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Originally published:

October 2018

Angewandte Chemie - International Edition 57(2018), 16036

DOI: https://doi.org/10.1002/anie.201811003

Towards Utilising Photocrosslinking of Polydiacetylenes for the Preparation of "Stealth" Upconverting Nanoparticles

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Abstract: *We demonstrate a novel strategy for preparing hydrophilic upconverting nanoparticles (UCNPs) by harnessing the photocrosslinking ability of diacetylenes. Replacement of the hydrophobic oleate coating on the UCNPs with 10,12-pentacosadiynoic acid, followed by overcoating with diacetylene phospholipid and subsequent photocrosslinking under 254 nm irradiation produces water-dispersible polydiacetylene-coated UCNPs. These UCNPs resist the formation of a biomolecular corona and show great colloidal stability. Furthermore, amine groups on the diacetylene phospholipid allow for functionalisation of the UCNPs with, for example, radiolabels or targeting moieties. These results demonstrate that this new surface-coating method has great potential for use in the preparation of UCNPs with improved biocompatibility.*

Lanthanide-doped upconverting nanoparticles (UCNPs) display a number of favourable characteristics for application in bioimaging such as tunable emission wavelengths, large anti-Stokes shifts, long emission lifetimes, high photochemical stability, and reduced autofluorescence interference.[1–7] Moreover, such nanomaterials (NMs) can be photoexcited in the near-infrared(NIR)-1 optical transparency window (650–950 nm), which is an optimal wavelength range for in vivo imaging.[2,3,5] All biomedical applications of UCNPs, however, rely on the synthetic ability to generate water-dispersible and colloidally stable material.[1,6,8,9] Conventional routes to the preparation of UCNPs involve the use of high-boiling organic solvents, and produce nanocrystals that are hydrophobic in nature.[4,7–12] Thus, further surface modification of the as-produced UCNPs is needed in order to make them hydrophilic.[4,8–18] For clinical applications, however, it is equally important that the UCNPs show minimal interaction with the bio-(macro)molecules (e.g., proteins, lipids) present in complex biological fluids.[1,19,20] With the adsorption of biomolecules, the NMs lose both their synthetic identity and inherent physicochemical properties.[2,19–25] This biomolecular corona formation on systemically circulating NPs also tends to make them highly prone to trapping by the mononuclear phagocyte system (MPS), which can lead to their rapid clearance from the bloodstream and eventual insufficient accumulation in the organs of interest.[26]

The inability to control the fate of UCNPs in vivo has hampered their clinical progress. Upconversion (UC) luminescence from the UCNPs is highly sensitive to alterations in both their size and the surface capping ligands.[1,2,27,28] This adds to the complexity since any surface modification on UCNPs should not result in any significant adverse effect on their optical properties.[1,2,4,10,11,28] In this work, we present photopolymerisation of diacetylenes (DAs) as an effective way to produce colloidally stable corona-resistant UCNPs. We also demonstrate that the photocrosslinked UCNPs maintain their UC luminescence, and have active sites for further functionalisation with therapeutic/diagnostic moieties, which makes them highly suitable for biomedical application. Photopolymerised polydiacetylenes (PDAs) are a special class of conjugated polymers produced from the topochemical 1,4-addition of the DA units under UV irradiation (Figure S1 in the Supporting Information).[29–35] Notably, several research groups have investigated the potential of PDAs for drug delivery, sensing, and imaging.[29–48] However, the use of PDAs for synthesising water-dispersible UCNPs remains unexplored.

Oleate-capped hydrophobic UCNPs were first synthesised employing our recently described co-precipitation procedure.[49,50] Given that the hexagonal (β-phase) $NaYF_4$-based UCNPs display high UC efficiency,[1,4,10,11] first the β-$NaYF_4$ host lattice was co-doped with Nd^{3+}/Yb^{3+}/Er^{3+} ions in the core, followed by construction of an active Nd^{3+} (25 %) shell (2 nm)[50] to suppress the surface-related quenching effects.[11,51] The introduction of Yb^{3+} as well as Nd^{3+} in the UCNPs allows their excitation at both $\lambda = 976$ and 793 nm.[49,50] The relative concentration of the Ln^{3+} dopants in as-prepared UCNPs was quantified by inductively coupled plasma mass spectrometry (ICP-MS). The produced UCNPs showed excellent dispersibility in hexane, cyclohexane, as well as chloroform, with a size of around 10 nm in solution (Figure S2 in the Supporting Information).[49]

To render these UCNPs water-dispersible, surface-bound oleate ligands were first substituted with 10,12-pentacosadiynoic acid (PCDA) in a ligand-exchange reaction with nitrosonium tetrafluoroborate ($NOBF_4$).[52] Next, the resulting PCDA-capped UCNPs were overcoated with photopolymerisable diyne phospholipid(s), 1,2-bis(10,12-tricosadiynoyl)-*sn*-glycero-3-phosphoethanolamine (PE) and 1,2-bis(10,12-trico-

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sadiynoyl)-*sn*-glycero-3-phosphocholine (PC) employing the procedure described by Lu et al.,[14] and the diacetylenic ligand assembly was subsequently photocrosslinked by UV irradiation (254 nm). The complete procedure for the preparation of crosslinked PDA-coated hydrophilic UCNPs is summarised in Figure 1.

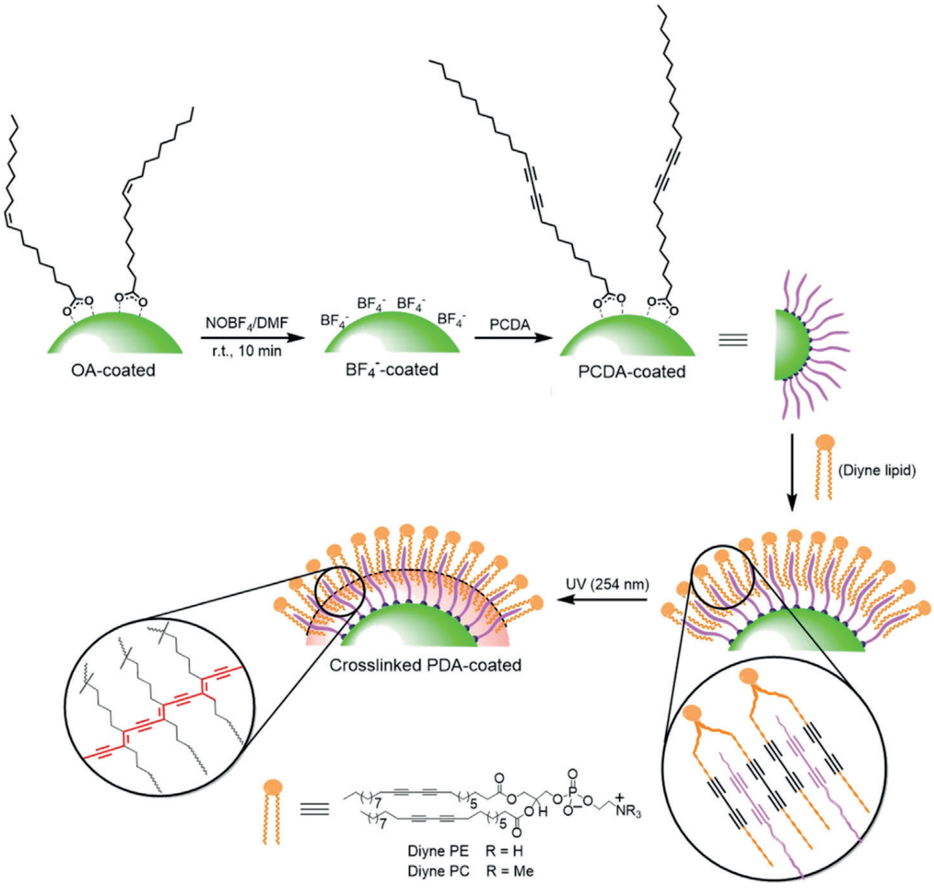

Figure 1. Synthesis of crosslinked PDA-coated UCNPs.

During the course of photocrosslinking, the colour of the UCNP dispersion changes from blue to red (Figure 2), accompanied by pronounced changes in the absorption spectra (Figures 3 a and S3 in the Supporting Information). Following irradiation at 254 nm for 50 min, the absorption band at 650 nm experiences a bathochromic shift (ca. 25 nm). Additionally, a structured band centred at around 550 nm grows in. After 90 min of irradiation, this band undergoes a further blue shift, along with a hypochromic shift of the 675 nm band.

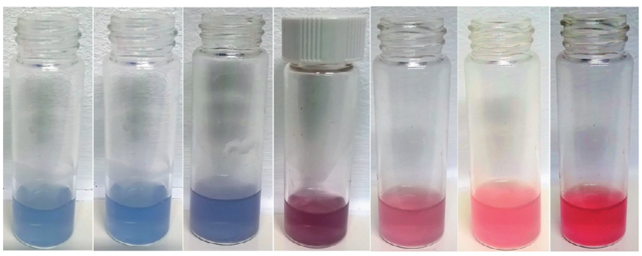

Figure 2. Observed blue-to-red colour change of the dispersion of PE/PC (1:1)-coated UCNPs in water, with increasing irradiation time (5–90 min) during the course of photocrosslinking (254 nm).

As for other PDA-based materials,[29,31,33,34] the blue-to-red transition observed here could be due to the conformational changes induced in the alkene–alkyne backbone that forms upon crosslinking. It can be expected that photopolymerisation results in a geometric stress on the PDA assembly covering the UCNP surface, which is then required to undergo reorganisation in order to overcome it. It should be noted, however, that the colour changes in PDA-based systems can also be invoked by several other factors.[29,31,34] The exact basis for the observed colour change in our case remains to be determined. As is typical of red-phase PDAs, the final UCNPs exhibited characteristic emission between 550–750 nm ($\lambda_{ex} = 450$ nm), with a maximum at around 625 nm (Figures 3 b and S4 in the Supporting Information).[29,31,33,34]

FTIR spectroscopy confirmed the assembly of diyne ligands on the UCNP surface (Figure 3 c). In particular, the UCNPs show overlapping C=C and C=O stretching vibrations (1733 and 1694 cm^{-1}) for the alkene and ester groups on the crosslinked PDAs as well as strong C−H stretches for the aliphatic chains (2849 and 2919 cm^{-1}). Characteristic band for surface bound carboxylate ($RCOO^-$) was observed around 1465 cm^{-1}. Furthermore, phosphate-related vibrations, originating from the diyne phospholipids, were located in the 1180–1000 cm^{-1} region. Dynamic light scattering (DLS) studies showed that the UCNPs prepared using only PE have a mean hydrodynamic diameter (D_h) of around 14 nm in water (Table 1). This is an increase of about 4 nm compared with the size of oleate-coated hydrophobic UCNPs in cyclohexane.[49] Moreover, these UCNPs showed a negative zeta potential (ζ) of −37.6 mV at physiological pH values (Table 1).

We and others have previously shown that maintaining surface charge neutrality is an effective way to inhibit the formation of biomolecular corona on the NPs.[26,49,53–58] Because one of our objectives was also to produce corona-resistant UCNPs, we explored the idea of using mixed-diyne phospholipids for overcoating the PCDA-capped UCNPs as a way to yield neutral UCNPs. For this, the synthesis was performed using 1:1, 2:1, and 3:2 (w/w) mixtures of PE and PC, respectively. Zeta potential and DLS measurements revealed that the use of mixed diyne lipid composition not

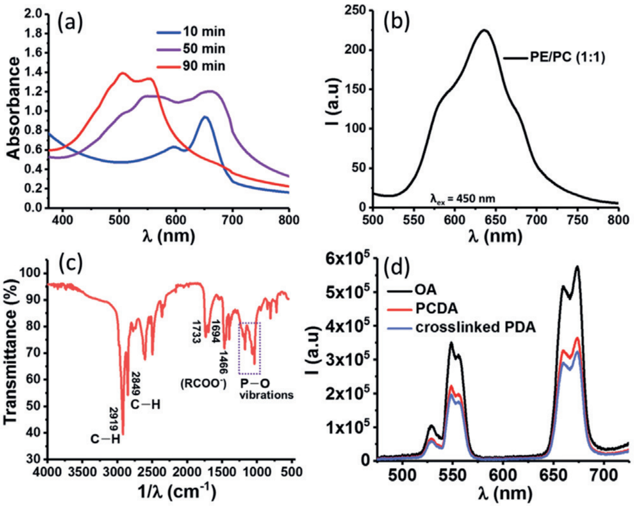

Figure 3. a) Changes in the UV/Vis absorption spectra of PE/PC (1:1)-coated UCNPs, following 254 nm irradiation. b) Emission spectra ($\lambda_{ex} = 450$ nm) of crosslinked PE/PC (1:1)-coated UCNPs. c) FTIR spectra of crosslinked PE/PC (1:1)-coated UCNPs. d) UC emission spectra of OA-, PCDA-, and crosslinked PE/PC (1:1)-coated UCNPs ($\lambda_{ex} = 800$ nm, $[UCNP_{OA\text{-}coated}] = 5$ mg mL^{-1} and $[UCNP_{PCDA\text{-}coated}] = 12$ mg mL^{-1} in $CHCl_3$, $[UCNP_{crosslinked}] = 12$ mg mL^{-1} in H_2O).

only results in a close to zero surface charge of the UCNPs at physiological pH ($\zeta = 3.4 \pm 1.2$, 1.6 ± 1.1 and -5.1 ± 1.8 mV for PE/PC = 1:1, 3:2 and 2:1 (w/w), respectively) but also the crosslinked UCNPs have almost same size distribution as before (Table 1). In general, ζ for all the four PDA-coated UCNPs increased with decreasing pH (Table 1). Notably, the UCNPs did not undergo any significant change in their size distribution for up to 21 days ($D_h < 16.5$ nm), which substantiates their good colloidal stability. Interestingly, overcoating the PCDA-coated UCNPs with only PC resulted in immediate aggregation of the particles in water. Likewise, all attempts to synthesise crosslinked hydrophilic UCNPs without using PCDA were also unsuccessful. These findings indicate that the use of both PCDA and PE diyne lipid is a prerequisite for preparing hydrophilic UCNPs with this method.

Upon excitation at $\lambda = 800$ nm with a femtosecond-pulsed laser, the crosslinked hydrophilic UCNPs exhibited characteristic sharp UC emission (Figure S5 in the Supporting Information), with intense bands at 528, 549, and 674 nm.[49,50] The representative spectrum from the PE/PC (1:1)-coated crosslinked UCNPs, in water, is shown in Figure 3 d. The observed emission bands correspond to the $^2H_{11/2} \rightarrow ^4I_{15/2}$, $^4S_{3/2} \rightarrow ^4I_{15/2}$, and $^4F_{9/2} \rightarrow ^4I_{15/2}$ transitions of Er^{3+} ions, respectively, and are in accordance with those observed for the OA- and PCDA-coated UCNPs (in $CHCl_3$). Introducing crosslinked diyne ligands on the UCNP surface, however, results in a noticeable drop in the UC intensity; a decrease of around 10% is observed for the crosslinked hydrophilic UCNPs compared with the PCDA-coated ones. The suppression of UC emission in crosslinked hydrophilic UCNPs can be reasonably assigned to the quenching of Nd^{3+} excited states by water molecules, as already reported for other water-dispersible UCNPs.[28,59–61]

To investigate whether the crosslinked UCNPs can resist surface adsorption of biomolecules, such as serum proteins, they were incubated with different concentrations of human serum (HS). The reaction mixtures were analysed for biomolecular corona formation using gel electrophoresis (see the Experimental Section in the Supporting Information for details).[49] Images of the gels for the UCNP-associated serum proteins, isolated after the incubation of all the four crosslinked UCNPs with HS for 1 h at 37 °C, are shown in Figures 4 and S6, S7 in the Supporting Information. As expected, owing to their overall neutral surface charge, the UCNPs with mixed diyne lipid (PE/PC) coating showed almost no adsorption of serum proteins, whereas substantial amount of protein corona was formed on the negatively charged PE/PC (1:0)-coated UCNPs. This behaviour is con-

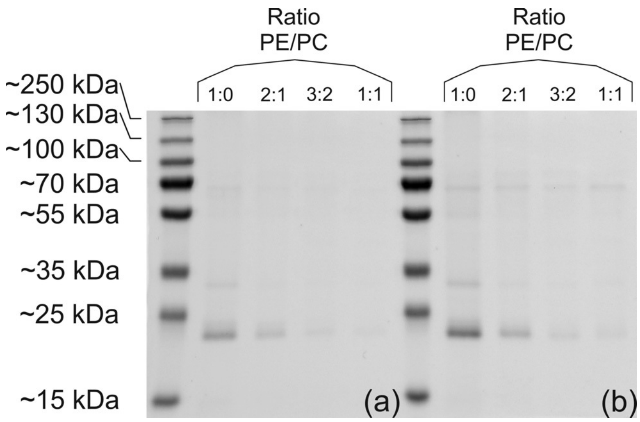

Figure 4. SDS-PAGE analysis of the proteins adsorbed by different crosslinked PDA-coated UCNPs upon incubation in a) 50% and b) 80% of human serum for 1 h at 37 °C.

Table 1: DLS particle size distribution (D_h) and zeta potential (ζ) of crosslinked PDA-coated $NaYF_4$:Nd^{3+}/Yb^{3+}/Er^{3+} (1/20/2%)@$NaYF_4$:Nd^{3+}(25%) core–shell UCNPs prepared in this work.

PE:PC[b]	Particle size (D_h) [nm][a]					Zeta potential (ζ) in mV					
	As synthesised	+3 days	+7 days	+14 days	+21 days	pH 4.3	pH 5.3	pH 6.3	pH 7.3	pH 8.3	pH 9.3
1:0	14.1 ± 2.1	14.2 ± 1.9	14.2 ± 2.2	15.1 ± 2.6	16.4 ± 2.2	−25.1 ± 2.3	−28.4 ± 2.2	−35.3 ± 2.2	−37.6 ± 3.7	−38.1 ± 3.7	−40.1 ± 3.7
2:1	14.3 ± 1.7	14.1 ± 2.1	14.3 ± 1.9	15.5 ± 2.7	15.9 ± 2.5	12.2 ± 1.1	−3.4 ± 1.1	−10.4 ± 1.1	−5.1 ± 1.8	−12.1 ± 2.5	−25.6 ± 5.2
3:2	14.2 ± 2.5	14.2 ± 2.3	14.2 ± 2.6	15.3 ± 2.2	15.8 ± 2.4	11.1 ± 1.6	6.2 ± 2.4	2.8 ± 1.9	1.6 ± 1.1	−9.1 ± 1.8	−12.1 ± 1.8
1:1	13.9 ± 1.8	13.9 ± 2.1	14.1 ± 2.3	15.3 ± 2.4	16.1 ± 1.6	25.3 ± 1.8	14.1 ± 2.1	5.2 ± 2.8	3.4 ± 1.2	−8.3 ± 2.5	−10.4 ± 2.1

[a] The measurements were performed in water at pH 7.4 (see Table S1 in the Supporting Information for polydispersity index (PDI) data). [b] PE = 1,2-bis(10,12-tricosadiynoyl)-*sn*-glycero-3-phosphoethanolamine and PC = 1,2-bis(10,12-tricosadiynoyl)-*sn*-glycero-3-phosphocholine.

sistent with our previous results on biomolecular corona resistance by other near-neutral charged NPs coated with zwitterionic polymers and low molecular weight compounds.[49,53,54] Interestingly, the PE/PC-coated crosslinked UCNPs could inhibit serum protein adsorption effectively even at high serum concentrations. These results point toward excellent stealth characteristics for the crosslinked UCNPs with PDA coating.

Furthermore, the availability of the peripheral primary amino groups on the UCNPs for later introduction of other functionalities through bioconjugation reactions was evaluated (Figure 5). The crosslinked PE/PC (3:2)-coated UCNPs

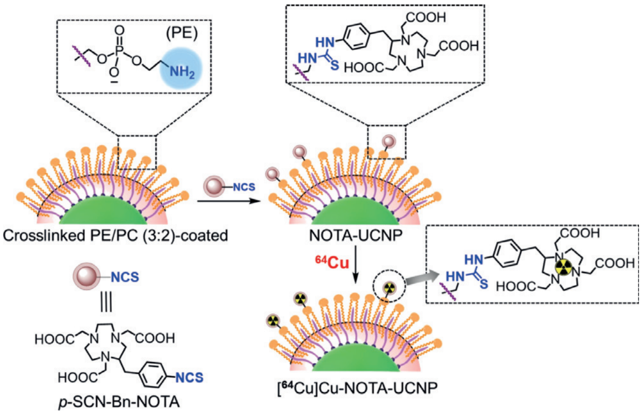

Figure 5. Surface functionalisation of crosslinked PDA-coated UCNPs with radiometal chelating NOTA motif, and ^{64}Cu labelling.

were reacted with isothiocyanate-functionalised radiocopper-chelating 1,4,7-triazacyclononane motif (*p*-SCN-Bn-NOTA). Indirect confirmation on attachment of NOTA to the UCNPs was obtained by performing ^{64}Cu labelling experiments on the resulting UCNPs. The radiocomplexation was confirmed by radio-thin layer chromatography (radio-TLC; Figure S8 in the Supporting Information).

In summary, we have presented a new strategy to obtain water-dispersible UCNPs by using photocrosslinkable DAs as surface coating agents. Covalent crosslinking of the diyne units on the UCNP surface affords rigid assemblies that are readily dispersible in aqueous medium and display high stability under physiological conditions. This crosslinking method also provides a way to tune the overall surface charge of the UCNPs without altering their size and optical properties. For instance, by using mixed diyne lipid compositions, UCNPs with overall neutral surface charge could be produced. The crosslinked NPs retain their photon UC property, albeit with a relatively small loss in UC luminescence intensity in aqueous environments. These UCNPs exhibit excellent resistance to biomolecular corona formation both under the physiological conditions and at high serum concentrations. Moreover, surface-exposed amino groups of PE phospholipid enable easy attachment of other bio- and radioactive moieties to the UCNPs by using bioconjugation methods. For example, radiocopper-labelled UCNPs could be successfully prepared by first reacting the crosslinked UCNPs with SCN-NOTA derivative, and then labelling the UCNP-NOTA conjugates with ^{64}Cu. Owing to the rigidity of the covalently-linked PDA assembly, the demonstrated coating strategy holds promising potential for generating "stealth" UCNPs with higher stability and improved biocompatibility in comparison to other surface coating approaches. With this in mind, we are currently conducting in-depth in vivo investigations of the pharmacokinetics and biodistribution of these crosslinked UCNPs.

Acknowledgements

We thank Dr Robin Steudtner (Institute of Resource Ecology, HZDR) for his support with the UC luminescence measurements. This work was supported by the Helmholtz Initiative and Networking Fund (Functional Nanomaterials for Multimodality Cancer Imaging (NanoTracking), project ID: VH-VI-421), a HZDR fellowship to M.S., and an Alexander von Humboldt Foundation research fellowship to T.J.

Conflict of interest

The authors declare no conflict of interest.

Keywords: crosslinking · diacetylenes · lanthanides · nanomaterials · upconversion

How to cite: *Angew. Chem. Int. Ed.* **2018**, *57*, 16036–16040
Angew. Chem. **2018**, *130*, 16268–16272

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