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In vivo Deep-Tissue Microscopy with UCNP/Janus-Dendrimers as Imaging Probes: Resolution at Depth and Feasibility of Ratiometric Sensing

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Abstract

Lanthanide-based upconverting nanoparticles (UCNPs) are known for their remarkable ability to convert near-infrared energy into higher energy light, offering an attractive platform for construction of biological imaging probes. Here we focus on *in vivo* high-resolution microscopy - an application for which the opportunity to carry out excitation at low photon fluxes in non-linear regime makes UCNPs stand out among all multiphoton probes. To create biocompatible nanoparticles we employed Janus-type dendrimers as surface ligands, featuring multiple carboxylates on one 'face' of the molecule, polyethylene glycol (PEG) residues on another and Eriochrome Cyanine R dye as the core. The UCNP/Janus-dendrimers showed outstanding performance as vascular markers, allowing for depth-resolved mapping of individual capillaries in the mouse brain down to a remarkable depth of ~1000 μm under continuous wave (CW) excitation with powers not exceeding 20 mW. Using *a posteriori* deconvolution, high-resolution images could be obtained even at high scanning speeds in spite of the blurring caused by the long luminescence times of the lanthanide ions. Secondly, the new UCNP/dendrimers allowed us to evaluate the feasibility of quantitative analyte imaging *in vivo* using a popular ratiometric UCNP-to-ligand excitation energy transfer (EET) scheme. Our results show that the ratio of UCNP emission bands, which for quantitative sensing should respond selectively to the analyte of interest, is also strongly affected by optical heterogeneities of the medium. On the other hand, the luminescence decay times of UCNPs, which are independent of the medium properties, are modulated *via* EET only insignificantly. As such, quantitative analyte sensing in biological tissues with UCNP-based probes still remains a challenge.

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Supporting Information. Additional experimental details, synthesis, optical spectroscopic data, materials characterization.

Introduction

Development of new probes remains one of the key objectives in the optical imaging field, driven by the ever-growing demand for higher imaging speed, better resolution and improved selectivity. Probes capable of multiphoton absorption are particularly useful for imaging in scattering biological tissues, where non-linear excitation can offer major improvements in depth resolution while simultaneously lowering the risk of photodamage.¹ Today multiphoton microscopy is the tool of choice for deep-tissue imaging, particularly in neuroscience, where relevant physiological processes in rodent brain can be observed just a few hundred microns under the tissue surface.^{1b, 2}

A variety of probes for two-photon (2P) imaging have been developed over the years, ranging from small-molecule chromophores and fluorescent proteins to various nanoscopic imaging agents.³ In all such materials 2P excitation (2PA) requires nearly simultaneous action of two photons, and consequently it has to be performed using pulsed ultrafast lasers in order to attain the necessary photon fluxes.⁴ However, there exists a class of agents, known as lanthanide-based upconverting nanoparticles (UCNPs), in which 2PA or even higher order absorption can occur under low-energy continuous wave (CW) excitation.⁵ In a UCNP crystal two infrared (IR) photons are absorbed sequentially, thanks to the intermediate energy storage provided by the long-lived excited states of lanthanide ions.⁶ The nanocrystal lattice facilitates energy migration between the ions, until two excitations meet and generate an upper excited state, which decays with emission of a higher energy photon. Consequently, UCNPs exhibit apparent 2PA cross-sections orders of magnitude higher than even the most efficient conventional 2P absorbers,⁷ eliminating the need for pulsed lasers and potentially bringing 2P microscopy methods to any laboratory possessing a regular light microscope.

CW excitation at near-infrared wavelengths (e.g. 980 nm for the most commonly used sensitizer ion Yb³⁺) results in virtually zero background fluorescence and exceptionally high signal/noise ratios (SNR). In addition, the luminescence decay times of UCNPs are on the order of 10⁻⁵-10⁻⁴ s, which enables time-gated imaging and affording even higher SNRs. Because lanthanide ions in UCNPs are firmly held in the crystal lattice, potential metal toxicity can be avoided.⁸ At the same time, UCNPs are exceptionally photostable, which is especially important for single molecule/particle type applications.⁹ Finally, in contrast to microsecond-scale luminescence of molecular probes, luminescence of UCNPs is insensitive to molecular oxygen, which can be advantageous for a number of biological applications. Overall, the above properties make UCNPs a very attractive platform for construction of imaging probes,^{5c, d, 10} especially when excitation needs to be performed at depths within tissue.^{7b, 10f, 11} However, examples of their use so far have been limited only to proof-of-principle demonstrations, suggesting that the niche for UCNPs in imaging and other biomedical applications is yet to be found.

Due to their relatively large size and intrinsic polyvalency UCNPs as labels for specific biological molecules¹² pose several challenges, which are common for all nanoscopic agents. Large probes inevitably alter properties of target bio-molecules and/or may act as centers for attachment of multiple molecules, causing aggregation and eventually loss of

solubility. Furthermore, most biological targets are located inside cells, where they may be visualized using genetically encodable probes, thereby avoiding complicated intracellular probe delivery issues. Hence, UCNPs are arguably more appropriate for extracellular imaging, where they can be used for sensing small-molecule analytes, pH, metal ions etc.

While the literature contains multiple examples of UCNPs as imaging probes, their application specifically as probes for high-resolution multiphoton microscopy remains underexplored. In our earlier work we have established that appropriately functionalized UCNPs indeed can operate as probes for two-photon depth-resolved microscopy *in vivo* under low-power CW excitation.¹³ The quadratic dependence of the excitation rate on photon flux allows confinement of UCNP luminescence to the immediate vicinity of the laser focus, dramatically increasing resolution, especially in the axial (Z) dimension. However, a significant drawback is the UCNPs extremely long emission lifetimes (many microseconds). In scanning microscopy applications long-lived emission can cause loss of resolution due to the excited state saturation and/or pixel smearing. In order to define the limits of applicability of UCNPs as probes for multiphoton microscopy we set out, in this account, to investigate the relationship between the resolution at depth, excitation fluxes and scanning speeds, attainable using UCNPs. To avoid complications associated with intracellular delivery, we focused on an application that requires highly stable blood-pool imaging agents, but does not depend on probes' cellular permeability and/or organ accumulation, namely on two-photon microangiography in the brain.

Our second goal was to evaluate the utility of a popular analyte-sensing scheme based on UCNP-to-ligand excitation energy transfer (EET)^{10g, 14} in the context of *in vivo* environments. EET has been widely claimed as a method to impart sensitivity of UCNPs' luminescence to external stimuli,^{10a, 15} whereby the UCNP emission bands are modulated by peripherally-coupled chromophores, whose own optical spectra are modulated upon interaction with analyte species.^{10b, 10d, 10g, 16} Our method of UCNP modification allowed us to position pH-sensitive chromophores at the particles' boundary (see below), thereby facilitating the EET and rendering the UCNP emission pH-sensitive. However, as we show below, the ratio of the UCNP emission bands, while being responsive to changes in pH, was also found to be very sensitive to the absorption by the medium, showing that quantitative sensing using the above ratiometric scheme in optically heterogeneous environments is not feasible.

In our original study we used hydrophilic carboxylate-terminated dendrimers as polydentate ligands to impart UCNP solubility.¹³ The choice of dendrimers was motivated by their intrinsic polyvalency and pseudo-globular shape, which was deemed to simultaneously enhance binding of the ligands to the surfaces and keep the nanoparticles stable in solution.¹⁷ However, the necessity to maintain the persistence of shape¹³ required construction of rather large dendrimers and hence was synthetically expensive. In addition, the stability of carboxylate-terminated dendrimers could be compromised by divalent cations, while the multiple negative peripheral charges could pose problems if the UCNPs were to be used as sensors of charged species.¹⁸

In the present study we extended our approach by employing Janus-type dendrimers,¹⁹ having multiple carboxylates on one ‘face’ of the ligand for binding to UCNP surfaces, neutral polyethyleneglycol (PEG) groups for aqueous solubility and a pH-sensitive dye Eriochrome Cyanine R (ECR) as the dendrimer core. While many approaches to UCNP solubilization have been developed over the years,²⁰ the dendrimerization method stands out as synthetically precise, tunable and potentially suitable for incorporation of multiple dye motifs near UCNP surfaces simultaneously with hydrophilic encapsulation. Compared to the previously developed dendrimers,¹³ the new Janus ligands offer several advantages, including significantly higher colloidal stability of the respective nanoparticles in aqueous solutions, especially those containing high concentrations of divalent metal cations. In addition, Janus ligands of lower dendritic generations can be used without jeopardizing the ligands’ solubilizing capacity. Lower generations entail smaller size and simpler less laborious synthesis.

The presentation below is organized as follows: (1) the development of Janus dendritic ligands and characterization of the UCNP/Janus-dendrimers’ stability, biocompatibility and bio-distributions; (2) two-photon brain angiographic imaging using UCNP/Janus-dendrimers; (3) evaluation of the EET-based analyte-sensing scheme. Our results demonstrate impressive practical advantages of UCNP-based probes, but also highlight challenges in the development of UCNPs into quantitative analyte-specific tissue-level imaging agents.

Results and Discussion

Synthetic development of Janus-type dendritic ligands.

In a Janus-type dendrimer one face of the molecule can be engineered to have high affinity for the nanoparticle surface, while another can be made hydrophilic and neutral to interface with the solvent. Conceptually similar ligands for UCNP modification have been prepared in the past, where PEG residues were appended to tricarboxylic or phosphoric acid binding arms.^{20a, 20e, 21} The key advantage of the dendritic system is its polyvalency, since multiple binding groups on the ligand serve to strengthen the contact between the ligand and the nanoparticle.

Polyglutamic dendrons, used by us previously,¹³ were chosen initially as the structural building blocks, while commercially available pH-sensitive dye Eriochrome Cyanine R (ECR)²² was chosen as a core of the scaffold. ECR has two carboxylic acid groups, thus providing a straightforward route to a Janus-type structure, and has deep color, which significantly simplifies detection of bands during chromatography. Absorption spectra of the protonated and deprotonated forms of ECR overlap differently with the emission spectra of UCNPs, and thus EET from UCNP onto ECR provides basis for ratiometric pH sensing.^{13, 23}

The structures of the dendritic Janus ligands synthesized in this study are shown in Scheme 1 along with a cartoon schematically representing a typical reaction sequence. The detailed scheme and complete description of the synthesis can be found in the SI (Schemes S3 and S4).

Two types of dendrons were used in our syntheses: glutamates and commercially available Newkome-type trifurcated dendrons.²⁴ Glutamic dendrons were assembled by the convergent method²⁵ as described earlier¹³ (SI, Scheme S2). *mono*-Amidation of the *bi*-carboxylic ECR core (Step 1), which is readily available, could be carried out simply using an excess of ECR with respect to dendron D1. After purification by size-exclusion chromatography (SEC) pure *mono*-dendronized ECR was isolated in 70–75% yield. Addition of the second orthogonally protected dendron D2 in Step 2 yielded the target Janus system. In this case, an excess of the reactant (D2) was used to facilitate coupling, since the remaining free carboxylic acid anchor point on the core ECR was shielded by the already attached branch.

Our previous work on dendritically protected oxygen probes²⁶ has shown that modification with polyethylene glycols (PEG) can be efficient for converting even extremely hydrophobic dendrimers into completely water-soluble inert macromolecules. At the same time, PEGylation is known, at least in some cases, to reduce the rate of systemic clearance. Based on these considerations, our Janus dendritic ligands were designed to possess a PEG-interface for aqueous solubilization. PEGylation of the Janus dendrimers was carried out using monomethoxy-PEG-amines,²⁷ ensuring that the resulting linkages can sustain base-mediated hydrolysis as well as action of non-specific esterases *in vivo*.

Removal of the more labile protecting group (*t*Bu) in Step 3 generated dendrimers having free carboxyl groups, which were introduced into the subsequent amidation reactions with monomethoxy-PEG-amines (Av. MW 1000, 2000 and 5000) in Step 4. UV/Vis absorption analytical measurements confirmed that complete PEGylation was achievable, but required longer reaction times as the PEG size increased. Finally, removal of the ethyl ester groups in Step 5 by basic hydrolysis gave the target Janus dendrimers **6** and **11**.

Modification of UCNPs with Janus-dendrimers.

The modification was performed simply by mixing dimethylformamide (DMF) or dimethylsulfoxide (DMSO) solutions of UCNP-BF₄⁻ with aqueous solutions containing excess of the dendrimers, ca ~10⁶-10⁷ dendrimer molecules per nanoparticle. Upon mixing, the solutions appeared optically clear, and after centrifugation (15 min, 8000 g) UCNP-dendrimers separated from the mother liquor (Fig. 1a). By repeating the washing/centrifugation cycle several times using deionized water, UCNP-dendrimers could be isolated as gel-like materials, easily detectable by their color as well as by their ability to emit green light upon illumination at 980 nm. The gels could be completely re-dissolved by agitation. However, if centrifugation was performed at greater speeds or for longer times, the UCNP morphology changed from gel-like to finely dispersed powder, which resisted re-dissolution in water or DMF. Similarly, drying of the UCNP-dendrimers led to the irreversible loss of solubility¹³ - an effect common for hydrophilically modified nanoparticles.

Because of the inability to dry UCNP-dendrimers without affecting their solubility, measurements of their exact concentrations were performed through quantification of the lanthanide ions, using inductively coupled plasma optical emission spectroscopy (ICP-OES) with a suitable set of calibration standards containing Er³⁺, Yb³⁺ and Y³⁺ ions (in the range

of 1–10 ppm). The concentrations obtained by this highly accurate method were very close to the theoretically predicted based on the masses of the precursor UCNP, assuming complete dendrimerization. Similarly, the absorbance values at the peak of the ECR core could be cross-referenced with the concentrations based on the atomic spectroscopy measurements, providing a rough concentration estimate.

Transmission electron microscopy (TEM) images of UCNP-dendrimers showed no changes neither in the particles' morphology nor in their size upon functionalization and dissolution in water (Fig. 1b and Fig. S1). The particles remained highly mono-disperse and free of aggregation. Based on the optical absorption measurements (for ECR $\epsilon_{576} \sim 40,000 \text{ M}^{-1} \text{ cm}^{-1}$) and taking into account the single particle mass ($\sim 6.36 \times 10^{-17} \text{ g}$ for a spherical particle, $\sim 30 \text{ nm}$ in diameter; density $\sim 4.5 \text{ g/cm}^3$) and the known total mass of the particles in a sample, the average number of the dendrimer molecules at the surface of the particle was estimated to be ~ 450 , which is in an excellent agreement with the number calculated by interpolation of the data obtained previously for other dendritic UCNP (~ 470).¹³ At such surface density, the average area per dendritic ligand is $\sim 630 \text{ \AA}^2$, which corresponds to a circle $\sim 28 \text{ \AA}$ in diameter - a reasonable footprint for an octacarboxylic Glu3 glutamic arm. These data suggest that the UCNP surfaces are covered by the dendritic ligands relatively densely, having a total of ~ 4000 PEG residues per particle.

Solubility and stability of UCNP-dendrimers in aqueous solutions.

The stability of UCNP-dendrimers in aqueous media was evaluated by recording optical absorption spectra of their solutions as a function of time (Fig. 1c and Fig. S2). For UCNP that are unstable in solution, aggregation should lead to a gradual increase in scattering, followed by a decrease in the absorbance due to the material precipitation. For stable particles no such changes in absorption/scattering are expected. Our tests were carried out in buffered aqueous solutions (HEPES, 50 mM) at near-neutral pH.

First and foremost, the presence of the PEG groups on the Janus dendrimers proved to be critical for achieving high solubility. UCNP modified with Janus-type ligands **6b,c** and **11** overall showed high stability in aqueous solutions (see below). On the other hand, modification of UCNP with fully polycarboxylate dendrimers (the counterparts of the Janus molecules **6** and **11**), which were obtained by complete hydrolysis of the peripheral esters in compounds **3** and **8** (SI, Scheme S3), proceeded readily, but the resultant adducts were prone to rapid precipitation (10–15 min). This result is consistent with our previous finding that a significant fraction of carboxylates in the dendrimer (or any polymer in general) has to remain free for interfacing with the solvent in order to impart UCNP solubility.¹³ In flexible dendrimers and polymers (e.g. polyacrylic acid) conformations are likely to be favored, in which the majority of the peripheral carboxylates are bound to the UCNP surface, making the outer layer insufficiently hydrophilic. In contrast, the high hydrophilicity of the PEGylated Janus molecules makes them efficient in UCNP solubilization.

Secondly, for a given dendritic ligand the length of the PEG group appeared to play a key role in the overall solubility. UCNP-**6a** (Glu³, PEG1000) rapidly formed a fine dispersion that gradually settled over the course of 1–2 h. In contrast, UCNP modified with dendrimers containing PEG2000, **6b** and **11**, retained solubility for months when kept either

at room temperature or at 4°C. UCNP, modified with **6c**, (PEG5000), behaved similarly to **6b** and **11**.

In order to evaluate the effect of PEGylation, the properties of the UCNP-dendrimers **6b** and **11** were assessed under a range of conditions that can be potentially encountered in physiological systems. The particles showed complete integrity in the physiological pH range (pH 6–8) (Fig. 1c and Figs. S2a) and appeared generally stable over a broader pH range, pH 4–10. Upon decrease in pH below 4, baseline absorbance sharply decreased, however it returned back to the original level upon bringing pH to more basic levels.

One of our goals was to overcome potential instability of hydrophilic UCNPs in the presence of divalent metal cations, such as Ca^{2+} , Mg^{2+} and Zn^{2+} that are common in biological systems. Exposed glutamic acid residues in purely polyglutamic UCNP-dendrimers¹³ readily form salts with these cations, causing aggregation and precipitation. Since neutral hydrophilic PEG groups do not have affinity to ions, PEGylation was presumed to resolve the aforementioned problem. Indeed, no changes in absorption were observed over ~17h when particles were dissolved in aqueous HEPES buffer containing 2 mM concentrations of Ca^{2+} , Mg^{2+} or Zn^{2+} salts. For reference, the average blood plasma concentration of free Ca^{2+} and Mg^{2+} is ~1 mM, and the concentrations of Zn^{2+} is orders of magnitude below that (see SI for details). Changes in the solutions' optical density were below 1% in all cases tested (Fig. S2b–d).

The particles were found to be stable in the temperature range of 22–44°C, and their luminescence decay kinetics changed slightly with temperature, as expected. UCNP luminescence-based nanothermometry is an active area of investigation,^{11i, 28} whereby the most common method to obtain temperature information is based on ratiometric sensing. As has been shown recently specifically in relation to sensing temperature, ratiometric measurements are subject to multiple distortions²⁹ and are unlikely to be able to provide quantitative information in optically heterogeneous environments, such as biological tissue. On the other hand, luminescence decay measurements are not affected by the medium optical properties, and therefore provide a superior alternative to ratiometric sensing. The dependence of the decay time of the UCNP/Janus-dendrimers on temperature (Fig. 1f) could be well approximated by a straight line with the slope of $-0.66 \mu\text{s}/^\circ\text{C}$. Thus, UCNP-based temperature sensing with adequate accuracy in the biological temperature range (25–40°C) would necessitate lifetime measurements with rather high accuracy (error<0.5%), considering that the decay time constants of UCNPs' are in the range of 10^{-4} s. Achieving such precision *in vivo* might be difficult.

All of the above experiments were conducted in solutions buffered by HEPES (50 mM), however when dissolved in phosphate buffers (pH 7.2) UCNPs gradually aggregated and precipitated. Phosphate groups, which are known to have higher affinity for UCNPs than carboxylates,^{20a, 20e, 21b, 30} are likely able to displace the dendritic ligands in spite of the latter having polyvalent contacts with the surface. The spectroscopic changes accompanying this process are shown in Fig. S3a–d. As expected, other water-stabilized UCNPs, prepared according to the published methods (e.g. UCNPs stabilized by PAMAM^{14a} or PEI³¹ dendrimers, or by polyacrylic acid³²) were found to be even more unstable and precipitated

in the presence of phosphate much more rapidly. For the UCNP-Janus dendrimers the rate of aggregation/precipitation was found to be dependent on the phosphate concentration, such that at ~1 mM level optical properties of solutions changed only slightly during the first 30–40 min (Fig. S3d). Nevertheless, for practical imaging such behavior could be still problematic, since phosphate is one of the most ubiquitous biological anions, present in the blood plasma at ~1 mM and in cellular cytoplasm in up to 10 mM concentrations.³³ Fortunately, addition of albumin was found to fully rescue UCNPs even at very high concentration of phosphate (up to 50 mM) (Fig. S3e,f). Albumin is present in blood plasma at a very high concentration (~2–4% by weight), using UCNPs for vascular imaging (see below) is therefore fully appropriate.

Since in many common types of cell growth media phosphate is used as a buffer, stabilizing UCNPs in such environments would require the presence of albumin. In the past, PEGylated dendritic imaging probes have been shown to lack affinity to cellular membranes,³⁴ exhibiting no traces of cellular internalization even after prolonged incubation in cell-culture medium. Similarly, incubation of HeLa cells with UCNP-11 in a standard albumin-containing cell growth medium at 37°C for over 24 h showed no traces of internalization, judged by the lack of detectable upconverted emission from inside cells. Cells exposed to UCNP-dendrimers appeared healthy and continued dividing at the same rate as controls.

Clearance and bio-distributions in vivo.

To measure the rate of clearance of the dendritic UCNP probes from the blood UCNP-11 as an aqueous solution (150 μ L, 25 mg/ml) was injected into the blood of a mouse *via* a catheter placed into the femoral vein, and the intensity of emission, integrated over a selected lateral plane, was measured as a function time (Fig. S8a). The retention half-time ($t_{1/2}$) was found to be ~12 min (averaged over two animals). In comparison to purely organic polymer-conjugated vascular markers, such as e.g. FITC-dextran, UCNP-dendrimers, in spite of their relatively large size and high per-particle weight (30 nm diameter, $\sim 6.4 \times 10^{-17}$ g per particle, ~38 MDa), were excreted from the blood rather rapidly. For example, $t_{1/2}$ for FITC-dextran with molecular weight of just 20 kDa was reported to be ~30 min, while for 40 kDa and 70 kDa conjugates it was well above 1h.³⁵ The higher excretion rates of larger PEGylated UCNP-dendrimers suggest that different mechanisms are responsible for removing them from the blood than for smaller organic polymers. Nevertheless, UCNP-11 effectively permitted imaging for ~40 min upon single injection (see below), and it is likely that the retention times can be further improved by using PEG-residues of different size.

Accumulation of UCNPs in different organs was evaluated by *ex vivo* imaging (Fig. S8b). The organs were removed from the animal and laid on a Petri dish. Imaging was performed with an intensified CCD camera, using a laser diode for excitation. As the excitation spot moved over an organ, the luminescence intensity, corresponding to the UCNP red emission band (660 nm) was recorded, giving an estimate of the particles' distribution throughout the body. The maximal accumulation was detected in the liver and spleen, while the signals from the kidney and bladder were much weaker, indicating, as expected, that the particles were too large to pass through the kidney dialysis system and be excreted in the urine. This behavior is quite common for nanoparticles having no specific targeting ligands.³⁶

Two-photon imaging of brain vasculature.

The non-linear advantage, which underpins the ability to perform high-resolution imaging at depth using UCNP-based probes, is gained due to the quadratic dependence of the UCNP emission rate on excitation flux (Fig. 1e). For microscopic imaging the flux at the focus must be maintained sufficiently low to avoid excited state saturation, which, if it occurs, would lead to growth of the excitation volume and subsequent loss of resolution. Probes with long excited-state lifetimes, such as UCNPs, are especially prone to saturation due to their inherently slow excited state deactivation. Secondly, imaging with long-decaying probes is prone to smearing, which can occur when the beam is scanned over the field of view at rates comparable to or exceeding those of the excited state decays.³⁷

Two-photon microscopy in mice was performed using UCNP-11 (0.15 mL, 25 mg/mL, aqueous solution) excited using a low-power CW laser diode ($\lambda_{\text{ex}}=980$ nm).

A set of 10 μm -thick Maximal Intensity Projection (MIP) image stacks of the brain vasculature taken at different depths is shown in Fig. 2(c–h) along with a wide-field image showing the brain surface (Fig. 2a) and a 30 μm -thick MIP close to the surface (Fig. 2b). The MIP is a composition of multiple lateral (X–Y) planes, mimicking a 3D view that an observer would see looking down at the brain from above. All the images in Fig. 2c–h relied solely on the UCNP luminescence as a source of contrast.

It is clear that the signal-to-background ratio and resolution are maintained at adequate levels throughout the entire imaging depth, allowing for detection of individual capillaries 1 mm-deep under the brain surface. This result is remarkable, considering that the peak incident power in this experiment did not exceed 22 mW for imaging at 1000 μm depth, and it was much lower closer to the cortical surface. Another set of images reaching down to 900 μm , is shown in the Supporting Information (Fig. S6). In that experiment the maximal CW power did not exceed ~16 mW, and the excitation gate duration was only 20 μs . Since power is significantly attenuated with depth and strongly affected by optical heterogeneities of the medium (i.e. tissue inhomogeneous absorption and scattering), it is difficult to calculate the photon flux at the laser focus. However, in zero-approximation, i.e. no absorption and scattering, at 20 mW power the photon flux ($\lambda=980$ nm) through the beam waist cross-section of e.g. 1 μm^2 would be $\sim 9.9 \times 10^{24}$ photons $\times \text{s}^{-1} \times \text{cm}^{-2}$. For comparison, in conventional two-photon microscopy with red-emitting fluorescent dyes, such as Alexa 680 (Fig. 2b), the time-averaged power required to observe structures ~1 mm under the brain surface is typically close to 100 mW,³⁸ which corresponds to the instantaneous power at the pulse peak in the range of ~4–8 kW (assuming 80 MHz laser pulse repetition rate and 150 fs pulse duration) and the photon flux of $\sim 4.1 \times 10^{30}$ photons $\times \text{s}^{-1} \times \text{cm}^{-2}$. Certainly, from the biocompatibility point of view reducing excitation flux by 5–6 orders of magnitude presents a major advantage.

The images shown in Fig. 2 were obtained by recording the ‘red’ emission of UCNPs ($\lambda_{\text{max}}=658$ nm), which is much less absorbed by endogenous tissue chromophores than the ‘green’ emission ($\lambda_{\text{max}}=539$ nm). In each pixel the luminescence was excited by a laser gate of a selected duration (20–50 μs), and the decay was recorded in time-resolved fashion. The integrated intensity of the decay was plotted as a function of the pixel position. In this

imaging mode, the majority of the photons produced by the pulse were collected prior to moving the focus to the next location. As a result, the cross-talk between the pixels was almost prevented, but the scanning rate was quite low. Indeed, even when only one excitation gate was executed per pixel acquiring a full e.g. 256×256 frame with pixel dwell time of 300 μ s still required ~20 s.

Fortunately, collection of complete decays was not a requirement in our experiments, since in this case we were not concerned with imaging of luminescence lifetimes. Instead, image acquisition could be performed by continuously scanning the beam over the FOV and synchronously recording the emission signal, as is done in conventional scanning fluorescence microscopy. Fluorescence, however, is emitted on the nanosecond time scale, and, therefore, it can be considered nearly instantaneous compared to the speed of scanning. When acquisition was performed in such a continuous mode with UCNPs as a source of contrast, the images, as expected, appeared smeared due to the UCNPs' long luminescence decays (Fig. 3a,c). With the pixel dwell times of just a few microseconds, the photons originating in preceding pixels continued to arrive at the detector, while the excitation spot has already moved to the next position. The smearing became less apparent at lower scanning speeds.

The smearing effect could be a significant drawback if UCNPs were to be used for vascular angiography. However, the resolution could be readily restored by image processing.³⁷ The point spread function (PSF) (or 'smearing function') was constant over the entire FOV, and it could be easily measured prior to acquiring an image. Indeed, as we mentioned above, the luminescence decays of UCNP/dendrimers are nearly insensitive to the environmental parameters (pH, temperature, local oxygenation etc), and hence the PSF could be simply calculated as $F(t/T)$, where $F(t)$ is the UCNP luminescence decay measured in any chosen pixel, and T is the pixel dwell time (the inverse of the scanning speed). It is important to mention that the decay profile is dependent on both the duration of the excitation pulse and average pulse power. Since these parameters may need to be adjusted during imaging, it can be beneficial to measure individual PSF's for each imaging depth. In the present demonstration, however, we used just a single decay profile, corresponding to the depth of 100 μ m. The subsequent deconvolution could be performed using standard algorithms (e.g. Richardson-Lucy, as implemented in MatLab: `deconvlucy()`). The raw images of a single lateral plane ($Z=100 \mu$ m), obtained at different scanning speeds, and their respective deconvolutions are shown in Fig. S7 as an example. The complete MIP images of a brain slice, extending down from the surface to a depth of 550 μ m, acquired at speeds corresponding to pixel dwell times of 10 and 40 μ s, are shown in Figs. 3a,c, along with processed (deconvoluted) images (Fig. 3b,d, respectively). It can be seen that high quality images could be restored almost irrespective of the scanning speed, although the section of the image that could not be reconstructed (the 'dead zone', shown outside the red rectangle), increased with the speed, as larger and larger part of the FOV contained incomplete decays.

Overall, the above experiments demonstrate that in spite of their long luminescence lifetimes, UCNP-based probes allow mapping of fine anatomical structures with high precision without sacrificing imaging speed. The depths accessible with UCNPs are comparable to or even exceed those achievable by conventional two-photon microscopy,

while the key advantage comes from the fact that imaging is possible with low-power CW excitation sources, thereby dramatically lowering cost of the required lasers and significantly reducing danger of photodamage. The latter could be especially important for retinal angiography, where photodamage presents a real concern. Use of low power lasers should also help avoiding unwanted overheating of tissue, which is not uncommon in conventional multiphoton microscopy. Overall, the ability to employ low-cost low-power lasers should dramatically increase accessibility multiphoton microscopy for research, clinical and educational use.

Excitation energy transfer (EET).

Over the past decade extensive literature has emerged on modulation of UCNP luminescence by various surface-bound chromophores, whose own properties change in response to external stimuli.^{10a, 15a, 39} Coupling between UCNPs and peripheral dyes is achieved *via* UCNP-to-ligand excitation energy transfer (EET), often referred to as 'luminescence resonance energy transfer (LRET)',^{14, 40} although resonant dipole-dipole type interactions are frequently postulated with no substantiation. Sensing schemes rely on differential modulation of UCNP luminescence bands by peripheral molecules with an optically-coupled response to a specific analyte.^{10d, 16, 40–41} Unfortunately, luminescence intensity ratios are also dependent on optical heterogeneities of the medium,²⁹ making quantitative intensity-based ratiometric measurements in heterogeneous environments problematic. On the other hand, sensing based on modulation of luminescence kinetics, which in principle is medium-independent and much more stable, is not readily achievable with UCNPs, as their luminescence decays are not easy to modulate. Indeed, in most cases EET-induced changes in the UCNP luminescence kinetics do not correlate with changes in intensity.^{10e, 13, 42}

The emission spectra of UCNP-**11** in water and of the parent particles UCNP-BF₄⁻ in DMF, taken at the same concentration (by weight of UCNP), are shown in Fig. 1d. The spectra exhibit the well-known features: three visible bands (λ_{max} =527, 538 and 651 nm), corresponding to $^2\text{H}_{11/2} \rightarrow ^4\text{I}_{15/2}$, $^4\text{S}_{3/2} \rightarrow ^4\text{I}_{15/2}$, and $^4\text{F}_{9/2} \rightarrow ^4\text{I}_{15/2}$ transitions of Er³⁺ ion. In water the emission is significantly reduced, especially the transitions near 520–540 nm, suggesting particularly strong quenching of the $^2\text{H}_{11/2}$ and $^4\text{S}_{3/2}$ states by the solvent molecules and/or organic ligands. Similar to the absorption spectra of the ligands, emission of UCNPs was unaffected by metal salts throughout the physiological pH range.

The absorption spectrum of Eriochrome Cyanine R (ECR) undergoes complex changes over a broad pH range (pH 4–11) due to protonation-induced equilibria between several forms of the dye (Fig. 4a,c). In the range pH 4–10, the primary changes involve bands centered at λ_{max} =445 nm and λ_{max} =578 nm. The shoulder of the latter overlaps with the 'green' emission band of UCNPs (λ_{max} =539 nm), but not with the 'red' band (λ_{max} =658 nm) (Fig. 4b). Hence, the EET from UCNPs to ECR would be expected to affect the ratio of the bands as a function of pH. Indeed, such changes could be observed (Fig. 4b,d) similarly to the previously reported systems.^{13, 23}

While the protonation range of ECR is somewhat too acidic for physiological pH measurements (apparent pK~5.5; Fig. 4c,d), the constructs present a useful model for evaluating the UCNP-based ratiometric scheme in the context of absorbing/scattering

environments. First, it can be readily seen that the absorption by the medium at the wavelengths overlapping with UCNP emission strongly affects ratiometric values. Simply decreasing the concentration of the UCNP/dendrimers by ~35% (in Fig. 4e the corresponding range in the absorption of ECR is marked by a gray bar) leads to a change in the emission ratio $Em(\lambda_{658})/Em(\lambda_{539})$ (Fig. 4f) comparable to that induced by changes in pH by more than one unit (in Fig. 4d the corresponding change in the emission ratio is marked by a gray bar). The ECR molecules throughout the medium are able to induce such changes because the extinction coefficients at the two emission bands (~540 nm and ~660 nm) are different, and hence the emitted photons are absorbed differently along the entire optical path. Of course, there exist dyes exhibiting larger changes in absorption upon protonation than ECR, but absorption by the medium (e.g. red blood cells) still cannot be neglected, as it strongly interferes with ratiometric detection. In this regard, absorbers with spectra resembling those of hemoglobin produce even stronger effects on the emission ratio (Fig. S4), and scattering further increases effects of absorption (Fig. S5). The corresponding illustrative experiments are discussed in the Supporting Information (SI, Section 4).

Luminescence lifetime measurements, especially in the microsecond domain, are independent of the optical properties of biological tissue and unobstructed by endogenous luminescence, since no endogenous chromophores emit light on the microsecond time scale. Therefore, *in vivo* analyte sensing by decay time presents a superior alternative to intensity-based ratiometric measurements. In this regard, attenuation of UCNP emission due to EET may be accompanied by a concomitant decrease in the lifetime, provided energy transfer occurs by a non-radiative mechanism. In contrast, if the EET is due to emission-reabsorption (known as *trivial* energy transfer⁴³), no changes in the excited state lifetime would be expected, but the intensity of the emission would still undergo changes. In the case of UCNP/Janus-dendrimers the latter scenario is clearly dominant. Upon the change in pH from 4 to 10, the intensity ratio changes by ~40% (from 1.5. to 3.5, Fig. 4d), while the decay time drops only by 6.7% (from 120 μ s to 112 μ s; Fig. 4g,h). This change accounts for a slope of only -1.4 μ s/pH unit, suggesting that for determining pH with e.g. ± 0.1 unit accuracy one would have to measure lifetimes with precision of $\pm 0.1\%$. Such accuracy is not easily achievable in the context of biological experiments.

Similar or slightly better results have been reported for a number of other UCNP-dye systems,^{13, 42a, 44}. However, in the cases where UCNPs were modified with ligands loaded with large quantities of dyes,⁴⁴ EET efficiencies, calculated from luminescence lifetimes, were reported to reach as high as 60%. Thus, placing more chromophores in close proximity to the emitter ions, increasing the spectral overlap between the donor and acceptor and, of course, decreasing the average emitter-acceptor distance⁴⁵ should favor the non-radiative pathway in the overall EET, potentially leading to more useful UCNP-based sensor systems. To this end, dendritic macromolecules could be especially useful as UCNP ligands, since they can be prepared having multiple chromophoric units throughout their structure,⁴⁶ while maintaining a highly hydrophilic periphery.

Conclusions

We presented the design and properties of UCNP's solubilized by way of modification of their surfaces with Janus-type dendrimers having peripheral PEG groups and containing an organic dye as a core. The resulting hydrophilic nanoparticles exhibit excellent biological compatibility and stability under physiological conditions. The unique photophysical properties of UCNP's enabled multiphoton high-resolution *in vivo* brain angiography, reaching nearly record-level depths, while using record-low excitation intensities. The demonstrated ability to perform vascular imaging *in vivo* at high scanning speeds, in spite of the UCNP's long luminescence lifetimes, suggests the UCNP-based probes present a viable practical alternative to conventional macromolecular vascular agents, especially in view of the possibility to carry out multiphoton imaging using environmentally safe low-power excitation sources.

Future efforts will have to focus on establishing efficient ways of modulating UCNP's luminescence lifetimes in response to biologically-relevant analytes or environmental parameters in order to enable quantitative sensing in scattering/absorbing environments. Steps in this direction are being made by creating smaller and yet brightly emissive nanocrystals,^{9a, 11e, 11g, 45, 47} whose luminescence decays may be more responsive to modulation *via* non-radiative energy transfer processes. Supplementing such small crystals with appropriate peripheral ligands, such as Janus-dendrimers described in this work, possibly having stronger binding termini (e.g. phosphoric acid residues),^{20a, 20e, 21b} should pave a way to UCNP-based probes for *in vivo* analyte-specific multiphoton imaging.

Experimental Methods

The synthetic protocols and characterization data for all new compounds can be found in the Supporting Information (SI).

General.

All chemicals were obtained commercially and used without further purification, unless otherwise stated. Dendrons NH₂-Glu³OEt¹³ and NH₂-Newk²O^tBu⁴⁸ were synthesized and characterized using previously published methods. UCNP's were synthesized as described previously.⁴⁹ Bio-Beads™ S-X1 (Bio-Rad, 200–400 mesh) were used for size-exclusion chromatography. NMR spectroscopy, mass-spectrometry, UV-vis and fluorescence spectroscopy, transmission-electron microscopy (TEM) were performed using standard instrumentation. NMR spectra were recorded at 300K using CDCl₃ and DMSO-*d*₆ as a solvent. Mass spectra were recorded using 1,8,9-trihydroxyanthracene (dithranol) as a matrix. TEM images were acquired using a 120 kV accelerating voltage. For steady-state measurements of UCNP emission *via* upconversion, a CW laser diode module (λ_{max} =980 nm, Newport) was used as an excitation source. For power-dependence measurements, the incident power on the sample was varied by using neutral density filters and measured by an optical power meter (Coherent). Time-resolved emission measurements were performed using in-house-constructed time-domain phosphorometer,²⁷ modified for modulation of the laser diode.

Synthesis of UCNPs.

UCNPs of well-defined shape and size today are routinely prepared by either hydrothermal⁵⁰ or thermal decomposition⁵¹ methods, which require so-called capping ligands (e.g. oleic acid or oleylamine)⁵² to ensure particles' stability in non-polar media. In the present study we used hexagonal phase β -NaYF₄-based nanocrystals, doped with Yb³⁺ (20%) and Er³⁺ (2%).⁵³ Highly monodisperse spherical nanoparticles, 30±1 nm in diameter, were prepared by thermal decomposition of trifluoroacetate salts in the presence of oleic acid.^{49b} The oleate capping ligands were removed upon treatment with NOBF₄, rendering UCNPs coordinated with BF₄⁻ ions.^{49a} The latter readily undergo exchange reactions with a variety of carboxylate ligands, driven by the oxophilic nature of lanthanides. The BF₄⁻-modified UCNPs were stored as solutions in DMF or DMSO. As shown previously, prolonged storage (several years) does not affect properties of these nanoparticles.

Modification of UCNPs with dendritic ligands.

In a typical procedure,^{49a} a sample of an organic ligand (**6a-c** or **11**) was dissolved in a mixture of water and DMF (~2:3 v/v) to the concentration of the ligand of ~10 mg/mL. A solution of UCNPs pre-treated with NOBF₄ in DMSO or DMF (~50 mg/mL) was added to the resulting mixture, so that the ratio (by mass) UCNP:ligand was ~1:2. The mixture was stirred for 1 h at r.t. and poured into distilled water (40 mL per 100 mg of UCNP). The UCNP-dendrimers were isolated *via* centrifugation (8,000 g, 15–30 min). After centrifugation the precipitated gel was separated from the supernatant, and the latter was re-centrifuged again until no further precipitation was observed. The centrifuge speed was kept such that the acceleration was below 10,000 g. To remove the excess of the ligand and residual organic solvents, the obtained gel-like precipitates were combined, re-dispersed in distilled water, and the target UCNP-dendrimers was precipitated again by centrifugation. This procedure was repeated several times, after which the gel was re-dissolved in distilled water and filtered through a syringe filter (pore size 0.4 µm). The final concentration of UCNP/dendrimer solution was ~0.5–1 mg/mL for spectroscopic measurements and ~25 mg/mL for animal imaging experiments.

In vivo imaging and bio-distribution studies.

In vivo imaging was performed in epi-fluorescence mode using the previously described multimodal microscope.⁵⁴ UCNPs were excited by a CW laser diode (λ_{max} =980 nm). In the case of imaging with conventional fluorescent dyes (e.g. FITC), excitation was provided by a standard Ti:sapphire laser oscillator (Insight; Spectra Physics). Beam focusing and collection of emission were accomplished using a water-immersion objective (20x; NA 0.95; XLUMPLFI; Olympus). Three-dimensional median filtering and histogram equalization were used for image processing. For vascular imaging, C57BL/6J mice (male; 25–30 g; 10–12 week-old) were anesthetized by isoflurane (1–2% in a mixture of air and O₂) under constant temperature (37°C). A cranial window was made in the parietal bone, the dura was removed, and the window was sealed with a 150 µm-thick microscope coverslip. During imaging, blood pressure and blood gases were monitored *via* a catheter inserted into the femoral artery, which also served for administration of probes. For biodistribution studies, the luminescence intensity in different organs was measured *ex vivo* in reflectance mode

using a gated intensified CCD camera (Picostar HRI, LAVision, 660V gain, 500 ms-1000 ms CCD exposure, D.C. mode) using a 650/40 nm band-pass filter. All experimental procedures were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(a) Aqueous solution of UCNP-**6b** before (left) and after (right) centrifugation. Excitation by a hand-held laser diode (980 nm) generates upconverted emission throughout the solution, but after centrifugation the emission is localized to the gel at the bottom of the tube. (b) TEM image of UCNP-**6b**. Bar: 30 nm. (c) Absorption spectra of a solution of UCNP-**11** (2.5 mg/mL, 50 mM HEPES, pH 7.1) recorded every 10 min for 17 h. Inset: peak near 980 nm, corresponding to the absorption of Yb^{3+} . (d) Emission spectra of UCNP/ BF_4^- in DMF and UCNP-**11** in aq. buffer (50 mM HEPES, pH 7.13) taken at equal concentrations. (e) Power dependencies of the integrated emission signals near 540 nm and 660 nm for UCNP-**11** in aq. buffer (2.5 mg/mL, 50 mM HEPES) upon excitation at 980 nm. (f) UCNP-**11** emission decays as a function of temperature in the physiological temperature range ($\lambda_{\text{ex}}=980$ nm; rectangular pulse, 50 μs -long; $\lambda_{\text{em}}=545\pm 10$ nm). The arrow and the vertical line indicate, respectively, the start of the excitation pulse and the beginning of the decay portion used for exponential analysis. Inset: change in the decay time constant with temperature and its fit with a straight line.

Figure 2.

Images of the mouse cortical microvasculature *in vivo* obtained using UCNP-**11** as a vascular marker and two-photon excitation by a CW laser ($\lambda_{\text{ex}}=980$ nm) in gated mode. (a) Image of the brain surface obtained with a CCD camera and green light illumination. The segment in red rectangle is shown in panels b-h. (b) Maximum Intensity Projection (MIP) image stack extending from the surface to the depth of 30 μm . Bar: 100 μm . (c-h) MIP images (10 μm -thick) of the same segment at different scanning depths. In each pixel an excitation gate (rectangular pulse, 50 μs -long) was followed by the emission collection period of ~ 250 μs ($\lambda_{\text{em}}=670\pm 35$ nm). The imaging depth and the laser power during the application of gate (measured after the objective) were as follows: (c) 200 μm , 2 mW; (d) 400 μm , 4.4 mW; (e) 600 μm , 6.3 mW; (f) 800 μm , 13.2 mW; (g) 900 μm , 16.3 mW; (h) 1000 μm , 22 mW.

Figure 3. Image deconvolution/de-smearing using UCNP luminescence decay-based point spread function. (a, c) MIP images of a mouse brain segment ($Z=0-550\ \mu\text{m}$), obtained with pixel dwell times of $10\ \mu\text{s}$ and $40\ \mu\text{s}$, respectively. Scanning direction: top-to-bottom. Bar: $50\ \mu\text{m}$. (b, d) The same images as in (a, c) after processing using the Richardson-Lucy algorithm. The image area restored by the deconvolution is shown within the red rectangle.

Figure 4.

Changes in the absorption (a) and emission (b) spectra of UCNP-11 with pH (HEPES, 10 mM; λ_{ex} =980 nm). (c) Change in the absorbance at 539 nm. (d) Change in ratio of the UCNP emission bands integrated intensities, $\text{Em}(\lambda_{658})/\text{Em}(\lambda_{539})$, with pH. The vertical gray bar shows the change corresponding to an increase in pH from 4.7 to 6. (e) Change in the absorbance upon dilution of the solution at a constant pH 6.95. The vertical gray bar indicates the change in the absorbance by 35%. (f) Change in the ratio $\text{Em}(\lambda_{658})/\text{Em}(\lambda_{539})$ upon dilution. The vertical bar corresponds to the change in the absorbance by 35% (as in e).

(g) Luminescence decays of UCNP-**11** upon change in pH. The luminescence was excited by rectangular 300 μ s-long pulses from a modulated laser diode (λ_{ex} =980 nm). The vertical bar indicates the end of the excitation pulse, and the small bar indicates the start of the decay segment that was used in single-exponential analysis. (h) Change in the luminescence decay times with pH.

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Scheme 1.

Janus-type dendritic ligands: structures and a cartoon of the synthetic sequence illustrating assembly of compound **11** as an example.

Synthetic steps: 1) attachment of New² dendron *t*Bu ester (D1); 2) attachment of Glu³ dendron Et ester (D2); 3) cleavage of *t*Bu esters; 4) PEGylation of D1 dendron and forming the solvent interface; 5) cleavage of Et esters and forming the UCNP-binding interface.