has been found to have a weaker coordination ability than the phosphate component of o-phosphorylethanolamine ligand which was used in the experiments presented in this thesis [98]. Ligand oxidation is another common method used for ligand functionalization but is limited by its applicability to only a few ligands. The most common example of this is the oxidation of double bonds in oleic acid to produce hydrophilic carboxylic acid ligands [95]. The ligand attraction method utilizes hydrophobic interactions to adsorb an amphiphilic co-polymer to the surface. This strategy is the basis of polyacrylic acid (PAA) coatings, which form hydrophobic

interactions with the octadecyl groups of oleylamine ligands [99]. Oleylamine is a common alternative to oleic acid during synthesis [95]. The primary drawback of this approach is that it imparts greater overall size to the NP. Layer-by-layer assembly or self-assembly methods are typically based on electrostatic attraction between the surface ligands and the incoming species. This method also results in larger overall nanoparticle size but has been shown to generate layers that are highly uniform in terms of shape and size [95]. Li *et al.* demonstrated this approach where the adsorption of poly(allylamine hydrochloride) (PAH) was alternated with poly(sodium 4-styrenesulfonate) (PSS) to generate nanoparticles with amino rich surfaces [100]. A further method is based on surface silanization, where hydrolysis and condensation of the siloxane monomers results in a silica shell [95]. This method is advantageous because it allows for the use of siloxane chemistry for further functionalization, as well as advantages typically associated with silica nanoparticles (See Table 9-1).

10.3. Experimental

10.3.1. Materials

10.3.2.

Tetramethylammonium hydroxide (>97%), o-phosphorylethanolamine (>99.0%), ninhydrin reagent (2% solution), ammonium fluoride (99.99%) 1-octadecene (90%), and oleic acid (90%) were from Sigma Aldrich (Oakville, ON, Canada). Sodium hydroxide (97%), methanol and hexane were from Caledon Laboratories (Georgetown, ON, Canada) and were reagent grade or better. Anhydrous ethanol was from GreenField Specialty Alcohols, Inc. (Brampton, Ontario, Canada). Deionized water was prepared using a Millipore Synergy UV R purification system (Millipore Corp., Mississauga, ON, Canada).

10.3.3. Instrumentation

Transmission electron microscopy (TEM) images of UCNPs were obtained from a Tecnai 20 Transmission Electron Microscope manufactured by FEI and located at the Mount Sinai Hospital and the Advanced Bioimaging Centre.

Inductively coupled plasma (ICP) atomic emission spectroscopy (AES) analysis was done with a Thermo Scientific iCAP6500 spectrophotometer ICP-AES (Perkin-Elmer).

10.3.4. Methods

10.3.4.1. Synthesis of Core Oleic β -NaYF₄: Yb, Tm (UCNP) nanocrystals.

In a typical procedure 0.45856 g of Y(CH₃CO₂)₃, 0.25269 g of Yb(CH₃CO₂)₃, and 0.00416 g of Tm(CH₃CO₂)₃, were combined in a 150 mL round bottom flask with 30 mL of 1-octadecene and 12 mL of oleic acid and mixed for several minutes. The reaction mixture was heated to 115 °C for 30 minutes under vacuum. The temperature was then cooled to 50 °C under an argon atmosphere. Subsequently 20 mL of a methanol solution with 0.29658 g of NH₄F and 0.19039 g of NaOH was added to the reaction mixture. The cloudy solution was heated to 70 °C to remove the methanol. The temperature was then raised to 300 °C and maintained for 1 hour. The product was collected by centrifugation (4500 rpm, 8 minutes) and was washed with anhydrous ethanol several times and dispersed in hexane.

10.3.4.2. Synthesis of Core-Shell Oleic β -NaYF₄: Yb, Tm (UCNP) nanocrystals.

In a typical procedure 0.45856 g of Y(CH₃CO₂)₃ was combined in a 150 mL round bottom flask with 30 mL of 1-octadecene and 12 mL of oleic acid and mixed for several minutes. The reaction mixture was heated to 115 °C for 30 minutes under vacuum. The temperature was then cooled to 80 °C under an argon atmosphere and the core nanoparticles from step 1 were added. After hexanes were removed the temperature was then cooled to 50 °C and 20 mL of a methanol

solution with 0.29658 g of NH_4F and 0.19039 g of NaOH was added to the reaction mixture. The cloudy solution was heated to 70 °C to remove the methanol and the temperature was then raised to 300 °C and maintained for 1 hour. The product was collected by centrifugation (4500 rpm, 8 minutes) washed with anhydrous ethanol several times and dispersed in hexane.

10.3.4.3. Synthesis of Amine Coated $\beta\text{-NaYF}_4$: Yb, Tm (UCNP) nanocrystals.

100 mg of oleic acid coated UCNP in ~1 mL of hexane were mixed with 0.4 g of o-phosphorylethanolamine, 1 mL of tetramethylammonium hydroxide and 4 mL of anhydrous ethanol at 70 °C overnight. The nanoparticles were precipitated with hexane. The nanoparticles were then separated and washed with deionized water by centrifugation at 4500 rpm for 8 minutes for a total of three washes. Amine functionalization was confirmed using ninhydrin reagent. The ICP-AES analysis of the nanoparticles revealed the elemental composition of UCNPs by weight. The nanoparticles were characterized using fluorescence emission spectroscopy and TEM (Figure 10-5)

10.4. Results and Discussion

The ICP-AES analysis of the nanoparticles revealed that they were 16.34% Na, 0.07% Tm, 13.25% Y, 4.95% Yb and 65.37% F percent by mass. Using these % ratios and 20.06 nm NP radius the MW of the nanoparticles was calculated to be 5066.27 g/mol. The nanoparticles were characterized using fluorescence emission spectroscopy and TEM. See Figure 10-5.

Figure 10-5. (a) Normalized PL spectrum of NaYF₄ 4.95% Yb, 0.08% Tm, core-shell UCNPs coated with oleic acid upon irradiation with a 980 nm laser. The spectrum is normalized to the most intense UV-vis emission at 454 nm. (b) Transmission electron micrograph of oleic acid capped UCNPs, and (inset) scale bars are 20 nm and 100 nm (c)

The PL spectrum shown in Figure 10-5 (a) displays three emission bands across the UV-vis region with maximum intensities at 364 nm, 454 nm and 484 nm. The spectrum was normalized to the most intense UV-vis emission at 454 nm. The relative ratio of the 364 nm peak to the 454 nm peak indicates good UV emission in the absorption range of the ONB-FU ligand, which will be introduced in the following section. The average size and homogeneity of the UCNPs were determined through electron microscopy. A TEM image of the oleic acid capped UCNPs is shown in Figure 10-5(b). The UCNPs adopted a hexagonal structure characteristic of β -UCNP, which was expected *a priori* due to the synthesis method chosen and the temperature of the reaction. The average size of the UCNPs was determined to be 20.1 nm by physical measurement of 100 particles. The spread of the ensemble was 3.0 nm as given by the standard

deviation and indicated good monodispersity. The size, shape and emission peaks were consistent with similar synthetic reports. [76, 77, 78, 79]

10.5. Conclusion

In summary, β -NaYF₄ 4.95% Yb, 0.08% Tm, core-shell UCNPs with uniform shape, size distribution and monodispersity were successfully synthesized through thermal decomposition. The PL spectrum of the synthesized UCNPs displayed three emission bands across the UV-visible region with maximum intensities at 364 nm, 454 nm and 484 nm. Water solubility was imparted, in an additional step following ligand exchange with *o*-phosphorylethanolamine.

11. Caging 5-Fluorouracil with *o*-Nitrobenzyl Bromide

11.1. Abstract

This section describes a drug release method that utilizes the ONB photolabile tether reported by the Rotello group [1] to allow caging of the well-known chemotherapeutic 5-FU. The ONB group is advantageous because it undergoes photolytic cleavage at 365 nm allowing for the controlled release of the covalently attached therapeutic. Moreover the ONB group can covalently bind to a large range of therapeutics and therefore this strategy is not limited to photosensitizing agents. The ONB group was synthesized in 6-step synthesis and characterized by NMR and MS. The kinetics of the phototriggered release of the 5-FU from the ONB-FU ligand were quantified using HPLC. A time-dependent increase in free 5-FU was observed with direct UV irradiation of the free ONB-FU ligand. The quantity of photolytic release was determined to be dependent on irradiation time. The maximum release of 5-FU from solution samples of ONB-FU occurred at 80 minutes of UV irradiation and represented 96% of potential

release based on the initial concentration of ONB-FU in solution. Negligible 5-FU release was observed in an analogous experiment with direct NIR irradiation, which was conducted as a control.

11.2. Introduction

The *o*-nitrobenzyl bromide (ONB) group was selected for experimentation because it can be used for the direct caging of biologically active molecules that contain carboxylic, phosphate or hydroxyl groups [101]. The drug or biologically active molecule can then be irreversibly released by UV irradiation at 365 nm [1]. ONB was also selected because the photolytic release mechanism has been investigated both theoretically and experimentally [102]. A six-step synthesis was performed to create ONB conjugated with 5-fluorouracil (5-FU). The anti-cancer drug 5-FU was selected because it is a well-known agent that has been used for the treatment of solid tumours associated with breast and colorectal cancers. The mechanism of action for 5-FU is also well known. 5-FU interferes with DNA and RNA synthesis after being converted to several cytotoxic forms by multiple biochemical pathways. Most commonly 5-FU is administered parenterally, but often has toxic side effects due to non-specific distribution [103]. Therefore the attachment of 5-FU to the ONB linkage would allow for controlled release. Further, by attaching the 5-FU ONB complex to a UCNP platform this could contribute to the building of a theranostic system.

11.2.1. Photolabile protecting groups

Photolabile protecting groups or “cages” are based on chemical modification of a molecule using a photoremovable protecting group. In the case of the caging of therapeutics, the chemical modification should also prevent drug activity. Pelliccioli and Wirz list criteria for the design of a good photoremovable protecting group [104]. The photolysis should be a clean reaction with

high quantum yield and an absorption coefficient at wavelengths above 300 nm to reduce biological damage by UV irradiation. Also, the photochemical by-products should be biocompatible and transparent at the irradiation wavelength to avoid competitive absorption, and the released compound, in this case the therapeutic agent, should be soluble in the target solution [104].

It is important to consider that the release of the substrate/therapeutic may occur after considerable delay since many photoreactions proceed through a cascade of reactive intermediates. In addition the time delay and rate of release are dependent on the solvent, temperature and chemical modification of the caging group and as such are difficult to predict [104]. The most common photolabile protecting groups are ONB derivatives.

11.2.2. ONB

Kaplan *et al.* pioneered the development and synthesis of the ONB photocage in 1978 [2]. Since that time over 40 nitrobenzyl derivatives have been synthesized [104, 105]. The general disadvantages of the ONB photolabile group include strongly absorbing by-products as well as relatively slow rates of release following excitation [104]. An additional concern traditionally associated with ONB groups has been the potential for evolution of toxic by-products. It was initially thought that NO₂ was a by-product of this reaction, however, more recent literature reports that photolysis of ONB follows a ‘cleaner’ reaction and that NO₂ is not released [105, 106]. A second NO₂ group is frequently added to facilitate the synthetic route (see Figure 11-1).

Figure 11-1. a) o-nitrobenzyl group, b) o-nitrobenzyl group with additional NO₂

Some examples of photolytic release utilizing the ONB group are described by Corrie and include the release of nucleotides, carboxylates, alcohols, phenols, phosphates, protons, calcium and fluorophores [105]. Scheme 11-1 illustrates the photochemical reaction of ONB-FU that is explored in this thesis, (adapted from reference [105, 106]).

Scheme 11-1 Photochemical Reaction of ONB-FU adapted from reference [105, 106]

11.2.3. 5-FU

5-FU is a well studied cancer chemotherapeutic that is frequently used as a representative drug in the development of delivery methods. Fluorine is a popular element in drug design as all known fluorinated natural products are toxic. Fluorine provides a good hydrogen mimic in organic molecules because the van der Waal's radius (1.35 Å) is close to that of hydrogen (1.10 Å), therefore minimizing steric strain [107]. It is for this reason that 5-bromouracil, is too large to be incorporated into the biosynthetic pathway and therefore does not have the same anti-cancer activity as 5-FU [107]. Fluorination is also beneficial because it increases enzyme binding through hydrophobic interactions and increases solubility in fats improving membrane partitioning [107].

In the 1950's it was found that uracil metabolism was a potential target for antimetabolite chemotherapy following the observation that rat hepatomas use uracil more rapidly than normal tissues [108]. Anti-metabolite agents, such as 5-FU, become incorporated into macromolecules, such as DNA and RNA, and/or inhibit essential biosynthetic processes [109]. 5-FU is digested intracellularly to into several active metabolites which disrupt RNA synthesis and inhibit the nucleotide synthetic enzyme thymidylate synthase (TS) by the enzyme dihydropyrimidine dehydrogenase (DPD) [109].

11.3. Experimental

11.3.1. Materials

N,N-dimethylformamide ACS reagent ($\geq 99.8\%$), tert-butylbromoacetate (98%), potassium carbonate (99.0%), pyridine (99.8%), phosphorus tribromide (99%), trifluoroacetic acid (TFA, $>99\%$), dichloromethane (DCM, 99.8%), sodium sulfate (99%), 5-fluorouracil (5-FU, $>99\%$), $\text{Y}(\text{CH}_3\text{CO}_2)_3$ (99.9%), $\text{Yb}(\text{CH}_3\text{CO}_2)_3$ (99.9%), $\text{Tm}(\text{CH}_3\text{CO}_2)_3$ (99.9%), sodium borohydride

(98%) were from Sigma Aldrich (Oakville, ON, Canada). Sodium hydroxide (97%), toluene (99.7%), nitric acid (68 – 70%), hydrochloric acid (16 M), methanol, acetonitrile, and dimethyl sulfoxide (DMSO) were from Caledon Laboratories (Georgetown, ON, Canada) and were reagent grade or better. 3-hydroxy-4-methoxybenzaldehyde (Isovanillin, 98%) and o,o'-bis(trimethylsilyl)-5-fluorouracil (97%) were from Alfa Aesar (Ward Hill, Massachusetts, USA). Deuterated dimethylsulfoxide (DMSO-d₆) and deuterated chloroform (CDCl₃) were from Cambridge Isotope Laboratories (Cambridge, MA). Deionized water was prepared using a Millipore Synergy UV R purification system (Millipore Corp., Mississauga, ON, Canada).

11.3.2. Instrumentation

¹H NMR and ¹⁹F NMR spectra were recorded on a Bruker 400 MHz NMR. CD₂Cl₂ and DMSO were used as the deuterated solvents as indicated. Peak multiplicities are designated by the following abbreviations: s, singlet; bs, broad singlet; d, doublet; t, triplet; q, quartet; dd, doublet of doublets, and the J coupling constant in Hz.

Electrospray ionization (ESI) mass spectra (MS) were recorded using a Q-TOF mass spectrometer equipped with Z-spray source manufactured by Waters (Milford, MA). The source temperature was kept at 100 °C. The sample was directly infused into the chamber at 50 µL/min. The spray voltage was kept at 3.20 kV and the cone voltage at 30 V. The ESI samples were dissolved in methanol.

Analytical High Pressure Liquid Chromatography (HPLC) was performed on an Agilent 1100 HPLC (Mississauga, Ontario). Detection was done with a UV detector at a wavelength of 256 nm. A SPHERI-5-RP-C18 5 µM 250 x 4.6 mm column supplied by PerkinElmer at 25 °C was used for separations (Waltham, MA). A solution of 50% methanol and 50% deionized water was used as the mobile phase at flow rate of 1 mL/min.

11.3.3. Experimental procedures and product characterization

11.3.3.1. Step 1: Synthesis of Compound 1 (3-hydroxy-4-methoxy-2,6-dinitrobenzaldehyde)

In a 250 mL red round bottom flask, 24 mL of HNO₃ was cooled to -10 °C in an ice salt bath for 30 minutes. 15.0 g (98.3 mmol) of isovanillin (3-hydroxy-4-methoxybenzaldehyde) was added over 6 hours. After completion of the addition the mixture was brought slowly to room temperature and stirred for a further 2 hours. The reaction mixture was then transferred to 200 mL of ice water. After filtration the solid residue was collected and dried under vacuum. The yield was 18.5 g (76.2 mmol, 77%). The product was a pale yellow powder. MS (ESI): 240.99[M – H]⁻, 296.94[M]⁺ 1H NMR (400 MHz, CDCl₃): δ 10.48 (s, 1H, CHO), 7.84 (s, 1H, H Ar), 4.09 (s, 3H, OCH₃).

Scheme 11-2 Step 1: Synthesis of Compound 1 (3-hydroxy-4-methoxy-2,6-dinitrobenzaldehyde)

Figure 11-2. Compound 1 (3-hydroxy-4-methoxy-2,6-dinitrobenzaldehyde) ^1H NMR (400 MHz, CDCl_3): δ 10.48 (s, 1H, CHO), 7.84 (s, 1H, H Ar), 4.09 (s, 3H, OCH_3).

11.3.3.2. Step 2: Synthesis of Compound 2 (3-(hydroxymethyl)-6-methoxy-2,4-dinitrophenol)

30.0 g of compound (1) were added to a 250 mL red round bottom flask with 150 mL of deionized water and 75 mL of ethanol and stirred. 5.0 g (124.0 mmol) of NaOH was added to the flask and the resulting solution became dark red and clear. 2.48 g (62.0 mmol) of NaBH_4 was then added slowly with stirring and then the mixture was allowed to stir for 3 hours at room temperature. The mixture was cooled to 0 °C and acidified with 3M HCl. The brown solution was extracted 3 times with ethyl acetate then washed with 150 mL of brine. The solution was

dried over Na₂SO₄, filtered and dried under vacuum. Compound 2 was obtained as a dark brown solid. The yield was 30 g (124.0 mmol, 93%). MS (ESI): 243.14[M – H][–] 1H NMR (400 MHz, CDCl₃): δ 7.70 (s, 1H, H Ar), 4.82 (s, 2H, PhCH₂O), 4.09 (s, 3H, OCH₃), 2.75 (br, 1H, Benzyl OH).

Scheme 11-3 Step 2: Synthesis of Compound 2 (3-(hydroxymethyl)-6-methoxy-2,4-dinitrophenol)

Figure 11-3 Compound 2 (3-(hydroxymethyl)-6-methoxy-2,4-dinitrophenol)¹H NMR of (400MHz, CDCl₃): δ 7.70 (s, 1H, H Ar), 4.82 (s, 2H, PhCH₂O), 4.09 (s, 3H, OCH₃), 2.75 (br, 1H, Benzyl OH).

11.3.3.3. Step 3: Synthesis of Compound 3 (tert-butyl 2-(3-(hydroxymethyl)-6-methoxy-2,4-dinitrophenoxy)acetate)

4.0 g of compound (2) (46.0 mmol) was added to a suspended solution of 16.85 g of K_2CO_3 in 50 mL of dry DMF. The mixture was stirred for 1 hour and then 3.61 g of tert-butylbromoacetate was added. The reaction was stirred at room temperature for 48 hours followed by filtration. The yield was 2.06 g (5.8 mmol, 35%). MS (ESI): 381.22[M + Na]⁺ 1H NMR (400 MHz, $CDCl_3$): δ 7.71 (s, 1H, HAr), 4.81 (s, 2H, $-PhCH_2O-$), 4.73 (d, 2J = 7.32 Hz, 2H, $-OCH_2CO-$), 3.99 (s, 3H, $-OCH_3$), 2.69 (t, 3J = 7.44 Hz, 1H, Benzyl-OH), 1.56 (s, 9H, $-C(CH_3)_3$).

Scheme 11-4 Step 3: Synthesis of Compound 3 (tert-butyl 2-(3-(hydroxymethyl)-6-methoxy-2,4-dinitrophenoxy)acetate)

Figure 11-4 Compound 3 (tert-butyl 2-(3-(hydroxymethyl)-6-methoxy-2,4-dinitrophenoxy)acetate) ¹H NMR (400 MHz, CDCl₃): δ 7.71 (s, 1H, HAr), 4.81 (s, 2H, -PhCH₂O-), 4.73 (d, 2J = 7.32 Hz, 2H, -OCH₂CO-), 3.99 (s, 3H, -OCH₃), 2.69 (t, 3J = 7.44 Hz, 1H, Benzyl-OH), 1.56 (s, 9H, -C(CH₃)₃).

11.3.3.4. Step 4: Synthesis of Compound 4 (tert-butyl 2-(3-(bromomethyl)-6-methoxy-2,4-dinitrophenoxy)acetate)

2.0 g of Compound (3) (5.6 mmol) was dissolved in toluene and placed in an iced bath. Five drops of dry pyridine were added to the solution. The reaction was stirred for 24 hours at room temperature. After 24 hours 5 mL of H₂O were added and stirred for 1 hour. The solution was then further diluted with 300 mL of water and treated with 10 mL of 1M HCl. The aqueous reaction mixture was then extracted with ethyl acetate (150 mL) 3 times. The organic layers were

combined and washed thoroughly with brine and dried over Na_2SO_4 . After removal of solvent, the residue was charged on a SiO_2 column for purification (DCM). The yield was 1.128 g (2.67 mmol, 47%). MS (ESI): 445.00 $[\text{M} + \text{Na}]^+$ NMR (400 MHz, CDCl_3): δ 7.77 (s, 1H, HAr), 4.82 (s, 2H, $-\text{PhCH}_2\text{Br}$), 4.70 (s, 2H, $-\text{OCH}_2\text{CO}-$), 4.00 (s, 3H, $-\text{OCH}_3$), 1.48 (s, 9H, $-\text{C}(\text{CH}_3)_3$).

Scheme 11-5 Step 4: Synthesis of Compound 4 (tert-butyl 2-(3-(bromomethyl)-6-methoxy-2,4-dinitrophenoxy)acetate)

Figure 11-5 Compound 4 (tert-butyl 2-(3-(bromomethyl)-6-methoxy-2,4-dinitrophenoxy)acetate) ^1H NMR (400 MHz, CDCl_3): δ 7.77 (s, 1H, HAr), 4.82 (s, 2H, $-\text{PhCH}_2\text{Br}$), 4.70 (s, 2H, $-\text{OCH}_2\text{CO}-$), 4.00 (s, 3H, $-\text{OCH}_3$), 1.48 (s, 9H, $-\text{C}(\text{CH}_3)_3$).

11.3.3.5. Step 5: Synthesis of Compound 5 (tert-butyl 2-(3-((5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)methyl)-6-methoxy-2,4-dinitrophenoxy)acetate)

In a 250 mL red round bottom flask 0.18 mL of *o,o*-bistrimethyl siloxyl 5-fluorouracil was mixed with 0.30 g of compound (4) (0.7 mmol) in 15 mL of acetonitrile. The reaction mixture was heated to reflux (80 °C) for 72 hours under N_2 atmosphere in the dark. After cooling to room temperature 5 mL of methanol was added to treat the product. After 1 hour the solvent was

removed under reduced pressure and the residue was loaded onto a SiO₂ column for purification.

A gradient elution of dichloromethane to ethyl acetate was used. Solvent was again removed

under reduced pressure to yield 0.05 g (0.106 mmol, 15%) of a grey powder. MS (ESI):

469.05[M]⁻ ¹H NMR (400 MHz, DMSO): δ 11.87 (1H, -NHFU-), 7.90 (s, 1H, HAr), 7.75 (d,

2J = 5.56 Hz, 1H, HFU), 4.96 (s, 2H, -PhCH₂N-), 4.85 (s, 2H, -OCH₂CO-), 3.95 (s, 3H,

-OCH₃), 1.39 (s, 9H, -C(CH₃)₃).

Scheme 11-6 Step 5: Synthesis of Compound 5 (tert-butyl 2-(3-((5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)methyl)-6-methoxy-2,4-dinitrophenoxy)acetate)

Figure 11-6 Compound 5 (tert-butyl 2-(3-((5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)methyl)-6-methoxy-2,4-dinitrophenoxy)acetate) ¹H NMR (400 MHz, DMSO): δ 11.87 (1H, -NHFU-), 7.90 (s, 1H, HAr), 7.75 (d, 2J = 5.56 Hz, 1H, HFU), 4.96 (s, 2H, -PhCH₂N-), 4.85 (s, 2H, -OCH₂CO-), 3.95 (s, 3H, -OCH₃), 1.39 (s, 9H, -C(CH₃)₃).

11.3.3.6. Step 6: Synthesis of Compound 6 (2-(3-((5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)methyl)-6-methoxy-2,4-dinitrophenoxy)acetic acid)

40 mg of Compound (5) was dissolved in 2 mL of DCM. 0.5 mL of TFA was added. The reaction mixture was stirred at room temperature for 4 hours, then transferred to a 100 mL round bottom flask and concentrated under vacuum. The crude mixture was then washed with diethyl ether three times and filtered. The white powder was further dried under reduced pressure. The

yield was 0.063 g (0.152 mmol, 72%). MS (ESI): 240.99[M – H]⁻, 296.94[M]⁺ ¹HNMR (400 MHz, DMSO): δ 11.86(s, 1H, -NH_{FU}-), 7.92(s, 1H, H_{Ar}), 7.89(d, 2, H_{FU}), 4.96 (d, 4H, -OCH₂CO-), 3.95 (s, 4H, -PhCH₂N-) 3.67 (s, 4H, -OCH₃)

Scheme 11-7 Step 6: Synthesis of Compound 6 (2-(3-((5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)methyl)-6-methoxy-2,4-dinitrophenoxy)acetic acid)

Figure 11-7 Compound 6 (2-(3-((5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)methyl)-6-methoxy-2,4-dinitrophenoxy)acetic acid) ¹HNMR (400 MHz, DMSO): δ 11.86(s, 1H, -NH_{FU}-), 7.92(s, 1H, H_{Ar}), 7.89(d, 2, H_{FU}), 4.96 (d, 4H, -OCH₂CO-), 3.95 (s, 4H, -PhCH₂N-) 3.67 (s, 4H, -OCH₃).

11.3.4. The photochemical reaction of free ONB-FU ligand

The uncaging of 5-FU from the free ONB-FU ligand was characterized by irradiating 200 μ L of 0.1 mg/mL ONB-FU solution at 365 nm with 0.5 mW UV radiation, and separately at 980 nm with 29 mW NIR irradiation. No more than 25% of solvent was evaporated during irradiation. Following irradiation the solvent was removed under pressure. The product was then redissolved in 200 μ L of 50:50 methanol:water and was vortexed for 2 minutes. Standard additions of 0.05 mg/ml, 0.10 mg/ml and 0.15 mg/ml of 5-FU were added. 10 μ L of the solution was injected into the column and eluted at 25 °C. The mobile phase was 50% methanol and 50% deionized water, and the flow rate was 1.0 mL/min. Absorption at 256 nm was monitored. The resultant concentrations were plotted as a percent of total possible 5-FU based on the initial ONB-FU concentration. The data is presented in Figure 11-8.

11.4. Results and Discussion

The purpose of the work described in this section was to prepare the ONB-FU compound and determine the kinetic rates of 5-FU release. The ONB-FU ligand was successfully prepared with an overall yield of 1.27% from a 6 step synthesis and characterized by NMR and MS. It was expected that the release of the 5-FU might occur after considerable delay since many photoreactions proceed through a cascade of reactive intermediates as described by Pelliccioli and Wirz [104]. Following this delay it was found that a time-dependent increase in free 5-FU was observed with direct UV irradiation of the free ONB-FU ligand. (Figure 11-8) This linear irradiation dependence from 30 minutes to 70 minutes demonstrated kinetics which were consistent with previously reported ONB derivatives [110, 111, 112]. The maximum release of 5-FU from solution samples of ONB-FU occurred at 80 minutes of UV irradiation and

represented 96% of potential release based on the initial concentration of ONB-FU in solution. Negligible 5-FU release was observed in analogous experiments with direct NIR irradiation using 30 mW power, which was conducted as a control. In the following section, a description is provided of how this ONB-FU ligand was covalently conjugated to the UCNP, and the kinetics of NIR triggered 5-FU release are compared to that of the free ligand.

Figure 11-8. Plot of the timescale of the release of 5-FU following irradiation of the ONB-FU complex using a 0.5 mW UV lamp with a wavelength centered at 365 nm.

11.5. Conclusion

In summary, the ONB-FU ligand was synthesized in a 6-step synthesis. All products were characterized by NMR and MS. The kinetics of the phototriggered release of the 5-FU from the ONB-FU ligand was observed and quantified using HPLC. A time-dependent increase in free 5-FU was observed with direct UV irradiation of the free ONB-FU ligand. The quantity of photolytic release was determined to be dependent on irradiation time which was consistent with previous experiments with ONB-FU derivatives. The maximum release of 5-FU from solution

samples of ONB-FU occurred at 80 minutes of UV irradiation and represented 96% of potential release based on the initial concentration of ONB-FU in solution

12. Externally triggered photochemical drug release from UCNPs

12.1. Abstract

This section describes the NIR-mediated release of 5-FU from a UCNP platform using a photocleavable linker strategy based on ONB. The ONB-FU complex was covalently bonded to the UCNP surface using N-hydroxysuccinimide (NHS) facilitated N,N'-diisopropylcarbodiimide (DIC) coupling. Conjugation was confirmed by UV-vis spectroscopy. Following NIR irradiation to produce upconversion in the UV range, the 5-FU therapeutic was released, and then quantified using HPLC analysis.

12.2. Introduction

12.2.1. Upconverting nanoparticles in photochemical drug release

The potential of UCNP for photochemical drug release has been discussed in previous chapters. At this time only two examples of the use of UCNPs for photochemical drug release have been published and these will be briefly reviewed here. Both of these examples lack direct conjugation of the therapeutic to the nanocarrier, making the methods highly susceptible to leakage. The first example by Yang *et al.* described a mesoporous silica coated UCNP conjugate for DOX delivery. The overall size of the monodisperse particles was 150 – 200 nm prior to the addition of the surface coating. DOX was caged within the mesoporous silica shell by a cross-linked polymer consisting of an ONB derivative. The drug release from the NP surface was determined to be 75%. *In vivo* studies demonstrated a 20% decrease in cell viability in dark conditions

attributed to leakage and a 80% decrease in cell viability following NIR irradiation [65]. In addition to leakage, a limitation of this approach was attributed to the overall size of the nanocarrier which could hinder conjugation of additional functionalities and reduce practicality for animal trials. A second example by Liu *et al.* also involved the coating of UCNPs with a silica shell. The final size of these nanocarriers was 54 nm. In this example the silica shell had azobenzene incorporated mesopores. In this approach the azomolecules acted as a molecular impeller that propelled the release of DOX. This impeller movement resulted from reversible azobenzene photoisomerization by simultaneous emission of both UV and visible light by the UCNPs that resulted in a continuous rotation–inversion movement [64]. This novel approach to drug delivery is limited by the potential for leakage resulting from diffusion of the therapeutic from the mesopores.

12.3. Experimental

12.3.1. Materials

N-hydroxysuccinimide (NHS, 99%), *N,N*-diisopropylcarbodiimide (DIC, 99%) were from Sigma Aldrich (Oakville, ON, Canada). Methanol and dimethyl sulfoxide (DMSO) were from Caledon Laboratories (Georgetown, ON, Canada) and were reagent grade or better. Deionized water was prepared using a Millipore Synergy UV R purification system (Millipore Corp., Mississauga, ON, Canada).

12.3.2. Instrumentation

¹H NMR and ¹⁹F NMR spectra were recorded on a Bruker 400 MHz NMR. CD₂Cl₂ and DMSO were used as the deuterated solvents as indicated. Peak multiplicities are designated by the following abbreviations: s, singlet; bs, broad singlet; d, doublet; t, triplet; q, quartet; dd, doublet of doublets, and the J coupling constant in Hz.

Electrospray ionization (ESI) mass spectra (MS) were recorded using a Q-TOF mass spectrometer equipped with Z-spray source manufactured by Waters. The source temperature was kept at 100 °C. The sample was directly infused into the chamber at 50 µL/min. The spray voltage was kept at 3.20 kV and the cone voltage at 30 V. The ESI samples were dissolved in methanol.

Analytical High Pressure Liquid Chromatography (HPLC) was performed on an Agilent 1100 HPLC. Detection was done with a UV detector at a wavelength of 256 nm. A SPHERI-5-RP-C18 5 µM 250 x 4.6 mm column supplied by PerkinElmer at 25 °C was used for separations. A solution of 50% methanol and 50% deionized water was used as the mobile phase at flow rate of 1 mL/min.

12.3.3. Experimental procedures and product characterization

12.3.3.1. Coupling ONB-FU to Amine Coated β -NaYF₄: Yb, Tm (UCNP)

0.0020 g of ONB-FU was combined with 0.08 g of N-hydroxysuccinimide in 0.5 mL of DMSO. The mixture was stirred and purged with N₂ until clear. 0.50 mL of a 14.7 mg/mL solution of amine coated β -NaYF₄: Yb, Tm UCNPs was added. The mixture was stirred for 5 minutes. 0.08 mL of N,N'-diisopropylcarbodiimide was diluted to 0.16 mL with DMSO and added dropwise to the mixture while stirring vigorously. The reaction was stirred for 24 hours at room temperature. The nanoparticles were then separated and washed with 50:50 methanol:deionized water by centrifugation at 11000 rpm for 30 minutes. The washing was repeated for a total of five times. The supernatant was run on the C-18 HPLC to ensure the absence of free 5-FU and that minimal free ligand was present.

Figure 12-1 UV-vis spectrum of UCNP-ONB-FU, UCNP, ONB-FU in dimethylformamide (DMF). The ONB-FU spectra is the difference spectrum of UCNP-ONB-FU and UCNP obtained by subtracting one from the other. The sharp peak at 274 nm is attributed to the o-phosphorylethanolamine ligand, with the broad peak at 340 nm attributed to the ONB-FU ligand.

12.3.3.2. The photochemical reaction of UCNP-ONB-FU

The uncaging of 5-FU from the UCNP-ONB-FU complex was characterized by irradiating 200 μ L of 2.8 mg/mL UCNP-ONB-FU solution with 0.5 mW UV at 365 nm, and separately with 29 mW IR irradiation at 980 nm. No more than 25% of solvent was evaporated during irradiation. After irradiation the solvent was removed under pressure. The product was then redissolved in 200 μ L of 50:50 methanol:water and was vortexed for 2 minutes. The nanoparticles were then separated by centrifugation at 11000 rpm for 30 minutes. The supernatant was collected and standard additions of 0.05 mg/mL, 0.10 mg/mL and 0.15 mg/mL of 5-FU were added. 10 μ L of the solution was injected into the HPLC column and was eluted at 25 $^{\circ}$ C. The mobile phase was 50% methanol and 50% deionized water, and the flow rate was 1.0 mL/min. Absorption at 256 nm

was monitored. The resultant concentrations were normalized to represent the average number of 5-FU molecules per nanoparticle (Figure 12-2 and Figure 12-3).

12.4. Results and Discussion

The purpose of this component of experimental work was to covalently conjugate the ONB-FU ligand to the UCNPs and characterize the kinetics of UC mediated 5-FU release. The conjugation reaction proceeded through the well established DIC reaction [113] and was confirmed by UV-vis absorbance (Figure 12-1). The broad peak at 340 nm was attributed to the ONB-FU ligand and was consistent with previous reports [1]. As a control experiment to confirm the activity of the ONB-FU ligand on the NP surface, the release of 5-FU with direct UV irradiation was performed. The kinetics of 5-FU release from the UCNPs were quantified by HPLC in an analogous experiment to the UV triggered 5-FU release from the ONB-FU ligand (Figure 12-2). The kinetic rates of 5-FU release from the NP surface were consistent with that of the free ligand. The rate of reaction was found to be faster from the NP surface than as a free ligand in solution which is consistent with previously reported surface effects [114]. The quantity of 5-FU collected following UV irradiation accounted not only for ONB-FU on the nanoparticle surface but also any free ligand which may have been present in the solution. The maximum number of 5-FU molecules released from a single nanoparticle surface was estimated to be 3.08. This was determined from the initial solution concentration of nanoparticles and the difference between the average amount of 5-FU released in the time from 20 - 80 minutes following UV irradiation and the base line free 5-FU. The difference was taken to account for the free ligand which may exist in solution and would be susceptible to the UV irradiation.

Figure 12-2. Time-based release of 5-FU following irradiation of the UCNP-ONB-FU complex using 0.5 mW UV lamp with a wavelength centered at 365 nm. HPLC was used to quantitatively monitor the release of 5-FU photocleavage and a linear response with time was observed for an irradiation of 10 to 20 min (inset).

Release of 5-FU by upconverted NIR irradiation should induce ONB-FU cleavage of only surface ligands and not any free ligands present in solution. It was hypothesized that increasing NIR intensity would increase UC emission intensity resulting in faster 5-FU release. Samples were prepared in water and were irradiated with 980 nm laser for various amounts of time. The completion of release of 5-FU from the UCNPs was determined to take about 14 minutes following initiation of irradiation with NIR light at 30 mW (Figure 12-3). The kinetic curve was fitted with bi-exponential equation and amplitude-weighted average rate constant was determined to be 0.03 min^{-1} . It was also observed, that the rate of 5-FU release could be modulated by varying NIR intensity by using 80 mW and 10 mW NIR laser powers. As shown in Figure 5, a complete photolysis was observed at 10 minutes followed NIR irradiation at 80 mW. An increase in the amplitude-weighted average rate constant was almost 6-fold. In contrast, decrease in NIR irradiation to 10 mW corresponded to decrease in amplitude-weighted average rate constant to 0.006 min^{-1} .

The average maximum number of 5-FU molecules released from a single UCNP by direct UV excitation was estimated to be 3.9 based on the initial solution concentration of nanoparticles and the concentration-response curve for 5-FU (Figure 4). The average maximum number of 5-FU molecules released per nanoparticle under NIR irradiation conditions was found to be 3.0, indicating that the efficiency of release from the UCNPs by NIR excitation was about 77% of that achieved by direct UV excitation. Extrapolating to potential practical application, non-targeted tissue structures are unaffected by NIR treatment provided they stay below 42 °C, and laser powers in the 2W – 15W range with similar wavelengths to that used herein are found in clinical photothermal therapy methods.³⁹ Therefore, the NIR power that is used to penetrate tissues appears to be sufficient to excite UCNPs for drug release.

Figure 12-3 Time-based release of 5-FU following irradiation of UCNPs that were conjugated to ONB-5-FU, using a 980 nm laser at 10 mW, 30 mW, and 80 mW. The amount of 5-FU released was dependent on both time and the power of the NIR source.

12.5. Conclusion

In this work, a common anti-cancer drug was conjugated to upconverting nanoparticles to prepare a photosensitive release system. By attaching the therapeutic to the ONB

photoactivatable linker, the UCNPs can serve as a photocaging nanocarrier. Following NIR irradiation with upconversion in the UV range, the 5-FU therapeutic was released and was subsequently quantified. Controlled rates of drug release could be achieved by selection of NIR excitation power. Excitation using NIR was found to deliver approximately 80% of the conjugated drug that was available on the UCNPs.

13. Conclusion and Future Work

Water soluble UCNPs and the ONB caging group for the chemotherapeutic 5-FU were synthesized and characterized. Following conjugation of the ONB-FU ligand to the UCNPs, the extent of photolysis of the ONB caging group caused by the UC emission was observed and quantified, suggesting potential for externally triggered drug delivery using NIR radiation.

Characterization of the UCNPs, synthesized by thermal decomposition, demonstrated that they were β -NaYF₄ 4.95% Yb, 0.08% Tm, core-shell UCNPs with uniform shape, size distribution and monodispersity. The PL spectrum of the synthesized UCNPs displayed three emission bands across the UV-visible region with maximum intensities at 364 nm, 454 nm and 484 nm. Water solubility was imparted, in an additional step following ligand exchange with o-phosphorylethanolamine.

In a separate step the photoactive ONB-FU ligand was synthesized. The kinetics of the UV phototriggered release of the 5-FU from the ONB-FU ligand was observed and quantified using HPLC. The maximum release of 5-FU from solution samples of ONB-FU occurred at 80 minutes of UV irradiation and represented 96% of potential release based on the initial concentration of ONB-FU in solution.

The photocleavage of the chemotherapeutic, 5-FU, was achieved from the surface of UCNPs. The water soluble core-shell UCNPs were coupled to the ONB-FU ligand. The UV PL of the UCNP was in resonance with the absorption band of the ONB-FU derivative and energy transfer resulted in photocleavage and subsequent release of 5-FU from the surfaces of UCNPs for these *in vitro* studies. Release of 5-FU was complete within minutes using a NIR laser source centered at 980 nm that operated below 100 mW power. The efficiency of triggered release was as high as 80% of the total conjugated ONB-FU. This work provides an important first step toward the development of a UCNP platform capable of targeted chemotherapy.

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