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Surface-Modified Upconverting Microparticles and Nanoparticles for Use in Click Chemistries

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Abstract: A method is described for modifying the surface of upconverting microparticles (UCμPs) and nanoparticles (UCNPs) such that they become amenable to click chemistry. Respective reagents are presented and used in both kinds of particles, either directly or in combination with tetraethoxysilane. The particles also were labeled by using the click reaction, a) with fluorophores to yield materials that have emission colors that depend on the wavelength of excitation; b) with maleinimido groups (so to obtain labels for thiols), and c) with biotin (to make them useful for affinity studies based on the biotin–streptavidin system). We believe that both the UCμPs and UCNPs have the potential of being used in numerous areas including upconversion imaging, biolabeling, and derivatization, but also in encoding and security.

Keywords: biotin · click chemistry · fluorescence · nanoparticles · upconversion

Introduction

Upconverting nanoparticles (UCNPs), sometimes also referred to as upconverting nanophosphors display the unique property of emitting photoluminescence at visible wavelengths under near-infrared (NIR) excitation.[1,2] Unlike conventional fluorophores, UCNPs convert low-energy NIR radiation to higher energy (visible) light by multiphoton absorption and subsequent emission of dopant-dependent luminescence. The large anti-Stokes shift allows easy separation of the discrete emission peaks from the excitation source. UCNPs are chemically stable and do not bleach, which is in striking contrast to most organic fluorophores. Applications of UCNPs (which are virtually invisible in low concentrations) include authentication in general, security,[3] counterfeit,[4,5] brand protection,[6] flow cytometry,[7] point-of-care diagnostics,[8] and bioarrays.[9] In bioanalytical terms, they have been demonstrated to be useful in immunoassays,[10] as luminescent labels,[11] in sensing pH,[12] and in imaging of cells and small animals.[13] UCNPs have the advantage of being photoexcitable at wavelengths at which the auto-absorption of any biological matter is quite weak, thereby reducing background of both absorption and luminescence (which would occur, along with Raman scatter, at wavelengths of >980 nm anyway) to virtually zero.

Upconverting microparticles (UCμPs), as opposed to UCNPs, clearly are much larger, but more efficient in terms of upconversion. They are commercially available and used, for example, in security inks or for the visualization of IR radiation. UCμPs also have been employed in homogeneous immunoassays[14] and enzyme activity assays,[15] following bead-milling to reduce the particle size to the sub-micron range. Low-energy laser diodes are adequate for photo-excitation, and their (visible) emission is rather bright. Unlike UCNPs, they cannot be well suspended (as a kind of colloidal dispersion) in aqueous or organic solutions.

To be of use in affinity studies (such as in high-throughput screening) and in bioassays, the surface of UCNPs and UCμPs has to be functionalized to facilitate covalent immobilization of biomolecules on their surfaces. Such surface chemistries are expected to be versatile, to enable immobilization of proteins, receptors, enzymes, or nucleic acid oligomers, to mention a few. Moreover, UCNPs can be suspended fairly well in certain organic solvents, but not in water unless their surface is appropriately modified. This is crucial, however, with respect to many bioapplications.[16] Only if proper surface modification is accomplished, can their bioanalytical potential can be fully exploited.

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The most common method to improve dispersibility involves the coating of NPs with a thin shell of silica (SiO_2). The resulting silica NPs are chemically stable, fairly biocompatible, nontoxic, and can be prepared in narrow size distribution. Silica is well documented as a coating agent for quantum dots,[17] metal oxides,[18] lanthanide nanoparticles,[19] and even upconverting particles.[11,20] In fact, NPs and μPs made from, or coated with silica have been used in various kinds of sciences including nanomedicine,[21] nanobiotechnology,[22] and fluorescence imaging.[23] Yet another benefit of silica-coated particles is based on the different types of functional groups that can be attached to the particle surface using appropriate silane reagents.[23,24]

The introduction of functional groups to the surface of almost any kind of micro- and nanoparticles is also required to enable bioconjugation. Various kinds of functionalized particles have been reported in the literature. Generally, linkers with terminal amino, thiol, or carboxy groups are preferred.[11,19,25] However, the functional groups required for these kinds of conjugation are quite abundant in proteinic biomolecules, a fact that compromises selective conjugation. Moreover, amino groups and carboxy groups are charged in pH 6–8 solution and thus give rise to electrostatic (i.e. unspecific) interactions including adsorption and particle aggregation. The so-called "click-chemistry" is an attractive alternative because the functional groups involved (azido and alkyne) are hardly present in biomolecules including proteins and oligomers. It is therefore said to be "bioorthogonal".[26]

The click reaction, otherwise known as the Huisgen ligation, involves the dipolar cycloaddition of an organic azido group to an alkyne group.[27] The catalytic effect of Cu^+ ions on this cycloaddition was independently discovered by Meldal and co-workers[28] and by Sharpless and co-workers,[29] and this has provided an enormous stimulus to the applications of this reaction.[30] The reagents used are often available in a reasonable number of synthetic steps. Cycloaddition proceeds in high yields, occurs at room temperature in many organic solvents and—most notably in terms of biological applications including bioconjugation[31]—also in aqueous solution at near-neutral pH. Generally, only simple purification steps are required, as a result of almost complete and regioselective conversion into the 1,4-disubstituted 1,2,3-triazole.[30] Furthermore, no protecting groups are needed for the click reaction, as it tolerates a variety of functional groups and shows high kinetic stability.

We perceived that the combination of click chemistry with upconversion technology will lead to new avenues in terms of affinity studies, sensing and labeling, and are reporting here a) on reagents for preparing NPs and μPs for use in click conjugation, b) on respective NPs and μPs with upconversion capability, and c) on how such particles can be labeled with fluorophores and other species by means of the click reaction. We believe that such particles have the potential of being used in numerous areas including upconversion imaging and assays, but can also be modified with various biomolecules to act as vectors for directed labeling in cellular studies. Such nanoparticles are believed to represent an attractive (and bioorthogonal) alternative to acid-functionalized upconverting nanophosphors.[32]

Results

Choice of micro- and nanoparticles: The upconverting microparticles used here (referred to as **μP-1** and **μP-2**) are commercially available and may be ball-milled to various sizes to facilitate their use in bioassays, as shown by Kuningas et al.[14] The surface click chemistry reported here was performed with un-milled particles, mainly for the purpose of completeness, as the main focus of this work is on nanoparticles (UCNPs). The two kinds of nanomaterials (referred to as **NP-1** and **NP-2**) were synthesized as described in the Experimental Section. Table 1 summarizes figures of merit of the materials used.

Table 1. Micro- and nanoparticles used in this work.

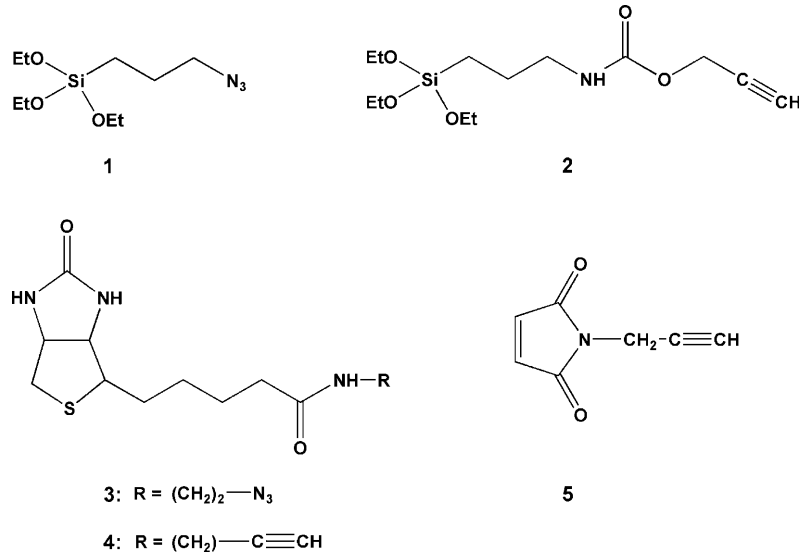
Code	Net formula	Size	Emission color[a]	Dispersible
μP-1	La_2O_2S: Yb,Er	5–15 μm	green and red	no
μP-2	Y_2O_2S: Yb,Tm	5–15 μm	blue and NIR	no
NP-1	$NaYF_4$: Yb,Er	60–90 nm	green and red	fairly well
NP-2	$NaYF_4$: Yb,Tm	50–90 nm	blue and NIR	fairly well

[a] Also see Figures 1 and 3.

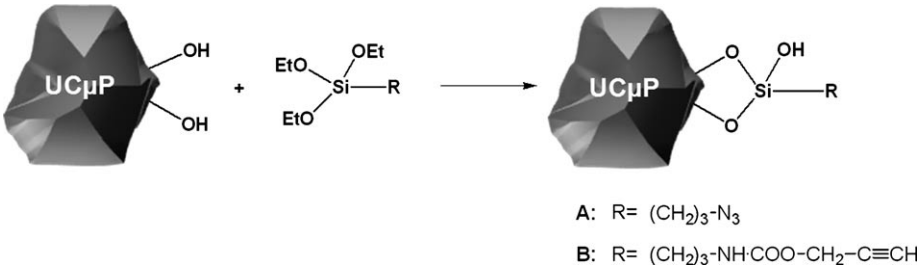
Synthesis of reagents for surface modification: The reagents shown in Scheme 1 were used to introduce an azido group or a terminal alkyne group onto the surface of both the UCμPs and UCNPs. Similar (but more complex) reagents have been used in the past[33] to modify the surface of silica nanoparticles. The introduction of biotin by means of reagents **3** and **4** warrants access to the biotin–(strept)avidin affinity system and the maleinimide **5** allows for selective binding to thiol moieties.

Synthesis and characterization of surface-modified upconverting microparticles (UCμPs): The UCμPs were functionalized by using silanes **1** and **2**. This is possible because of the (partially) oxidic nature of practically all microparticles. Metal oxides at the surface of solid materials usually are present in hydrated form (more precisely as hydroxides; see Scheme 2) in a way comparable to silicium dioxide. Silane reagents, such as aminopropyl-triethoxysilane (APTS),[34] have previously been allowed to react with numerous surfaces including uncommon ones, such as titanium, graphite, iron, or materials used for medical implants, to render them reactive towards biomolecules. This is a quite direct way of functionalization and also does not cause any aggregation of particles.

Figures of merit of the UCμPs thus obtained (type **μP-1** or **μP-2**) are given in Table 2. The UCμPs were characterized by SEM, fluorescence spectroscopy, and IR spectroscopy. Both kinds of particles have a diameter of about 12 μm, but are rather irregular in shape and size. Surface modifica-



Scheme 1. Click reagents used in this work.



Scheme 2. Synthetic route to azido-modified and alkyne-modified upconverting microparticles (UCμPs), respectively.

Table 2. Overview on the surface-modified upconverting microparticles (μPs) used in this work.

Code	Functionality	Preparation
μP-1-A	azido	**μP-1** reacted with **1**
μP-2-B	alkyne	**μP-1** reacted with **2**
μP-1-A-4	biotin	**μP-1-A** clicked with **4**
μP-1-A-6	fluorescent label	**μP-1-A** clicked with label **6**

tion virtually had no effect on the size or morphology of the particles. As can be seen from the SEM image shown in the Supporting Information, the UCμPs are not spherical and show a wide variety in terms of size and shapes.

Infrared spectra were acquired next in order to verify the presence of azido or alkyne groups on the modified UCμPs (see Figure S2 in the Supporting Information). Those of the azido-modified **μP-1** showed a strong peak at 2098 cm^{-1}, which is characteristic of the asymmetric stretch vibration of the azido group.[35] Further peaks are found at 2965 and 2879 cm^{-1} for the asymmetric stretching vibrations of the C–H bonds. The IR spectrum of the alkyne-modified **μP-2** (see Figure S2 in the Supporting Information) reveals a strong peak at 1710 cm^{-1} that is attributed to the C=O bond vibration of the urethane carbonyl group, and a peak at 1538 cm^{-1} characteristic of the amide. The weak peak at 2126 cm^{-1} is assigned to the C≡C stretching vibrations, and peaks at 2977, 2944, and 2887 cm^{-1} to C–H stretches.

The strong green and red intrinsic emission of **μP-1** upon excitation with a $\lambda = 980$ nm diode laser is clearly visible to the naked eye. The emission peaks of **μP-1** are found at $\lambda =$ 546 and 660 nm (Figure 1, solid line). Strong blue (along with considerable near-infrared emission) is observed for microparticles of type **μP-2**, with peaks located at $\lambda = 475$ and 800 nm, respectively (Figure 1, dashed line). The rather sharp and discrete emission lines of the upconverters represent another attractive feature that has a large potential in terms of multiplex signaling and sensing.

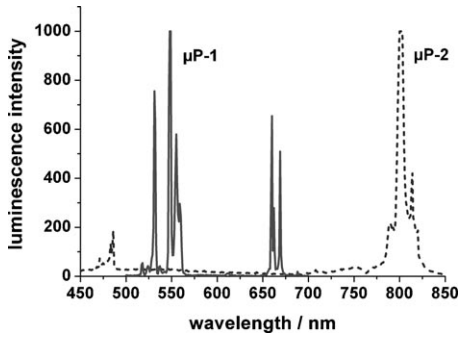
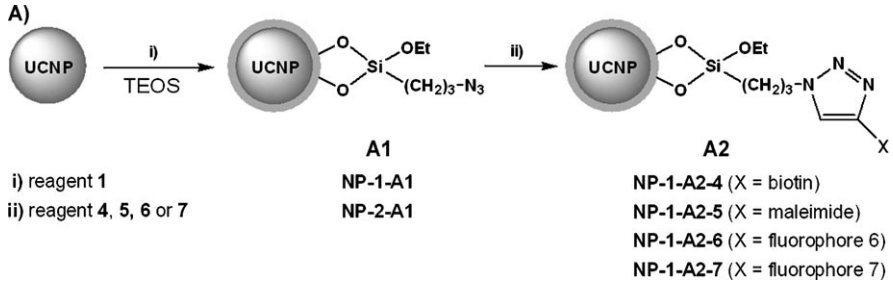


Figure 1. Emission spectra of UCμPs of type **μP-1** (solid line) and **μP-2** (dashed line) following excitation with a 10 mW 980 nm diode laser.

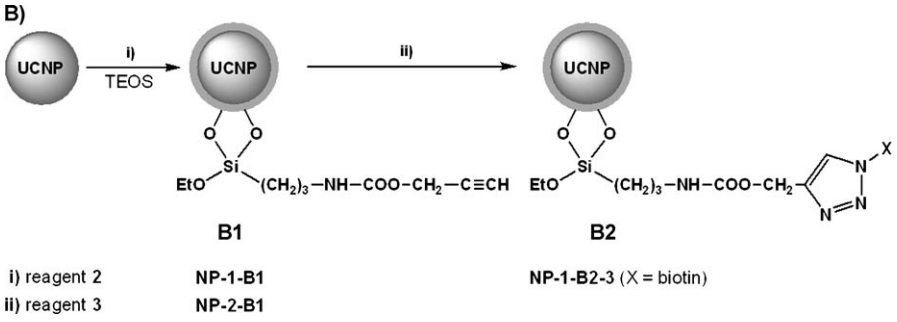
Synthesis and characterization of surface-modified upconverting nanoparticles (UCNPs): The surface chemistry of UCμPs was extended to the respective nanoparticles. The highest upconversion efficiencies to date have been observed for doped hexagonal phase $NaYF_4$.[36] The co-precipitation[37] method chosen here yields cubic $NaYF_4$, which has a moderate upconversion efficiency. A (partial) phase transfer to the more efficiently upconverting hexagonal form was accomplished by tempering the material at 400 °C, as described in the literature.[37] The UCNPs

were first directly functionalized, as described for the UCμPs, owing to the presence of hydroxy functions on the surface of an otherwise non-oxidic material. It is found, however, that UCNPs coated with a (hydrophilic) silica coating are more stable in dispersion. Therefore, the UCNPs were simultaneously coated with silica and click-functionalized by employing a mixture of tetraethoxyorthosilicate (TEOS) and the respective triethoxysilane (**1** or **2**) by using a modified Stöber method.[38] A schematic of this one-step procedure[39] for simultaneous coating and functionalization of UCNPs with either azide or alkyne is given in Schemes 3 and 4, respectively.

The UCNPs were characterized by using TEM, elemental analysis, IR spectroscopy, and fluorescence spectroscopy. The diameter of the nanoparticles (see Table 1) after the annealing process is 60–90 nm for **NP-1** and 50–90 for **NP-2** as determined by TEM (Figure 2, left and Table 3). They are crystalline and almost spherical in shape. Owing to their small size



Scheme 3. Synthetic route to azido-modified nanoparticles (**NP-1-A1** and **NP-2-A1**) possessing a silica shell, and respective click conjugations.



Scheme 4. Synthetic route to alkyne-modified core–shell nanoparticles (**NP-1-B1** and **NP-2-B1**), and respective click conjugations.

Table 3. Figure of merit of the surface-modified UCNPs of type **A1** and **B1**.

Code	Functionality	Size [nm]	Preparation
NP-1-A1	azido	70–100	**NP-1** coated with a mixture of TEOS and **1**
NP-1-B1	alkyne	80–130	**NP-1** coated with a mixture of TEOS and **2**
NP-2-A1	azido	60–100	**NP-2** coated with a mixture of TEOS and **1**
NP-2-B1	alkyne	90–130	**NP-2** coated with a mixture of TEOS and **2**

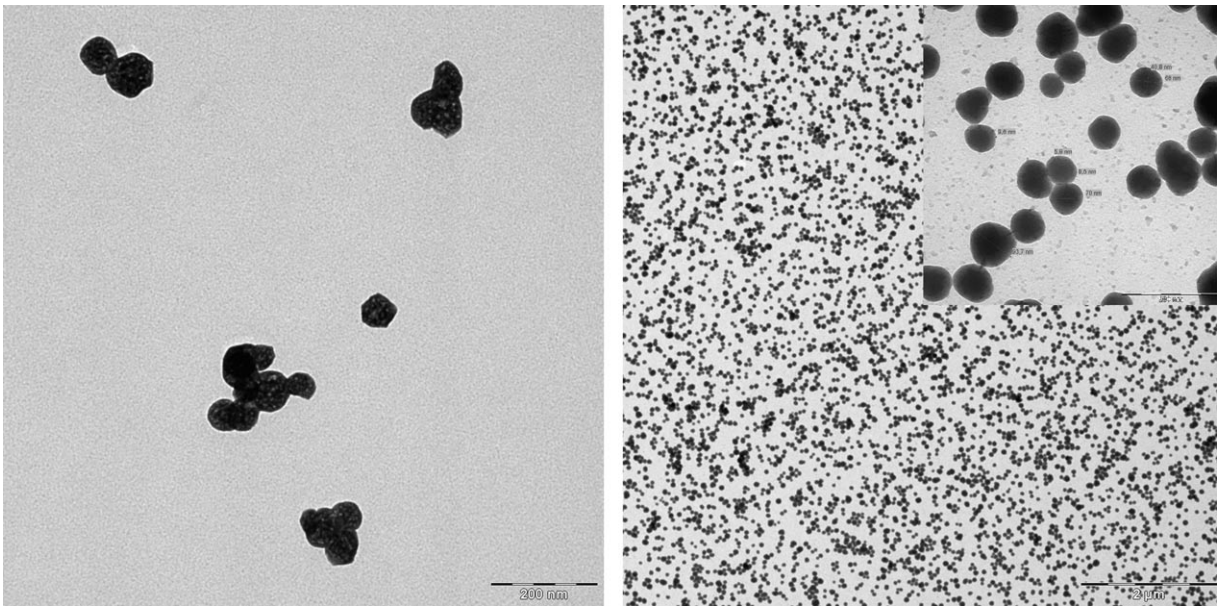


Figure 2. TEM images of annealed UCNPs of type **NP-1** (left) and of silica-coated and azido-modified UCNPs of type **NP-1-A1** (right).

they can be dispersed in water or ethanol, but they tend to aggregate in water solution. The silica-coated UCNPs are almost spherical and uniform in size (Figure 2, right). They can be easily dispersed in water and, in contrast to particles not coated with silica, show little tendency towards aggregation. The particles are stable for several months in aqueous or ethanol dispersions.

The core–shell structure of the particles is visible in the upper right insert of Figure 2. The thickness of the SiO_2 coating is about 10 nm for **NP-1-A1** (azido-modified), 20 nm for **NP-1-B1** (alkyne-modified), 20 nm for **NP-2-A1** (azido-modified) and 20 nm for **NP-2-B1** (alkyne-modified) according to TEM measurements. The thickness of the shell seems to depend on the quantities of TEOS and ammonia employed, but no systematic study was performed on this.[40]

The presence of the azido or alkyne groups was verified by elementary analysis. The fractions of C, H, and N, respectively, are 3.1, 1.2, and 2.4% for **NP-1-A1**, and 3.5, 1.3, and 0.7% for **NP-1-B1**. The high nitrogen content of **NP-1-A1** clearly indicates the presence of azido groups. The presence of nitrogen in **NP-1-B1** results from the presence of the urethane group in the linker. This is equivalent to around 0.57 mmol of azido groups, and to around 0.50 mmol of alkyne groups, respectively, per gram of particles.

As in case of microparticles, IR spectra were acquired to detect the presence of functional groups. The spectra of the azido-NPs (**NP-1-A1**), and the alkyne-NPs (**NP-1-B1**) are shown in Figure S3 in the Supporting Information. The peaks for the azido group, and the alkyne group, respectively are found at the same wavenumbers as discussed for the microparticles.

The emission spectra of the modified nanoparticles were acquired using a 5 W, $\lambda = 980$ nm continuous wave diode laser as the excitation light source. Particles doped with ytterbium and erbium (type **NP-1**) give green and red emissions (Figure 3), those doped with ytterbium and thulium (type **NP-2**) give blue and NIR emission (Figure 3). Peaks maxima are at the same wavelengths as those of the UCμPs with surface modification (type **μP-1** and **μP-2**). The UCNPs require stronger laser excitation than the UC-μPs in order to cause efficient upconversion.[41]

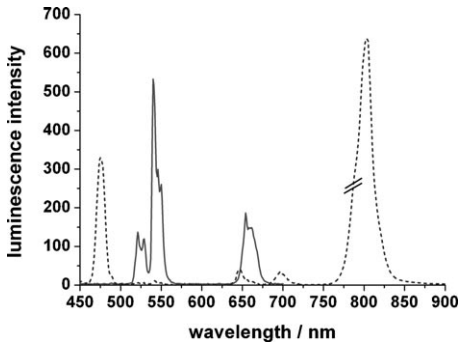


Figure 3. Emission spectrum of upconverting nanoparticles of type **NP-1-A1** (solid line) and **NP-2-A1** (dashed line) suspended in aqueous solution and photoexcited with a 980 nm laser.

Functionalization of the particles by using click chemistry: Next, the nanoparticles were functionalized with groups to make them conjugatable to biomolecules. Such "particle labels" are highly-attractive alternatives to the widely used, but toxic quantum dots of the CdSe type. Two versatile functions were chosen. The first is the maleinimide group, which undergoes addition to thiol groups at room temperature and in aqueous solution. It is widely used to label proteins. The second is the biotin group which is the "work horse" in numerous labeling protocols, because of its very-strong interaction ($K_D \approx 10^{-14}$ M) with (strept)avidins. The functionalized UCNPs listed in Table 4 were obtained by clicking the nanoparticles of Table 3 to the click reagents **3**–**5** of Scheme 1.

Table 4. Figure of merit of the bioreactive UCNPs.

Code	Functionality	Reaction
NP-1-B2-3	biotin	**NP-1-B1** clicked to **3**
NP-1-A2-4	biotin	**NP-1-A1** clicked to **4**
NP-1-A2-5	maleinimide	**NP-1-A1** clicked to **5**
μP-1-A-4	biotin	**μP-1-A** clicked to **4**

The azido-modified biotin **3** was clicked to alkyne modified **NP-1-B1** to give **NP-1-B2–3**. The presence of the functional groups was verified by means of diffuse reflectance FT-IR spectroscopy, as shown in Figure S4 in the Supporting Information. The spectra were acquired by using the respective alkyne- or azido modified **NP-1** as a reference material. The negative peak at 2131 cm^{-1} in the difference spectrum (see Figure S4a in the Supporting Information) indicates that the alkyne functionality has disappeared. The C–H stretch vibration is visible at 2983 and 2908 cm^{-1}. The peaks at 1665 and 1598 cm^{-1} are assigned to the C=O and C–N amide stretches.

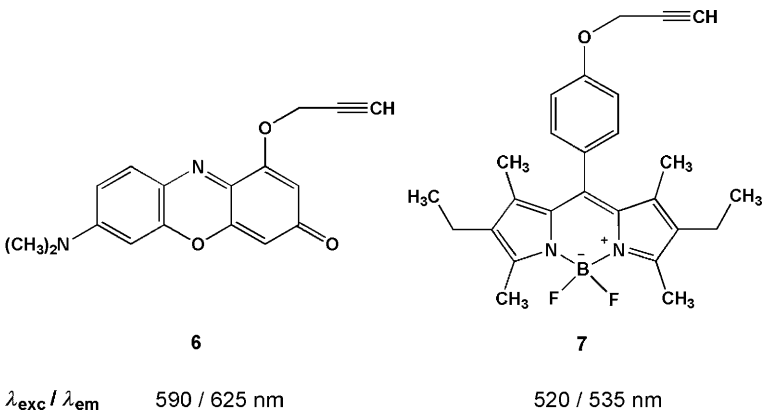
Alkyne-modified biotin **4**, in turn, was clicked to azido-modified **NP-1-A1** to give **NP-1-A2–4**. The disappearance of the azido group is indicated by the negative peak at 2108 cm^{-1}.The C–H stretches are found at 3003 and 2906 cm^{-1}. The C=O stretch vibration is located 1709 cm^{-1}, and the C–N amide stretch at 1542 cm^{-1} (see Figure S4b in the Supporting Information).

Finally, the alkyne-modified maleinimide **5** was clicked to azido-modified **NP-1-A1** to give **NP-1-A2–5**. The absence of the azido functionality is indicated by the negative peak at 2114 cm^{-1}. The C–H stretches are located at 3998 and 2906 cm^{-1}. The maleinimidic C=O stretches are found at 1788 and 1724 cm^{-1}. The C=C stretching peak is located at 1582 cm^{-1} (see Figure S4c in the Supporting Information). The alkyne-modified biotin **4** also was clicked to azido-modified microparticles (**μP-1-A**) to give **μP-1-A-4**. Its differential IR spectrum (see Figure S5 in the Supporting Information) indicates that the same surface chemistry has been accomplished as with nanoparticles **NP-1-A2–4**.

Fluorescent labeling of the particles by using click chemistry: Azido- and alkyne-modified particles offer another at-

tractive possibility in that they may be click-labeled with organic fluorophores to obtain particles that emit various colors depending on whether the upconverting (inorganic) core is photoexcited, or the (organic) fluorophores of the shell. The luminescence of the upconverter is generated under NIR excitation, here at $\lambda = 980$ nm, whereas the luminescence of the organic fluorophore appears under excitation with visible light. Dually excitable particles with dual emissions are likely to have even more sophisticated applications than those outlined in the Introduction for conventional upconverters.

To investigate this, two clickable fluorescent labels (see Scheme 5) were chosen for performing the following experiments. First, the alkyne-modified purple fluorescent dye **6** (Table 5) was clicked to azido-modified UCμPs of type **μP-1-A** to give **μP-1-A-6**, which was investigated by fluorescence microscopy and fluorescence spectroscopy. A reference sample, obtained without using the copper(I) catalyst, showed no trace of the dye after washing the particles. In contrast, the UCμPs clicked to the fluorophore **6**, display the typical orange fluorescence of the oxazine label. A fluorescence microscopy image of the labeled microparticles under visible-light excitation ($\lambda = 550$ nm) is given in Figure S6 in the Supporting Information. The orange-colored emission of the fluorophore covalently attached to the surface of the particles is clearly visible.



Scheme 5. Alkyne-modified fluorescent dyes for click labeling of UCμPs and UCNPs.

Table 5. Figure of merit of the fluorescently labeled UCμPs and UCNPs.

Code	Functionality	Preparation
μP-1-A-6	fluorophore **6**	**μP-1-A** clicked with **6**
NP-1-A2-6	fluorophore **6**	**NP-1-A1** clicked with **6**
NP-1-A2-7	fluorophore **7**	**NP-1-A1** clicked with **7**

Similarly, the azido-modified nanoparticles of type **NP-1-A1** were clicked to **6** and **7** to give **NP-1-A2–6** and **NP-1-A2–7**, respectively. The upconversion emission spectrum (under $\lambda = 980$ nm excitation) and the conventional emission spectrum of the organic label (under $\lambda = 520$ and 590 nm excitation, respectively) are given in Figure 4. The absorption and emission of fluorophore **6** (like that of all oxazines of the Nile Red type) is strongly solvatochromic, but if covalently bound to the surface of particles of type **NP-1-A2**, the excitation and emission maxima are rather invariable and found at $\lambda = 590$ and 625 nm, respectively, both in water and ethanol suspension, indicating stronger interaction with the surface than with the solvent.

Discussion

The method described here for chemically modifying the surface of upconverting μPs and NPs is straightforward and versatile. It is likely to be applicable to various other materials possessing oxidic (and thus hydroxylic) surfaces. It makes surfaces compatible with click chemistry, which again is highly versatile, particularly with respect to bioconjugation because it is bioorthogonal and thus is not troubled by functional groups often present in biological samples. In fact, click chemistry has been applied to NPs made from silica,[24,42] gold,[43] iron oxide,[44] and various polymers.[45,33] The one-step method introduced here is primarily intended for uses in context with bioassays. It is much simpler than two-step methods, such as those used for modifying UCNPs with amino groups.[11] The purification of UCNPs by size exclusion chromatography (SEC) is another attractive feature of the work presented here. When applying SEC, the NPs can be kept in solution at any time and aggregation is minimized.

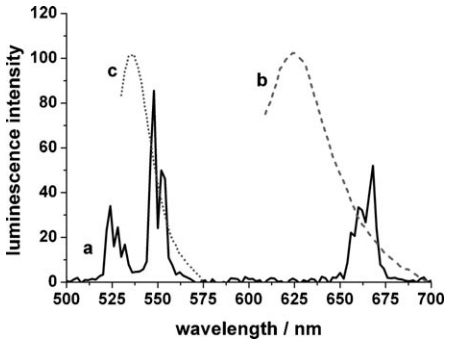


Figure 4. Dual emission of nanoparticles of type **NP-1-A2–6** and **NP-1-A2–7**. a) Upconversion emission (at 980 nm excitation); b) emission of label **6** (photoexcitation at 590 nm); c) emission of label **7** (photoexcitation at 520 nm).

Click reaction of the reactive micro- and nanoparticles gave particles that can be conjugated to a) (strept)avidin[39, 46] (via the biotin group), and b) thiols[47] (via the maleinimide groups). In a further version, it leads to particles carrying organic fluorescent labels. Thiol labeling is clearly to be favored in bioconjugation over amine labeling, simply because most proteins possess numerous free amino groups (this leading to random labeling and to varying dye-to-protein ratios), but often only one to three thiol groups. Human serum albumin (HSA), for example, has one free thiol group only, but more than 50 amino groups that may be labeled.

The UCμPs and UCNPs have attractive spectral features in giving dual emissions depending on the wavelength of excitation. If excited with NIR light, dual emission of the inorganic core is observed (green and bright red, or blue and dark red). If excited in the visible, the (single) emission of the organic fluorophore (green or orange) is being seen (Figure 4). Moreover, by varying the quantity of organic label, a wide range of intensities can be adjusted for both the "inorganic" and "organic" emission, thus giving a 2-dimensional matrix of two (or three) intensities (and possibly also lifetimes) that enables unambiguous encoding of particles. We assume that if organic dyes are used that absorb at one of the two emission bands of the upconverters, various ratios of intensities of the dual emission may be adjusted.

Experimental Section

(3-Azidopropyl)triethoxysilane (1): Dry(!) sodium azide was added to a solution of (3-chloropropyl)triethoxysilane in dry acetonitrile. Tetrabutylammonium azide[48] was added, as phase transfer catalyst, and the mixture was stirred under reflux for 70 h. The solid was filtered off and the solvent removed on a rotary evaporator. The colorless residue was dissolved in dichloromethane and washed three times with doubly distilled water. The organic phase was dried over disodium sulfate and the solvent removed on a rotary evaporator to give the product as a colorless liquid. Yield 3.46 g (76%). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 3.82$ (q, $J = 7.0$ Hz, 6H), 3.26 (t, $J = 6.9$ Hz, 2H), 1.71 (m, 2H), 1.23 (t, $J = 7.0$ Hz, 9H), 0.67 ppm (m, 2H); ^{13}C NMR (300 MHz, $CDCl_3$): $\delta = 58.5$, 53.8, 22.7, 18.3, 7.6 ppm; IR(neat): $\tilde{\nu} = 2978$, 2934, 2892, 2095 cm^{-1}; CI-MS: m/z: M^+ calculated: 247.1; found MH^+ 248.1.

***O*-(Propargyloxy)-*N*-(triethoxysilylpropyl)urethane (2)**: Compound **2** was purchased from ABCR (www.abcr.de) and used without further purification.

Azido-biotin (3): Compound **3** was synthesized by a modification of the method described in the literature[49] using 2-azidoethylamine[50] as the amino linker. Commercially available biotin NHS ester was dissolved in dry dimethylformamide (DMF), and 2-azidoethylamine was added. Triethylamine was added and the solution stirred overnight at room temperature. The solvent was evaporated to give the crude product as a yellow, oily substance. It was crystallized from ethanol to give the product **2** as a colorless solid. The mass and NMR-spectra are in agreement with literature data.

Propargyl-biotin (4): Compound **4** was synthesized by dissolving biotin, dicyclohexylcarbodiimide, and 1-hydroxybenzotriazole in equimolar quantities in dry DMF. After stirring for 10 min, an equimolar quantity propargylamine was added dropwise and the mixture stirred for another 4 h at room temperature. The solvent was removed under reduced pressure to give an orange oil. Addition of little methanol resulted in the precipitation of *N*,*N*′-dicyclohexylurea, which was filtered off. The methanol in the filtrate was then removed under reduced pressure. The crude product was then purified by column chromatography using a gradient of methylene chloride and methanol as eluents to yield propargylbiotin (**4**) as a white solid. The mass and NMR spectra were in agreement with literature data.[51]

Propargyl-maleimide (5): Compound **5** was synthesized from maleic acid anhydride and propargylamine in a two-step synthesis.[52] Maleic acid anhydride (4.9 g, 50 mmol) was dissolved in 25 mL of acetone and heated to reflux. Propargylamine (2.75 g, 50 mmol) was separately dissolved in acetone and added dropwise to the refluxing solution of the anhydride. The solution was stirred under reflux for 1 h and the solvent then removed on a rotary evaporator to give a purple solid. The crude product was recrystallized from a 1:4 mixture of diethyl ether and methanol to give the respective (open-chain) amidic acid as a colorless solid. Yield: 4.58 g (60%). ^{1}H NMR (300 MHz, [D_6]DMSO): $\delta = 13.88$ (s, 1H), 9.15 (s, 1H), 6.32 (d, $J = 12.3$ Hz, 1H), 6.26 (d, $J = 12.1$ Hz, 1H), 3.97 (q, $J = 2.5$ Hz, 2H), 3.19 ppm (t, $J = 2.6$, 1H); CI-MS: m/z: M^- calculated: 153.1, found 153.0.

The ring closure of *N*-propargylmaleamic acid to give maleimide **5** was accomplished by dissolving *N*-propargylmaleamic acid (3.5 g, 23.5 mmol) in xylene (100 mL) and stirring under reflux for 8 h by using a water trap. The solution was allowed to cool to room temperature and filtered. The solvent was removed on a rotary evaporator to obtain the product as a pale-yellow solid. Yield: 0.97 g (31%). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta =$ 6.76 (s, 2H), 4.29 (d, $J = 2.5$ Hz, 2H), 2.21 ppm (t, $J = 2.6$ Hz, 1H); ^{13}C NMR (300 MHz, $CDCl_3$): $\delta = 169.1$, 134.5, 71.6, 26.8 ppm. EI-MS: m/z: M^+ calculated: 135.1, found 135.1.

Upconverting microparticles (UCμPs): Two types of commercially available UCμPs, referred to as **μP-1** and **μP-2** (see Table 1) were used. Microparticles of type **μP-1** are composed of La_2O_2S and doped with ytterbium and erbium ions, those of type **μP-2** consist of Y_2O_2S and are doped with ytterbium and thulium ions. Both were obtained from Mol-Tech GmbH Berlin (www.mt-berlin.com). Owing to their relatively large size, they cannot be solubilized in water or organic solvents. Therefore, when using the microparticles in solution, vigorous stirring is required to prevent sedimentation of the particles. Alternatively, the particles may be placed in viscous aqueous solutions of hydrogels such as polyurethane, poly(vinyl alcohol), or polyacrylamide.

Surface modification of upconverting microparticles (UCμPs): The UCμPs were washed with acetic acid prior to silanization to remove ammonium ion (which is ubiquitous on the surfaces of particles). For example, UCμPs (200 mg) of type **μP-1** were rinsed three times with acetic acid (2 mL, 0.1 M) and washed several times with doubly distilled water. The particles were filtered by using a suction filter and then dried overnight in a drying furnace. The activated UCμPs were then suspended in dry toluene (20 mL) in a 100 mL Schlenk flask and flushed with dry nitrogen. The respective silane (**1** or **2**, 200 μL) were added and the mixture was stirred for 48 h at 90 °C. The mixture was allowed to cool and the particles were separated by centrifugation for 15 min at 4000 rpm. After removal of the supernatant, the particles were washed several times with ethanol and acetone, and finally dried in a furnace at 60 °C.

Synthesis of upconverting nanoparticles (UCNPs): The UCNPs (referred to as **NP-1** and **NP-2**; see Table 2) were synthesized using the established[37] co-precipitation method. Solutions (0.2 M) of the trichlorides of the ions Y^{III} and Yb^{III}, along with either Er^{III} or Tm^{III} were combined with an EDTA solution to form the respective EDTA complexes. This solution (around 40 mL) was injected quickly into a 3.5% aqueous solution of sodium fluoride. The resulting mixture was stirred for 1 h at room temperature to give a colorless precipitate that was separated by centrifugation at 4000 rpm for 30 min, washed 3 times with water and then once with ethanol. The precipitate was dried in a drying furnace and under vacuum. The colorless powder was tempered at 400 °C for 4.5 h under argon atmosphere.

Coating and surface modification of UCNPs: The particles were coated with a silica shell by means of a modified Stöber process.[38] In a typical experiment, UCNPs (75 mg, **NP-1** or **NP-2**) were dispersed in ethanol (10 mL) by ultrasonication. Subsequently, the mixture was heated to 40 °C, and water (500 μL) and ammonia (500 μL, 25 wt%) were added,

followed by tetraethoxysilane (TEOS, 375 μL). The solution was stirred for 30 min. The respective functionalized silane (**1** or **2**; 25 μL) was added and the mixture stirred for another 2.5 h. After cooling to room temperature, the particles were purified by size exclusion chromatography (on Sephadex LH-60) by using ethanol as the eluent to remove unreacted TEOS and ammonia. The proper fraction can be recognized by its slight turbidity and by illuminating it with a $\lambda = 980$ nm laser, which results in visible luminescence. The resulting silica-coated UCNPs were stored in ethanol solution to avoid aggregation. To transfer the particles into aqueous solution, the reaction mixture from the Stöber coating process was diluted with water and purified again by size exclusion chromatography using Sephadex G-75 (which is better water-compatible than LH-60) with distilled water as the eluent. The resulting suspensions in either ethanol or water are stable for several months.

General procedure for performing click reactions: Click reactions were generally performed in water/ethanol mixtures (1:10 to 10:1; v/v) by adding a mixture of copper(II) sulfate and ascorbic acid. Catalytic quantities of triethylamine accelerate cycloaddition. In a standard procedure, 0.5–1 mg of one of the clickable reagents (**3**–**7**) is added to a dispersion of the respective UCNP (2 mL, 1 mg mL^{-1}) in a water/ethanol (1:1) mixture. Thereafter, an aqueous solution of copper sulfate (10 μL, 20 mM), a solution of sodium ascorbate (10 μL, 0.1 M), and triethylamine (10 μL) are added. The mixture is stirred at room temperature for 14 h. Experiments without the copper catalyst were performed in parallel at all stages. In case of microparticles, the solutions were centrifuged and washed several times with water and ethanol. In case of nanoparticles, work up was accomplished by size exclusion chromatography (Sephadex LH-60) to remove unreacted dyes, any biomolecules, and catalyst.

Instrumental techniques: Scanning electron microscopy (SEM) images (such as Figure S1 in the Supporting Information) were acquired by using a Jeol (www.jeolusa.com) instrument (JSM 800). Transmission electron microcopy (TEM) images were acquired by using a 120 kV Zeiss (www.smt.zeiss.com) instrument of type Leo 912AB equipped with a Proscan CCD (www.proscan.de) camera. Fluorescence microscopy images (see Figure S6 in the Supporting Information) were acquired by using a Leica DMRE microscope (www.leica.de). A mercury lamp acted as the light source, and the excitation band ($\lambda = 515$ to 560 nm) was adjusted by using a bandpass filter. Fluorescence was collected after it had passed a longpass filter with a cut-off wavelength of 590 nm. ^{1}H NMR and ^{13}C NMR spectra were recorded by using an Avance 300 NMR-spectrometer from Bruker Bio Spin (www.bruker-biospin.com). Tetramethylsilane (TMS) was used as the internal standard. DR-FTIR spectra (see Figures S2–S5 in the Supporting Information) were acquired on a FT/IR 6100 IR spectrometer distributed by Jasco (www.jascoinc.com). A diffuse reflectance accessory (EasiDiff) provided by Pike Technologies (www.piketech.com) was used for sample preparation. Luminescence spectra of particles and fluorescence spectra of dyes were recorded by using a Cary Eclipse fluorometer (from Varian; www.varianinc.com). A 980 nm fiber-optic diode laser (5 mW cw; from Roithner Lasertechnik; www.roithner-laser.com) was used as the light source for upconversion photoexcitation.

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