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Biotinylated CdSe/ZnSe nanocrystals for specific fluorescent labeling

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A set of two new surface ligands is presented for the preparation of water-soluble, biotinylated CdSe/ZnSe core/shell nanocrystals, suitable for fluorescent biological labeling. It consists of a thiolated diethyleneglycol derivative and an alkylthiol substituted biotin molecule, which replace the initial capping ligands at the nanocrystal surface. Successful ligand exchange and long-term photostability of the modified nanocrystals as well as their highly specific binding to neuronal cells are demonstrated in different labeling experiments.

Introduction

Fluorescent semiconductor nanocrystals have attracted much attention in the field of biological labeling due to their unique optical properties combined with high photo-stability. Core/shell structures of CdSe/ZnS, CdSe/ZnSe or CdSe/CdS type are of special interest, as they exhibit efficient photoluminescence in the visible part of the spectrum.[1–4] Synthesized by organometallic routes in non-aqueous solvents, these nanocrystals are hydrophobic because surfactant molecules (*e.g.* trioctylphosphine oxide, TOPO) cap their surface and prevent them from aggregating. In order to achieve nanocrystal hydrosolubility – indispensable for their use in biological labeling – different strategies have been applied in the literature:

1.) Substitution of the original surface ligands by (di)mercaptocarboxylic acids; nanocrystals can be dispersed in water after deprotonation of the carboxylic acid group by increasing the pH.[5,6] Disadvantages of this procedure are the pH-sensitivity of the colloidal dispersion and the high tendency for non-specific interactions of the (charged) carboxylate groups with biomolecules such as proteins, DNA, *etc.*

2.) Growth of an additional silica shell on the semiconductor shell.[7,8] Though providing very high nanocrystal stability, a rather complicated series of synthesis and purification steps and a difficult control of the resulting surface chemistry are drawbacks of this approach.

3.) Encapsulating the nanocrystals with micelles,[9] cross-linked polymers[10] or charged proteins.[11] These techniques do not replace the original surface ligands and therefore preserve essentially the initial fluorescence quantum yield (Q.Y.). On the other hand, the diameter of the nanocrystals increases drastically and lies typically in the range of 12–25 nm. This may be too bulky for certain applications due to the reduced capability of the nanocrystals to access target systems. Furthermore, as a consequence of their large size, the number of nanocrystals which can bind to a target molecule is reduced, which may decrease imaging sensitivity.

In this article, we present a simple and straigthforward method to overcome the cited problems. Two new surface ligands are synthesized, which minimize non-specific interactions because of uncharged solubilization functions, while maintaining essentially the original nanocrystal diameter.

Results and discussion

CdSe/ZnSe nanocrystals[12] are dispersed in water after functionalization with the specially designed ligand **1** (Scheme 1), namely a derivative of diethyleneglycol where one alcohol group has been replaced by a thiol group. The latter binds to the nanocrystal surface, while the two ether groups as well as the remaining alcohol function ensure hydrosolubility. The substantial role of the ether groups becomes clear in control experiments where 1-mercaptohexanol is used instead of ligand **1**, leading to nanocrystals which can be dispersed in alcohols but not in water. The neutral alcohol group in **1** minimizes non-specific electrostatic interactions, but does not allow for the conjugation of nanocrystals with biomolecules *via* crosslinking agents. For this reason we synthesized ligand **2** (Scheme 1), an alkylthiol substituted biotin molecule.

Once "biotinylated" by means of this ligand, nanocrystals can easily be conjugated with biomolecules, using the specific and strong interaction between biotin and streptavidin. As a result of the relatively short chain length of ligand **1**, the biotin moieties of ligand **2** can stick out the organic capping layer, enhancing its accessibility by streptavidin binding sites. In order not to affect the hydrosolubility of the nanocrystals, we used low molar fractions (typically 5 mol%) of ligand **2** with respect to ligand **1** during nanocrystal functionalization. For molar fractions exceeding 10%, nanocrystals do not redisperse completely in buffer solutions.

Functionalization of CdSe/ZnSe nanocrystals with ligand **1** enables their dispersion in water or in buffer solutions. To study the influence of the buffer system, different 50 mmolar solutions have been compared. Our results indicate that TRIS (pH 8), HEPES (pH 7.4) and especially borate buffers (pH 8.5) provide better colloidal and photophysical stability of the functionalized nanocrystals than phosphate based buffers such as phosphates (pH 7.4 and 8.5) and phosphate buffered saline (PBS; pH 7.4, 10 mmolar solution). Colloidal stability has been verified by repeated centrifugation of the samples in intervals of several days in order to detect partial precipitation. After one month keeping the functionalized nanocrystals in borate buffer solution, no precipitate and no altering of the fluorescence properties could be observed. It should be underlined here that the pH of the solution can be chosen in a range varying from

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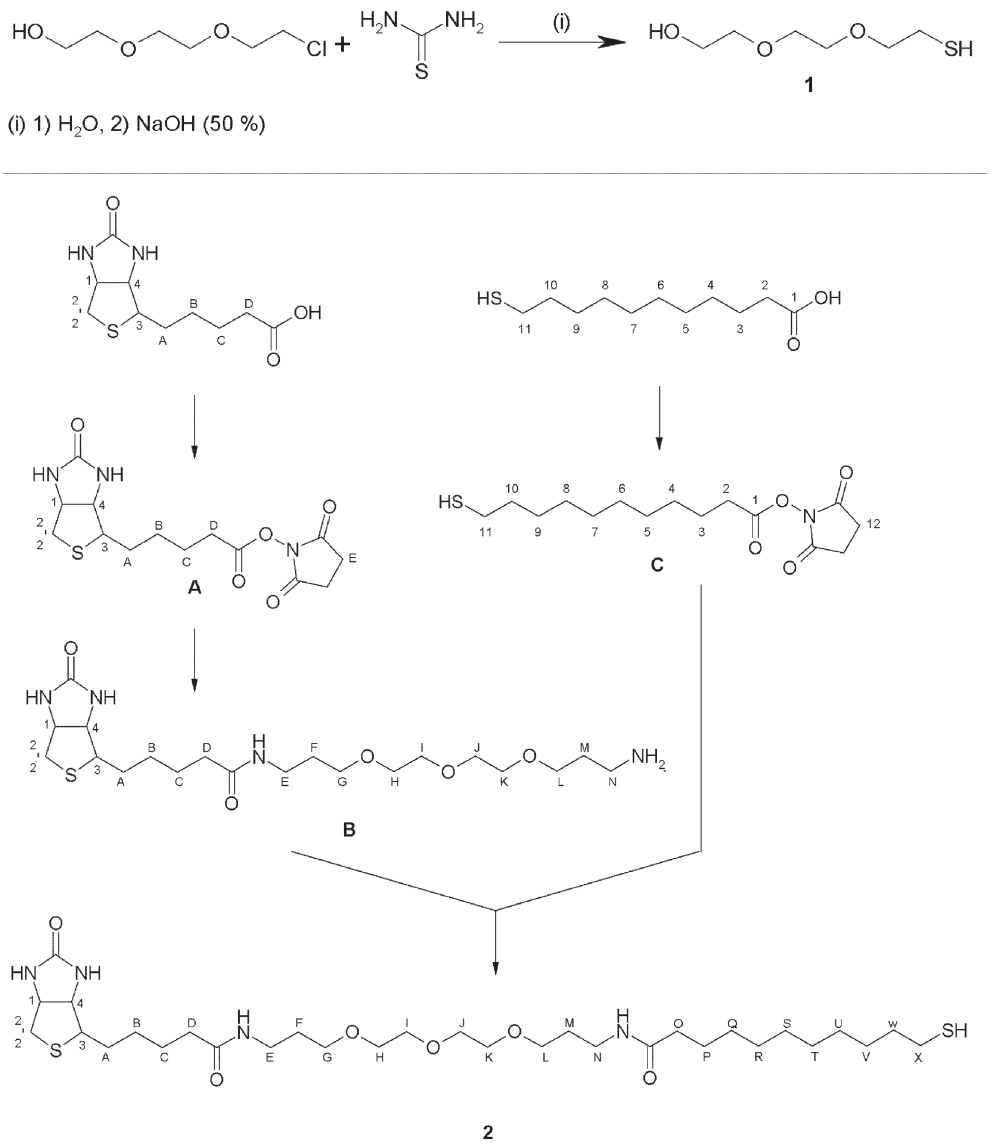

Scheme 1 Synthesis pathways of ligands **1** and **2**, used for the preparation of water-soluble, biotinylated core/shell nanocrystals. For details and characterization data see Experimental section.

slightly basic to slightly acidic values (pH 5–9) without precipitation of the nanocrystals. This is an important advantage over methods where hydrosolubility is assured by charged groups, such as carboxylate or ammonium groups. On the other hand, a decrease of the fluorescence Q.Y. of the CdSe/ZnSe nanocrystals upon ligand exchange and dispersion in water is observed.[13] We attribute this to hole trapping by the thiol ligands, which could be additionally favoured by the relatively weak confinement of the holes in the CdSe/ZnSe core/shell structure. As will be shown in the following, the fluorescence efficiency remains however largely sufficient and stable for labeling experiments. In Fig. 1, optical properties of the nanocrystals before and after functionalization are displayed.

If the nanocrystals are successfully functionalized with both ligand **1** and ligand **2**, they can selectively bind to streptavidin. As a control experiment, biotinylated agarose beads with a mean size of 50 μm are first incubated with streptavidin and then with the functionalized nanocrystals. After thoroughly rinsing, the agarose beads exhibit a bright fluorescence, which clearly proves the successful binding of the nanocrystals to the beads. To exclude non-specific interaction of the nanocrystal surface ligands with the beads, a large excess of biotin has been added without any detectable decrease of the beads' fluorescence. If, however, nanocrystals carrying exclusively ligand **1** and not ligand **2** (no biotin groups) are used for incubation, no fluorescence of the beads is detectable after washing. In an additional experiment the stability against photobleaching of the nanocrystal labeled beads is compared to the one of beads, which are labeled with R-Phycoerythrin, one of the most commonly-used fluorescent dyes. Both samples are continuously irradiated and photos are taken at different time intervals (Fig. 2). While the fluorescence of the Phycoerythrin labeled beads is practically extinguished after 15 minutes, the emission of the nanocrystal labeled ones can still be detected after 8 hours, demonstrating a significantly higher photostability.

Finally we present first results where the water-soluble, biotinylated nanocrystals are used for labeling of nerve cells in primary cell culture. For this issue, neuronal cells from mouse embryo cerebellum (stage E13), grown for 5 days on polylysin glass covers, are fixed in 4% paraformaldehyde and are made permeable with triton X100. The primary antibody TuJ1 from neuronal microtubules is reacted with a biotinylated secondary antibody, which allows, after incubation with streptavidin, the binding of biotinylated nanocrystals. Positive control experiments are carried out either by replacement of the streptavidin–biotin–nanocrystal complex by Cy3 labeled streptavidin or by direct revelation of TuJ1 antibody with a common fluorescent dye (Alexa-488, Molecular Probes), fixed on the secondary antibody. Negative control experiments are realized by omission of the TuJ1 antibody from the experiment. Fig. 3a reveals

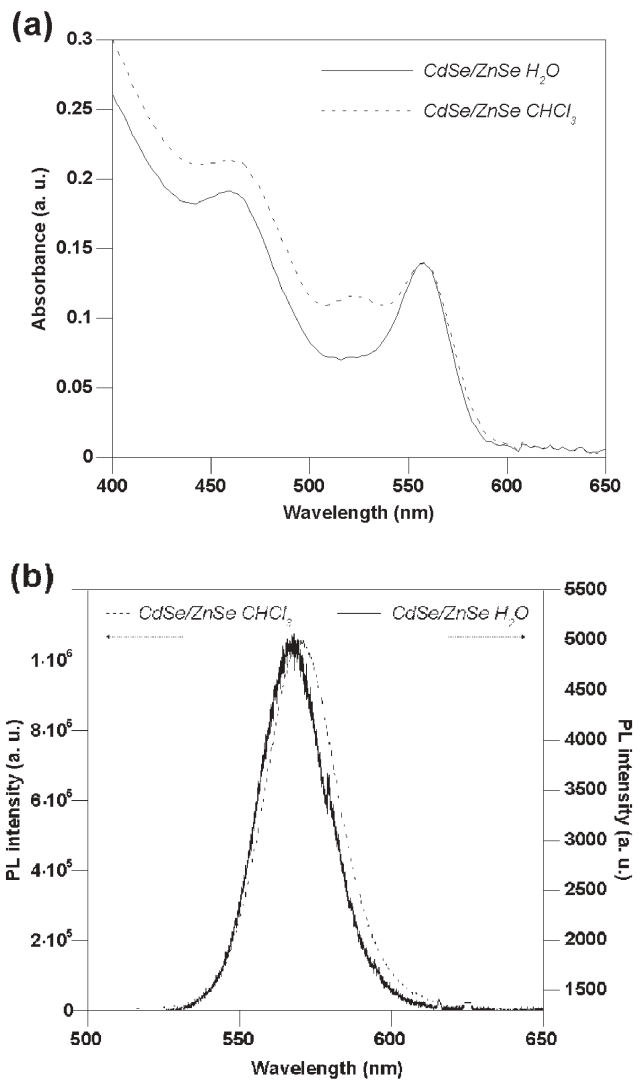

Fig. 1 (a) UV-vis absorption spectra of the CdSe/ZnSe core/shell nanocrystals used in this study. (b) Photoluminescence spectra of the same nanocrystals (excitation wavelength 365 nm).

unequivocally the specific targeting of the nerve cells by the nanocrystals, as can be concluded from the comparison with the control (Fig. 3b). In contrast to a similar experiment where carboxylated CdS nanocrystals were attached to neurons,[14]

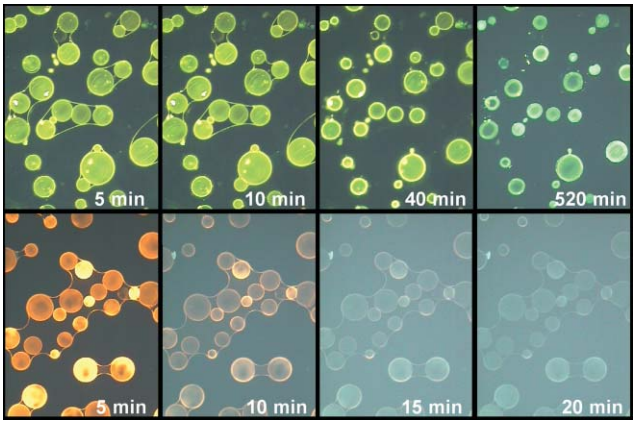

Fig. 2 Fluorescence images of agarose beads labeled with biotinylated nanocrystals (top row) and with biotinylated R-Phycoerythrin (bottom row). The mean size of the beads is 50 μm. Under continuous irradiation with a mercury lamp (100 W), photos are taken at different time intervals using an Olympus Microscope equipped with an U-MWU band pass filter (cut-off 330–385 nm).

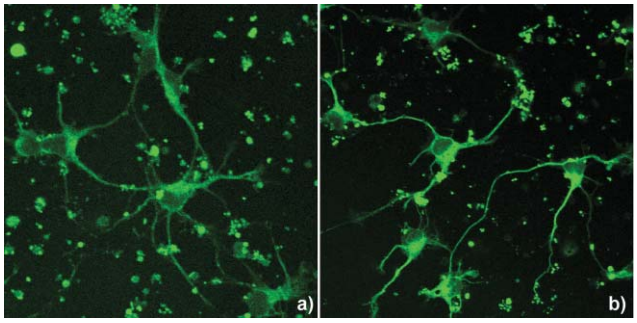

Fig. 3 (a) Fluorescence image of nerve cells in primary culture labeled with biotinylated nanocrystals (excitation wavelength 458 nm). (b) Control experiment with Alexa-488 labeled cells.

fluorescent labeling occurs also on the cell interior, where the TuJ1 antibodies are located. This underlines the potential of functionalized nanocrystals to act as efficient intracellular fluorescent probes after triton X100 permeabilization.

Conclusion

We have presented a set of two new ligands, specially designed for the use of CdSe/ZnSe and similar types of nanocrystals in biological labeling: the first one providing hydrosolubility and the second one allowing for specific binding to streptavidin *via* a biotin group. The functionalized nanocrystals exhibit long-term colloidal stability in common buffer systems such as borate solutions. Successful functionalization of nanocrystals with the two ligands has been shown by labeling of streptavidin modified agarose beads. The stability of the fluorescence of these beads against photobleaching is increased by more than one order of magnitude as compared to R-Phycoerythrin labeled ones. Staining of nerve cells demonstrates the very low tendency for non-specific binding of the biotinylated nanocrystals as a consequence of the use of uncharged groups to assure hydrosolubility. This underlines the high potential for their use in intracellular labeling.

Experimental

Synthesis of ligand 1 (8-thio-3,6-dioxaoctanol) (ref. 15)

In a 50 mL two-neck flask equipped with a condenser, 5 g (29.6 mmol) of 2-(2-(2-chloroethoxy)ethoxy)ethanol (Aldrich, Ref. 16,297-3, 98%) and 2.97 g (39 mmol) of thiourea (Aldrich, Ref. 24,025-7, 99+%), dissolved in 20 mL of ultrapure water, are stirred under reflux for 2 hours. The mixture becomes homogeneous after a few minutes. A solution of 2.5 g of sodium hydroxide in 25 mL of water is added and the mixture is refluxed for a further 2 hours. After cooling, the aqueous layer is acidified with a solution of 6 mL of concentrated sulfuric acid in 4 mL of water, and extracted with ethyl acetate (5 × 25 mL). The collected organic phases are dried over sodium sulfate, filtered, evaporated under reduced pressure and purified by flash chromatography on silica (chloroform/methanol, 97.5/2.5, R_f: 0.5) to give compound **1** as a colourless and odourless oil (2.43 g, 50%).

δ^1_H (200.13 MHz, $CDCl_3$) 3.76–3.60 (10H, m, CH_2O), 2.71 (2H, dt, J = 8.06 Hz and J = 6.19 Hz, H_6), 2.52 (1H, m, OH), 1.60 (1H, t, J = 8.06 Hz, SH).

δ^{13}_C (56.32 MHz, $CDCl_3$) 72.87 (C_2), 72.48 (C_5), 70.36 (C_3), 70.27 (C_4), 61.78 (C_1), 24.21 (C_6).

m/z (Fab+) 167 $(M + H)^+$, 184.

$C_6H_{14}O_3S$, found: C, 43.32%; H, 8.32%; S, 19.30%, requires C, 43.35%; H, 8.49%; S, 19.29%.

Synthesis of ligand 2

Biotin *N*-hydroxysuccinimide ester A (ref. 16). To a stirred solution of biotin (Sigma, Ref. B-4501, 99%, 5 g, 20.47 mmol) and *N*-hydroxysuccinimide (Aldrich, Ref. 13,067-2, 97%, 2.36 g, 20.47 mmol) in anhydrous DMF (150 mL), 1.3 eq. of 1,3-dicyclohexylcarbodiimide (Aldrich, Ref. D8,000-2, 99%, 5.49 g, 24.44 mmol) is added. The mixture is stirred at room temperature for 48 h. The formed dicyclohexylurea is filtered off and the solvent is evaporated under reduced pressure to dryness. 500 mL of diethyl ether are added to the residue and the solution is stirred for 2 h, giving a white precipitate. After filtration, this solid is recrystallized in isopropanol, yielding the pure product as a white powder (6.5 g, 93%).

δ^1_H (200.13 MHz, $CDCl_3$) 4.31 (1H, m, H_1), 4.16 (1H, m, H_4), 3.10 (1H, m, H_3), 2.89 (1H, m, H_2), 2.81 (5H, s, $H_E + H_2'$), 2.67 (2H, m, H_D), 1.80–1.35 (6H, m, $H_{A,B,C}$).

m/*z* (DCI NH_3 + Isobutane) 342 $(M + H)^+$.

***N*-(13-Amino-4,7,10-trioxatridecanyl) biotinamide B (ref. 17).** Biotin *N*-hydroxysuccinimide ester **A** (4 g, 11.71 mmol) is dissolved in 100 mL of dry DMF. Under inert atmosphere, this solution is added dropwise within 1 h to a solution of 4,7,10-trioxa-1,13-tridecanediamine (Aldrich, Ref. 36,951-9, 97%, 12.91 g, 58.6 mmol) in 4 mL of triethylamine. After stirring the reaction mixture at room temperature for 72 h, the solid formed is filtered off and DMF is evaporated under reduced pressure. The resulting oil is added dropwise to 1 L of hexane. A white precipitate is formed after a few minutes. It is recrystallized in isopropanol (2 h at reflux, 12 h without heating and stirring), yielding 3.55 g of the pure product as white crystals (67%).

F_p: 111–113 °C

δ^1_H (200.13 MHz, D_2O) 4.40 (1H, m, H_1), 4.25 (1H, m, H_4), 3.60–3.35 (12H, m, H_{G-L}), 3.25–3.10 (3H, m, $H_3 + H_E$), 2.90–2.81 (2H, m, H_2), 2.67 (2H, m, H_N), 2.12 (2H, m, H_D), 1.75–1.50 (8H, m, $H_{A,B,C,M}$), 1.42 (2H, m, H_F).

m/*z* (DCI NH_3 + Isobutane) 447 $(M + H)^+$.

11-Mercaptoundecanoyl-*N*-hydroxysuccinimide ester C (ref. 18). *N*-Hydroxysuccinimide (Aldrich, Ref. 13,067-2, 97%, 1.00 g, 8.7 mmol) is stirred in dichloromethane (500 mL) for 30 min. 11-Mercaptoundecanoic acid (MUA) (1.88 g, 8.62 mmol), dissolved in 10 mL of dichloromethane, is poured into the first solution. Then 1,3-dicyclohexylcarbodiimide (DCC) (Aldrich, Ref. D8,000-2, 99%, 1.96 g, 9.5 mmol), dissolved in 50 mL of dichloromethane, is added dropwise within 30 min. The resulting mixture is stirred at room temperature for 24 h. The dicyclohexylurea formed is filtered off and the solvent is evaporated under reduced pressure. The resulting residue is purified by flash chromatography (pentane/diethyl ether, 50/50, R_f: 0.4) to yield compound **C** as a white solid (1.63 g, 60%).

δ^1_H (200.13 MHz, $CDCl_3$) 2.84 (4H, s, H_{12}), 2.60 (2H, t, J = 7.40 Hz, H_2), 2.52 (2H, pseudo dt, H_{11}), 1.85–1.50 (4H, m, $H_{10} + H_3$), 1.50–1.10 (12H, m, H_4–H_9).

δ^{13}_C (56.32 MHz, $CDCl_3$) 169.1, 168.6, 34.0, 30.9, 29.3, 29.2, 29.0, 28.7, 28.3, 25.6, 24.6, 24.5.

m/*z* (DCI NH_3 + Isobutane) 218 (MUA), 236 (MUA + NH_4^+), 316 $(M + H)^+$, 333 $(M + NH_4^+)$.

Ligand 2 (ref. 18). Under inert atmosphere, *N*-(13-amino-4,7,10-trioxatridecanyl) biotinamide **B** (446 mg, 1 mmol) is added to a solution of 11-mercaptoundecanoyl-*N*-hydroxysuccinimide ester **C** (315 mg, 1 mmol) in 50 mL of dry chloroform and 200 μL of triethylamine. The reaction mixture is stirred at room temperature for 1.5 h (control by thin layer chromatography). Chloroform is evaporated under reduced pressure and the residue is purified by flash chromatography (dichloromethan/methanol, 90/10, R_f: 0.26). The pure product is isolated as a colorless oil which solidifies with time (187 mg, 30%).

δ^1_H (200.13 MHz, $CDCl_3$) 6.75, 6.55, 6.44, 5.93 (4 × 1H, 4 × br, NH, SH), 4.52 (1H, m, H_1), 4.35 (1H, m, H_4), 3.70–3.50 (12H, m, H_{G-L}), 3.40–3.30 (4H, m, $H_N + H_E$), 3.16 (1H, m, H_3), 2.96–2.88 (2H, m, H_2), 2.52 (2H, m, H_X), 2.17 (2H, m, H_O), 1.85–1.50 (14H, m, $H_{A,B,C,F,M,P,W}$), 1.50–1.20 (12H, m, H_{Q-V}).

δ^{13}_C (56.32 MHz, $CDCl_3$) 173.4, 173.2, 172.6, 164.0, 70.4, 70.0, 69.8, 61.9, 60.3, 55.5, 40.5, 37.8, 37.6, 36.8, 35.9, 34.0, 29.4, 29.3, 29.1, 29.0, 28.3, 28.1, 28.0, 25.8, 25.5, 25.4, 24.6.

m/*z* (DCI NH_3 + Isobutane) 647 $(M + H)^+$.

Preparation of water-soluble, biotinylated nanocrystals

Ligand **2** (20 μL, 250 g L^{-1} in dry chloroform) and ligand **1** (24.4 mg) are mixed with CdSe/ZnSe nanocrystals[3] (core diameter 3.7 nm, shell thickness 0.7 nm; 500 μL, 8 g L^{-1} in dry chloroform) in a 2 mL microtube under argon (molar ratio = 95 mol% of ligand **1** and 5 mol% of ligand **2**). The mixture is stirred for 48 h at 30 °C in the dark. Then water (500 μL) and acetone (100 μL) are added and the microtube is vigorously shaken for 1 h. The aqueous layer is separated and evaporated to dryness to give a colored solid, which is redispersed in water (200 μL) and centrifuged (45 s, 10,000 rpm). The supernatant is filtered with a 30000 d Vivaspin© filter, rinsed twice with water (100 μL), vacuum-dried and stored at 4 °C.

Labeling of agarose beads with biotinylated nanocrystals

Biotin–agarose beads (Sigma, Ref. B 0519) in PBS (50 μL) are rinsed twice with 100 μL of PBS (Sigma, Ref. P 4417). After centrifugation, the surpernatant is removed. A solution of streptavidin (Sigma, Ref. S 4762) in PBS (50 μL, 2.5 mg mL^{-1}) is added to the beads and the reaction mixture is kept at room temperature during 15 minutes. The beads are then rinsed twice with PBS (100 μL). Biotinylated nanocrystals are dispersed in 50 μL of ultrapure water. 5 μL of this dispersion are poured into the solution of streptavidin-conjugated beads. After 5 minutes, the beads are rinsed twice with PBS (30 μL) and placed on microscopy slides.

R-Phycoerythrin labeled agarose beads are prepared in the same way, but using 2 μL of a solution of R-Phycoerythrin-biotin conjugate (Molecular Probes, Ref. P 811) instead of biotinylated nanocrystals.

Labeling of neuronal cells with biotinylated nanocrystals

Neuronal cells from mouse embryo cerebellum (stage E14) are grown for 5 days on polylysin glass covers in neurobasal medium supplemented with glutamine. The cells are then fixed for 20 minutes in 4% paraformaldehyde in phosphate-buffered saline (PBS in mM: 3.16 NaH_2PO_4, 6.84 Na_2HPO_4, 0.15 NaCl, pH 7.2). Fixation and all subsequent steps are performed at room temperature. After permeabilization for 30 minutes in PBS + 0.1% Triton X-100, the cells are blocked for 2 h with reagent 2 from a CSA amplification kit (DAKO), supplemented with 0.01 mg mL^{-1} streptavidin. Subsequently, the cells are washed 3 times with PBS + 0.1% Tween20 and incubated for 30 minutes with the neuron specific anti-class III β-tubulin antibody (TuJ1, BAbCO, dilution 1/500 in PBS + 0.1% Tween20 + 5 μM biotin). In the following, the buffer used for all incubation and washing is PBS + 0.1% Tween20. The cells are washed again 3 times and incubated for 30 minutes with biotinylated anti-(mouseIgG)-antibody (reagent 4 of CSA kit, DAKO). After three additional washes, the cells are incubated either with Cy3-streptavidin (Amersham) or with unlabeled streptavidin (10 μg mL^{-1}) for 30 minutes and washed again 3 times. Cells labeled with Cy3-streptavidin can then be mounted in an antifading medium (DAKO) and directly observed, whereas cells incubated with unlabeled straptavidin are further incubated for 30 minutes with

biotinylated nanocrystals before a final washing step and mounting. Negative control is realized by omission of the TuJ1 antibody in the first labeling step.

Labeling of neuronal cells with Alexa-488

After permeabilization for 30 minutes in PBS + 0.1% Triton X-100, the cells are blocked for 2 hours with reagent 2 from a CSA amplification kit (DAKO), supplemented with 0.01 mg mL^{-1} streptavidin. Subsequently, the cells are washed 3 times with PBS + 0.1% Tween20 and incubated for 30 minutes with the neuron specific anti-class III β-tubulin antibody (TuJ1, BAbCO, dilution 1/500 in PBS + 0.1% Tween20 + 5 μM biotin). The cells are washed again 3 times and then incubated for 30 minutes with Alexa488-anti-(mouseIgG)-antibody (Molecular Probes) (1/1000 dilution in PBS + 0.1% Tween20). Finally, the cells are washed 3 times and are mounted in an antifading medium (DAKO).

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