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Effect of surface coating of $\text{KYb}_2\text{F}_7:\text{Tm}^{3+}$ on optical properties and biomedical applications

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Abstract

This project aims to provide an insight on the effects of biocompatible polymers on the optical properties and the nanoparticle-cell interaction of $\text{KYb}_2\text{F}_7:\text{Tm}^{3+}$ nanocrystals that exhibit strong near infrared (NIR) fluorescence. $\text{KYb}_2\text{F}_7:\text{Tm}^{3+}$ nanocrystals were synthesized with a diameter of 20–30 nm and surface modified with poly(ethylene glycol), Pluronic[®] F-127, and poly(N-vinylpyrrolidone), due to the associated advantages. Some of these include biocompatibility and biodistribution in the instance of agglomeration and hydrophobicity as well as the addition of a targeting agent and drug loading by further functionalization. Despite the decrease in fluorescence intensity induced by the surface modification, thulium's emission fingerprint was easily detected. Moreover, surface modified $\text{KYb}_2\text{F}_7:\text{Tm}^{3+}$ nanocrystals failed to induce a toxic response on endothelial cells following a 24 h uptake period up to concentrations of $100 \mu\text{g ml}^{-1}$. *In vitro* toxicity and confocal imaging have demonstrated the versatility of these NIR fluorescence nanocrystals in biomedical imaging, drug delivery, and photodynamic therapy.

Keywords

lanthanide ions; rare Earth ions; near infrared imaging; contrast agents; surface modification

1. Introduction

In recent years, materials have been scaled down to nanometer sizes, possessing novel properties and multifunctional capabilities. As a result, biomedical research has been focused in nanomedicine, bringing many advantages including personalized medicine, minimally invasive and quick procedures, resulting in a more efficient diagnosis and treatments for various diseases. Therefore, many materials have been used as imaging contrast agents including fluorophores [1] and quantum dots [2, 3], but possess adverse qualities. Fluorophores are commonly used due to their extensive range in excitation and emission, as well as their biocompatibility. Their drawbacks include a small stokes shift, low emission, and efficiency. Although the emission of quantum dots is intense and tunable, their absorption is located within the UV region, which causes irreversible photodamage, and

susceptible to the blinking effect [4], hindering their use in deep tissue imaging. Furthermore, the elemental composition of quantum dots is the pairing of groups II–VI or III–V, resulting in high cytotoxicity [5]. Alternatively, lanthanide-based nanoparticles (LBNP) could circumvent the drawbacks associated with current contrast agent.

The lanthanide elements played a critical role in the development of lighting and light conversion technologies, more importantly as laser materials. Hence, lanthanide ions (e.g. neodymium, erbium, thulium, etc) are famous for their extraordinary optical properties, with well-defined energy levels arising from the shielding of the 4f orbitals by the $5s^25p^6$ subshells. Currently, LBNP are being employed in a plethora of applications including security [6], electrical displays [7, 8], nanothermometry [9, 10], and most widely, the biomedical field, including biosensing [11–13], therapy [14–16], and imaging [11, 17–19]. This is due to the many advantages they possess: (1) emission of LBNP are color tunable, which can be achieved by simply varying the dopant ion and/or concentration [6, 19, 20], (2) resiliency to photodegradation and stability over prolonged periods of time due to the emission arising from real energy level transitions [21], and (3) large Stokes and anti-Stokes shift. Lanthanide ions are capable of producing anti-stokes emission, commonly referred to as upconversion, a multi-photon process in which lower energy photons are absorbed and higher energy photons are emitted, usually being a near-infrared (NIR) to visible transition, figure 1. Upconversion requires a combination of two lanthanide ions, a synthesizer (Yb^{3+}) and an emitter (Ho^{3+} , Er^{3+} , Tm^{3+}), absorbing at the same wavelength to be produced. Once both ions absorb incoming photons, the synthesizer ion transfers its energy to the emitter further exciting it to higher energy levels. Thulium (Tm^{3+}) is one of the lanthanide ions that has been used as laser material due to its intense blue emission [22, 23]. Furthermore, Tm^{3+} is able to produce blue and UV upconversion, which is useful in photodynamic therapy due to the overlap with most photosensitizers. Most importantly, Tm^{3+} is able to produce NIR to NIR upconversion emission, allowing the use of the biological window. In addition, the minimum excitation power threshold for upconverting nanoparticles is lower than that of fluorophores, and on par with quantum dots, permitting the use of lower power densities [24].

A nanoparticle's behavior within a biological system is of utmost importance when designing a nanoparticle for any biomedical application. In order to prevent undesirable effects of certain nanoparticles, including aggregation and hydrophobicity, the use of biocompatible coatings has been implemented. Nanoparticle coatings have been shown to provide many advantages such as drug encapsulation, functionalization, but most vitally, biocompatibility. Moreover, surface modification opens new capabilities, including highly targeted therapeutics [25, 26]. The main problem of systemic drug administration is achieving even biodistribution of pharmaceuticals. Poor biodistribution results in the increase of the administered drug load; however a high local concentration increases the probability of toxicity and adverse side effects. Targeted delivery aims to provide a solution to the problem and improve systemic and local administration of pharmaceuticals. Poly(ethylene glycol) (PEG) is used as a linker between the targeting agent, folic acid, and the nanoparticle. Certain cancerous cells, including breast cancer, overexpress folate receptors increasing cellular uptake of folic acid functionalized nanoparticles [27–29]. The LBNP is then loaded with doxorubicin, a common anti-cancer drug, which is released

through a pH trigger. Due to the simple adsorption mechanism, the dissolution of doxorubicin is increased in an acidic environment, typical of cancerous cells. Surface modification has been shown to provide many advantages but most significantly, improved biocompatibility and biodistribution. Increasing nanoparticle circulation increases the efficiency of an encapsulated by lowering the possibility of drug metabolism and increasing its half-life.

Coatings used were carefully selected due to the advantages they grant, such as PEG. It is the most widely used and successful coating due to the many advantages it offers. PEG, aside from being able to dissolve in polar and nonpolar media, increases blood circulation of nanoparticles, which is attributed to the elimination of protein absorption on the nanoparticles [30]. It has also been demonstrated that PEG modified surfaces are able to penetrate the cell membrane through non-specific cellular uptake [31]. Another often overlooked polymer coating is poly(N-vinylpyrrolidone) (PVP). PVP is a biocompatible, non-toxic, water-soluble, neutral charged, polymer block. It is a commonly used nucleating agent during nanoparticle synthesis, regulating nanoparticle growth and preventing agglomeration [32–34]. Although PVP is generally removed immediately following the synthesis process, it is found in many drug delivery mechanisms [35–37]. PVP has been shown to outperform PEG in antitumor drug delivery [38]. Furthermore, liposomes-coated with PVP have displayed a prolonged blood circulation after IV administration [36]. Pluronic® F-127 (PF127) is another polymer consisting of three different polymer blocks, poly(ethylene oxide) (PEO)–poly(propylene oxide) (PPO)–poly(ethylene oxide) (PEO). The outer polymer block, PEO, prevents nanoparticle aggregation, biofouling, and increases cellular uptake [39], whereas PPO, a hydrophobic polymer, can be adapted for drug delivery due to the ease of drug conjugation, commonly with hydrophobic anticancer agents [40–43]. In addition, PF127 has self-assembly properties, allowing the option of drug loading through conjugation or encapsulation. Recently, a delivery system comprised of PF127 coated iron oxide nanoparticles have been developed for the delivery of doxorubicin [44].

2. Experimental methods

2.1. Synthesis of lanthanide-doped nanocrystals

The nanocrystals were synthesized through a one-pot solvothermal route. The precursors (KF, Yb(NO₃)₃, and Tm(NO₃)₃) were stoichiometrically added to a 60% ethanol and 40% deionized water solution and thoroughly stirred until dissolution was achieved. The solution was then placed in an autoclave and heated for 24 h at 200 °C. The nanocrystals were then washed thrice with ethanol and twice with deionized water in order to remove any excess precursors. The nanoparticles were centrifuged at 12 000 rpm for 10 min, followed resuspension and thorough sonication to prevent any nanoparticle agglomeration. Finally, the solution was then freeze-dried using a Virtis Freezemobile 12XL to obtain a nanocrystal powder.

The polymer coatings were placed by adding 4 ml of hexane to uncoated nanocrystals and sonicated for 10 min. 0.706 mg of the polymer coatings (PEG, PF127, PVP) was dissolved in 20 ml of 10x phosphate buffer saline. Equal volumes of the two solutions (4 ml) were stirred

together and covered lightly allowing the hexane to evaporate. After 24 h, the solution was washed and centrifuged collecting coated nanocrystals.

2.2. X-ray diffraction

X-ray powder diffraction (XRD) was performed using a Rigaku Ultima IV x-ray diffractometer with Cu K α ($\lambda = 1.5 \text{ \AA}$) to determine the nanocrystal structure and phase. The samples were uniformly deposited on a substrate, and a beam opening of 10 mm was used. The experimental results were then compared to a reference database to accurately determine the nanocrystal structure and composition of the nanoparticle powder.

2.3. Electron microscopy

The nanocrystals were suspended in ethanol and thoroughly sonicated until uniform dispersion was achieved. They were then deposited on a mesh copper grid with a carbon film. The samples were then taken to a JEOL 2010F high resolution transmission electron microscope with a high-resolution pole piece $1.9 \text{ \AA}$, operated at 200 kV to determine their size and morphology. Electron dispersive spectroscopy was done using a Hitachi STEM-5500 has a field-emission gun with a 0.4 nm of spatial resolution operated at 30 kV.

2.4. Optical characterization

The upconversion emission data of the synthesized nanocrystals was collected at room temperature using a custom QuantaMaster 51 spectrofluorometer from Photon Technology International Inc. with a wavelength corrected 920C cooled PMT (Horiba) visible detector. The sample was excited at the wavelength of 980 nm using a laser diode (Axcel Photonics) fiber coupled to a power tunable controller (Thorlabs).

To investigate the surface modification effects on the emission intensity, a barium sulfate coated (203 mm in diameter) integrating sphere from Oriel (Model 70451) was used with a mounted spectrofluorometer sample chamber on the side, opposite to the excitation source. The powder sample was held in a specially designed sample holder with a quartz window to hold the powder in place and mounted at the sample port of the integrating sphere. The diffuse fluorescence spectrum from the nanocrystals was recorded with the spectrofluorometer by exciting the sample at 980 nm excitation. The fluorescence output from the sphere was collected using a liquid light guide (LLG) with a specially designed baffle that prevents the direct entry of the exciting laser beam into the detector. The LLG collects the light from the sphere and feeds to the cooled PMT through an emission monochromator. The data was collected using FelixGX with identical scan settings, increments of 0.5 nm and a 1 s integration time. OriginPro 8 was used to create a baseline to remove noise and detector inconsistencies.

2.5. Dynamic light scattering (DLS) characterization

DLS measurements were done using a Zetasizer Nano ZS (Malvern Instruments) with a 4 mW He-Ne laser (632 nm) at $25 \text{ }^{\circ}\text{C}$. The sample was diluted in deionized water at a very low concentration and then sonicated for 1 h to ensure even nanoparticle distribution. 1 ml of each solution was deposited on a clear plastic cuvette. Data was obtained at 12.8° and 175° measurement angles and averaged over 12 scans.

2.6. Nanoparticle cytotoxicity studies

In the interest of investigating the nanocrystal-induced cytotoxicity, rhesus monkey retinal endothelial cells (RhRECs) were cultivated under standard conditions (5% CO₂ flow at 37 °C), and using minimum essential media α (MEM α) containing 10% fetal bovine serum as growth media. After reaching confluency, cells were plated on to a 24-well plate at a concentration of 20 000 cells per well with 1 ml of MEM α . They were then incubated for 24 h and then treated with nanoparticles at different concentrations (10, 50, 100 $\mu\text{g ml}^{-1}$) in triplicate for a more precise statistical analysis. Following a 24 h uptake period, the cells were washed with Hank's balanced salt solution (HBSS) removing any excess nanocrystals and non-viable cells. Afterward, the cells were detached from the plate using trypsin (0.25% Trypsin-EDTA) obtaining a media and cell solution. 100 μl of the solution was taken out and mixed with Trypan Blue Stain (4%) in a 1:1 ratio staining any residual dead cells. Viable cells from each well were counted twice under an inverted microscope using a hemocytometer.

2.7. Cell imaging

In order to further study the internalization of the nanoparticles into cells, RhRECs were cultivated as described above and plated onto cover slips ($d = 10$ mm) located in the bottom of wells. Following a 24 h incubation period, the cells were treated with nanoparticles suspended in media, and given a 24 h uptake period. The media was then removed and the cells fixed using 4% formaldehyde and incubated for 15 min. The cells were then washed three times with HBSS. The labeling solution was made using 9.947 ml of MEM α with the addition of 33 μl (3.3 μM) of Hoechst (nuclear stain), and 20 μl (10 μM) of BODIPY (cytoplasm stain). The cells were then incubated for 10 min at room temperature then washed twice with HBSS. The coverslip was removed and 10 μl of vectashield mounting media was added to prevent photobleaching, and the coverslip was then placed upside down onto a microscope slide. The samples were then taken to a Zeiss 710 Live 2P System, which was chosen due to the tunable Chameleon Ultra II Ti:Sa for nanoparticle excitation. The images were processed using Imaris 3D Interactive Analysis Software.

3. Results and discussion

The KYb₂F₇:Tm³⁺ nanoparticles were synthesized through a solvothermal procedure as opposed to the most common method, thermal decomposition. Although, both synthesis methods are able to control the size of the nanoparticles [45], thermal decomposition yields nanoparticles with a narrower size distribution. Unlike thermal decomposition, which requires organic solvents, a solvothermal synthesis utilizes biocompatible nucleating agents to control nanoparticle growth, producing nanoparticles more suitable for biomedical applications. Additionally, the low cost and high yield of solvothermal is preferable over the high cost and low yield of thermal decomposition. Lanthanide ions are subject to emission quenching due to cross-relaxation between dopant ions resulting in energy loss through non-radiative processes [46]. Therefore, to prevent any emission quenching between Tm³⁺ ions, thulium was judiciously doped into the KYb₂F₇ host crystal at a concentration of ~1 mol%. Precursors were thoroughly mixed in a 60:40 ethanol and water solution in a Teflon lined container. The solution was then placed in an autoclave, heated to 200 °C and held for 24 h.

Immediately following the synthesis process the nanoparticles were washed twice with ethanol and thrice with water. The nanoparticles were centrifuged at 12 000 rpm for 10 min and resuspended. Finally, the solution was freeze dried removing any adsorbed water and obtaining a nanoparticle powder.

XRD data was collected to accurately determine the structure and composition of the nanoparticles. The experimental diffraction pattern obtained was compared to a reference database confirming the nanocrystals to be orthorhombic α -phase KYb_2F_7 which is part of the Pna21 space group (figure 2(a)). The size and morphology of the nanoparticles was determined using electron microscopy, where the nanoparticle powder was placed in ethanol and thoroughly sonicated to ensure uniform dispersion. A portion of the solution was deposited on a lacey carbon film-coated copper grid and taken to a JEOL 1230 transmission electron microscope, revealing the nanocrystals to be spherical in shape and to having a diameter of 20–30 nm (figures 2(b) and (c)). The size-dependent nanoparticle distribution in biological environments and cellular interaction have been thoroughly studied; where a size of approximately 25 nm has been reported to be most efficient due to having the fastest cellular internalization time [47]. DLS was used to verify the presence of the various coatings (figure 3). Coated and uncoated samples were diluted in deionized water at a very low concentration required by the equipment. The solutions were then sonicated in a water bath for a 1 h in order to ensure even nanoparticle distribution. DLS measurements reveal successful PEG, PF127, and PVP coatings on the nanocrystals due to the size distribution peaks shifting to a bigger size. Uncoated samples had an average size distribution centered at 132 nm, whereas PEG, PF127, and PVP peaks were at 147.6, 175, and 186.1 nm respectively. The increase in size compared to the TEM images corresponds to agglomeration when suspended in solution as well as the hydrodynamic shell around the nanocrystals [48, 49]. The second peak appearing at larger sizes corresponds to big nanoparticle aggregates that failed to dissociate. Despite the fact that some modified particles appear to be uncoated, the overall increase in particle size is indicative of the presence of the biocompatible coatings. Studies regarding the refinement of the modification procedure and coating size will be performed in the future.

To investigate the effects of the biocompatible coatings on the optical properties, the emission spectra of coated (figure 4(a)) and uncoated (figure 4(b)) samples were collected. The data shows the characteristic Tm^{3+} peaks, including the NIR 800 nm peak ($^1\text{G}_4 \rightarrow ^3\text{F}_4$), 478 nm ($^1\text{G}_4 \rightarrow ^3\text{H}_6$), 451 nm ($^1\text{D}_2 \rightarrow ^3\text{F}_4$), and the two UV emission peaks at 375 nm ($^1\text{D}_2 \rightarrow ^3\text{H}_6$), and 350 nm ($^3\text{P}_0 \rightarrow ^3\text{F}_4$). The excitation wavelength at 980 nm and the emission at 800 nm, which has the highest emission cross section, are situated within the first optical window. This region of the spectrum spans from 620 to 980 nm and penetrates deeper into soft tissue due to the lower absorption and scattering by tissue as compared to visible light. This phenomenon allows the use of lower power densities which result in a lower probability of photodamage to the material and tissue. The different coatings did induce a change in the nanoparticle upconversion emission (figure 4(b)). Following the coating procedures, emission spectra was collected showing a decrease in emission from the coated samples. The expected emission decrease agrees with the inevitable absorption and emission radiation interaction with the various coatings. Emission quenching was calculated to be 31.1% for PEG coated samples, 58.6% for PF127, and 65.4% for PVP with respect to

uncoated samples. Similar emission quenching for PVP coating has been reported [34] as well as PEG coating [50]. Although PF127 and PVP coated samples had a considerable impact on the emission intensity, the Tm^{3+} emission fingerprint was easily detected.

Biocompatibility is the most crucial feature when considering a material for any biomedical application. Although nanoparticles are known to induce a cytotoxic response, it can be silenced by coating the nanoparticle with biocompatible polymers or biomolecules. For the purpose of exploring the effects of the various coatings on the nanoparticle cytotoxicity, RhRECs were acquired and cultivated under standard conditions (37 °C with 5% CO_2). The cells were then plated, incubated for 24 h, then treated with nanoparticles at different concentrations (10, 50, 100 $\mu\text{g ml}^{-1}$) in triplicates, for a higher statistical precision. 24 h were given as an uptake period, for a thorough nanoparticle-cell interaction. Following the 24 h uptake period, the cells were detached from the wells and the number of viable cells counted using a hemocytometer under an inverted microscope. Cytotoxicity appeared to be dependent on the coating or lack thereof. Uncoated samples showed the highest cytotoxicity with an average drop of ~15%, whereas PEG completely suppressed any cytotoxicity with a viability of 100%. PF127 coated samples did not exhibit a cytotoxic response at 10 $\mu\text{g ml}^{-1}$, contrary to 50 and 100 $\mu\text{g ml}^{-1}$, with a 5% viability reduction. PVP coated samples showed a drop in viability of 3% at 10 $\mu\text{g ml}^{-1}$, a 4% increase at 50 $\mu\text{g ml}^{-1}$, and a decrease in viability to 90% at the highest concentration. Overall, surface modification had a positive effect on the cytotoxicity minimizing any cytotoxicity exhibited by uncoated $\text{KYb}_2\text{F}_7:\text{Tm}^{3+}$ (figure 5(a)).

Cell growth rate was determined by the number of viable RhREC after treatment with nanoparticles at a concentration of 100 $\mu\text{g ml}^{-1}$ for periods of 24 and 48 h. Following the 24 h mark, growth rate was consistent through the samples, showing a slight increase in cellular population. After 48 h, the cellular populations had increased by factors of 2.3, 2.3, 2.6, and 2.8 respectively for PEG, PF127, PVP, and uncoated samples, whereas the control population increased by a factor of 3.1 (figure 5(b)). The reduced cell growth rate is attributed to the nanoparticles restricting cell division and motility. This is congruent with the results, as coated samples further reduced cell grow compared to uncoated samples.

In vitro imaging was conducted by fixing the PF127 coated nanoparticle-treated cells following a 24 h uptake period onto circular glass cover slips using 4% formaldehyde. The cells were then washed thoroughly with HBSS and stained using Hoechst for the nucleus and BODIPY for the cytoplasm. The nuclear stain was excited at 405 nm using a diode laser, the cytoplasm at 561 nm using a He–Ne laser, and the nanoparticles at 980 nm using a Chameleon Ultra II Ti:Sa. A z-stack scan was performed with the middle slice indicating that the nanoparticles are located inside and around the cells, with a small percentage not neighboring a cell (figure 5(c)). Based on the localization of signal associated with coated nanoparticle, it can be conclude that surface modified $\text{KYb}_2\text{F}_7:\text{Tm}^{3+}$ nanoparticles with PF127 effectively was internalized onto cells over the treatment period of 24 h.

4. Conclusions

In conclusion, we have synthesized highly fluorescent $\text{KYb}_2\text{F}_7:\text{Tm}^{3+}$ nanocrystals with potential applications in biomedical imaging. We have demonstrated that surface modification of $\text{KYb}_2\text{F}_7:\text{Tm}^{3+}$ nanoparticles diminished the fluorescence, yet, the emission fingerprint was easily detected. Furthermore, biocompatible coatings have positive effects on the biocompatibility, diminishing any toxicity and enhancing the possibilities of the nanoparticles to be used in a biological

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Figure 1.

Energy level diagram of ytterbium–thulium co-doped system. Upconversion emission under 980 nm excitation. In this system, multiple ytterbium ions excite an already excited thulium ion to higher energy levels. The process can be repeated, resulting in the generation of highly energetic visible and UV photons.

Figure 2.

(a) X-ray powder diffraction pattern of $\text{KYb}_2\text{F}_7:\text{Tm}^{3+}$ nanoparticles (top) plot comparing with KYb_2F_7 reference (bottom). TEM images of (b) uncoated and (c) PVP coated $\text{KYb}_2\text{F}_7:\text{Tm}^{3+}$ nanocrystals.

Figure 3. DLS size measurements of coated and uncoated KYb₂F₇: Tm³⁺ nanoparticles suspended in deionized water.

Figure 4.

(a) Detailed emission of uncoated KYb₂F₇:Tm³⁺. (b) Emission comparison of uncoated and coated KYb₂F₇:Tm³⁺ nanoparticles under constant excitation power. As expected, emission quenching is observed after the addition of a biocompatible coating. application due to the low to null toxicity. It is noteworthy to mention future work includes surface modification refinement, coating thickness effects, and *in vivo* imaging. In summary, surface modified KYb₂F₇:Tm³⁺ nanocrystals are strong candidates in several biomedical applications including NIR fluorescence imaging, photodynamic therapy, and targeted drug delivery.

Figure 5.

(a) Cell viability of RhRECs following uncoated, PEG coated, PVP coated, FA coated, and uncoated $\text{KYb}_2\text{F}_7:\text{Tm}^{3+}$ nanoparticle treatment at different concentrations (10, 50, 100 $\mu\text{g ml}^{-1}$). (b) Growth rate of treated at 100 $\mu\text{g ml}^{-1}$ and untreated RhRECs at 24 and 48 h. (c) Confocal image form Zeiss 710 of RhREC incubated with surface coated nanoparticles.