Biofunctionalization, cytotoxicity, and cell uptake of lanthanide doped hydrophobically ligated NaYF₄ upconversion nanophosphors

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Surface biofunctionalization of the hydrophobic lanthanide ion doped hexagonal phase NaYF₄:Yb,Er upconversion nanophosphors (UCNPs) was achieved by introducing amino and carboxyl groups, respectively. Amino groups were added by using the 3-aminopropyltrimethoxysilane reaction after a thin layer of SiO₂ coating. The carboxyl groups on surface were added directly by coating modified amphiphilic polyacrylic acid polymer. Experimental studies of cytotoxicity and cell uptake of UCNPs were conducted. The cytotoxicity analysis of the functionalized UCNPs was conducted by methylthiazol tetrazolium assays. Cell uptake was accomplished by incubating the UCNPs with human osteosarcoma cells and proved by transmission electron microscopy. The results showed that the functionalized UCNPs had very low toxicity compared with the control group, while UCNPs taken into the cells indicated that they had very high biocompatibility. The imaging of UCNPs, which were incubated with AB12 mouse mesothelioma cells and excited by 1 W 980 nm light, showed individual particles with visible light emission. These results exhibited promising applications of functionalized UCNPs in cell imaging and photodynamic therapy. © 2008 American Institute of Physics. [DOI: 10.1063/1.3008028]

I. INTRODUCTION

Lanthanide ion doped upconversion nanophosphors (UCNPs) upconvert two or more near infrared (NIR) photons (e.g., 980 nm) to one visible photon via sequential electron excitation and energy transfer processes at 4f orbitals, which bring UCNPs many advantages over the conventional organic dye markers and quantum dots for biological applications. For example, the NIR excitation allows deeper light penetration in tissue and has much higher signal-to-noise ratio due to the absence of autofluorescence and the reduction in light scattering. In addition, UCNPs do not have photobleaching due to their atomiclike emissions and have sharp luminescence bands (~10 nm). Moreover, high power NIR sources are inexpensive and easy to use. Thus, the biological applications of using UCNPs have attracted much attention in recent years.²,³

The current UCNPs employ Yb³⁺ ions as sensitizers, which collect and transfer energy to codoped Er³⁺, Ho³⁺, or Tm³⁺ ions, and emit green, red, and blue light. These ions need to be doped into hosts such as oxides, oxy sulfides, and fluorides to enhance the upconversion efficiency, and among them, hexagonal phase (β) NaYF₄ crystal in bulky state has been reported to be the most efficient matrix for NIR-to-green or blue upconversion.¹-⁶ Recently, significant progresses have been made in the synthesis and physical studies of nanoscale NaYF₄ based UCNPs via either hydrothermal or cothermolysis methods.⁴-¹⁴ The cothermolysis method of using trifluoroacetate precursors has achieved 10-200 nm hydrophobically ligated lanthanide ion doped NaYF₄ based UCNPs by using high boiling point organic solvents such as oleic acid (OA),⁸,¹⁴,¹⁵ oleylamine,⁹ oleylamine/trietylphosphine (OA-TOP),¹⁶ and trioctylphosphine oxide (TOPO).¹⁷ Similar to the counterparts of the quantum dots, the as-synthesized UCNPs are capped by organic ligands corresponding to the coordination solvents being used in the synthesis. In particular, the small, hexagonal phase, hydrophobic UCNPs have made it possible to use similar methods that have been developed for quantum dots for biofunctionalization.¹⁸,¹⁹ However, due to the strong negative ion property of fluoride host, the conversion of hydrophobic UCNPs to hydrophilic ones without particle agglomeration remains challenging.¹⁰,¹⁶ Moreover, very few reports are available for toxicity and cell uptake although these data are essential for any potential biomedical applications of UCNPs.

Since amino and carboxyl are two basic functional groups for further bioconjugations and bioapplications, in this work, we selected to functionalize OA-TOP capped hexagonal phase NaYF₄:YbEr UCNPs with amino and carboxyl groups for preliminary biological studies. We explored the conditions and mechanisms to control the SiO₂ growth without causing particle aggregation. After the SiO₂ coating, surface addition and quantification of biofunctional amine group was achieved by introducing amino and carboxyl onto UCNPs. The introduction of the amino and carboxyl groups to UCNPs provided them biocompatibility properties for future bioconjugation. During the preparation of this paper, we noticed one latest

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paper using polyethyleneimine (PEI) coated NaYF4 UCNPs for cell imaging, viability, and biodistribution studies.\textsuperscript{20} As far as we know, there are still no work reporting the systematic cytotoxicity studies and imaging work originated from hydrophobically ligated UCNPs, and there are no reports that these UCNPs having ever been taken into the cells and no individual particles ever been imaged. In this work, the cytotoxicity properties of the amino and carboxyl group functionalized UCNPs were characterized by using the methylthiазol tetrazolium (MTT) assay. The biocompatibility and cell uptake were studied by incubating the above functionalized UCNPs with human osteosarcoma (HOS) cells. Finally, NIR cell imaging on individual particle was also demonstrated.

II. EXPERIMENTAL DETAILS

A. Preparation of upconversion nanophosphors and surface biofunctionalization

Trioctyolphosphine (TOP) (90%), octadecene (90%), sodium trifluoroacetate (98%), trifluoroacetic acid (CF\textsubscript{3}COOH), reagent grade), APS (98%), tetraethyl orthosilicate (TEOS), and dimethylamine (DMA) were purchased from Sigma-Aldrich. Polyoxymethylene nonylphenol (NP-9) was purchased from Chemistry Store. OA was purchased from Fisher Scientific. 99.99\% Ln\textsubscript{2}O\textsubscript{3} (Ln=Y, Yb, Er, and Tm) were provided by Sunstones Bioscience Inc. CF\textsubscript{3}COOLn precursors were prepared by dissolving corresponding lanthanide oxides in trifluoroacetic acid and heated at reflux temperature. After clear solutions were obtained, the solvent was removed under vacuum. The resulting solids were dried under vacuum at room temperature overnight and used without further purification.

Synthesis of OA-TOP capped UCNPs and silica coating were reported in our work.\textsuperscript{18} The silica coated UCNPs were prepared based on the method developed by Darbandi et al.\textsuperscript{18} with two modifications. First, NP-9 was used instead of NP-5. Second, due to the limited solubility of UCNPs in cyclohexane, there was no stock solution prepared. OA-TOP capped UCNPs dissolved in cyclohexane need 20–30 min sonication before the silica coating. Typically, 40–50 mg OA-TOP capped UCNPs were dissolved in 25–100 mL cyclohexane by sonicating for 30 min, then 2 mL NP-9 and 0.1 mL TEOS were added, which were followed by vigorous stirring for 30 min to form water-in-oil (W/O) microemulsion system. 50–100 \textmu L DMA was then added to initiate hydrodrolis of TEOS to form SiO\textsubscript{2} layer onto UCNPs. The SiO\textsubscript{2} growth was stopped after 12–24 h reaction. The nanoparticles were destabilized from the microemulsion using ethanol and precipitated by centrifugation. The resulting UCNP/SiO\textsubscript{2} composite particles were washed with absolute ethanol three times. For each washing step, followed by centrifugation, a sonicator bath was used to completely disperse the precipitate in the ethanol and remove any physically adsorbed molecules from the particle surface. Finally, UCNP/SiO\textsubscript{2} nanoparticles, which were dispersible in ethanol and water, were obtained.

To add amino functional groups, 10 mg UCNP/SiO\textsubscript{2} were resuspended in 50 mL isopropanol by sonicaton. 0.1–0.4 mL APS was then added dropwise under vigorous stirring, which was allowed to react at room temperature for 12 h. Then, the amino coated UCNPs nanoparticles were collected by centrifugation and washed three times in pH=7.4 phosphate buffer. Quantification of amino concentration on UCNPs was made by using ninhydrin test.\textsuperscript{21}

Carboxyl coated UCNPs were obtained by mixing OA-TOP capped UCNPs with octylamine and isopropylamine modified PAA directly. The modified PAA was prepared in a similar way to that in Ref. 22.

B. MTT assay, cell uptake, and cell imaging

To evaluate cytotoxicity of the nanoparticles, HOS cells (ATCC, Manassas, VA) were incubated with the nanoparticles for 2, 5, and 9 days. The HOS cells were cultured in 25 \textsuperscript{cm\textsuperscript{2}} flasks (Becton-Dickinson, Franklin Lakes, NJ) and maintained in an incubator at an incubation temperature of 37 °C regulated with 5% CO\textsubscript{2}, 95% air, and saturated humidity. A Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin/amphotericin B was used as the cell culture medium (Quality Biological, Gaithersburg, MD). At confluence, the cells were subcultured by splitting. A cell suspension at a concentration of approximately 5 \times 10\textsuperscript{4} cells/ml was then prepared, as determined by a hemocytometer count. The cells were seeded into the 24-well culture plate, 10\textsuperscript{4} cells (200 \textmu L \times 5 \times 10\textsuperscript{3} cells/ml in each well. Then 50 \textmu L of each kind of nanoparticle solution was added to each of the three wells and kept at 37 °C in a fully humidified atmosphere at 5% CO\textsubscript{2} in air. Three wells without nanoparticles were used as a control group. After inoculation for 2, 5, and 9 days, MTT assay was performed to evaluate cell viability in each well. Briefly, the culture medium was removed, and the cultures were washed with phosphate buffered saline twice. 400 \textmu L of dimethylsulfoxide (MTT detergent reagent; ATCC, Manassas, VA) were added to each well including control group, followed by incubation at 37 °C for 4 h to allow MTT formazan formation. When the purple precipitate was clearly visible under the microscope, 400 \textmu L of dimethylsulfoxide (MTT detergent reagent; ATCC, Manassas, VA) were added to each well. After leaving the plate with cover in the dark for 4 h at room temperature, the dye solution with the cells in each well was transferred into a 1.5 mL Eppendorf tube and centrifuged at 13 000 rpm for 5 min. The supernatant was transferred into a new Eppendorf tube. Absorbance of the converted dye was measured at a wavelength of 570 nm with background subtraction at 670 nm using a UNICO 1200 series spectrophotometer (United Products & Instruments, Inc., NJ). The ratios of absorbance from each parallel experimental group (n=3) to the absorbance of control group after 2 day cell culture were used to assess the cell viabilities with each kind of nanoparticle.

Cell uptake experiments were performed by incubating amine and carboxyl functionalized UCNPs with HOS cells (ATCC, Manassas, VA). The HOS cells were cultured in 25 \textsuperscript{cm\textsuperscript{2}} flasks (Becton-Dickinson, Franklin Lakes, NJ) and maintained in an incubator at an incubation temperature of 37 °C regulated with 5% CO\textsubscript{2}, 95% air, and saturated hu-
midity. A DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin/amphotericin B was used as the cell culture medium (Quality Biological, Gaithersburg, MD). At the confluence, the cells were subculturated by splitting. A cell suspension at a concentration of approximately 5 × 10^4 cells/ml was then prepared, as determined by a hemocytometer count. The cells were seeded into the 24-well culture plate, 10^4 cells (200 μL × 5 × 10^4 cells/ml) in each well. Then, 50 μL of UCNP solution was added to each of three wells and kept at 37 °C in a fully humidified atmosphere at 5% CO_2 in air. After incubation, the cells were grown as a monolayer and fixed for 2.5 h with 2% glutaraldehyde in 0.2M sodium cacodylate buffer, pH 7.2, rinsed with 0.2M sodium cacodylate buffer, pH 7.2, postfixed with 1% OsO_4 in sodium veronal buffer, for 1 h at 4 °C, rinsed with sodium veronal buffer. Samples were then incubated with 0.25% toluidine blue for 60 min in 0.2M sodium cacodylate buffer, pH 5.1, incubated overnight with 2% uranyl acetate in 0.05M sodium maleate buffer, and then into a 1:1 dilution of EtOH:resin, 1:2 for 2–3 h each, then into straight resin overnight. Unstained 70 nm sections were obtained using a diamond knife on a Leica UC6 ultramicrotome and observed at 80 kV on a Zeiss912AB transmission electron microscope (TEM).

For cell imaging, 1.5x10^5 AB12 murine malignant mesothelioma cells were incubated with increasing doses of carboxyl functionalized UCNP solution up to 25 μg/ml for 24 h in a 5% CO_2, 32 °C incubator. UCNPs were excited with approximately 1 W of 980 nm light delivered by a 15 W solid-state diode laser with a diffusing fiber.

### C. Instrumentation and characterization

TEM images of silica coated UCNPs were obtained using LEO/Zeiss 910 TEM equipped with a PGT-IMIX Energy Dispersive X-ray (EDX) system (100 keV). With a field-emission-gun, this microscope provides a point-to-point resolution of 0.2 nm and an electron probe of 0.7 nm with an energy of up to 200 KeV, respectively. Cell uptake TEM images were taken on an 80 kV Zeiss912AB TEM equipped with an Omega energy filter. Micrographs were captured using a digital camera from Advanced Microscopy Techniques and saved as Tagged Image File Format files onto a Dell PC computer. The energy dispersive spectrometer (EDS) analysis was performed using FEI XL30 Field Emission Gun Scanning Electron Microscope (FEG-SEM) equipped with a PGT-IMIX Position Tagged Spectrometry (PTS) EDX system. The photoluminescence (PL) measurements are performed at room temperature. A 980 nm laser diode (1 W maximum, Lasermate Group, Inc.) was used as the excitation source and the beam was focused (12 cm focal length) to a spot size of approximately 0.5 mm. The PL signals were focused to the end of a optical fiber and then delivered into the slit of a monochromator (SP-2500i, Princeton Instruments) with a 2400 g/mm grating (holographic, 400–700 nm). The signal was detected by a photomultiplier module (H6780–04, Hamamatsu Corp.) and was amplified by a lock-in amplifier (SR510, Stanford Research Systems) together with an optical chopper (SR540, Stanford Research Systems). The signal was recorded under computer control using the SPECTRASENSE software data acquisition/analyzer system (Princeton Instruments). Cell imaging was conducted under 10× magnification on an inverted microscope connected to a charge coupled device camera for image capture. UCNPs were excited with approximately 1 W of 980 nm light delivered by a 15 W solid-state diode laser with a diffusing fiber.

### III. RESULTS AND DISCUSSION

#### A. Silica coating of hydrophobically ligated UCNPs

The technique of the W/O microemulsion has been applied to coat a layer of silica onto hydrophobically ligated, such as oleate and TOPO capped quantum dots. Recently, we transferred the method to OA-TOP capped UCNPs. In this method, reversed micelles were formed by water nanodroplets in an organic medium and further used for synthesis or surface modification of nanoparticles. The formation of SiO_2 starts from the hydrolysis of TEOS at the oil/water interface catalyzed by bases such as DMA. Comparing with oleate and TOPO coated quantum dots, the hexagonal phase UCNPs usually have larger particle sizes (~100 nm) and less solubility in organic solvent. For example, OA-TOP capped UCNPs can be dissolved in hexane to form a stable solution but the solution is not transparent. In our previous work, we have shown that SiO_2 was coated onto OA-TOP capped NaYF_4:Yb,Er UCNPs. However, we also observed that independent SiO_2 particles were also formed during the SiO_2 coating, which caused particle agglomeration. Note that the clean SiO_2 coated UCNPs are crucial for further biofunctionalization and conjugations. Thus, in this work, we first investigated the conditions of SiO_2 coating onto UCNPs without forming individual SiO_2 particles.

Figure 1 depicts TEM images of the as-synthesized UCNPs and the products of the SiO_2 encapsulation in the pres-
ence of DMA as catalyst for TEOS polymerization. To investigate the conditions of forming SiO$_2$ layer, we started by adjusting UCNPs and DMA concentrations. In the first two comparison experiments, the same amounts of UCNPs, NP-5, TEOS, and DMA were used with 40 mg, 2 ml, 100 µl, and 0.12 ml, respectively, while solvent cyclohexane was 25 and 50 mL. Under the same reaction time of 24 h, reaction of the monodisperse UCNPs [Fig. 1(a)] with TEOS in 25 ml solvent resulted in agglomerated SiO$_2$ particles [Fig. 1(b)] and the average SiO$_2$ layer thickness on the UCNP surface was 12 nm. However, the reaction in 50 mL cyclohexane led to clean SiO$_2$ coated UCNPs in which neither independent SiO$_2$ particles nor particle agglomeration were observed, and the average coating layer thickness is 8 nm. In another experiment, we kept cyclohexane solvent at 25 mL while reducing the DMA to 0.06 mL to perform the same TEOS hydrolysis reaction. Clean UCNP/SiO$_2$ particles with SiO$_2$ layer approximately 5 nm were also obtained [Fig. 1(d)]. The results above indicate that the concentrations of the DMA have played an important role to control the growth speed of SiO$_2$ layer growth. High concentrations of base intend to induce fast SiO$_2$ growth, as a consequence, excess individual SiO$_2$ particles appear. By carefully adjusting DMA concentrations to control hydrolysis speed, the formation of SiO$_2$ particles and particle agglomeration could be avoided in coating UCNPs, and furthermore, the desired SiO$_2$ layer thickness could be obtained too.

It was reported that SiO$_2$ coatingonto oleate capped PbSe quantum dots involved the spontaneous ligand exchange between oleate and TEOS. As a consequence, excess individual SiO$_2$ particles appear. By carefully adjusting DMA concentrations to control hydrolysis speed, the formation of SiO$_2$ particles and particle agglomeration could be avoided in coating UCNPs, and furthermore, the desired SiO$_2$ layer thickness could be obtained too.

Coating onto TOPO capped CdSe quantum dots. For biological applications, the elemental compositions of the particles could affect cytotoxicity behavior, which have been reported on quantum dots. So, exploring the coating mechanism while confirming the particle compositions are important for further biological study. Since OA-TOP ligands contain phosphine, we measured the P/Y atomic ratios of the samples before and after SiO$_2$ coating by EDS. The EDS results are shown in 2.

FIG. 2. (Color online) EDS results of UCNPs (a) before SiO$_2$ coating and (b) after SiO$_2$ coating.

FIG. 3. (Color online) (a) Standard curves of amine concentrations and (b) ninhydrin tests of amino functionalization of SiO$_2$/NCs (left and middle) and control (right).

FIG. 4. (Color online) Comparisons of upconversion emission spectra of the (a) initial UCNPs, (b) after UCNPs with amino group coating, (c) and UCNPs with carboxyl group coating.
Figures 2(a) and 2(b) present the EDS results of the samples in Figs. 1(a) and 1(c) in which both samples show strong phosphine intensity. Figure 2(b) also presented a strong Si peak indicating the successful SiO$_2$ coating. Calculation shows that the P/Y ratios are 0.082 and 0.083 before and after SiO$_2$ coating, respectively. Additional EDS measurements of the sample in Fig. 1(b) showed that the P/Y ratio is 0.080. The above results indicated that there were no losses of phosphine during the SiO$_2$ growth onto the hydrophobically ligated UCNPs, and that there was no ligand exchange with TEOS. Thus, all original OA-TOP capped UCNPs have been encapsulated by the SiO$_2$ layer.

B. Amine and carboxyl functionalization of UCNPs

After introducing the biocompatibility by coating a SiO$_2$ layer onto the hydrophobic UCNPs, we performed amino functionalization by reacting UCNP/SiO$_2$ with APS and quantified the amine concentrations on the particle surface. Qualitative testing amino groups could be observed directly from the appearance of the blue color when ninhydrin reagent reacts with amino group on the UCNPs. Quantitative determination of the amine concentration could be conducted by measuring the light absorption at 565 nm.\(^{21}\) In this work, the color changes in the three tests including control are shown in Fig. 3(b). In this work, the two tests resulted in amine concentrations of 24.8 $\mu$mol [Fig. 3(b), left] and 42.9 $\mu$mol [Fig. 3(b), middle] on 10 mg UCNPs, respectively.

In addition to adding amino groups onto the hydrophobic UCNPs, we developed an alternative method by directly coating carboxyl groups to transfer the hydrophobic UCNPs to hydrophilic. The carboxyl group functionalization was achieved by mixing amphiphilic modified PAA with the UCNPs.\(^{25}\) The successful addition of carboxyl groups could be confirmed by that the hydrophobic UCNPs were dispersible in phosphate buffer.

C. Upconversion emission spectra

After coating amino and carboxyl groups, the impact of surface properties on the UCNP upconversion luminescence intensity was investigated. Figure 4 shows the comparisons of the emission spectra of the UCNPs before and after coating the amino and carboxyl groups with a NIR excitation at 980 nm. Our results showed that both amino/SiO$_2$ coatings and the direct carboxyl coating have very little effect on the emission intensity, which indicating that upconversion luminescence of the UCNPs will not be sacrificed after further bioconjugation such as specific antibodies conjugation for biological applications.

FIG. 5. (Color online) Cytotoxicity assay of UCNPs coated with amine and carboxyl (1.0, 0.25, and 0.0625 mg/ml) groups. The results show that cytotoxicity of the UCNPs is very low compared with control.

![Graph showing the effect of amino and carboxyl functionalization on cell viability over time](image-url)
D. MTT assay, cell uptake, and cell imaging

For biomedical applications, the cytotoxicity and bio-compatibility of the functionalized UCNPs are the most important factors needed to be investigated. In addition, it is also necessary to minimize the particle aggregation since aggregated particles will affect not only the particle circulation inside human body but also the particle delivery to the targeted cancer cells. In this work, the cell toxicity and the cell uptake were studied by incubating the above functionalized UCNPs with human osteosarcoma cells. The results are shown in Figs. 5 and 6, respectively.

Figure 5 shows the MTT toxicity measurements of the amino and carboxyl groups functionalized UCNPs. At the concentration of 1 mg/mL, after nine days incubation, cells incubated with amino functionalized UCNP were 96.2% alive compared with the control group, while 92.8% for cells incubated with carboxyl functionalized UCNPs. The results demonstrate that UCNPs functionalized by both amino and carboxyl groups show almost no toxicity.

It has been reported that the cytotoxicity of quantum dots was influenced by quantum dot concentration. Similar in this work, we also examined concentration effects of UCNPs by using carboxyl functionalized UCNPs. Two additional samples with 0.25 mg/mL and 0.0625 mg/mL UCNPs were incubated with cells at the same conditions. The results showed that 93.0% and 96.3% survived, respectively. Therefore, the slight difference among the three concentrations indicated that the concentration effects of functionalized UCNPs on toxicity were negligible under the above assay conditions.

Recently, Chatterjee et al. tested cell biocompatibility of PEI/UCNPs and proved cell imaging using folic coated PEI/UCNPs. Their cell imaging work only proved that UCNPs were attached onto cell surface, meanwhile there were significant particle agglomeration shown in their imaging pictures. In our work, we performed direct probe of the UCNP biocompatibility by examining whether the functionalized UCNPs can penetrate the cell wall and be fed into the cells. Cell uptake experiments were conducted by incubating 1 mg/mL carboxyl coated UCNPs with human osteosarcoma cells employing the same route as the above MTT assay. To conduct cell TEM image analysis, cell slice analysis was performed. In this method, an incubated cell was washed and cut in half and then the TEM microscopy image inside the cell was directly taken and the results are shown in Fig. 6. In Fig. 6, there were many individual UCNPs observed inside the cells, which supported a high biocompatibility of the carboxyl functionalized UCNPs and indicated that the aggregated particle clusters cannot enter into the cells. To the best of our knowledge, the reported work usually imaged UCNPs in clusters, while the result in this work achieved cell uptake presented the possibility to image a single UCNP. To confirm cell imaging application using our UCNPs, we incubated the carboxyl functionalized UCNPs

FIG. 6. (Color online) Cell uptake of carboxyl group functionalized UCNPs. Left: low magnification TEM; Right: High magnification TEM of the left part. Scale bar=500 nm.
with the AB12 mouse mesothelioma cells and imaged the results using 980 NIR excitation source and the results are presented on Fig. 7.

It was not surprising to observe the dominant green emission from the clusters in Fig. 7. While an enlarged picture also showed emission from individual UCNP, which supported our above conclusion on single particle imaging.

IV. CONCLUSION

In conclusion, biofunctionalization of hydrophobically ligated UCNP with amino and carboxyl groups have been reported in this work. Experimental studies of cytotoxicity, cell uptake, and cell imaging of these UCNP have been investigated. The low cytotoxicity, nonclustered UCNP inside cells and cell imaging under 980 laser excitation indicated that these UCNP have promising biological applications. Besides using for cell imaging applications in our future work, our next step is to encapsulate porphyrins within the bio-functionalized UCNP and further conjugate specific antibodies aimed at specific UCNP delivery for photodynamic therapy applications.27

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