Biofunctionalization

Biofunctionalization of Fluorescent Rare-Earth-Doped Lanthanum Phosphate Colloidal Nanoparticles**

*Felix Meiser, Christina Cortez, and Frank Caruso**

In recent years, the utilization of nanoparticles (NPs) for conjugation with biomolecules has attracted widespread interest.[1–7] In particular, fluorescent semiconductor colloidal nanoparticles, or quantum dots (QDs), have played an important role in the application of NPs in biomedical applications.[1–3] Compared with their organic fluorophore counterparts, QDs can be prepared with high chemical stability, high quantum yield, and can exhibit high resistance to photobleaching. In addition, the optical properties of QDs can be tuned by controlling their size through synthesis. A range of biomolecules, such as deoxyribonucleic acid (DNA) and proteins, have been conjugated to QDs and used in labeling studies.[1] For example, QD bioconjugates have been used in the fluorescent labeling of cells,[2,3] agglutination assays,[3] in vitro detection assays,[8] and most recently, in selective and generalized imaging of live cells.[9–11] Despite the wide and successful use of QDs in diverse biomedical studies, commercial preparations of QDs face challenges associated with reproducible QD preparation, suitable surface coatings, and, in certain cases, cytotoxicity issues, particularly in vivo.[12,13]

An alternative class of colloidal NPs that are potentially promising for biolabeling studies are those based on rare-earth-doped lanthanum phosphates ($LaPO_4$). Recent work by Haase and co-workers reported the synthesis of monodisperse fluorescent $LaPO_4$ NPs.[14–16] These NPs, approximately 7 nm in size, have fluorescence that originates from their bulk properties—transitions between d and f electron states and their local symmetry—and is independent of their size.[14–16] The optical properties of the $LaPO_4$ NPs can also be tuned by the rare-earth dopant used—different colors are available by varying the dopants used in their synthesis (e.g., Ce, Tb, Eu, Dy).[14–17] The high chemical stability, high quantum yield (up to 61%),[16] and expected low toxicity[18] of these NPs make them potentially suitable for biological labeling applications. In addition, the application of rare-earth-doped $LaPO_4$ thin films (from micron-sized powders) as coatings for luminescent lamps points to a high photostability of such materials.[19,20] Herein, we report the first demonstration of the biofunctionalization of nanometer-sized colloidal $LaPO_4$ NPs. Green (Ce/Tb-doped) $LaPO_4$ NPs[17] were conjugated to the model protein avidin, a tetrameric protein that can bind with high affinity to four biotin molecules. NP functionalization was examined by using a suite of techniques, including analytical ultracentrifugation (AUC), microelectrophoresis, absorption and fluorescence spectroscopy, dynamic light scattering (DLS), and transmission electron microscopy (TEM).

Figure 1 illustrates the conjugation of $LaPO_4$ NPs with avidin. A primary requirement was to first obtain stable, aqueous colloidal dispersions of the NPs. This was achieved by dispersing the $LaPO_4$ NPs in an aqueous solution contain-

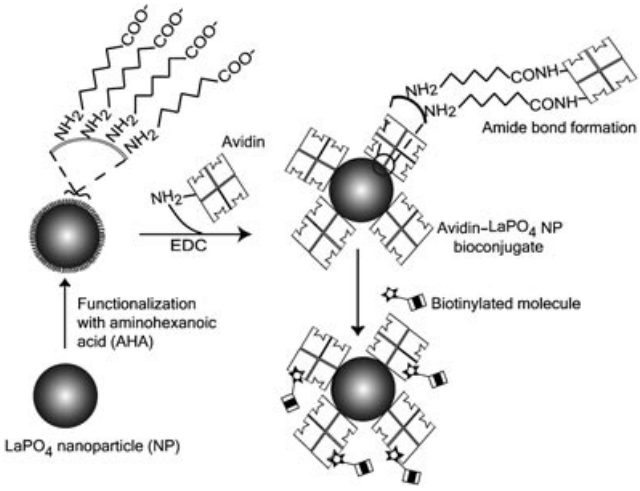

Figure 1. Schematic illustration showing the biofunctionalization of fluorescent Ce/Tb-doped $LaPO_4$ NPs with avidin. The NPs were first modified with AHA, and the carboxy groups of AHA were then activated by EDC to conjugate avidin through the formation of amide bonds. The binding of avidin–NP bioconjugates with biotinylated molecules is also shown.

[*] F. Meiser, C. Cortez, Prof. F. Caruso
Centre for Nanoscience and Nanotechnology
Department of Chemical and Biomolecular Engineering
The University of Melbourne
Victoria, 3010 (Australia)
Fax: (+61) 3-8344-4153
E-mail: fcaruso@unimelb.edu.au

[**] This work was supported by the Australian Research Council, the Victorian State Government Science, Technology, and Innovation initiative, and the BMBF. The Particulate Fluids Processing Centre is acknowledged for infrastructure support. S. Haubold and C. Meyer from Nanosolutions GmbH, Hamburg (Germany) are thanked for providing the nanoparticles. We thank H. Zastrow, A. Völkel, and C. Pilz (MPI Potsdam (Germany)) for technical assistance, and S. Crawford (The University of Melbourne) for assistance with the TEM measurements.

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

  DOI: 10.1002/ange.200460856

ing the bifunctional spacer 6-aminohexanoic acid (AHA). AHA imparts a negative charge on the surface of the NPs, as confirmed by the strongly negative ζ-potential at basic pH values (Figure 2). The isoelectric point (pI) of 4.5 obtained for

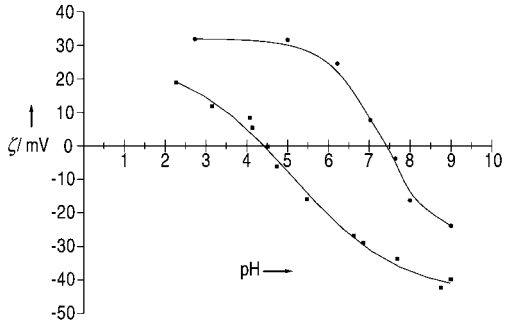

Figure 2. ζ-potential of the functionalized Ce/Tb-doped $LaPO_4$ NPs as a function of pH: after modification with AHA (■); and after AHA-modification and avidin bioconjugation (●). The plot shows the shift in isoelectric point upon AHA modification and avidin coating. The curves drawn are to guide the eye.

the AHA-modified $LaPO_4$ NPs is close to values reported for particles bearing carboxy groups,[21,22] which suggests that the amine group of AHA is attached to the particle surface, whereas the carboxy group is directed into the surrounding solution. Modification of the $LaPO_4$ NPs with AHA serves two important purposes. First, it confers sufficient colloidal stability to the nanoparticles where subsequent biofunctionalization is to be performed (pH 7). Support for this is also provided by examination of the AHA-coated $LaPO_4$ NPs by TEM, which reveals no signs of significant NP aggregation (Figure 3). Second, the terminal carboxy group at the particle surface allows the immobilization of amine-containing ligands, such as proteins. The protein avidin was chosen in this study to make use of the highly specific interaction between avidin and biotin ($K_d = 10^{-15}$ M),[23] since a wide variety of biotinylated molecules are commercially available. Avidin is composed of four identical subunits with each subunit folded in the form of a barrel (β-barrel).[24] The biotin-binding site is positioned near one end of the avidin barrel, hence two biotin-binding sites are present on each end of the protein.

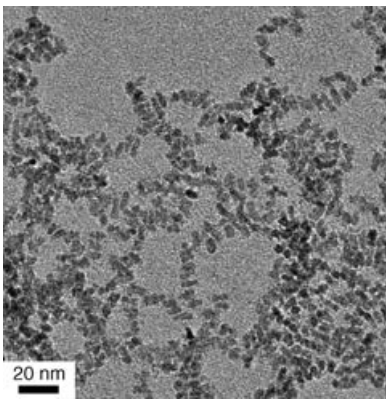

Figure 3. TEM image of AHA-modified Ce/Tb-doped $LaPO_4$ NPs.

The covalent coupling of avidin to the NP surface was facilitated by the crosslinker 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDC), which activates the carboxy groups on the NPs and leads to the formation of amide bonds with avidin. The avidin-coated AHA-modified $LaPO_4$ NPs showed a pI of 7.5, with a positive ζ-potential (30 mV) at pH 4.75 (Figure 2). The shift in ζ-potential after exposure to avidin (pI of 10–10.5)[25] indicates the successful conjugation of avidin to the NP surface.

Figure 4 shows the absorption and fluorescence spectra of the $LaPO_4$ NPs before and after conjugation with avidin. Three washing steps after the reaction ensured the removal of

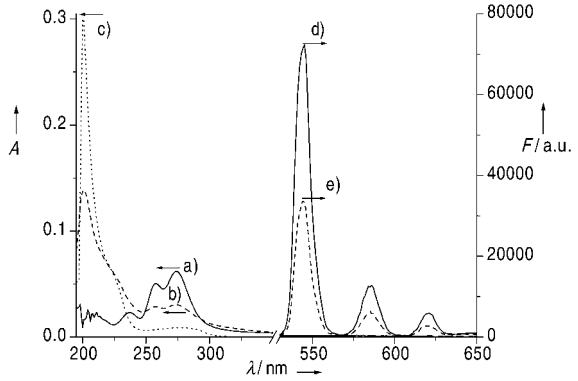

Figure 4. Absorption (*A*; a, b, c) and fluorescence (*F*; d, e) spectra of AHA-modified Ce/Tb-doped $LaPO_4$ NPs before (——) and after (-----) avidin functionalization. The absorption peaks of the NPs at 257 and 274 nm are evident. The absorption spectrum of pure avidin (c, •••••) is also shown.

unconjugated avidin in solution. The absorption spectrum of the AHA-modified Ce/Tb-doped $LaPO_4$ NPs (Figure 4, spectrum a) shows intense peaks at 257 and 274 nm, which are due to the absorption of the cerium dopant.[16] As expected, the avidin/AHA-$LaPO_4$ NP bioconjugates (Figure 4, spectrum b) show absorption features characteristic of both the AHA-functionalized NPs (peaks at 257 and 274 nm) and of pure avidin (major peak at ~200 nm, Figure 4, spectrum c). These results support the microelectrophoresis data for avidin conjugation to the NP surface. For the AHA-modified NPs three distinct fluorescence peaks are observed at 545, 585, and 622 nm (Figure 4, spectrum d). These fluorescence peaks are due to the d–f orbital transitions of the dopants.[14–16] An additional peak was also observed at 490 nm (not shown).[26] The same characteristic fluorescence peaks can also be observed after avidin biofunctionalization (Figure 4, spectrum e). These data are in agreement with our previous work where the characteristic fluorescence peaks of $LaPO_4$ NPs interacting with polyelectrolytes in multilayer thin films appear at the same spectral positions as those for the corresponding NPs in solution.[17]

To verify the increase in diameter as a result of bioconjugation, AUC was used to measure the size distribution of the NPs before and after avidin coating (Figure 5a). Assuming a density of 5.0 g cm^{-3} for the $LaPO_4$ NPs,[27] we determined an average diameter of 7.6 nm for the AHA-

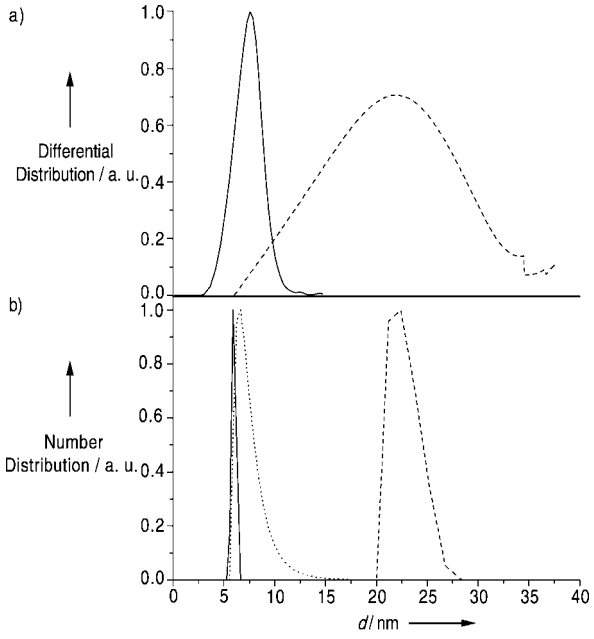

Figure 5. Diameter distribution of AHA-modified Ce/Tb-doped $LaPO_4$ NPs before (——) and after (-----) avidin functionalization, as determined by a) AUC and b) DLS methods. In the AUC measurements (a), a particle density of 5.0 $g\,cm^{-3}$ was used. In (b), the diameter distribution of avidin is also shown (•••••).

modified NPs. This value is close to that derived from TEM (mean size 6–8 nm; see Figure 3 and ref. [17]).[28] After bioconjugation, if the same density is assumed, the diameter increases to 21.8 nm, which is consistent with having a monolayer of avidin molecules, approximately 7 nm in size. This value is slightly higher than the size of avidin (dimensions $6.0 \times 5.5 \times 4.0$ nm[29]). Since the exact density of avidin-coated NPs is not known, the method of DLS was also employed to yield information on the hydrodynamic radius of the NPs. Figure 5b shows the DLS diameters of the individual AHA-modified $LaPO_4$ NPs and avidin, as well as the avidin–$LaPO_4$ NP bioconjugate. The measured diameter for the AHA-modified NPs (~7 nm) is in good agreement with that determined from TEM and AUC, while the measured diameter of avidin (~7 nm) is similar to that determined from AUC experiments. The DLS diameter of about 22 nm for the bioconjugate closely agrees with the AUC data when assuming a density of 5.0 $g\,cm^{-3}$ for the AUC experiments (Figure 5b). Both the AUC and DLS data further support avidin functionalization of the $LaPO_4$ NPs.

We also performed an assay utilizing biotin covalently linked to the organic dye fluorescein (biotin-FITC) to test the binding capacity of the avidin-functionalized NPs to biotin. The bioconjugates were mixed with biotin-FITC in the presence of 2-[*N*-morpholino]ethanesulfonic acid (MES) buffer (pH 4.75) and phosphate buffer (PB, pH 9) in a 9:1 volume ratio, and then centrifuged to sediment the particles, leading to the formation of a pellet. The supernatant fluorescence was measured and compared against a standard of known biotin-FITC concentration. A decrease in the amount of biotin-FITC in the supernatant (i.e., decrease in fluorescence intensity) was observed after incubation with the bioconjugates, indicating biotin-FITC binding to the avidin-functionalized $LaPO_4$ NPs. The decrease in fluorescence intensity is directly correlated to the amount of biotin-FITC bound, and the point at which the fluorescence begins to appear in the supernatant corresponds to saturation binding of the avidin-coated $LaPO_4$ NPs. From these experiments, we calculated that 29 biotin molecules are bound per particle (see Supporting Information). Assuming that each avidin can bind four biotin molecules, the number of avidin molecules conjugated to each NP is calculated as about seven.

A second approach, the micro BCA protein assay, was performed to determine the amount of avidin conjugated to the NPs. This method is based on a colorimetric measurement and involved the comparison of the amount of avidin per NP with a standard concentration series of avidin (see Supporting Information). Approximately 6.2×10^{17} avidin molecules were calculated to be present in a sample of 7.7×10^{16} avidin-functionalized NPs, which equates to approximately eight avidin molecules per NP. This value is in good agreement with that determined from the fluorescence assay. The protein-to-nanoparticle ratio obtained is similar to that reported for bioconjugation of a maltose binding protein, which is slightly smaller than avidin (size $3 \times 4 \times 6.5$ nm), with 6-nm diameter QDs, where it was found that about ten proteins were coupled to each QD NP.[30]

In conclusion, fluorescent Ce/Tb-doped $LaPO_4$ NPs with a diameter of about 7 nm have been prepared as stable colloidal dispersions by modification with AHA, and subsequently biofunctionalized with the model protein avidin. Assays show that approximately eight avidin molecules are conjugated to each $LaPO_4$ NP. The avidin molecules on the NP surface remain active, as demonstrated by the ability of these avidin–NP conjugates to bind biotin. Since biotinylated molecules are easily obtained, we envisage the applicability of these bioconjugates in biosensing and biolabeling applications where their photostability and fluorescent properties should prove useful. Binding to biotinylated antibodies would demonstrate their use in immunofluorescence assays, particularly in ELISA-type applications. The successful bioconjugation of the AHA-functionalized $LaPO_4$ NPs with avidin suggests that, in principle, coupling of other biomolecules (such as protein A, antibodies, and DNA) to the rare-earth-doped $LaPO_4$ NPs through the same protocol is possible. Other features, such as dopant-tunable emission,[14–17] photon-upconversion properties,[31] and the possibility to detect multiple labels using a single excitation wavelength with no spectral overlap,[17] offer considerable promise for the use of biofunctionalized rare-earth-doped lanthanide phosphate NPs in various biotechnological applications.

Experimental Section

Ce/Tb-doped $LaPO_4$ NPs, as a powder, were kindly provided by Nanosolutions GmbH, Hamburg, Germany. The NPs were synthesized according to the method of Haase and co-workers.[16] Modification of the NPs was achieved by dispersing them with AHA in 0.1M NaOH. The NPs were washed several times in 0.1M tris(hydroxymethyl)aminomethane hydrochloride (Tris) buffer (pH 9) and ethanol, and then redispersed in 0.1M Tris buffer (pH 9). For details see Supporting Information. This dispersion was colloidally stable for at

least two months when stored at 4 °C. The AHA-modified NPs were functionalized with avidin in a reaction mixture containing EDC dissolved in *N*-methyl-imidazole buffer (0.05 M, pH 7), and then redispersed in 0.05 M MES buffer, pH 4.75. For details see Supporting Information.

ζ-potentials of the NPs in aqueous solution were measured by using a ZetaSizer 2000, Malvern Instruments. DLS data were obtained with a High Performance Particle Sizer from Malvern Instruments. AUC measurements were performed on a Beckman-Coulter Optima XL-1 ultracentrifuge. UV/Vis spectra were obtained with a 8453 spectrophotometer from Agilent Technologies. The $LaPO_4$ NP molar extinction coefficient at a wavelength of 274 nm was calculated as $9.0 \times 10^5\,M^{-1}\,cm^{-1}$, using a density of $5.0\,g\,cm^{-3}$ and a radius of 3.8 nm. Fluorescence spectral measurements were performed by using a Cary Eclipse fluorescence spectrophotometer. The excitation wavelength for the $LaPO_4$ NPs was set at 254 nm. Excitation of biotinylated fluorescein was at 494 nm. TEM experiments were carried out on a Philips CM120 BioTWIN TEM operated at an acceleration voltage of 120 kV. Samples for TEM experiments were prepared by placing 3 μL of the NPs diluted 1/100 in 0.01 M Tris buffer (pH 9) on a TEM copper grid and allowing them to air dry overnight.

Binding of biotinylated fluorescein (biotin-FITC) to the avidin-functionalized NPs was tested by mixing the two components in the presence of buffer and centrifuging at 15000 g for 15 min to sediment the NPs containing bound biotin-FITC. The fluorescence of the supernatant was measured and compared against a standard containing the same initial amount of biotin-FITC. The concentration at which the avidin-coated NPs were saturated with biotin was determined by performing the assay with final concentrations of biotin-FITC ranging from 0.05 μM to 2.5 μM. An adsorption isotherm was plotted and the amount of biotin bound to the avidin-coated NPs was determined, and hence the amount of avidin per NP (see Supporting Information). The Micro BCA Protein Assay Reagent Kit was obtained from Pierce (Rockford, IL) and used as described in the protocol (see Supporting Information)

Received: June 3, 2004

Keywords: biolabeling · colloids · lanthanides · luminescence · nanoparticles

[1] W. J. Parak, D. Gerion, T. Pellegrino, D. Zanchet, C. Micheel, S. C. Williams, R. Boudreau, M. A. Le Gros, C. A. Larabell, A. P. Alivisatos, *Nanotechnology* **2003**, *14*, R15.
[2] M. Bruchez, M. Moronne, P. Gin, S. Weiss, A. P. Alivisatos, *Science* **1998**, *281*, 2013.
[3] W. C. W. Chan, S. Nie, *Science* **1998**, *281*, 2016.
[4] H. Weller, *Adv. Mater.* **1993**, *5*, 88.
[5] A. P. Alivisatos, *Science* **1996**, *271*, 933.
[6] Y. Xiao, F. Patolsky, E. Katz, J. F. Hainfeld, I. Willner, *Science* **2003**, *299*, 1877.
[7] C. M. Niemeyer, *Angew. Chem.* **2001**, *113*, 4254; *Angew. Chem. Int. Ed.* **2001**, *40*, 4128.
[8] E. R. Goldman, E. D. Balighian, H. Mattoussi, M. K. Kuno, J. M. Mauro, P. T. Tran, G. P. Anderson, *J. Am. Chem. Soc.* **2002**, *124*, 6378.
[9] X. Y. Wu, H. J. Liu, J. Q. Liu, K. N. Haley, J. A. Treadway, J. P. Larson, N. F. Ge, F. Peale, M. P. Bruchez, *Nat. Biotechnol.* **2003**, *21*, 41.
[10] B. Dubertret, P. Skourides, D. J. Norris, V. Noireaux, A. H. Brivanlou, A. Libchaber, *Science* **2002**, *298*, 1759.
[11] J. K. Jaiswal, H. Mattoussi, J. M. Mauro, S. M. Simon, *Nat. Biotechnol.* **2003**, *21*, 47.
[12] B. Ballou, B. C. Lagerholm, L. A. Ernst, M. C. Bruchez, A. S. Waggoner, *Bioconjugate Chem.* **2004**, *15*, 79.
[13] A. M. Derfus, W. C. W. Chan, S. N. Bhatia, *Nano. Lett.* **2004**, *4*, 11.
[14] H. Meyssamy, K. Riwotzki, A. Kornowski, S. Naused, M. Haase, *Adv. Mater.* **1999**, *11*, 840.
[15] K. Riwotzki, H. Meyssamy, A. Kornowski, M. Haase, *J. Phys. Chem. B* **2000**, *104*, 2824.
[16] K. Riwotzki, H. Meyssamy, H. Schnablegger, A. Kornowski, M. Haase, *Angew. Chem.* **2001**, *113*, 574; *Angew. Chem. Int. Ed.* **2001**, *40*, 573.
[17] P. Schuetz, F. Caruso, *Chem. Mater.* **2002**, *14*, 4509.
[18] A. J. Hutchison, *Peritoneal Dial. Int.* **1999**, *19*, S408. Based on this and other studies using lanthanum ions for medical applications.
[19] J. C. Bourcet, F. K. Fong, *J. Chem. Phys.* **1974**, *60*, 34.
[20] N. Hashimoto, Y. Takada, K. Sato, S. Ibuki, *J. Lumin.* **1991**, *22*, 893.
[21] N. Kato, P. Schuetz, A. Fery, F. Caruso, *Macromolecules* **2002**, *35*, 9780.
[22] S. E. Burke, C. J. Barrett, *Langmuir* **2003**, *19*, 3297.
[23] M. Wilchek, E. A. Bayer, *Methods Enzymol.* **1990**, *184*, 14.
[24] O. Livnah, E. A. Bayer, M. Wilchek, J. L. Sussman, *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 5076.
[25] A. L. Lehninger, *Biochemistry*, 2nd ed., Worth Publishers, New York, **1975**.
[26] The spectral range from 480 to 520 nm is not shown, as the use of a filter was required to eliminate excitation light.
[27] For calculation, a density of $5.0\,g\,cm^{-3}$ was used, as this is the mean value for the mineral monazite, which has the same constituents as the $LaPO_4$ NPs. *Encyclopedia of Minerals* (Eds.: W. L. Roberts, G. R. Rapp, Jr., J. Weber), Van Nostrand Reinhold, New York, **1974**, p. 143.
[28] Both spherical and ellipsoidal nanoparticle shapes are observed by TEM, in agreement with previous work.[16]
[29] N. M. Green, M. A. Joynson, *Biochem. J.* **1970**, *118*, 71.
[30] I. G. Medintz, A. R. Clapp, H. Matoussi, E. R. Goldman, B. Fischer, J. M. Mauro, *Nat. Mater.* **2003**, *2*, 630.
[31] S. Heer, O. Lehmann, M. Haase, H.-U. Güdel, *Angew. Chem.* **2003**, *115*, 3288; *Angew. Chem. Int. Ed.* **2003**, *42*, 3179.

