Upconversion Nanoparticles

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Versatile Synthetic Strategy for Coating Upconverting Nanoparticles with Polymer Shells through Localized Photopolymerization by Using the Particles as Internal Light Sources**

Selim Beyazit, Serena Ambrosini, Nataliya Marchyk, Emilia Palo, Vishal Kale, Tero Soukka, Bernadette Tse Sum Bui, and Karsten Haupt**

In memory of Daniel Thomas

Abstract: *We present a straightforward and generic strategy for coating upconverting nanoparticles (UCPs) with polymer shells for their protection, functionalization, conjugation, and for biocompatibility. UCPs are attracting much attention for their potential use as fluorescent labels in biological applications. However, they are hydrophobic and non-compatible with aqueous media; thus prior surface modification is essential. Our method uses the internal UV or visible light emitted from UCPs upon photoexcitation with near-infrared radiation, to locally photopolymerize a thin polymer shell around the UCPs. In this way, a large variety of monomers with different chemical functionalities can be incorporated. If required, a second layer can be added on top of the first. Our method can provide a large spectrum of surface functional groups rapidly and in one pot, hence offering a platform for the preparation of libraries of functional polymer-encapsulated UCPs for applications in bioassays, biosensing, optical imaging, and theranostics.*

Lanthanide-doped upconverting nanoparticles (UCPs) have attracted much attention because of their potential use as fluorescent labels in biological applications, such as bioimaging and bioassays.[1–3] UCPs are excited by low-energy near-infrared (NIR) or infrared light and emit UV or visible light in a process based on the sequential absorption of two or more photons, termed upconversion.[4,5] NIR excitation has several advantages, as it has a high penetration depth in tissues and causes no photodamage to living cells. In contrast, with traditional fluorophores, such as organic dyes and semiconductor quantum dots, which show downconversion fluorescence, the lower wavelengths of excitation induce high autofluorescence background in most biological materials and cause other drawbacks, such as photobleaching and poor chemical stability.[1,2,6] Moreover, UCPs possess low cytotoxicity, a narrow emission bandwidth, a large anti-Stokes shift, and high photostability, which make them ideal as luminescent labels.[7] Despite all these advantages, they have been limited in their bioapplication because of their hydrophobic nature and their nondispersity in aqueous media. Indeed, UCPs are preferentially synthesized in highly hydrophobic solvents (octadecene) with oleates as stabilizing ligands.[6,8–10] Thus, in order to be applied in biological media, they have to be surface-modified, and additional functionalization is required to enable the covalent immobilization of biomolecules, such as proteins, nucleotides, and antibodies.[1–4,11] Currently, one of the most efficient and widely used strategies is to encapsulate the UCPs in a cross-linked silica shell by the Stöber or reverse-microemulsion method.[1,4,10–14] Although this strategy results in quite stable nanoparticles, post-treatment is not straightforward, and certain factors, such as the inability to remove the surfactants completely, might be a problem for in vivo applications.[9] Encapsulation with amphiphilic polymers, whereby the hydrophobic moiety of the polymer intercalates with the oleate ligands on the surface, and the hydrophilic part remains exposed to the solution, is another strategy and yields stable colloids.[15–17] To impart increased stability, the coated amphiphilic polymer shell can be further cross-linked, usually on its periphery.[9,18–20] Neither method is very versatile or generally applicable.

Herein, we report a facile and generic strategy to create a stable cross-linked polymer coating on UCPs, with a large choice of functional groups. The method consists of using the light generated by the visible or UV emissions of the UCPs when excited with a near-infrared (980 nm) light source to construct a thin polymer shell around the UCPs by photopolymerization. Since the emission from the UCPs is weak as compared to direct light, polymerization is confined to the close proximity of the UCPs and thus yields core–shell particles (Figure 1). The polymer shell can be made hydrophilic or hydrophobic, charged or neutral, chemically reactive

[*] S. Beyazit, Dr. S. Ambrosini, Dr. N. Marchyk, Dr. B. Tse Sum Bui, Prof. K. Haupt
Compiègne University of Technology
CNRS Enzyme and Cell Engineering Laboratory
Rue Roger Couttolenc, CS 60319, 60203 Compiègne Cedex (France)
E-mail: jeanne.tse-sum-bui@utc.fr
karsten.haupt@utc.fr
Homepage: http://www.utc.fr/mip
E. Palo, V. Kale, Prof. T. Soukka
University of Turku, Department of Biotechnology
Tykistökatu 6A, 20520 Turku (Finland)

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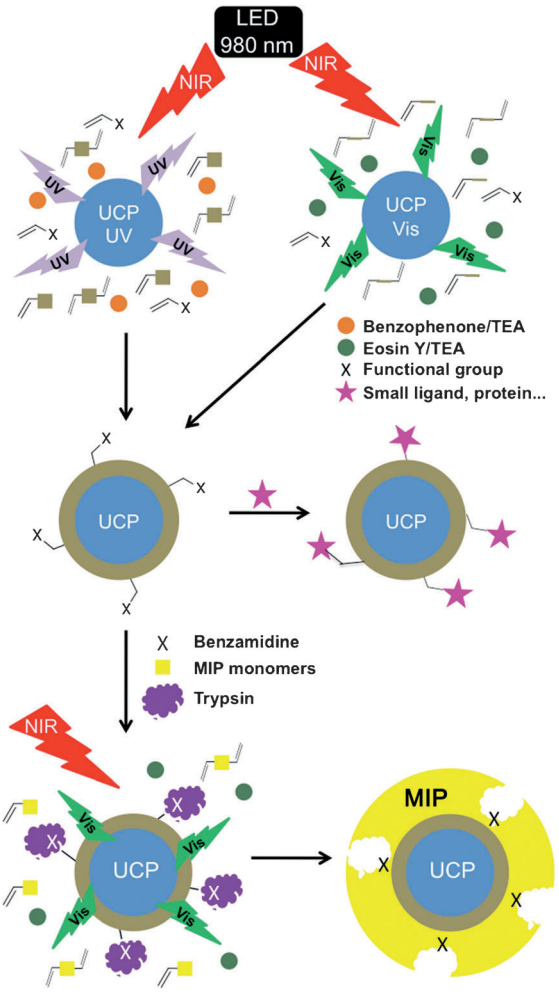
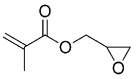
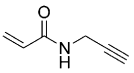
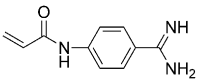
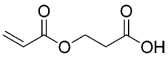


Figure 1. UV or visible light emitted from upconverting nanoparticles excited in the near-infrared region (980 nm) is used to create a polymeric shell in situ around the particles by photopolymerization. Benzophenone and eosin Y are used as UV and visible initiators, respectively. The incorporation of functional monomers enables the subsequent attachment of small ligands, proteins, or other moieties. A second shell, for example, a molecularly imprinted polymer (MIP), can be synthesized by re-initiation in the presence of different monomers and a molecular template (trypsin). LED = light-emitting diode.

Table 1: Functionalized water-compatible core–shell UCPs obtained by photopolymerization using the internal visible or UV emitted light of UCPs irradiated at 980 nm. UCP-Vis and UCP-UV represent visible and UV emitters, respectively.

Core–shell UCPs	Functional monomer[a]	Conjugation partner
UCP-Vis-GA	 glycidyl methacrylate	dichlorofluorescein
UCP-Vis-PA UCP-UV-PA	 propargyl acrylamide	fluorescein-N_3
UCP-Vis-AB	 *N*-acryloyl-*p*-amino-benzamidine	trypsin
UCP-UV-CEA	 2-carboxyethyl acrylate	FITC-streptavidin

[a] All polymers also contain HEMA and EbAM.

or inert, depending on the nature of the monomers used. In the following we demonstrate through several examples the versatility of our approach.

Typically, water-compatible polymer shells are obtained with hydrophilic monomers, in this case 2-hydroxyethyl methacrylate (HEMA) and the cross-linker *N*,*N*′-ethylenebis-(acrylamide) (EbAM). For the introduction of functional chemical groups for further bioconjugation, no major modifications to the existing strategy or conditions are required; functional monomers are simply added together with HEMA/EbAM to the prepolymerization mixture. For example, glycidyl (to link nucleophilic moieties), carboxy (for the attachment of amino groups by amide coupling), and propargyl[21] functionalities (for the attachment an azido group[22] by click conjugation) were introduced on the UCPs.

Moreover, a second polymer shell can be synthesized on top of the first shell by re-initiation in the presence of different monomers, as exemplified by the synthesis of a molecularly imprinted polymer shell for trypsin recognition.[23,24] The different water-compatible core–shell UCPs that were synthesized are listed in Table 1. If a hydrophobic coating is required, the same strategy can be adopted, as demonstrated by the photopolymerization of a divinylbenzene (DVB) or an ethylene glycol dimethacrylate (EGDMA) cross-linked shell around the UCPs.

For this study, we used two different UCPs: a visible-light emitter ($NaYF_4$:Yb^{3+},Er^{3+}),[25] hereafter referred to as UCP-Vis (see Figure S1 in the Supporting Information), and a UV-light emitter ($Na_{0.6}K_{0.4}YF_4$:Yb^{3+},Tm^{3+}),[26] hereafter referred to as UCP-UV (see Figure S2). Both were used with appropriate initiator systems, whereby the emission wavelength of the UCPs must overlap with the absorption wavelength of the initiator (see Figures S3 and S4).

After some optimization, we found that in the case of the UCP-Vis particles, an initiating system composed of eosin Y/triethylamine (TEA) (see the Supporting Information for its mechanism of action) was the most effective for our purpose. The emitted visible light from the UCPs ($\lambda_{em} \approx 520$–540 nm from $^2H_{11/2} \rightarrow {}^4I_{15/2}$ and $^4S_{3/2} \rightarrow {}^4I_{15/2}$ transitions from Er^{3+}) could excite the photosensitizer dye eosin,[27,28] whose maximum excitation wavelength is 535 nm (see Figure S3). The polymerization mixture contained the UCP-Vis, eosin/TEA, HEMA/EbAM, and the functional monomer, either glycidyl methacrylate (GA) or propargyl acrylamide[21] (PA, see the Supporting Information for its synthesis) (Table 1). Polymerization with a 980 nm LED light source (150 mW) was carried out in a mixture of dimethyl sulfoxide (DMSO) and toluene (1:1). After polymerization for 4 h, the UCP core–shell

nanoparticles were sedimented by centrifugation, washed with toluene/DMSO (1:1) and with water, and dried. The final pellet was resuspended in water and ultrasonicated to yield a well-dispersed clear solution. Successful grafting of the polymer shells can be seen in the TEM images (Figure 2). Dynamic light scattering (DLS) measurements showed that the shell thickness increased as a function of polymerization time (see Figure S5). Other parameters that influence the shell thickness are the type and concentration of monomers, and the amount of initiator (not shown).

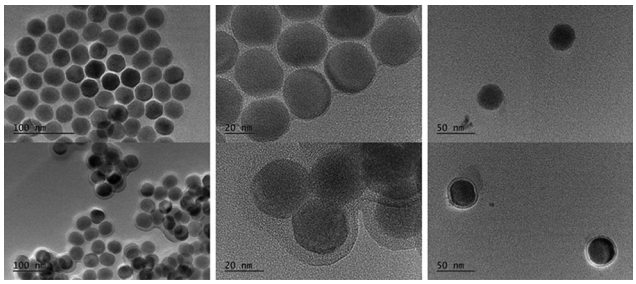


Figure 2. TEM images of non-modified UCP-Vis (top) and UCP-Vis core–shell nanoparticles (bottom) at different magnifications (scale bars, left: 100 nm, middle: 20 nm, right: 50 nm).

To demonstrate conjugation, we used the fluorescent dyes dichlorofluorescein and azidofluorescein[22] (see the Supporting Information for synthetic details) as models and attached them to the glycidyl- and propargyl-functionalized core–shell particles by nucleophilic substitution and by click chemistry, respectively. To prove that the conjugation was successful in each case, we recorded the emission spectra of fluorescein ($\lambda_{ex} = 495$ nm) and observed the characteristic emission peak of fluorescein (see Figures S6 and S7).

To demonstrate that a second shell can be photopolymerized on top of the first shell by again using the visible light emitted by the UCP, we synthesized a molecularly imprinted polymer (MIP) shell on top of a first shell surrounding the UCPs. MIPs are tailor-made synthetic antibody mimics that are able to specifically recognize and bind target molecules. They are synthesized by copolymerizing functional and cross-linking monomers in the presence of a molecular template, thus resulting in the formation of binding sites with affinities and specificities comparable to those of natural antibodies.[29–31] Their molecular-recognition properties combined with a high chemical and physical stability make them interesting alternatives to biological antibodies for use in immunoassays, biosensors, bioseparation, and other applications.[32]

The first shell (UCP-Vis-AB; Table 1) was obtained with a polymerization mixture containing the UCPs, eosin/TEA, HEMA/EbAM, and an anchoring monomer, *N*-acryloyl-*p*-aminobenzamidine (AB; see the Supporting Information for its synthesis[33,34]) in a mixture of toluene and DMSO (1:1). Aminobenzamidine is an inhibitor of serine proteases and was used in this study as an anchoring ligand for the imprinting template, trypsin (Figure 1).[23,24] After polymerization, the UCP core–shell nanoparticles were washed with toluene/DMSO (1:1) followed by water, and resuspended in 25 mM sodium phosphate buffer (pH 7.0) to yield a clear solution. The second shell (UCP-Vis-MIP) was then obtained by irradiation with NIR light in a MIP polymerization mixture containing trypsin, HEMA/EbAM, and the initiator eosin/TEA. After polymerization, the nanoparticles were washed with a 5% sodium dodecylsulfate (SDS) solution in water/acetic acid (9:1) and with ethanol, to eliminate trypsin and SDS, respectively.[23]

The recognition properties of the MIPs were evaluated by equilibrium binding experiments with fluorescein isothiocyanate labeled trypsin (FITC-trypsin;[35] see the Supporting Information for synthetic details). FITC-trypsin was incubated with UCP-Vis-MIP in 5 mM Tris-HCl buffer containing 10 mM $CaCl_2$ at pH 8.0. After incubation, the particles were separated by centrifugation, and the free FITC-trypsin in the supernatant was quantified by fluorescence measurements. The same procedure was applied to non-imprinted (NIP) UCPs, which were prepared in the same way as the MIPs, but in the absence of the protein template. Another control, MIP without AB, was also synthesized. The recognition of trypsin was found to be specific, since binding to the MIP was higher than that with the control NIP (Figure 3a). The apparent high binding to the NIP can be explained by the presence of benzamidine moieties, which act as affinity ligands. Nevertheless, the presence of AB is necessary; when the MIPs were

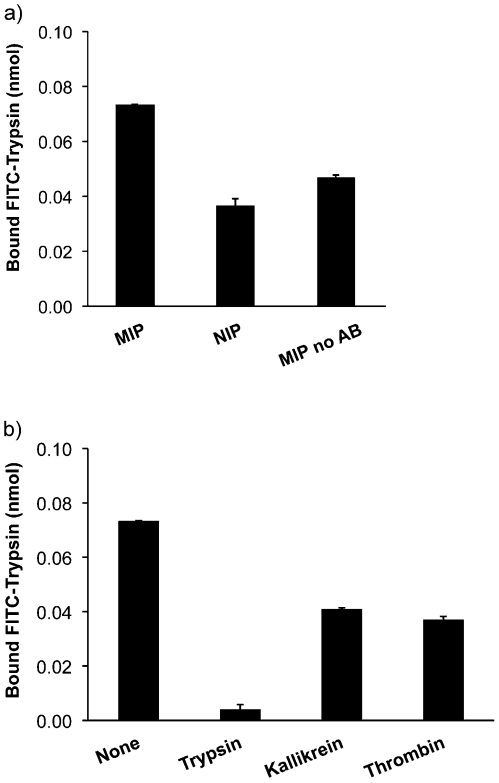


Figure 3. a) Binding of MIP, NIP, and MIP without AB to FITC-trypsin (100 nM) in 5 mM Tris-HCl buffer containing 10 mM $CaCl_2$ (pH 8.0). b) Displacement of bound FITC-trypsin (100 nM) from MIP by 500 nM kallikrein, thrombin, and trypsin in 5 mM Tris-HCl buffer containing 10 mM $CaCl_2$ (pH 8.0). The measurements were made at least in triplicate, and the error bars represent the standard deviation.

prepared in the absence of the anchoring monomer, less binding was observed. The selectivity of UCP-Vis-MIP for trypsin was investigated by performing competitive binding experiments with thrombin and kallikrein, two other serine proteases inhibited by *p*-aminobenzamidine. UCP-Vis-MIP was incubated with 100 nM FITC-trypsin together with 500 nM nonlabeled trypsin, kallikrein, or thrombin. After incubation, the particles were separated by centrifugation, and the free FITC-trypsin was quantified as described above. The binding of FITC-trypsin to UCP-Vis-MIP was almost totally suppressed in the presence of trypsin, whereas kallikrein and thrombin displaced much less of the fluorescent probe (Figure 3b). These results indicate that the MIP contains imprinted cavities that are highly selective for the target trypsin. We believe that other MIPs for any given analyte of interest could be prepared in a similar way.

We used the same approach as described above to coat UV-emitting UCPs (UCP-UV). In fact, radical photopolymerization mostly uses UV initiation; therefore, it may be possible to use existing polymerization protocols to coat these UCPs, which would be a great advantage. We first tested three different initiators, benzophenone/triethylamine, 2,2-dimethoxy-2-phenylacetophenone (DMPA), and benzyl-*N*,*N*-dimethyl dithiocarbamate[36] (BDC) (see the Supporting Information for the initiation mechanisms) to investigate whether the internal UV light emitted by the UCPs ($\lambda_{em} \approx 360$ nm from the $^1D_2 \rightarrow {}^3H_6$ transition from Tm^{3+}) could initiate the polymerization of a polymeric shell. The absorption spectra of the three initiators overlap with the emission band of UCP-UV (see Figure S4). Indeed, core–shell nanoparticles were effectively obtained with the three initiators. In the following, only results obtained with benzophenone/TEA initiation are shown.

Hydrophobic and hydrophilic shells were synthesized around UCPs. Figure 4 shows as an example the TEM images of bare and hydrophobic ethylene glycol dimethacrylate (EGDMA)-coated UCPs. To obtain hydrophilic functionalized UCP-UV nanoparticles for further conjugation, we irradiated UCP-UV at 980 nm in the presence of EbAM and HEMA in toluene/DMSO (1:1) with either 2-carboxyethyl acrylate (CEA) or propargyl acrylamide (PA) as an additional monomer. CEA and PA allow for amide coupling or click chemistry, respectively (Table 1). UCP-UV-CEA nanoparticles were then derivatized with FITC-labeled streptavidin after activation with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)/*N*-hydroxysulfosuccinimide (NHS). Figure 5 shows the emission spectra of UCP-UV-CEA before and after conjugation with FITC-streptavidin. The presence of FITC ($\lambda_{em} = 520$ nm) is clearly visible after the reaction. When the particles were excited at 980 nm, the red emission band of the UCPs was clearly visible; these nanoparticles can be used, for example, in imaging applications. This emission band is also an additional evidence of the presence of polymer-coated UCPs. UCP-UV-PA nanoparticles were conjugated with FITC-N_3 by click chemistry. The typical fluorescein emission peak observed in the fluorescence spectra after conjugation (see Figure S8) indicates the presence of a polymer shell around the UCP.

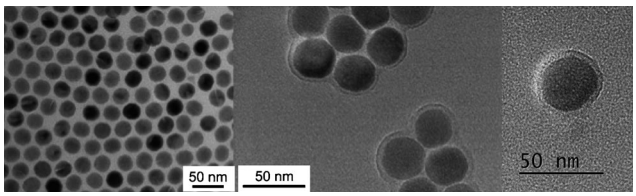


Figure 4. TEM images of bare UCP-UV (left) and UCP-UV core–shell nanoparticles (middle and right, EGDMA shell). Scale bars: 50 nm.

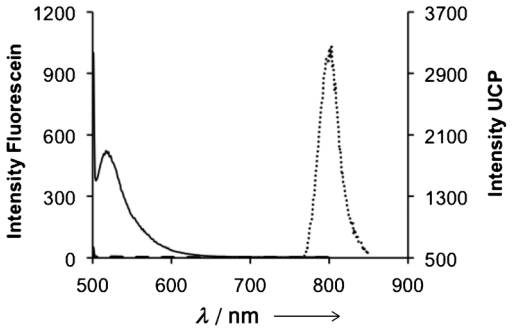


Figure 5. Dual-emission spectra proving the presence of the polymer around the UCP. Emission ($\lambda_{ex} = 495$ nm) of UCP-UV-CEA in 10 mM sodium acetate buffer (pH 6.5) before (dashed line) and after (solid line) FITC-streptavidin coupling. The presence of FITC ($\lambda_{em} = 520$ nm) is clearly visible after the reaction. The upconversion emission ($\lambda_{ex} = 980$ nm) of UCP-UV-CEA after FITC-streptavidin coupling is shown by the dotted line.

In conclusion, we have presented an elegant, straightforward, and generic strategy for the coating of upconverting nanoparticles with thin polymer shells for their protection, functionalization, conjugation, and biocompatibility. The method uses the internal UV or visible emitted light from near-infrared photoexcited UCPs. This approach enables the incorporation into the shell of a large variety of monomers, hydrophilic or hydrophobic, with different chemical functionalities, in a straightforward manner. A second layer, exemplified by a molecularly imprinted polymer shell specific for trypsin, can also be synthesized. We believe that our method offers great advantages, such as ease of application, rapid surface modification, and a large spectrum of possible surface chemistries for further functionalization, and can provide a platform for the preparation of libraries of functional polymer-encapsulated UCPs for applications in bioassays, biosensing, optical imaging, and drug delivery.

Experimental Section

Experimental details (reagents and materials, synthesis of propargyl acrylamide, FITC-N_3, *N*-acryloyl-*p*-aminobenzamidine, and fluorescein-labeled trypsin, synthesis of core–shell polymers and MIP, TEM and DLS analysis, binding studies, and bioconjugation of labeled entities) can be found in the Supporting Information.

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