Supramolecular Assemblies

Lipid-Coated Nanocrystals as Multifunctionalized Luminescent Scaffolds for Supramolecular Biological Assemblies**

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Supramolecular assemblies of self-organized, functionally cooperating molecules are essential for living matter, and have broad potential to downscale and control (bio)chemical

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[**] This work was supported by the Swiss National Science Foundation (NRP47). We thank Dr. D. Stamou for micropatterning instructions, Dr. J. Wiedmann for plasmids encoding eqFP611-His_6, E. Guignet for help in culturing bacteria and purifying proteins, Dr. C. Duschl for discussions, and Dr. D. Abankwa for reading the manuscript.

 © 2005 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim DOI: 10.1002/ange.200461491

and (bio)technological processes on the nanometer scale.[1–3] An interesting recent development in this context is to combine semiconducting nanocrystals (NCs) of well-known, exceptional optical properties[4,5] with various biomolecular recognition sites to produce supramolecular assemblies for in vitro and in vivo bioanalytical applications.[6–15]

Herein, we present a general and versatile procedure to make NCs water-soluble and simultaneously decorate them with a diverse range of multiple functionalities. Our approach is based on the self-assembly of single lipid monolayers on hydrophobic NCs. The lipid coat shields the NCs perfectly from physical and chemical modification. Interestingly, it is possible to tune the physical (electrical charges, structures, entropic shielding) and chemical (reactive group, biological recognition elements) surface properties of the NCs by varying the polar head groups of the lipid. This effect can be simply controlled by the composition of the lipids used for self-assembly. We demonstrate that such multifunctionalized lipid-coated NCs can bind different proteins per particle specifically and subsequently position themselves on micropatterned surfaces to form nanometer-sized supramolecular structures of higher complexity. Although lipids have already been used for coating (sub)micrometer containers and spheres as well as nanoparticles,[7,16,17] their potential for multifunctionalization and formation of complex supramolecular structures have not yet been exploited in this field.

Trioctylphosphine oxide (TOPO) stabilized CdSe NCs were cosolubilized with lipids in organic solvent and transferred in one step to an aqueous solution, thereby coating them with a monolayer of (functionalized) phospholipids. Aqueous solutions of these so-called lipid-NCs remain remarkably stable for several weeks. The lipid-NCs featured virtually identical spectral shape and photostability in water to those of the original NCs in organic solution, except for a twofold decrease in the fluorescence quantum yield. Lower fluorescence quantum yields of nanoparticles in water as compared to those in organic solvents have been reported before (see for example, ref [18] and references therein). However, the molecular basis of this effect is not yet understood. As shown recently, the organic coating layer can play an active role in the creation or prevention of fluorescence-quenching surface states of fluorescent NCs.[19]

The lipid-NCs appear in cryo-TEM images as particles composed of a central black spot and a gray outer ring, which represent the dense inorganic CdSe core and the organic TOPO/lipid layer, respectively (Figure 1a). The average diameter of the lipid-NC particles is 9 nm, which corresponds to the CdSe core (3 nm) plus twice the thickness of the TOPO (1 nm[20]) and lipid (2 nm[21]) layers (Figure 1b and c).

The simple and versatile introduction of functional groups into the lipid coat of the NCs is demonstrated for two prototypical examples, biotin and nitrilotriacetic acid (NTA). The fluorescence resonant energy transfer (FRET) is measured upon specific interaction between the functionalized lipid-NCs, named biotin-NCs and NTA-NCs, and fluorescent streptavidin (SA) or oligohistidine-tagged proteins, respectively (Figure 2a).

First, addition of Alexa568-labeled SA (SA-A568) to a solution of biotin-NCs resulted in a progressive decrease in the fluorescence intensity of the biotin-NCs and a simultaneous increase of the SA-A568 fluorescence intensity as a result of FRET (Figure 2b). A FRET efficiency of 50% was obtained for a molar ratio of two SA units per biotin-NC. Higher concentrations of SA resulted in aggregation of the biotin-NCs because of the multivalent binding capability of SA. Control experiments performed with lipid-NC lacking biotinylated lipids did not show any change in the fluorescence intensities of the NC and SA-A568, which confirms the specific interaction between biotin-NCs and SA. Binding of SA-A568 to biotin-NCs resulted in a small blue-shift of the NC emission (also visible in Figure 3 with Alexa633-labelled SA (SA-A633)). This shift might be caused by changes in the electrostatic environment of NCs as a result of the proximity of negatively charged SA, since it is also observed for nonlabeled SA.

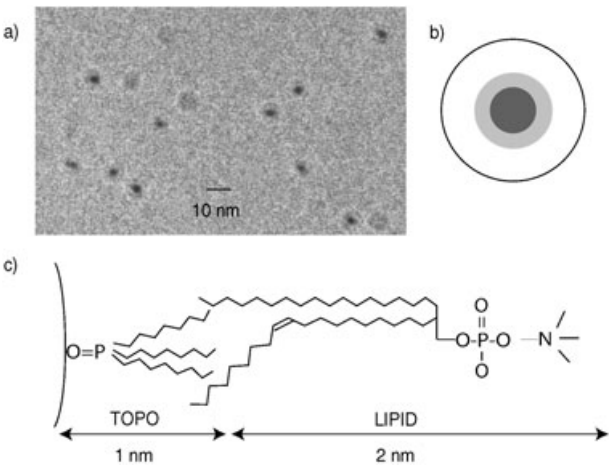

Figure 1. Composition of a lipid-NC. a) Cryogenic electron microscopy image of a vitrified solution of lipid-NCs. More than 80% of the NCs are monomeric. b) Schematic representation showing a CdSe nanocrystal (dark gray) covered by a monolayer of TOPO (light gray) which is surrounded by a self-assembled lipid monolayer (white). c) Schematic structure and orientation of TOPO and POPC with respect to the NC surface (curved line).

Second, specific binding of an oligohistidine-tagged protein to NTA-NCs was investigated using eqFP611-His_6,[22] a red fluorescent protein with an N-terminal hexahistidine sequence. Addition of eqFP611-His_6 to NTA-NCs resulted in a saturating decrease in the NC fluorescence and a concomitant increase in the eqFP611-His_6 fluorescence intensity (Figure 2c). Subsequent addition of ethylenediamine tetraacetate (EDTA) reversed this effect. Control experiments using lipid-NCs lacking NTA did not show any interaction between the NCs and eqFP611-His_6. These findings show that eqFP611-His_6 binds specifically to NTA-NCs. A stoichiometry of four to five fluorescent proteins per NC could be estimated from the concentration dependence of the FRET efficiency. The maximal FRET efficiency obtained is determined by the optical properties of the donor–acceptor pair, and the number of accessible binding sites on the NCs.

Supramolecular assemblies of higher complexity were formed using lipid-NCs that were functionalized simultaneously with biotin and NTA in the lipid coat (biotin/NTA-

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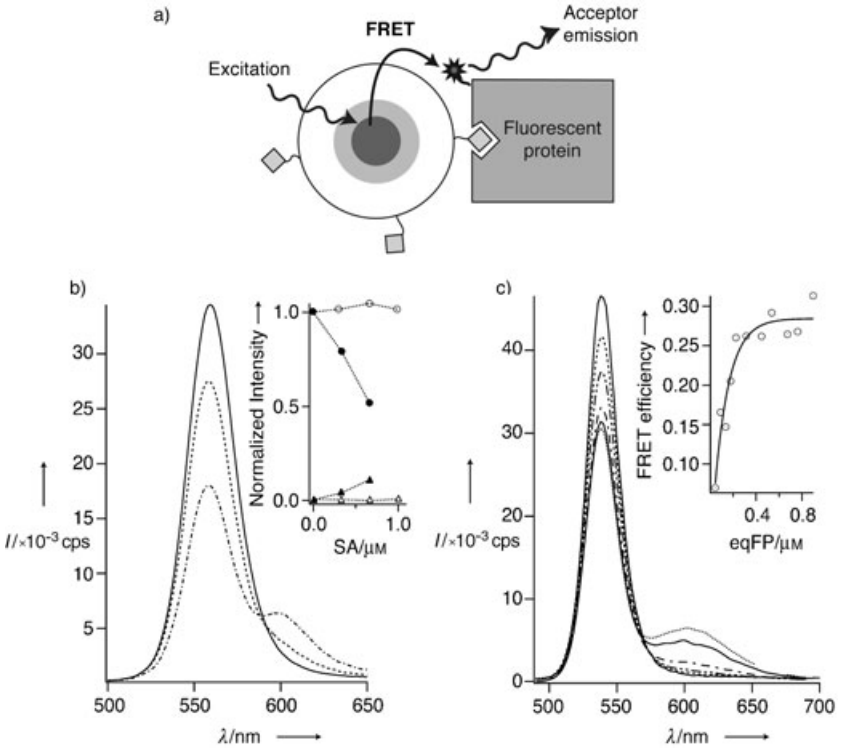

Figure 2. a) Specific interaction between a functionalized lipid-NC and a protein revealed by FRET. b) Fluorescence spectra of biotin-NCs (1 μM) in the absence (——) or presence (----- and –•–•) of increasing concentrations of SA-A568. Excitation was at 440 nm. Insert: Intensities at the maximum of the fluorescence spectrum of biotin-NCs (560 nm, ●) and SA-A568 (601 nm, ▲) and for the control experiment of nonfunctionalized lipid-coated NCs (560 nm, ○ and 601 nm, △) at increasing concentrations of SA-A568. The Förster distance R_0 for the couple NC/SA-A568 is 5.8 ± 0.2 nm. c) Fluorescence emission spectra of NTA-NCs (50 nM) in the absence (——) or presence (dotted and dashed lines) of increasing concentrations of eqFP611-His$_6$. Excitation was at 410 nm. Insert: FRET efficiency increased with the eqFP611-His$_6$ concentration to reach a maximum value of $E = 0.28$. The R_0 value for the couple NC/eqFP611-His$_6$ is 6.5 ± 0.2 nm. The distance between NC and eqFP611-His$_6$ is 9.7 ± 0.3 nm for 4–5 eqFP611-His$_6$ moieties per NC. All spectra shown were corrected for direct excitation of the fluorescent proteins.

NCs). The presence of both functionalities on the same NC could be demonstrated by sequential FRET from the NC via eqFP611-His$_6$ to SA-A633 upon addition of these proteins to biotin/NTA-NCs (Figure 3). Control experiments showed that eqFP611-His$_6$ and SA-A633 do not interact with each other or with lipid-NCs lacking both biotin and NTA. The average distances between the NC and either eqFP611 or SA, and between eqFP611 and SA, as determined by FRET are 9, 7, and 10 nm, respectively.

These results demonstrate two important features of multifunctionalized NCs. First, they can act as nanometer-sized scaffolds for the confined self-assembly of non-interacting macromolecules. Second, they serve as local excitation sources that transmit their excitation energy sequentially over distances greater than 10 nm by FRET to particular components within the supramolecular nanoassemblies.

In the next step, supramolecular nanostructures were built on top of 2D microstructured surfaces by exploiting the two above-mentioned concepts (Figure 4a). The micropatterning of SA on glass allowed the binding of biotin/NTA-NCs to surface areas of predefined shape (Figure 4b). Subsequent addition of eqFP611-His$_6$ led to the specific immobilization of this protein on the biotin/NTA-NCs areas, as demonstrated by co-localization of the fluorescence of both components and FRET between the biotin/NTA-NCs and eqFP611-His$_6$ (Figure 4c). The eqFP611-His$_6$ remained immobilized on surface-patterned nanostructures even after multiple washing with buffer, but was released after adding EDTA, which shows that the interaction between oligohistidine sequences and the NTA groups is stable and fully reversible. No binding to micropatterned SA was observed for lipid-NCs lacking biotin or for the biotin/NTA-NCs upon preincubation of the surface with excess biotin, which confirms specific binding of the NCs to the structured surface.

Patterned nanometer-sized local light sources offer attractive applications, for example, for DNA or protein arrays, where nonspecific binding of labeled analyte molecules impairs the high sensitivity with standard illumination techniques (resolution: hundreds of namometers). The NCs, as a

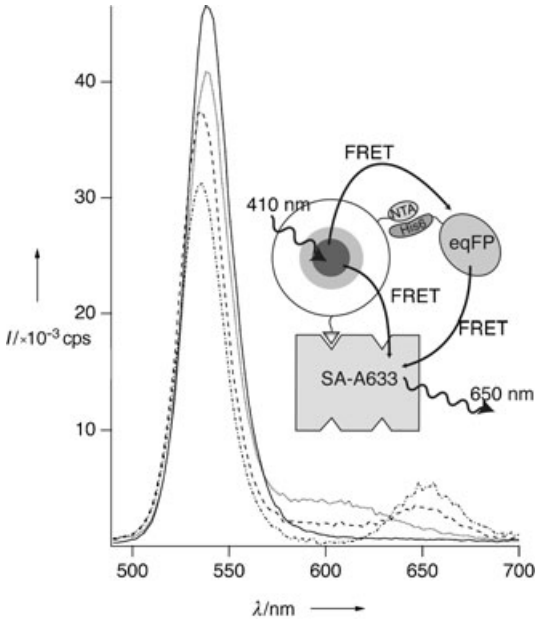

Figure 3. Schematic representation of a biotin/NTA-NC interacting with both eqFP611-His$_6$ and SA-A633. Fluorescence spectra of a 100-nM solution of the biotin/NTA-functionalized lipid-NCs in the absence (——) or presence of 0.4 μM eqFP611-His$_6$ (·····), and after subsequent addition of 47 nM (-----) and 156 nM (–•–•) SA-Alexa633 are also shown. Excitation was at 410 nm. FRET was first observed from the biotin/NTA-NCs to eqFP-His$_6$ and subsequently from the NCs to Alexa633, both directly and indirectly through eqFP611-His$_6$. The values of R_0 for the FRET couples NC/eqFP611, NC/SA-A633, and eqFP611/SA-A633 are 6.5 ± 0.2, 5.1 ± 0.2, and 10.2 ± 0.2 nm, respectively. The distances between these FRET couples are 8–9, 6–7, and 10–11 nm, respectively.

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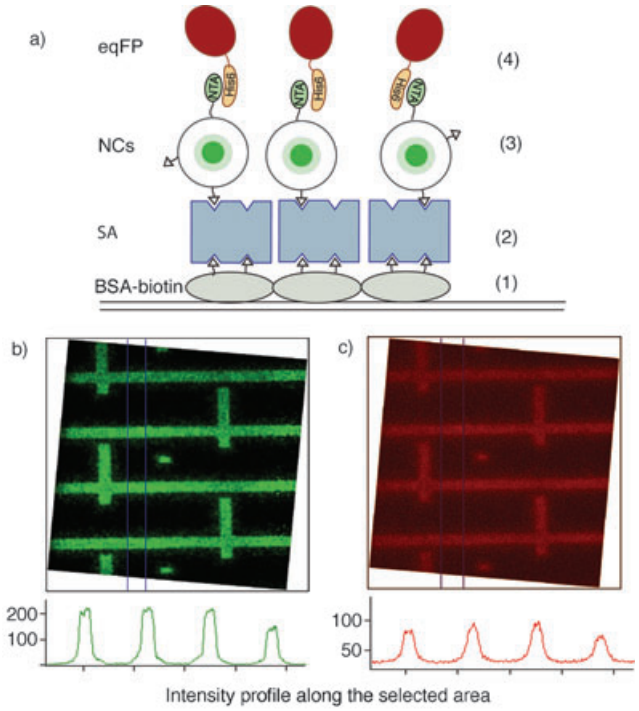

Figure 4. Directed supramolecular self-assembly on micropatterned surfaces using multifunctional NCs. a) The process is composed of the following sequential steps: 1) microcontact printing of biotinylated BSA (BSA-biotin) in 4-μm-wide lines on a glass surface; 2) binding of SA; 3) addition of biotin/NTA-NCs; and 4) incubation with eqFP611-His_6. b,c) Fluorescence confocal microscopy images and intensity profiles demonstrate specific binding of biotin/NTA-NCs to surface-patterned SA (b), step 3 (excitation: 458 nm; emission: 505–565 nm band-pass filter) and binding of eqFP611-His_6 onto surface-bound biotin/NTA-NC (c), with observation by FRET from biotin/NTA-NCs to eqFP611-His_6, step 4 (excitation: 458 nm; emission: 585 nm long-pass filter).

local light source, would only excite specifically bound analyte molecules within the FRET range (about 10 nm), thus enhancing the signal-to-noise ratio.

In summary, we have presented a versatile method to place multiple functionalities on NCs through lipid coating while minimizing nonspecific interactions. The physical and chemical long-term stability (weeks) of lipid-NCs is comparable to that of NCs covered by multilayered polymer coats.[23] If required, the chemical stability of the lipids can be increased even further by using, for example, lipids comprising ether instead of ester groups or by using lipids isolated from thermophilic bacteria.[24] Compared to the existing polymer-coated NCs, which are commercially available, the lipid-NCs offer complementary novel applications. Specific interaction between such NCs and different proteins through multiple high-affinity binding sites enabled site-selective controlled formation of supramolecular assemblies. The multifunctional NCs serve as local light sources which can be positioned with nanometer precision within supramolecular assemblies and can emit light over distances from 1 to more than 10 nm, again controlled with nanometer precision. Such concepts might be of importance for the development of biotechnology and bioanalytics on a nanometer and attoliter scale. Challenging examples are the use of single NCs for investigating substrate tunneling on multienzyme complexes,[25,26] or (bio)chemical syntheses and biomolecular reactions in submicrometer-sized containers.[27–29]

Experimental Section

Lipid-coated NCs: TOPO-stabilized CdSe NCs, synthesized in TOPO according to Peng,[30] were precipitated, centrifuged (4000 rpm, 5 min), and suspended in chloroform (2 volumes) containing lipids (1 mg mL^{-1}) composed of: a) 1-palmitoyl-2-oleyl-*sn*-glycerophosphatidylcholine (POPC; 90 mol%) and 1-palmitoyl-2-oleyl-*sn*-glycerophosphatidylglycerol (POPG; 10 mol%) for producing lipid-NCs; b) POPC (80 mol%), POPG (10 mol%), and either *N*-{[6-(biotinoyl)amino]hexanoyl}-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (DHPE-X-biotin, 10 mol%) or 1,2-dioleoyl-*sn*-glycero-3-[*N*-(5-amino-1-carboxypentyl)iminodiacetic acid succinyl] nickel salt (DOGS-NTA-Ni, 10 mol%), or an equimolar mixture of both, to produce biotin-, NTA-, or biotin/NTA-NCs, respectively. After adding β-octylglucopyranoside (OG, 26 mM) in water (4 volumes), the chloroform was evaporated under vacuum. The aqueous lipid-coated NC solution was dialyzed twice against 2000 volumes of water or phosphate-buffered saline (PBS) buffer to remove the detergent.

Absorbance and fluorescence spectra were recorded on a Perkin Elmer Lambda 35 spectrometer and a SPEX-fluorolog II instrument (Jobin Yvon, Stanmore, UK), respectively. Fluorescence quantum yields were determined as described elsewhere[31] using perylene-imide as standard.[32] The CdSe NCs exhibited fluorescence quantum yields of 20–25% and maximal fluorescence intensities between 540 and 560 nm, which correspond to CdSe core diameters between 2.9 and 3.3 nm;[33] the spectral halfwidths of less than 30 nm indicate that the particles are uniform with less than 10% variation in the particle size.

Samples for cryo-TEM were prepared as described elsewhere;[34] images from samples at −172 °C were recorded at a nominal magnification of 45000 on Kodak 4489 film in a Phillips CM12 electron microscope operated at 80 kV.

The Förster distance R_0 was calculated as previously described[31,35] assuming isotropic distribution of the transition dipoles of donor and acceptors, by taking the refractive index as 1.4 and quantum yield values of 0.1 and 0.45 for the NC and eqFP, respectively. The number n of acceptors associated with one NC and the distance r between donor and acceptor were calculated from the FRET efficiency E using the relation $E = nR_0^6/(nR_0^6 + r^6)$[6] and assuming that all acceptors are equidistant to the NC donor.

Surface patterning of NCs: NCs (40 nM) in PBS were overlaid on SA, which was patterned on glass slides in 4-μm-wide line structures as described elsewhere.[27,36,37] As a control experiment for specific binding, the surface was preincubated with biotin (1 μM) before adding the biotin/NTA-NCs. The formation and binding of molecules onto NC micropatterns were observed with a Zeiss LSM 510 confocal fluorescence microscope using appropriate laser and filter settings.

Received: July 30, 2004
Revised: November 11, 2004
Published online: January 26, 2005

Keywords: FRET (fluorescence resonant energy transfer) · lipids · luminescence · micropatterning · nanostructures

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