Self-Assembled Dual Dye-Doped Nanosized Micelles for High-Contrast Up-Conversion Bioimaging

Sara Mattiello, Angelo Monguzzi, Jacopo Pedrini, Mauro Sassi, Chiara Villa, Yvan Torrente, Roberto Marotta, Francesco Meinardi,* and Luca Beverina*

1. Introduction

Fluorescence imaging is the most common approach used on biological specimens for visual diagnostics.[1–4] However, even if this technique is very well established, its application on human living patients is still unpractical. Living tissues strongly absorb at the UV–vis excitation wavelengths required by most of the available and affordable fluorescent probes.[5] As a consequence, practical applications require the use of high intensity sources, with two important consequences: an increased scattering of the excitation light and a stronger background autofluorescence from tissues, which significantly reduce the signal-to-noise ratio of the optical detection, resulting in low-contrast images. Moreover, an intense excitation light greatly increases the potential damage of the tissues under investigation, thereby making harmful an otherwise noninvasive technique.

Many of the most advanced researches in this field aim at overcoming this critical issue. As depicted in Figure 1a, a possible solution is the use of materials showing anti-Stokes photoluminescence, namely the emission of photons at higher energy than the absorbed ones.[6] This effect is known as photon up-conversion (UC), and it is commonly achieved through non-linear optical phenomena like the second harmonic generation or the two-photon absorption (TPA). By exploiting this peculiar emission is thus possible to eliminate the autofluorescence background, present only at energies lower than the excitation, with a gain in the image contrast enabling the use of low power, innocuous light sources.

The field of anti-Stokes fluorescence imaging was traditionally dominated by inorganic crystals doped with luminescent lanthanide ions and by organolanthanide complexes, where the UC scheme requires the sequential absorption of two or more photons exciting the metastable states of the emitting ions.[7–10] The anti-Stokes imaging enforced by lanthanide enables the use of infrared light excitation, matching the transparency window of the biological tissue, but, due to the small UC cross section of the exploited lanthanide electronic transitions, it requires the use of extremely intense excitation sources.[10] The same consideration is valid for TPA materials, where the up-converted light is generated by the simultaneous absorption of two photons. Such approaches were successfully validated over the years for both in vitro and in vivo applications. Unfortunately, in both cases the required excitation irradiances are typically in the order of mW cm$^{-2}$.[11]

Conversely, the UC based on sensitized triplet–triplet annihilation (TTA-UC), recently proposed as an efficient photon management technique for solar applications, can be observed using excitation irradiances of few mW cm$^{-2}$.[8,11–14] The TTA-UC is a stepwise process which exploits the annihilation of optically dark, metastable triplets of an emitter, indirectly populated...
from proper sensitizers via energy transfer, to produce high-energy singlets, from which the up-converted emission originates. As detailed in Figure 1b, the TTA-UC requires a pair of selected chromophores with suitable electronic properties. Here, we used the Pt octaethylporphyrin (PtOEP) as sensitizer and the 9,10-diphenylanthracene (DPA) as annihilator/emitter. The PtOEP can be excited to its first singlet excited state by irradiation at 532 nm, which quickly converts to the low-lying triplet via intersystem crossing (ISC). If within the lifetime of this latter, the sensitizer collides with a ground state DPA, the two molecules undergo a triplet–triplet energy transfer (ET) resulting in the excitation of the emitter in its first triplet state. Finally, the collision of two emitter triplets leads to the promotion of one of them in the first, fluorescent singlet excited state through TTA. It is worth pointing out that, since the triplets of the dyes usually employed as emitters are long-living states with typical natural lifetime in the range of hundreds of milliseconds and over, the annihilation is exceptionally efficient, resulting in effective UC also at extremely low excitation light intensity. This allowed proving the TTA-UC scheme with a range of molecule pairs operating in different spectral windows in organic solvents.[15–21] UC-light activated drug release was also demonstrated.[16,22,23] The need to extend this phenomenon to water environment and to biological specimen triggered remarkable efforts in the preparation of TTA-UC structures. Among them, active polymeric structures,[24–29] ionogels,[30] liposomes,[21,31,32] different supramolecular approaches,[24–29] oil in water microemulsions,[33–35] dendrimers,[36] micelles,[37] and water disperisible nanoparticles[38,39] and nanocapsules[40–46] led to demonstration of the applicability of TTA-UC both in vitro and in vivo, within the tissue transparency window.[9,10,38,40,42]

Nowadays, the full exploitation of the TTA-UC scheme in biorelated applications remains challenging due to two characteristic features of this process. First, the molecular oxygen is an excellent quencher of triplet states, switching off quickly the TTA-UC process. This sets a very critical requirement as in biological tissues the oxygen tension is around 0.5–2.5 kPa, to be compared with its equilibrium value in air of 21 kPa.[47] The TTA-UC is actually so sensitive that it was recently used in the preparation of oxygen sensing materials having a particularly broad dynamic range.[16] Second, the sensitizer and emitter counterparts need to be in close proximity in order to efficiently undergo short-range interactions such as ET and TTA. The literature reports several approaches to tackle this limit. The encapsulation of the two active molecules within a polymeric shell provided working UC capsules, having dimensions ranging from hundreds of nanometers to microns.[40,43] The step by step assembly of core/shell structures featuring a liquid, or in any case low viscosity core surrounded by a silica shell provides a very elegant approach to solve at the same time both issues, while maintaining the dimensions of the final TTA-UC nanostructure within the nanometric range.[35] Encapsulation in other inorganic matrices like tungsten oxide, in this case for photocatalysis applications, was also demonstrated.[19] The approach is general and versatile, yet somewhat complicated by its multistep nature. Oil in water microemulsions, requiring an organic solvent based inner phase, also offer a viable and general way to prepare colloids displaying efficient TTA-UC in oxygenated environment.[33,34] Very recently Yanai and co-workers demonstrated that the synthesis and water phase self-assembly of amphiphilic cationic acceptor molecules with anionic donor (sensitizer) molecules provides an efficient way to address the oxygen quenching issue by a purely supramolecular approach.[25] Finally, König and co-workers demonstrated efficient TTA-UC in large unilamellar vesicles loaded with suitably functionalized diphenylanthracene derivatives as well as both PtOEP and a Ru bipyridine complex.[31] Unfortunately, all of the aforementioned approaches require either the synthesis of specifically designed chromophores, the incorporation of organic solvents, or a complex multistep fabrication of appropriate nanovectors, and therefore they can be impractical in many cases.
In this work, we demonstrate an exceedingly simple procedure for the preparation of efficient TTA up-converting nanomicelles (UC-NM) based on the use of the commercially available surfactant Kolliphor EL. The latter is one of the most commonly employed emulsifier for water insoluble drugs and is the key excipient of several FDA (Food and Drug Administration) approved preparations including Paclitaxel and Miconazole.[49]

Our synthetic protocol enables the synthesis of stable nanomicelles loaded with a proper dye pair for green-to-blue UC, showing an unprecedented UC efficiency of 6.5% for encapsulated materials. The inclusion of the dyes in the micelles preserves the UC performance in oxygenated deionized water as well as in the phosphate-buffered saline (PBS) solution used in biological research. In vitro fluorescence imaging experiments on murine fibroblasts (3T3 cell line) confirmed the high biocompatibility of these supramolecular optical probes, therefore providing evidence of the full compatibility with their use as anti-Stokes markers. Moreover, we envisage that the very nature of the assembly protocol we propose, makes possible to include additional payload molecules alongside with the TTA-UC couple. The disappearance of the UC signal due to the nanomicelles wreckage by an external stimulus would thus give a direct and precise indication of when and where the payload is released within the biological specimen.

2. Up-Conversion Nanomicelles Preparation

For this experiment, we selected PtOEP and DPA as a model sensitizer and emitter because they are amongst the most investigated and performing chromophores used for TTA-UC.[44] Figure 2a sketches the self-assembly strategy followed for the preparation of the UC-NMs aqueous dispersion. In the first step, we prepared a tetrahydrofuran (THF) solution of PtOEP (40 × 10−6 m) and DPA (4000 × 10−6 m), in the stoichiometric ratio of 1:100 that is the preferred feeding ratio to observe efficient TTA-UC in solution.[50] To this, we added Kolliphor EL and 1,2-propanediol under sonication and we maintained the solution in the ultrasound bath for 30 min. All volatiles were evaporated and the oily residue was taken up either with deionized water or with PBS (Phosphate-buffered saline) solution to give a UC-NMs stable dispersion with no appreciable haze (see Figure S1, Supporting Information), suggesting the absence of undesired aggregates. A precise estimate of the micellar size has been obtained by means of high-resolution Cryo-TEM (Transmission Electron Microscopy) measurements. This technique has been used in order to avoid the collapse of the micellar structure due to the removal of the dispersing medium. Indeed, the Cryo-TEM images (Figure 2b) show a series of spherical objects, with a monodisperse diameter distribution peaked at 6.2 nm (inset). These spheroids, given the affinity of DPA and PtOEP with the emulsifier environment and considering their insolubility in the aqueous medium, likely correspond to the amorphous blend of Kolliphor EL, PtOEP, and DPA. Figure S2 in the Supporting Information shows a 3D tomography confirming the micellar nature of the nano-objects.

In order to verify the presence of both the sensitizer and the emitter chromophores, we recorded the UV–vis absorption of the dispersion. As reported in Figure 2c (black line), the UC-NMs spectrum shows the fingerprint absorption peaks of DPA, namely the broad band centered at 380 nm characterized by a well-defined vibronic structure,[51] and of PtOEP, with a weak narrow band centered at 353 nm.[52] These data suggest that both molecules form a stable solution within the Kolliphor EL matrix, and the UC-NMs spectrum can be precisely fitted with a convolution of the single dye spectra weighted according to the initial feeding ratio. Consequently, we can assume that the incorporation of DPA and PtOEP is not selective toward one of the two components (Figure S3, Supporting Information). The final demonstration of the successful chromophores loading in the Kolliphor EL nanomicelles was obtained by recording the photoluminescence (PL) spectrum of the suspension under

![Figure 2](https://www.materialsviews.com)

Figure 2. a) Outline of the UC-NM self-assembly reaction scheme and sketch of the TTA-UC process in a single UC-NM. The up-converted photons are generated thanks to the diffusion (black arrows) and annihilation (TTA) of sensitized dark triplet excitons among the network of acceptors (DPA, blue dots) embedded in the Kolliphor EL micelles. b) Cryo-TEM images of UC-NM loaded with a 100:1 DPA:PtOEP cargo. The inset reports the size distribution of the micellar diameter, peaked at 6.2 nm. c) Absorption (black line) and PL (blue line) spectrum of UC-NM under 532 nm laser excitation. The red spectra show the sensitizer (PtOEP, red dots) phosphorescence in presence (solid line) and in absence (dashed line) of the acceptors in the NC. The inset reports a digital picture of the UC-NM dispersion in PBS under 532 excitation with (left) and without (right) a high-pass blue filter.
a laser excitation at 532 nm, matching the PtOEP absorption. Figure 2c shows that, beside a residual sensitizer phosphorescence at 645 nm due to an incomplete ET (red line), we can detect the typical blue emission of DPA (blue line) thanks to the effective UC of the harvested photons. This suggests that the sensitizer and emitter moieties are closely packed in the UC-NMs core, allowing the TTA-UC process through the diffusion of excitons in the dyes framework defined by the micellar volume, without the need of any molecular motion, as sketched in Figure 2a. To support this view, we performed a simple stochastic simulation of the spatial distribution of DPA molecules in a sphere of 6.2 nm radius, in order to have an approximate estimation of the intermolecular distance. Figure S4 in the Supporting Information shows the result of the Monte Carlo calculations performed using 56 DPAs per UC-NM (see Supporting Information) and imposing a minimum center-to-center intermolecular distance of 1.0 nm (in accordance with the steric hindrance of the DPA whose molecular radius is 0.45 nm). All the dyes have a nearest-neighbor closer than 1.3 nm and a next-nearest-neighbor closer than 1.5 nm. These values are comparable with the distances at which the exchange interactions are effective,[50] confirming that the excitation energy can migrate via exchange-mediated homomolecular hopping within the ensemble of emitters exploring the micellar volume to experience TTA. The resulting TTA-UC luminescence can be observed also by the naked eyes using a high-pass optical filter, as showed by the digital pictures of the sample (inset of Figure 2c).

3. Performance Analysis of Up-Conversion Nanomicelles

One of the most critical property required to a UC-NM operating in an aqueous medium is a strong ability to shield the chromophores from the environmental oxygen. In order to test this characteristic, we recorded the $I_{uc}$ (up-conversion intensity) at different times while leaving the sample in aerated conditions (Figure 3a). After a 20% drop observed during the first...
4 h, $I_{\text{uc}}$ remains constant up to 10 h demonstrating the remarkable resistance of the UC-NMs as also observed for other self-assembled structures.\textsuperscript{[53]} The initial decrease of $I_{\text{uc}}$ can be simply ascribed to a progressive consumption of the oxygen embedded in the UC-NM during the synthesis.

Dealing with the UC yield ($Q_{\text{uc}}$) in a TTA-UC system, the latter is generally poor at very low excitation intensity $I_{\text{exc}}$, and then it raises by increasing the irradiance of the incident light saturating at its maximum value. Conversely, we measured a noteworthy $Q_{\text{uc}}$ of $\approx 6.5\%$ in UC-NM filled with the largest tested concentration of POEP and DPA (400, and $4000 \times 10^{-6}$ M respectively; in this case we used a 1:10 ratio instead of the 1:100 so far employed due to the limited solubility of DPA in the micelles). Moreover, importantly, the UC-NMs are in the saturation regime for every irradiance above few tens of mW cm$^{-2}$ as it is demonstrated by the linear dependence of the TTA-UC emission versus $I_{\text{exc}}$ which also corresponds a constant $Q_{\text{uc}}$ (Figure 3b, triangles). Even if such a value is definitely high enough to enable in vitro fluorescence imaging experiments (vide infra), it is lower than that of a liquid solution of POEP/DPA with the same concentration in which the $Q_{\text{uc}}$ is above 20%.\textsuperscript{[54]} This finding requires some additional comments especially in view of a future optimization of this class of materials.

For an ideal system, the maximum $Q_{\text{uc}}$ obtained in the high excitation limit depends only on the sensitizer-to-emitter ET efficiency and on the statistical probability $f$ to obtain a singlet state upon annihilation of two triplets:\textsuperscript{[54]}

$$Q_{\text{uc}} = 0.5 \phi_{\text{ET}}$$

(1)

In Equation (1) the 0.5 factor simply indicates that two low energy states are required for generating a high energy one, and $f$ is $\approx 1/2$. In our system we measured $\phi_{\text{ET}} = 55\%$ (see Figure S7, Supporting Information), which implies a $Q_{\text{uc}}$ around 12.5%, two times larger than the observed value. In order to justify this discrepancy, and considering that also $\phi_{\text{ET}}$ is not as high as expected for the employed dyes concentrations, we investigated the dyes distribution within the nanomicelles.

To this aim, we prepared two distinct series of UC-NMs. In both of them we varied the sensitizer concentration $C_{\text{sens}}$ within the $20 \times 10^{-6} - 400 \times 10^{-6}$ M interval. In the first series (series A) we did not introduce any DPA molecule whiles in the second one (series B) its concentration was $4000 \times 10^{-6}$ M. All samples pertaining to series A show the typical red emission of POEP (Figure S6, Supporting Information), but with striking differences in their respective luminescence decay dynamics. Figure 3c reports the time-resolved PL decay recorded at 645 nm as a function of $C_{\text{sens}}$. At the lowest concentration ($20 \times 10^{-6}$ M), the PL decay is sizably faster ($\approx 3$ ms) than POEP intrinsic radiative decay time (120 ms).\textsuperscript{[55]} This effect is typical of oxygen quenching, meaning that even though the POEP molecules are effectively embedded in the NMs, they do not localize within the dense and hydrophobic core where—according to our data—no oxygen is present, but rather probably at the interface between the oleic core and the polyethylene glycol hydrophilic shell (inset of Figure 3a). By increasing the POEP concentration its PL lifetime progressively lengthens and becomes clearly nonexponential. In the sample with $C_{\text{sens}}$ of $400 \times 10^{-6}$ M the PL long-time component is $\tau_{\text{long}} = 82$ μs (green solid line). Such a $\tau_{\text{long}}$ is comparable to the POEP decay time usually observed in de-oxygenated organic solvents.\textsuperscript{[56]} This behavior suggests that the POEP molecules preferentially accommodate on, or very close to, the micelle surface where the interaction with the ambient oxygen are not fully prevented. The need to include a higher number of POEP molecules per single nanomicelle pushes more POEP within the micelle’s anoxic core, as showed in the inset of Figure 3a. The same phenomenology is expected to hold also for the DPA molecules, even if it is not directly detectable because the oxygen sensitive triplet state of this dye is completely dark and no upconversion occurs in the presence of quenching agents. Following this picture, not all the porphyrins are effective as sensitizer for the UC process, but only those inside the NM core can efficiently transfer the excitation to properly included DPA molecules.

Based on the results we obtained with series A samples, we expected a substantial enhancement of the nanomicelles average TTA-UC capabilities by using high $C_{\text{sens}}$. The measurements on series B samples support our general view. Figure 3d reports the $Q_{\text{uc}}$ values measured as a function of $C_{\text{sens}}$ showing that the conversion yield increases from less than 1% in the sample with $C_{\text{sens}} = 20 \times 10^{-6}$ M to 6.5% for the $400 \times 10^{-6}$ M sample. These findings suggest that the limited $Q_{\text{uc}}$ observed is not related to an inefficient TTA-UC photophysics in the UC-NM environment, but rather on a still not completely optimized distribution of the POEP within each single NM structure. This conclusion is strongly supported by the time-resolved data of the TTA-UC emission reported in the inset of Figure 3d, which show that the dynamic of the upconverted PL is exactly the same regardless $C_{\text{sens}}$, i.e., regardless the overall efficiency of the system. These data proves that, if the incident photons are absorbed by molecules correctly embedded in the NMs structure, the TTA-UC process occurs always in the same, highly efficient way. In contrast, the NMs embedding unprotected dyes are basically inactive as up-converters.

4. In Vitro Fluorescence Imaging with Up-Conversion Nanomicelles

In order to demonstrate the applicative potential of our NMs, and to verify their stability in biological media, we performed in vitro labeling of 3T3 murine fibroblast cell. First of all we proved the absence of any cytotoxic effects induced by the UC-NMs by performing a cell viability assessment by means of Live/Dead staining on cells labeled with increasing nanomicelle concentrations (Figure S8, Supporting Information). The micelles do not show any sizeable degradation within the time frame investigated both in the dark and under sunlight exposure (see Figure S9, Supporting Information). The 1:50 dilution of the mother solution gave the best trade-off between good cell viability, without signs of stress and cell injuries, and UC-NMs concentration providing satisfactory fluorescence imaging. Accordingly, we tested this concentration for further experiments aimed at evaluating the UC-NMs cytocompatibility throughout the time. One of the most frequently reported nanoparticle-associated toxicities is the generation of reactive oxygen species (ROS). ROS are chemically reactive compounds
that are formed as a by-product of the cellular oxygen metabolism. However, environmental stress factors such as exposure to intense light or excessive heating, common consequences of a prolonged irradiation in diagnostic circumstances, can boost the intracellular ROS concentration to cytotoxic levels, causing damage to cell structures and possible cell death.[57] Moreover, oxidative stress induced by the staining with nano-objects can cause further pathophysiological effects including genotoxicity, inflammation, and fibrosis as demonstrated by activation of associated cell signaling pathways.[58] Since oxidative stress is a key determinant of UC-NM-induced injury, it is necessary to characterize the ROS response resulting from the labeling. Cell Titer-Glo viability and ROS evolution assessments have been performed on labeled 3T3 cells throughout 72 h. Figure 4a,b show the result of the viability and ROS tests, respectively. Although the CellTiter-Glo shows a slower proliferation rate for the UC-NM labeled cells compared to the unlabeled control sample (P value < 0.005, for 24, 48, and 72 h), the overall labeled cell viability has been maintained constant through the experiment course. In the same time, the amount of ROS released from labeled 3T3 cells has been kept on physiological levels, since the ROS production test did not display differences between control and stained samples, confirming the highly biocompatible composition of the UC-NMs.

Finally we proceeded to acquire fluorescence confocal microscope images of paraformaldehyde fixed 3T3 cells stained with UC-NM (blue) and phalloidin positive F actin (red) using a green laser excitation at 532 nm. As shown in Figure 4, we can clearly observe that the blue UC light is generated in the cytoplasm region mostly around the cell nucleus, as expected for dyes not functionalized with target-specific ligands. In contrast, the phalloidin is a well-known standard dye employed for the specific staining of the cytoskeleton. Therefore, thanks to the overlay of the blue and red channels of the optical detector, we are able to record a nice, high-contrast, dual-channel image of the 3T3 cells by using the same excitation source (Figure 4e). This colocalization staining clearly shows that the UC-NMs are effectively internalized by the 3T3 cells, leading to a perinuclear localization and confirming their good stability in the biological medium. This result is the first demonstration of the efficient staining of cells with a TTA-UC based anti-Stokes emitter prepared through such a simple formulation approach employing FDA approved materials. Importantly these results are promising in the perspective of UC-NM applicability as optically active systems for advanced theranostic applications. The observation of UC emission indicates not only that the nanomicelles effectively work as photon up-converters, but it is also an evidence of the structural stability of the micellar structure. As TTA-UC and the corresponding signal would be lost in the case of UC-NMs collapsing, this optical feedback could be used to image the controlled release of water-insoluble additional payloads embedded within the micelles.[2]

5. Conclusion

In summary, we demonstrated a simple and general approach for the preparation of water dispersible, self-assembled nanomicelles loaded with a sensitizer/emitter chromophores pair, which show efficient sensitized up-conversion emission at low excitation power. Thanks to a deep analysis of their optical properties, we were able to optimize the synthesis protocol and obtain nano up-converters with an efficiency of 6.5% in the aqueous, oxygenated environment. The stability of these anti-Stokes emitters in the biological medium in conjunction with suitable biocompatibility enabled us to obtain a multichannel,
high-contrast optical imaging of 3T3 cells using a single excitation wavelength. Notably, our material has several advantages with respect to the traditional up-converting systems, such as the low excitation intensity required, limiting the damage to living tissues, and the shielding effect of nanomicelles from oxygen that protect the embedded dyes from quenching and photodegradation. More importantly, the nanomicelles synthesis is based on a simple formulation with a commercially available surfactant already employed in the formulation of several FDA-approved drugs. To our knowledge, this is the first example of efficient bi-component up-converters prepared by using biocompatible materials approved by the international authority, paving the way for the development of more complex, multicomponent, and multifunctional materials for advanced theranostics.

6. Experimental Section

UC-NM Synthesis: 1,2-propanediol and KH₂PO₄ were purchased from Merck. Na₂HPO₄ was purchased from Alfa Aesar. Deionized water was purchased from Carlo Erba. All the others starting materials were purchased from Sigma-Aldrich. All materials and solvents were used as received.

13.50 mg of DPA and 0.30 mg of PTOEP were dissolved in 10 mL of THF, then 220 mg of 1,2-propanediol/Kolliphor EL v/v mixture were added to 2 mL of the starting solution. The obtained solution was sonicated for 30 min in a SONICA 3200 EP ultrasonic cleaner, then THF was removed under reduced pressure and the oily residue was taken up sonicated for 30 min in a SONICA 3200 EP ultrasonic cleaner, then THF, then 220 mg of 1,2-propanediol/Kolliphor EL 3:10 v/v mixture purchased from Sigma-Aldrich. All materials and solvents were used as received.

PBS solution was prepared dissolving 80 g of NaCl, 14.4 g of Na₂HPO₄, 2.4 g of KH₂PO₄, and 2 g of KCl in 1 L of deionized water, and diluting 10 times before use.

UC-NM Structural Analysis: Up-conversion nanoparticles once applied in thin film on glow discharged holey TEM grids were plunge frozen in liquid ethane cooled at liquid nitrogen temperature using a FEI Vitrobot liquid ethane cooled at liquid nitrogen temperature using a FEI Vitrobot. Cryoelectron tomograms were calculated and filtered using Imod 3.8.40 (Mastronarde 1997). The 3D model was obtained using Amira software (FEI Visualization Group).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

L.B. and S.M. gratefully acknowledge Università degli Studi Milano-Bicocca (grant no. 2016-ATESP-0047). A. M. acknowledges support from Università degli Studi Milano-Bicocca (grant no. 2016-ATESP-0052 ) and from Cariplo Foundation (grant no. 2016-0925). C.V. and Y.T. acknowledge support from the Associazione Amici del Centro Dino Ferrari and from UNISTEM—Centro di ricerca sulle cellule staminali, Università degli Studi di Milano.