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Technical Note

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Uncoated, Broad Fluorescent, and Size-Homogeneous CdSe Quantum Dots for Bioanalyses

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In the present study, we describe the synthesis of highly luminescent uncoated water-soluble CdSe quantum dots (QDs) possessing the following characteristics: ∼2 nm in diameter, with very good size distribution (in 95% homodispersed) accompanied by a broad-band photoluminescent spectrum. The synthetic procedure is simple, is conducted at room temperature, in the absence of the most popular coordinating ligands (as TOPO or HDA), and is highly reproducible. The obtained CdSe core QDs possessed a comparatively long fluorescence half-life (∼30−90 ns, depending on the emission wavelength) detected by time-resolved spectroscopy. These QDs were further conjugated with antibodies and applied in several biochemical analyses.

The application of quantum dots (QDs) in life science research increased enormously[1−8] in the last years, starting from 1998 with the reports of Chan and Nie[9] and Bruchez and colleagues[10] about the possibility of using them as novel fluorophores in multiplex bioanalyses. The synthesis of highly luminescent water-soluble QDs became a priority. At present, the synthetic process has several shortcomings: it is time- and cost-consuming, still complicated, and conducted at high temperature; the preservation of the photoluminescent (PL) properties of the synthesized QDs requires a covering with several coats—currently, the most popular QDs consist of a CdSe core, followed by a ZnS shell and one or more organic coats. The encapsulation of QDs and their primary ligands with macromolecules such as lipids and polymers preserves their quantum yield (QY); however, the resulting overcoated nanocrystals become large—their size is commensurate with or larger than the size of biomolecules that have to be conjugated.[11,12] The efforts are directed to the development of procedures resulting in a synthesis of highly luminescent water-soluble QDs with diameters of 10−15 nm or lower, consisting of a thin inorganic/organic cover.[13−17]

The CdSe QDs are confirmed as the most useful in life science research, at least for the time being. However, a successful application of uncoated CdSe QDs in biochemical experiments has still not been shown, because the surface exchange of their primary ligands (as TOPO or HDA) and subsequent transfer in aqueous solutions results in a significat lose of their PL properties. Several groups have been described a synthesis of CdSe QDs directly in aqueous solution.[18,19] The crude product contains nanocrystals with heterogeneous size in the range from 2 to 5 nm, and in all cases, the QY is too low (less than 0.1%) to be used directly for conjugation with immunocompatible molecules. Procedures, including additional coatings, are required to improve their PL characteristics before applying them in life science experiments. An excellent review, summarizing all known synthetic and capping strategies in the quantum dot field, can be

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The major characteristic of the commonly described CdSe QDs is their narrow symmetric PL spectrum. The broadband PL spectrum of CdSe QDs is usually associated with the presence of different size nanocrystals in the crude solution. Despite this generally held view, recently, Chen et al.[21] reported that CdSe nanocrystals with homodispersed and uniquely stable 2-nm size manifest also a broad photoluminescence in organic solvents. Unfortunately, the authors did not report the quantum efficiency of the synthesized nanocrystals, as well as the possibility for water solubilization with preservation of their PL properties.

In the present study, we describe the synthesis of highly luminescent CdSe QDs possessing unusual characteristics: small size (~2 nm in diameter), very good size distribution (in 95% homodispersed), broad PL spectrum, and comparatively long fluorescence half-life (~30−90 ns, depending on the emission wavelength). The synthetic procedure was simple, was conducted at room temperature (~22 °C), occurred in the absence of the most popular coordinating ligands such as TOPO or HDA, and was highly reproducible. The synthesized uncoated CdSe core QDs were easily subjected to water solubilization (using mercaptosuccinic acid as a surface-modifying reagent) with enhancement of their QY (from ~40% in chloroform to ~50% in phosphate-buffered saline, PBS). Moreover, the water-soluble CdSe core QDs showed an excellent transparency and stability after long-term storage in full lack of aggregation. These QDs were further conjugated with peptides (antibodies) and applied in several biochemical analyses using flow cytometry and immunoblotting.

EXPERIMENTAL SECTION

Synthesis of CdSe Core Nanocrystals. The stock solution of tri-*n*-octylphosphine selenide (TOP-Se) was prepared by dissolving Se shots (0.7896 g, 10 mM, Aldrich) in TOP (7.413 g, 20 mM, Lancaster) at 100 °C for 2 h in argon atmosphere and then cooled to room temperature. Cadmium acetate (1 g, 3.75 mM, Wako) was dispersed in 2 mL of TOP in a round-bottom flask in argon atmosphere by stirring intensively for 20 min at 100 °C with subsequent cooling to room temperature. Four milliliters of Se stock solution was injected into the Cd stock solution. This provided a homogeneous reaction, in which all monomers exist in a liquid phase. Volume of the flasks was 100 mL. The reaction mixture was stirred intensively during 2−4 h at room temperature. Formation of CdSe nanocrystals was monitored by UV/visible absorption and fluorescent spectroscopy. The reaction was stopped by addition of ~30 mL of chloroform. The crude solution (dissolved in chloroform) showed a slight turbidity as a result of the insolubility of nonreacted cadmium acetate in chloroform. After centrifugation at 16 000 rpm, the crude solution showed an excellent transparency. The obtained nanocrystal solution in chloroform (supernatant) was kept at −20 °C in the dark for a long time. No changes in the absorption and PL spectra were observed after 11 months.

Calculation of Photoluminescence Quantum Yield.[22] For determination of room temperature fluorescence QY, the spectrally integrated emission of a nanocrystal dispersion in chloroform or PBS was compared to the emission of an ethanol solution of rhodamine 6G (Fluka) of identical optical density (<0.015) at the extinction wavelength 365 nm.

Table 1. Quantum Yield (QY) of Water-Soluble CdSe Core QDs after Surface Modification with Different Substances

surface-modifying substance	QY (%) of water-soluble QDs[a]
mercaptosuccinic acid	~50
dimercaptosuccinic acid	~8
mercaptopropionic acid	<1
mercaptoacetic acid	<1
seleno-L-methionine	<1
cysteamine hydrochloride	0
DL-cysteine	0

[a] CdSe QDs were dissolved in 10 mM PBS, pH 7.4.

Characterization of CdSe Core QDs. UV/visible absorption and PL spectra were recorded using the Hitachi U-4100 spectrophotometer and Hitachi F-4500 fluorescence spectrometer, respectively. Crystal structure was determined using an X-ray diffraction (XRD) technique. XRD patterns were recorded using a Rigaku (model RINT 2100) X-ray diffractometer with nickel filtered Cu Kα radiation. Purified CdSe core nanocrystals were dispersed in methanol, and drops of this solution were dried in a standard sample holder till a thickness of ~1 μm was reached. Surface morphology and crystal defects were determined using high-resolution transmission electron microscopy (HRTEM). HRTEM pictures were recorded using a JEOL (model JEM 3010) transmission electron microscope operating at 300 kV. Samples for HRTEM were prepared by ultrasonically dispersing nanocrystals in methanol and allowing a drop of this solution to dry on a carbon-coated copper grid.

Water Solubilization of CdSe Core QDs and Verification of Their Size. CdSe QDs were kept in chloroform in the dark at −20 °C before solubilization in water. The water-soluble CdSe core nanocrystals were obtained using mercaptosuccinic acid as a surface-modifying agent. The mercaptosuccinic acid was selected as a most appropriate agent among D,L-cysteine, cysteamine hydrochloride, mecraptopropionic acid, dimercaptosuccinic acid, mercaptoacetic acid, and seleno-L-methionine. The results in Table 1 show that highly fluorescent water-soluble CdSe core QDs were obtained using mercaptosuccinic acid (QY ~50%) as a surface-modifying agent. Significantly less fluorescent water-soluble QDs were obtained using dimercaptosuccinic acid (QY ~8%). With all other surface-modifying agents, CdSe core QDs lost almost completely their fluorescence in aqueous solution.

The fact that the exchange of TOP with mercaptosuccinic acid does not change PL QY is probably due to the slow rate of the exchange process, so only replacement of a few ligands is enough to provide water solubility of CdSe core QDs. Another observation supporting this assumption is that TOPO-capped CdSe core nanocrystals loose their PL efficiency on exchange with mercaptosuccinic acid since TOPO is a weaker ligand than TOP,[23] so more mercaptosuccinic acid ligands can bound to the surface of the nanocrystals.

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Briefly, the water-solubilization protocol consisted of the following steps. The QDs were dissolved in chloroform (3 mL with OD_{430nm} ~0.5 unit). Three milliliters of mercaptosuccinic acid (Sigma-Aldrich, dissolved in 100 mM PBS, pH 7.3 at concentration 30 mg/mL) was added to 3 mL of QDs in chloroform. Both phases (aqueous and nonaqueous) were shaken intensively for about 5−10 min until the aqueous phase became yellow. The mixture was centrifuged at 80*g* for 10 min to separate both phases. The uncoated CdSe QDs were easily modified and simply extracted (in ~100%) from the organic to aqueous phase. The aqueous phase, containing water-soluble CdSe core QDs, was decanted carefully and subjected immediately to ultrafiltration to remove free (nonreacted) mercaptosuccinic acid. The ultrafiltration was carried out in several steps using Vivaspin concentrators (Vivascience, Sartorius). The first step was carried out on 3 000 MW size filter and centrifugation at 3000*g* for 15 min (4 °C). The QDs were washed three times with 6 mL of 100 mM PBS (pH 7.3) on this filter. The upper phase was decanted, dissolved in 6 mL of PBS, and subjected additionally to a second ultrafiltration step on a 10 000 MW filter (3000*g* for 10 min, 4 °C). After the second ultrafiltration step, the lower phase was concentrated on a 3000 MW filter to a concentration corresponding to OD_{430nm} ~0.1 (~7.6 μM QDs, calculated by Yu et al.[24]). PBS consisted of 100 mM $Na_2HPO_4 \cdot 12H_2O$, 100 mM KH_2PO_4, 136 mM NaCl, 2 mM KCl. The purified water-soluble QDs possessed a bright fluorescence (QY ~50%) and a very good stability in a buffer (10−100 mM PBS, pH 6.8−10.8, at 4 °C)—no significant changes in the PL intensity were detected during five months.

It is necessary to note that the water solubilization of CdSe QDs was carried out at soft conditions avoiding precipitation and high-speed centrifugation, which minimized the possibility for aggregation and reduction of their quality. For example, it was established that the QY of water-soluble CdSe QDs obtained by precipitation and high-speed centrifugation (using mercaptosuccinic acid as a surface-modifying agent) was ~30%, which was significantly lower than the QY of water-soluble CdSe QDs obtained by ultrafiltration (~50%). Moreover, the water-soluble CdSe QDs obtained by ultrafiltration showed a better transparency and lack of aggregation than the water-soluble nanocrystals obtained by precipitation and high-speed centrifugation.

Conjugation of Water-Soluble, Broad Fluorescent CdSe QDs with Antibodies. The water-soluble, broad fluorescent QDs were further conjugated with antibodies. Details of the conjugation procedure are described in our previous reports.[25,26]

Briefly, 1.2 mL of water-soluble CdSe QDs (in 100 mM PBS, pH 7.3) were mixed with 150 μL of antibody solution (containing 1 mg of antibody in 150 μL of 100 mM PBS, pH 7.3). Then 150 μL of freshly prepared 6.4 mg/mL EDC stock solution in water was added to the mixture. EDC solution was kept at 4 °C for no more than 30 min before the conjugation procedure. The samples were incubated 2 h at room temperature under shaking in the dark and then kept overnight at 4 °C. The free nonconjugated nanocrystals as well as the isourea byproduct of the conjugation reaction were removed by two-step ultrafiltration using Vivaspin concentrators of appropriate size: 1.5 mL of the mixture (containing 1.2 mL of QDs, 150 μL of antibody, and 150 μL of EDC) was subjected to ultrafiltration using 30 000 MW filter (3000*g* for 12 min); the lower phase, containing free CdSe QDs and isourea byproducts, was removed; the upper phase, containing QD−antibody conjugates, was decanted, diluted to 5 mL by PBS, and additionally subjected to ultrafiltration using 300 000 MW filter (3000*g* for 10 min) to remove cross-linked antibody molecules (if cross-linking happens) holding in the upper phase. The lower phase was decanted and concentrated on 30 000 MW Vivaspin filter (3000*g* for 12 min) to concentration, corresponding finally to ~1 mg of antibody/mL.

Anti-c-abl antibody was purchased from Sigma.

Application of QD−Antibody Conjugates in Biochemical Analyses: Preparation of Cells. Human leukemia cell lines Jurkat (derived from acute lymphoblastic leukemia) and K-562 (derived from chronic myelogenous leukemia) were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 μg/mL streptomycin, and 100 units/mL penicillin, in a humidified atmosphere (5% CO_2) at 37 °C. The cell lines were a generous gift from Dr. J. Minowada (Hayashibara Biochemical Laboratories, Inc., Okayama, Japan).

The cells used for assay were in a logarithmic phase. They were sedimented by centrifugation (1000 rpm, 10 min) and washed three times with PBS(−) (Ca^{2+} and Mg^{2+} free, 4 °C) before experiments.

Flow Cytometry: Quantification of c-abl Protein in Fixed K-562 Cells Using QD−Anti-c-abl Antibody. Leukemia cells K-562 were treated with anti-c-abl siRNAs for 6 days as described by Ohba et al.[27] Cells were collected by centrifugation, washed three times with PBS(−), and the level of c-abl protein (p150) was analyzed using anti-c-abl antibody (rabbit, Sigma) conjugated with QDs. Permeabilization of cells for QD−anti-c-abl antibody was carried out with IntraPrep Permeabilization Reagent (Immunotech) following the manufacturer's instruction. The antibody−antigen interaction was detected by flow cytometry (Beckman Coulter-Epics XL). Data were collected and analyzed by XL System II software. No cells were excluded from the analysis, and ~10 000 cells were counted. Data were presented as a dot plot of QD fluorescence (side scatter, SS—*y*-axis in 0−128 scale, vs QD−conjugated antibody fluorescence—*x*-axis in −1−1000 scale) with quadrant markers drawn to distinguish the cells, containing different levels of QD-labeled antibody−antigen complexes (Figure 5).

QD-Based Western Blot Analysis ("Mono-Type" Protocol). Soybean agglutinin (SBA) was suspended 1:1 (v/v) in 2× Laemmli buffer (1.1 M Tris-HCl, pH 6.0, 3.3% SDS, 22% glycerol, 10% β-mercaptoethanol, 0.001% bromphenol blue). Samples were heated at 95 °C for 10 min and were applied (in different protein concentrations) to 5% stacking, 4−12% resolving SDS-polyacrylamide gel. Electrophoresis was carried out at room temperature in two steps: at 80 V for 15 min and 120 V for 2 h. BioRad Kaleidoskop protein standards were applied for comparison. After electrophoresis, the protein fractions were transferred to a Hybond-P PVDF membrane (Amersham Bioscience) using XCell

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II Blot Module (Novex). The transfer was carried out at 35 V for 18 h at 4 °C. The membranes were incubated under agitation for 1 h at room temperature in a blocking solution (PBS, containing 5% dry skimmed milk and 0.1% Tween-20) and then at room temperature for 1 h in the QD−conjugated antibody (anti-SBA, 1 μg/mL). After incubation, the membranes were washed three times with PBS, incubated for 10 min in 3 mM glutathione, and the fluorescence of the blotted antigens was detected immediately using a fluorescence gel imaging system (FluorChem, AlphaInnothech).

"Sandwich-type" western blot analysis of SBA was carried out following the previously described protocol.[28]

RESULTS AND DISCUSSION

In the present study, we described a comparatively simple and highly reproducible procedure for synthesis of highly luminescent uncoated water-soluble CdSe QDs possessing the following characteristics: ~2 nm in diameter, in 95% size-homogeneous, with broad-band PL spectrum, and ~30−90-ns fluorescence half-life (depending on the emission wavelength).

XRD measurements (Figure 1A) show that in the described synthetic protocol CdSe seems to crystallize in a cubic close-packed (zinc blende) structure. The characteristic zinc blende planes of 111, 220, and 311 located at 25.38°, 42.06°, and 49.76° in the 2θ range of 20°−70° for CdSe were observed. There are also weak peaks representing wurtzite facets observed in the XRD patterns. However, estimated from the relative intensity in the XRD patterns, the wurtzite CdSe nanocrystals are not dominant (less than 10%). Presumably, the formation of cubic phase of CdSe in our synthetic procedure is attributed to the low temperature of synthesis (~22 °C). It is generally accepted that CdSe crystallizes predominantly in cubic phase when the annealing temperature is below 200 °C, and the hexagonal phase becomes dominant when the annealing temperature is higher than 300 °C.[21,29−31] However, in some cases at low temperature of synthesis, the observed X-ray diffraction patterns can be explained also by the presence of zinc blende stacking faults along the (002) direction that is very typical for wurtzite II−VI nanocrystals.[31,32]

Figure 1B shows a representative HRTEM image. The observed nanocrystals are of uniform size and shape, with good crystallinity. It was established that the majority of CdSe QDs (~96%) were between 1.8 and 2 nm in diameter (Figure 1C,D). The mean diameter was 1.9 nm with a size distribution of ~3% from the histogram (Figure 1D). Similar size was calculated from XRD results—1.88 ± 0.07 nm in diameter. The high size homogeneity was also approved from the sharp absorbance spectra of CdSe QDs dispersed in organic solvent, as well as in aqueous solution (Figure 1E). The first exciton peak in the absorption spectrum exhibited fwhm ~26 nm, which corresponds to a size distribution of 2.7% and is similar to the results mentioned above.

The major advantage of the described CdSe QDs was their easy water solubilization with preservation and even enhancement of their QY in aqueous solutions without additional inorganic/organic coatings. The highly luminescent water-soluble CdSe QDs were obtained using mercaptosuccinic acid as a surface-modifying agent. The size and homogeneity of the water-soluble CdSe QDs, as well as a lack of aggregation, were approved indirectly by two-step ultrafiltration, using 10 000 and 3000 MW filters (Figure 2). Approximately 90−95% of the water-soluble QDs passed through 10 000 MW (retaining particles with diameters of >2.5 nm). The lower fraction was completely retained on 3000 MW filter, which corresponds to particles with diameters of >1.5 nm. Proceeding from the absorbance spectra of water-soluble CdSe QDs (Figure 1E), the size was calculated to be ~2 nm in diameter.

In the absorbance spectra (Figure 1E), a small hump at 360 nm was detected that is attributed to the formation of an exciton corresponding to HOMO−LUMO transition (first exciton). The absorption peak of water-soluble QDs was slightly red-shifted in comparison with that in chloroform. Surprisingly, the PL intensity of the purified water-soluble CdSe QDs was higher than that in chloroform (Figure 1F). Their PL QY enhanced from ~40% in chloroform to ~50% in PBS after purification from the excess mercaptosuccinic acid.

The PL spectrum of the synthesized CdSe QDs was broad-band (fwhm ~150 nm), despite their high size homogeneity. Obviously, this broad and bright fluorescence is not from a large size distribution, and therefore, it probably is from trap states. It could be due to the replacement of TOPO by TOP. To explain the possible reason for this bright broad fluorescence, we compared the spectral characteristics of CdSe QDs synthesized at room temperature in the absence of TOPO (Figure 1F) or after addition of TOPO in the end of the synthesis (Figure 3), as well as of TOPO-capped CdSe QDs growing at 120 °C (Figure 1S, Supporting Information). The PL spectrum of TOPO-capped CdSe QDs synthesized at room temperature consisted of two parts: a shoulder that probably corresponds to band edge fluorescence (this shoulder is also slightly expressed in PL spectrum of room temperature synthesized CdSe QDs in the absence of TOPO; Figure 1E, black line) and the other part that corresponds to deep trap fluorescence as a result of surface defects (this fluorescence is broad and with high intensity) (Figure 3).

To clarify the factors that might be responsible for the observed PL spectrum profile, we synthesized CdSe QDs at 120 °C in the presence of TOPO or HDA. All precursors and other experimental conditions were the same as in the room temperature synthesis. The results are presented in Supporting Information (Figure 1S). Initially, the PL spectrum of TOPO-capped CdSe QDs growing at 120 °C (Figure 1S, red line) was similar to the spectrum of room temperature synthesized QDs; however, the PL intensity was significantly lower than the PL intensity of room temperature synthesized QDs. After 2 min at 120 °C, a band edge peak appeared in the PL spectrum (Figure 1S, black line), instead of a shoulder. With increasing size of TOPO-capped CdSe QDs increased at 120 °C, the band edge peak attenuated and red-shifted, and the bright deep trap fluorescence decreased markedly (Figure 1S, blue line). Finally, we obtained TOPO-capped CdSe nanocrystals with narrow fluorescence, with broad absorption spectrum. Moreover, these QDs almost completely lost their

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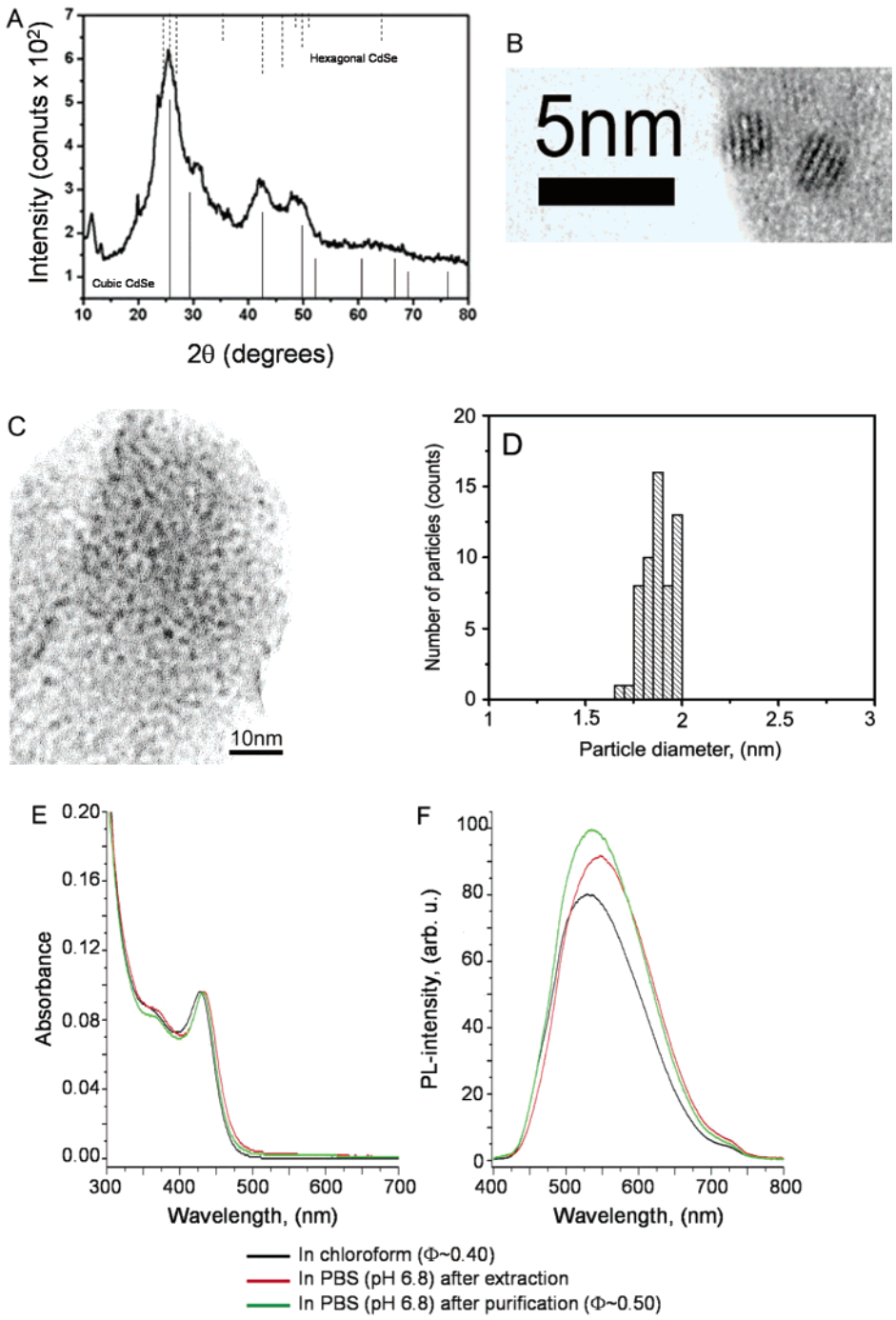

Figure 1. Spectral characteristics of the synthesized CdSe nanocrystals. (A) XRD pattern of CdSe nanocrystals. For comparison, the standard powder diffraction patterns are indicated in solid lines for the cubic phase (lower axis) and in dashed lines for the hexagonal phase (upper axis); (B) HRTEM image of a particle indicating a good crystallinity; (C) HRTEM image of large population of CdSe nanocrystals; (D) size histogram; (E) absorbance spectra; (F) fluorescence spectra (λ_{ex} = 420 nm).

luminescence after water solubilization using mercaptocompounds (QY <1%).

It seems that the bright broad spectrum of room temperature synthesized CdSe QDs is a result of the bright deep trap fluorescence, which dominates over the narrow band edge fluorescence in this type of nanocrystal. It is most likely the bright broad fluorescence is also intrinsic to low-size CdSe QDs despite the temperature of synthesis and the presence of TOPO. Even the small-size TOPO-capped CdSe core QDs synthesized at 120 °C possessed a very low QY and almost completely lost their luminescence after water solubilization (QY <1%), while CdSe QDs synthesized at room temperature in the absence of TOPO possessed a bright luminescence in organic and aqueous solutions (QY ~50%). Therefore, the temperature of synthesis (~22 °C) and absence of TOPO are probably responsible for obtaining QD cores, which surface guarantees bright luminescence and the possibility for direct solubilization in aqueous solutions with preservation of their high QY (~50%).

The broad and high-intensity deep trap fluorescence of CdSe QDs synthesized at room temperature is also responsible for the

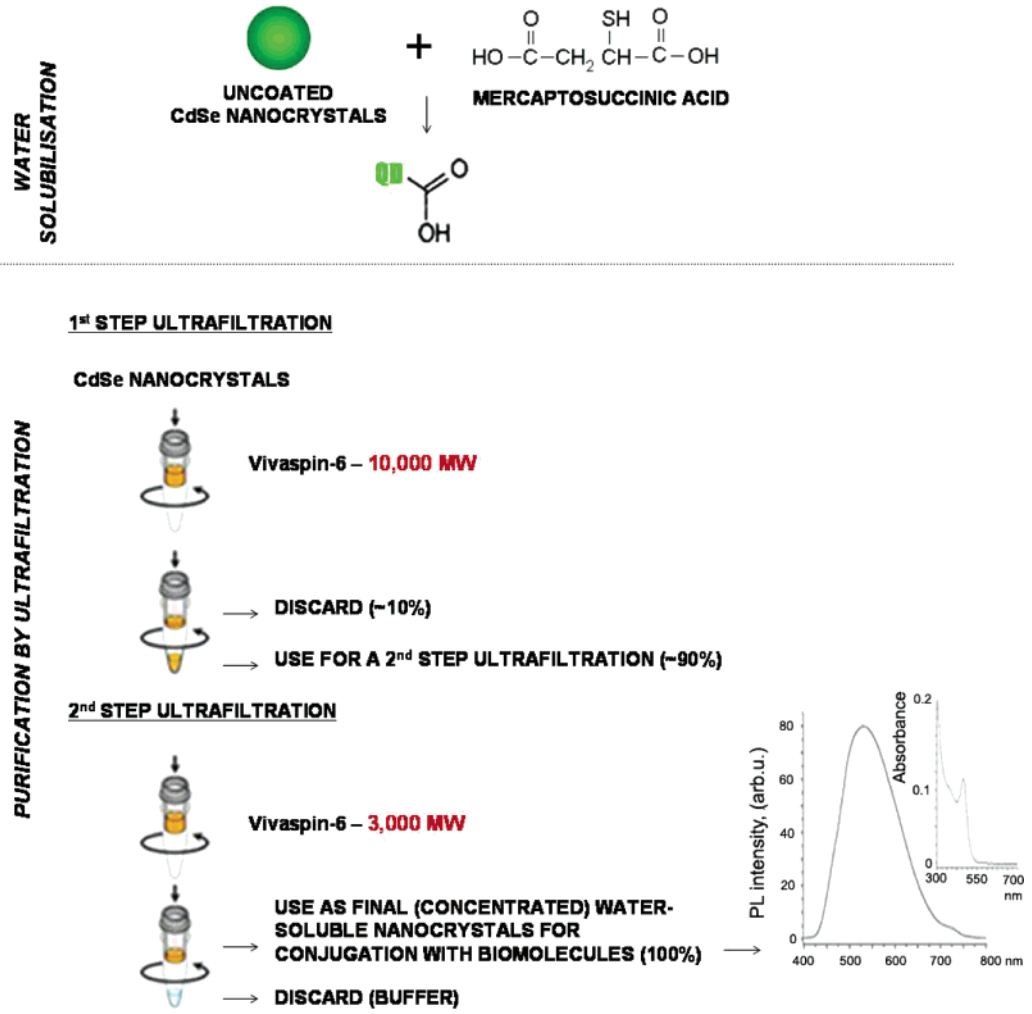

Figure 2. Scheme of purification of the synthesized uncoated, water-soluble CdSe QDs and verification of their size homogeneity.

comparatively high fluorescence half-life detected by time-resolved PL spectroscopy (Figure 4). Two main contributions are present in the data (Figure 4A): a fast decay (characteristic of CdSe/ZnS core/shell QDs synthesized at high temperature as well as of room temperature synthesized QDs recorded at λ_{em} = 475 nm corresponding to the band edge shoulder in their PL spectrum) and a slow decay (characteristic of room temperature synthesized QDs at λ_{em} = 525−650 nm corresponding to the broad part of their PL spectrum). The fluorescence half-life at 525−650 nm of the PL spectrum was ~27−92 ns for room temperature synthesized CdSe QDs dispersed in chloroform, while their fluorescence half-life at 475-nm emission was ~10−12 ns—commensurate with the fluorescence half-life of our CdSe/ZnS core/shell QDs synthesized from the same precursors at high temperature (240 °C). Similar fluorescence half-life (10 ns) was recently reported for commercial CdSe/ZnS core/shell QDs dispersed in organic solvents.[33] For CdSe/ZnSe core/shell QDs synthesized at high temperature and dispersed in organic solvents, the reported fluorescence half-life was lower than 5 ns.[34,35] In the present study, the fluorescence half-life values of the room temperature synthesized water-soluble CdSe core QDs were a bit lower (Figure 4B) than the values obtained in chloroform—at 525−650-nm emission, the fluorescence half-life was 20−40 ns.

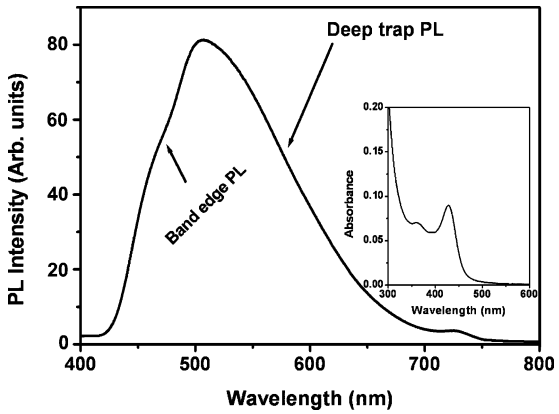

Figure 3. Fluorescence spectrum of TOPO-capped CdSe QDs synthesized at room temperature. TOPO was added to the chamber in the end of synthesis and TOPO-capped CdSe QDs were dissolved in chloroform (final concentration of TOPO, 5%). The PL spectrum was recorded at λ_{ex} = 365 nm.

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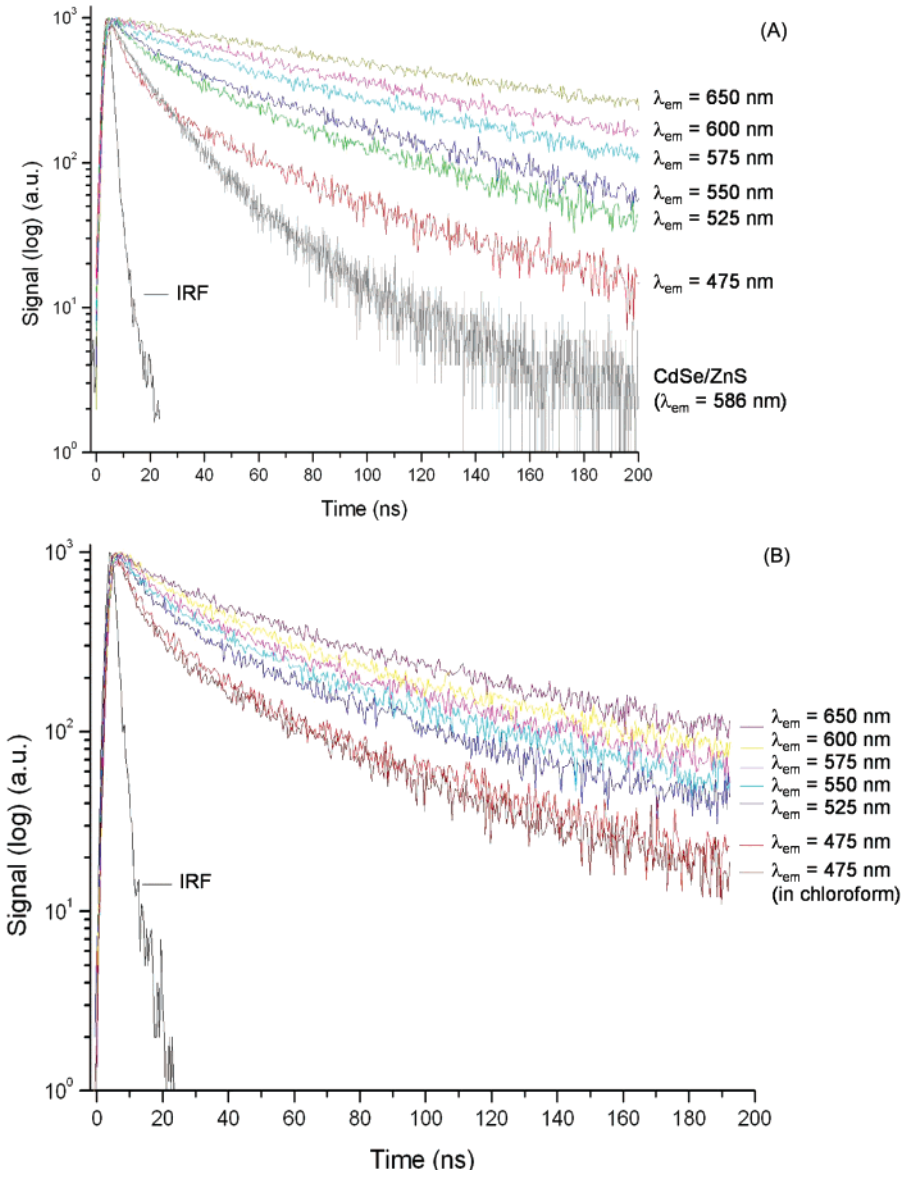

Figure 4. Time-resolved photoluminescence lifetime spectra of CdSe core QDs synthesized at room temperature: (A) dispersed in chloroform; (B) dispersed in PBS, pH 7.4. For comparison, a time-resolved PL lifetime spectrum of CdSe/ZnS core/shell QDs synthesized at 240 °C was recorded at emission wavelength corresponding to the PL maximum (586 nm). All data were recorded at $\lambda_{ex} = 365$ nm, frequency 40 kHz, using a Hamamatsu FLS920S spectrometer. IRF, instrumental response function of the nanosecond flashlamp.

Usually, the broad fluorescence spectrum of CdSe QDs is considered as a shortcoming. However, in the context of their small and homogeneous size and comparatively simple and highly reproducible synthetic protocol, we consider these QDs attractive for homemade production, biolabeling, and application in bioimaging experiments. Because of their small size and high QY, it is possible to conjugate several QD particles on one macromolecule (e.g., protein, DNA, etc.) and thus to increase significantly the fluorescence intensity, giving a possibility for highly sensitive analyses (even for detection of single molecules). Currently, the described QD bioconjugates consist usually of one QD particle bound to one macromolecule. The detection of room temperature synthesized broad fluorescent QDs can be realized using a fluorescence imager supplied with any filter within the range 450−600 nm that can be economical. The comparatively high fluorescence lifetime of these QDs (~30−90 ns in chloroform and 20−40 ns in PBS, recorded at 525−650-nm emission) also gives the possibility for development of comparatively low-cost time-resolved fluorescent bioanalyses rather than conventional sharp fluorescent QDs and Eu complexes.

In the present study, the broad fluorescent water-soluble CdSe QDs were conjugated with antibodies and applied for detection of specific antigens (proteins) in fixed cells and cell lysates, using flow cytometry or western blot analysis.

The results in Figure 5 demonstrate a flow cytometric analysis of c-abl in siRNA-treated and untreated K-562 cells using anti-c-abl antibody conjugated with the described CdSe QDs (Figure 5B,C) or FITC (Figure 5D,E).

The control cells showed a characteristic QD fluorescent peak in the right part of the histogram (Figure 5B), corresponding to QD-labeled antibody−antigen complexes. The treatment of cells with siRNAs (antisense to c-abl mRNA) resulted in the appearance of a second QD fluorescent peak in the left part of the histogram (Figure 5C) that is representative for cells with a decreased

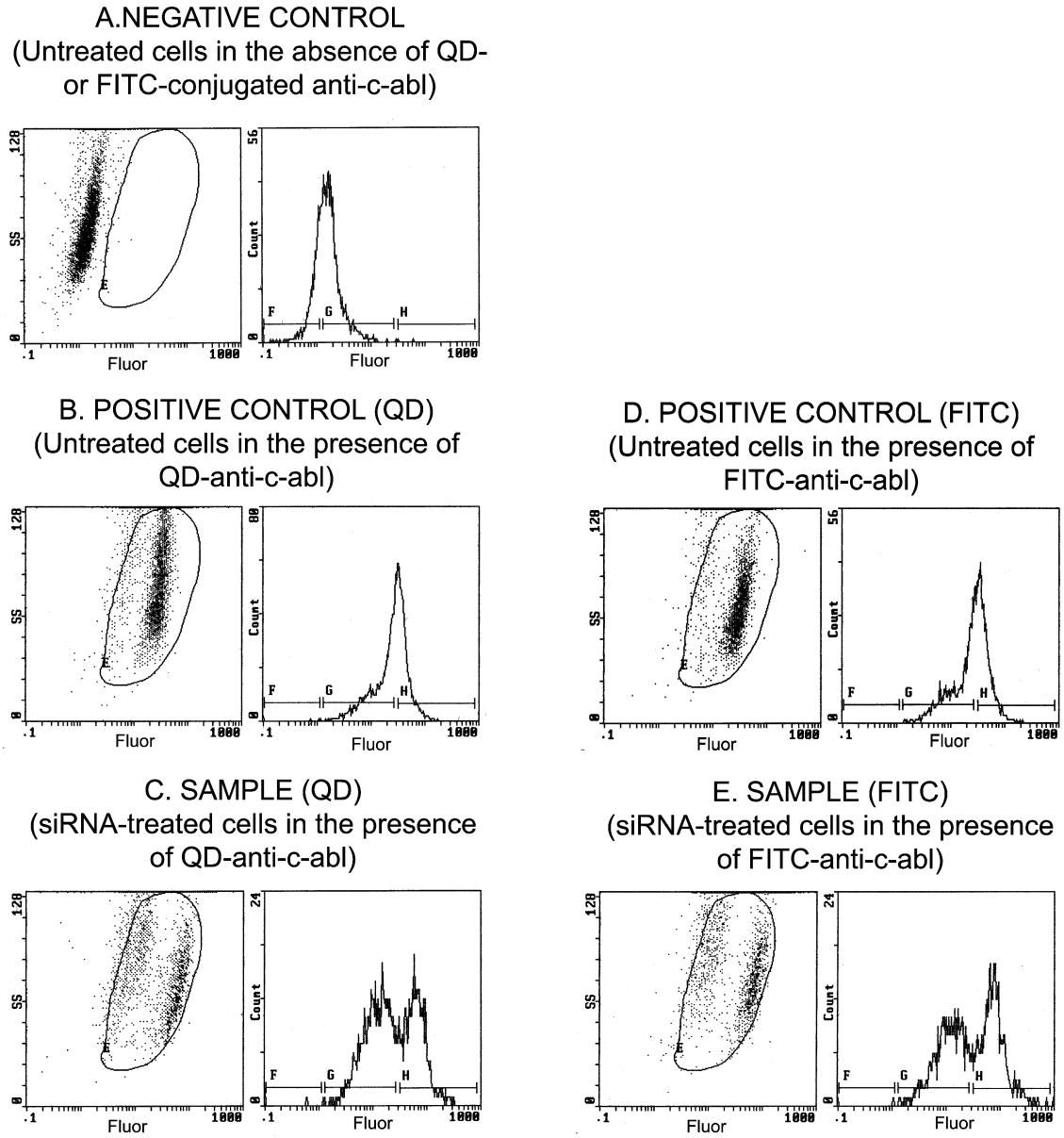

Figure 5. Application of QD−antibody or FITC−antibody conjugates for quantification of protein levels in K-562 cells, using flow cytometry. The cells were treated with anti-c-abl siRNAs for 6 days to decrease the amount of c-abl and bcr-abl proteins. The antibody−antigen complexes localized in cytoplasm were detected by flow cytometry. In the histograms, quadrants H correspond to cells, containing a maximum level of c-abl (positive control), quadrants G correspond to cells without fluorescent marker and therefore without c-abl−antibody complexes (spontaneous cell fluorescence, negative control), and quadrants E (between H and G) correspond to cells containing moderate or low levels of QD- or FITC-conjugated c-abl antibody and therefore expressing moderate or lower levels of c-abl protein in comparison with the positive control. To the right of each histogram QD or FITC, fluorescent curves are drawn. Histograms from one typical experiment are shown in the figure.

amount of c-abl protein. In the case of c-abl flow cytometric analysis using FITC-labeled antibody, the histograms had the same profile (Figure 5D,E). Obviously, there are no artifacts in our QD-based flow cytometric protein assay, which is in contrast with the commonly accepted opinion that there is a risk of artifacts using QDs in flow cytometry as a result of their large size. The described QDs are too small and almost commensurate with conventional dyes to give such artifacts.

One of the most promising applications of QDs in life science experiments is QD-based western blot analysis of protein expression in cells and tissues, which is still a major analytical approach in proteomic studies. Currently, it is also one of the basic methodologies (together with northern blot analysis and Light Cycler technology) for microarray data validation and verification in functional genomics. Despite the widespread and long-time use of western blot analysis, the method has not been much improved from its initial state 20 years ago. The conventional western blot technology possesses several shortcomings: a semiquantitative nature; a long duration (usually more 24 h); a poor reproducibility; a low sensitivity for detection of "tracer proteins"—including an impossibility to use directly cell lysates for protein separation and immunoblotting, as well as a necessity to use preliminary procedures for immunoprecipitation and concentration of these proteins (each step keeps a risk for accumulation of analytical errors). In the life science field, almost every researcher has been faced with the difficulties of obtaining reproducible high-quality

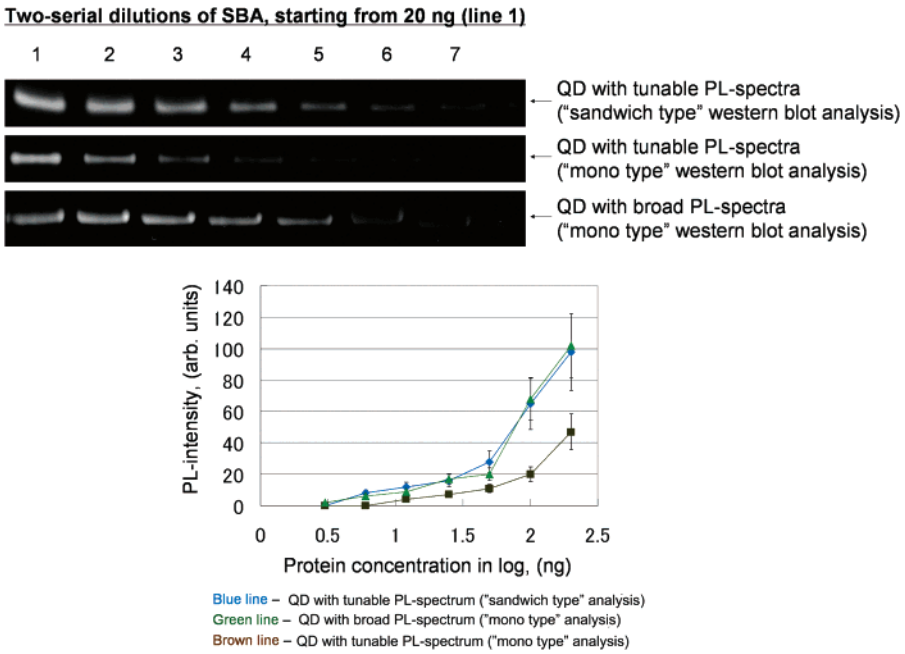

Figure 6. QD-based western blot analysis of pure soybean agglutinin (SBA). The graphic represents mean ± SD data from four independent experiments for sharp fluorescent QDs and five independent experiments for broad fluorescent QDs.

immunoblot images. The development of QD-based western blot analysis increases our expectations to decide, at least partially, all these problems. Ornberg et al. have described the potential of QDs in immunoblotting technology for multiplex detection of proteins or protein states (as phosphorylated/dephosphorylated) from a single western blot.[36] Recently, we reported one more privilege of QD-based immunoblot analysis—the possibility for ultrasensitive detection of "tracer" proteins directly in cell lysate, avoiding the preliminary procedures of their immunoprecipitation and concentration.[28] The described procedures enable a simplified fluorescent image acquisition; they are highly sensitive, less time-consuming than the standard immunoblot analysis using chemiluminescence detection of the blotted proteins, and the obtained blot images are with higher quality.

In our previous study,[28] we used QDs synthesized at high temperature, with tunable emission spectrum (emission maximum at 535 nm). Two QD-based western blotting protocols were applied, named as "mono type" and "sandwich type". The mono-type protocol consisted of the following steps: pure protein (or cell lysate) was subjected to protein fractionation on SDS−PAGE; the fractionated proteins were transferred on PVDF membranes; the membranes were subjected to immunoblotting, using QD-conjugated primary antibody and several washings with buffer. The sandwich-type protocol consisted of the following steps: pure protein (or cell lysate) was subjected to protein fractionation on SDS−PAGE; the fractionated proteins were transferred on PVDF membranes; the membranes were subjected to immunoblotting, using biotinylated primary antibody and several incubations with NeutrAvidin and QD−conjugated biotin. The blot fluorescence was detected directly by MultiImager (Alpha-InnoTech) at excitation with broad bandwidth UV light and emission at 530 nm. It was established that the sandwich-type protocol markedly improved the quality of blot images. The sensitivity of sandwich type was ~650 pg of pure protein/gel patch versus 1.25 ng of pure protein/gel patch for the mono-type protocol.[28] It is possible to increase the sensitivity of QD-based western blot analysis up to 20 pg, using higher quality sharp fluorescent QDs as has been described by Ornberg et al.[36]

In the present study, we try to clarify the possibility for application of broad fluorescent, water-soluble CdSe QDs (which are less expensive than the high-temperature synthesized water-soluble QDs) in western blot analysis of proteins and to compare the quality and stability of the blot images with our previously described procedures.

The results in Figure 6 demonstrate a concentration-dependent western blot analysis of pure soybean agglutinin, using primary antibody or biotin conjugated with sharp or broad fluorescent QDs. The broad fluorescent QDs increased the sensitivity of the mono-type protocol up to ~650 pg of pure protein/gel patch, which was analogous to sandwich-type immunoblot analysis using biotin conjugated with sharp fluorescent QDs. All images in Figure 6 were generated using identical exposure time (15 min) and excitation/emission settings. The fluorescent signals were stable during continuous scanning in the gel imager. No changes in the fluorescence intensity were registered between 5 and 30 min of scanning—a time enough for membrane imaging and data acquisition. Because of simple synthetic protocol and low cost of water-soluble room temperature synthesized CdSe QDs (in contrast to sharp-fluorescent QDs synthesized at high temperature and covered with organic coatings), they could be recommended for application in this most widely used methodology in proteomics studies.

CONCLUSIONS

In this study, we describe a simple and highly reproducible synthesis of CdSe QDs possessing unique size homogeneity accompanied with a broad fluorescent spectrum. These QDs can

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be easily solubilized in aqueous solutions with enhancement of their QY and preservation of their stability. The obtained CdSe QDs possessed a comparatively high fluorescent half-life (~30−90 ns in chloroform and 20−40 ns in PBS, depending on the emission wavelength), which makes them appropriate for time-resolved spectroscopy. The broad fluorescent QDs are appropriate for application in flow cytometric and western blot analyses. Moreover, it was observed in mono-type western blotting protocol that primary antibody conjugated with broad fluorescent QDs gives a higher quality images than the antibody conjugated with sharp fluorescent CdSe/ZnS QDs synthesized at high temperature using the same precursors.

SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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