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Comparative Examination of the Stability of Semiconductor Quantum Dots in Various Biochemical Buffers

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Due to their greater photostability compared to established organic fluorescence markers, semiconductor quantum dots provide an attractive alternative for the biolabeling of living cells. On the basis of a comparative investigation using differently sized thiol-stabilized CdTe nanocrystals in a variety of commonly used biological buffers, a method is developed to quantify the stability of such a multicomponent system. Above a certain critical size, the intensity of the photoluminescence of the nanocrystals is found to diminish with pseudo-zero-order kinetics, whereas for specific combinations of particle size, ligand, and buffer there appears to be no decay below this critical particle size, pointing out the necessity for thorough investigations of this kind in the view of prospect applications of semiconductor nanocrystals in the area of biolabeling.

Introduction

Due to their tuneable luminescence wavelength and high photostability compared to organic dyes, II−VI semiconductor quantum dots (QDs)[1] are attractive candidates for fluorescence markers in biolabeling. They are small enough to be incorporated by cells and even seem to be transferred into cells on their own just by adding a solution of QDs. Due to the size quantization effect, only one material is necessary to mark different proteins or cell compartments simply by employing different size fractions of one particle synthesis.

Apart from the organometallic synthesis of II−VI semiconductor QDs based on the thermolysis of precursors in highly coordinating, nonpolar solvents (trioctylphosphine/trioctylphosphineoxide, TOP/TOPO), a synthetic route using water as a solvent and thiols as ligands has been established.[2] QDs prepared by this method incorporate the sulfur of the thiol groups as part of their crystalline structure. When using Se or Te as the chalcogenide, one can speak of a core/shell nanocrystal (NC) containing a metal sulfide shell.[3] To keep the particles in solution, the ligands have polar moieties such as carboxylic groups. This facilitates the interest in such QDs for biological applications, as they are water-soluble as formed and can be transferred into living cells without prior ligand exchange. Furthermore, these groups may be functionalized by coupling with proteins for labeling. Successful conjugation of semiconductor QDs to proteins such as bovine serum albumin (BSA) and IgG has already been reported.[4,5] To use QDs for labeling in a living organism, knowledge of how the particle's luminescence depends on the concentration, pH value, concentration of salts in solution, and time is essential. Since CdTe QDs may slowly release the toxic Cd^{2+} and Te^{2-} ions into the solution, the particles must be as inert as possible for any in vitro application. As Parak et al. have shown,[6,7] the toxicity of CdTe QDs not only depends on the concentration of free Cd^{2+} ions but also depends on whether the particles are ingested by a cell and where they are stored. The release of Cd^{2+} from the particles' surface can be reduced by employing core/shell particles or coating of the particles with silica or a polymer.

Also, the photoluminescence (PL) of the QD must not be influenced by desorption of the ligands, destruction of the NC, or creation of traps for radiationless recombination of an excited electron/hole pair.

In this work, buffers commonly used for the indirect immunofluorescence labeling of cells were tested.[8] To do so, several fractions of the size-selective precipitation of thioglycolic acid (TGA)- and mercaptopropionic acid (MPA)-stabilized particles were mixed with different buffers of a defined concentration. The particle PL decay was observed over 1 week by emission spectroscopy.

Experimental Section

Synthesis of Thiol-Capped CdTe Nanocrystals.[2] A 3.06 mmol portion of the stabilizer and 0.985 g (2.35 mmol) of $Cd(ClO_4)_2 \cdot 6H_2O$ was dissolved in 125 mL of deionized water. The pH was adjusted to a value of 12 using a 1 M solution of NaOH. The solution was stirred and deaerated by bubbling nitrogen through it for 30 min. Under stirring, H_2Te gas generated by adding 10 mL of 0.5 M H_2SO_4 to 0.2 g (0.46 mmol) of Al_2Te_3 was passed through the solution with a slow stream of nitrogen. Depending on the stabilizer, a red or orange solution of CdTe precursors was formed. Afterward, the solution was refluxed

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under open-air conditions for several hours, until the particles had grown to the desired size. The progress of the reaction was followed by absorption spectroscopy.

Size-Selective Precipitation of CdTe QDs. The colloidal solution of CdTe QDs was evaporated until the first particles precipitated. A 500 μL portion of 2-propanol was then added to the solution. The resulting precipitate was removed by centrifugation for 5 min at 4500 rpm and redissolved in water. The procedure was repeated with a stepwise increase of the amount of 2-propanol until most of the CdTe was precipitated.

Chemicals and Methods. Milli-Q water (Millipore) was used as bidistilled water. All chemicals were purchased from Merck and used as received. Dulbecco's Modified Eagle Medium (DMEM) (1X) liquid (low glucose) containing 1000 mg/L D-glucose and L-glutamine and 110 mg/L sodium pyruvate from Invitrogen was used as the cell medium.

For analysis of the QDs, a Cary 100 absorption spectrometer (Varian) and a Fluoro-Log emission spectrometer (Instruments SA) were employed. The wellplates for the stability assay were measured with a Cary Eclipse emission spectrometer (Varian).

Results and Discussion

To quantify the stability of the CdTe QDs in aqueous buffers, all variables must be collected and a method must be found with which the variation of all pertinent variables can easily be measured without changing the measurement. The independent variables in a system containing nanoparticles and a buffer are the following: the type of the QDs, the ligands, the size of the QDs, the concentration of the QDs, the type of buffer, the concentration of the buffer, the pH value, and time. The dependent variables are the following: the intensity of the PL, the full width at half-maximum of the emission signal, and the wavelength at the emission maximum. In this work, the particle size and the type of ligands and buffer were varied and the dependent variables were measured over a time span of 5 days. The particles employed were CdTe QDs with TGA or MPA as ligands after application of size-selective precipitation.

To obtain a comparable signal, the amount of particles which were added to 100 μL of buffer was calculated to result in an optical density (OD) of 0.2 at the first absorption maximum. A varying concentration of QDs was accepted in favor of a constant OD, although a lower concentration of QDs results in diffusion of ligands from the particle surface and therefore a decreased stability.[3] In a living cell, it is desirable to maintain the concentration of CdTe as low as possible without a decrease of the particles' stability. The parameters are therefore similar to the parameters that were expected in biolabeling.

The measurement of a full wellplate took 20 min, which is the limiting factor in the determination of the accuracy of the time. On the day the buffers were added to the QDs, the wellplates were measured three times: immediately after the experiment was started and again after 2 and 5 h. After that, the luminescence was measured every 24 h.

The particle size was determined from the first absorption maximum using an empirical formula derived for particles synthesized via an organometallic route by Peng et al.:[9]

$$D = (9.8127 \times 10^{-7})\lambda^3 - (1.7147 \times 10^{-3})\lambda^2 + (1.0064)\lambda - (194.84)$$

where D is the diameter of the particles and λ is the wavelength of the first electronic transition.

The buffers were chosen to represent a wide spectrum of the pH scale between 5.0 and 11.0 and to test the most frequently used biochemical buffers (Figure 1). For extreme pH values, buffers sold by Merck were used instead of biochemical buffers: a citrate/NaOH buffer for pH 5.0 and $B(OH)_3$/KCl/NaOH buffers for pH 9.0 and 11.0. The buffers were systematically tested for the application of indirect immunofluorescence labeling with CdTe nanocrystals. In such an experiment, cells are fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 30 min. Their membranes are made permeable using PBS/glycine/saponine for 30 min at 37 °C after which free binding spaces are blocked using BSA for 30 min. After each step, the cells are washed with PBS. The fluorescence marker, linked covalently to a specific antibody, is then transferred into the cells over a period of 45 min after which the cells are placed under a microscope where the location of the marker shows the location of the specific antigen. Often, a secondary antibody that binds to an antigen-specific primary antibody is used, because this strategy simplifies the experiment by requiring only one type of marker-linked antibody. Other buffers tested were cell medium, morpholinethansulfonic acid (MES), and a tris(2-hydroxyethyl)-amine (Tris) buffer (pH 8.0, 200 mM).

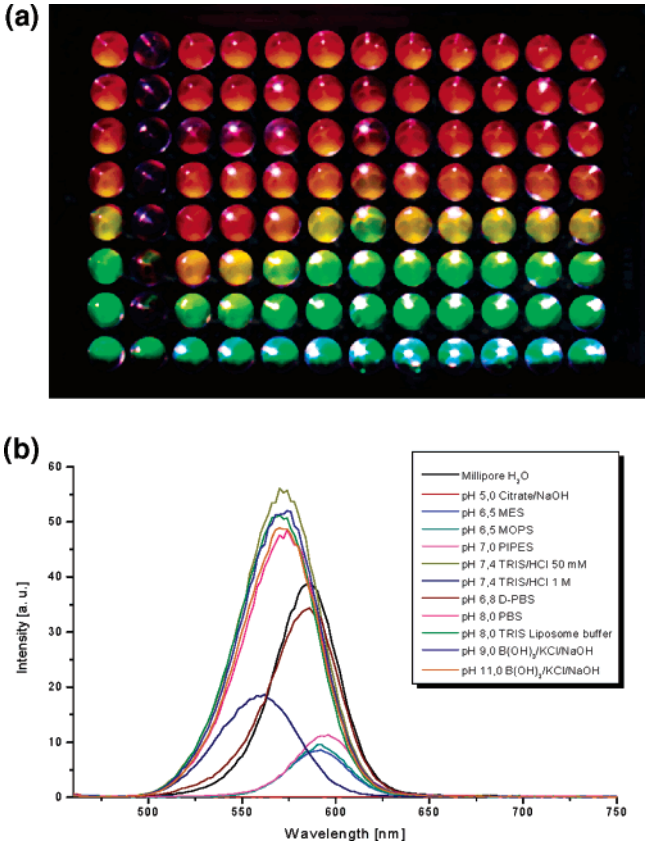

Figure 1. (a) True color photography of an 8 × 12 wellplate with TGA-capped CdTe QDs under a UV lamp after 1 day of incubation. The particle size decreases from top to bottom and lies between 3.46 and 2.42 nm. The buffers used are, from left to right, Milli-Q H_2O, pH 5.0 citrate/NaOH, MES, MOPS (3-(*N*-morpholino)propansulfonic acid), PIPES (piperazine-1,4-bis(2-ethanesulfonic acid)), 500 mM Tris/HCl, 1 M Tris/HCl, pH 6.8 PBS, pH 8.0 PBS, pH 8.0 Tris, pH 9.0 $B(OH)_3$/KCl/NaOH, and pH 11.0 $B(OH)_3$/KCl/NaOH. (b) Fluorescence spectra of fraction TGA 5 (3.22 nm) after 20 h in the named buffers.

Usually, organic markers have very little photostability and bleach in the order of minutes under illumination. Semiconductor QDs are more robust and show longer durability which makes them an interesting alternative.[10]

Large semiconductor QDs have a greater resistance to photobleaching than smaller ones. Regarding the stability toward

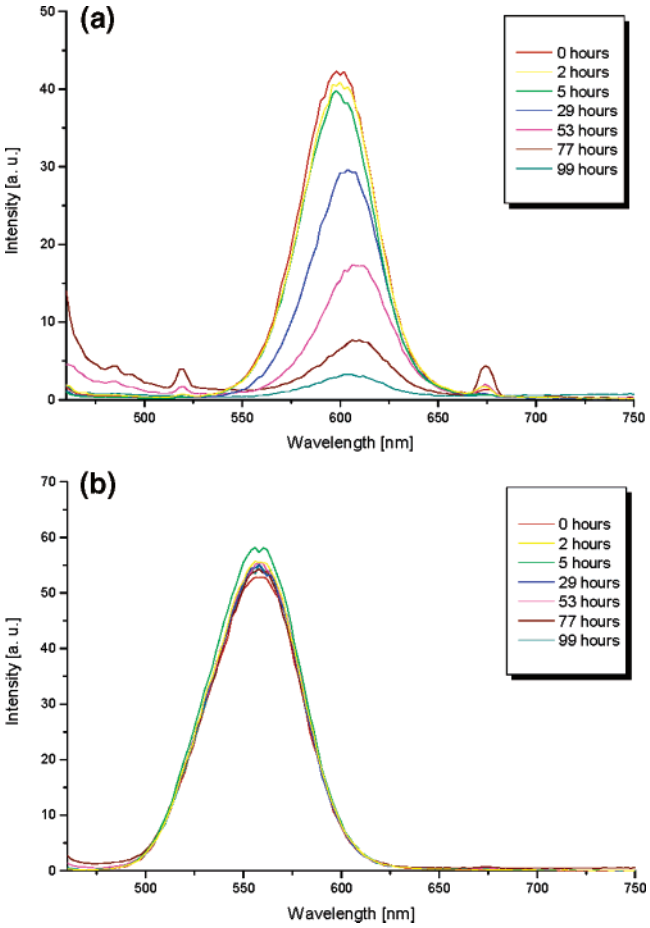

Figure 2. Fractions TGA 1 (a) and TGA 6 (b) in PBS (pH 7.4), observed over a time span of 1 week.

their chemical environment, the reverse tendency was observed. The smaller the QDs, the more stable they are under acidic conditions and low buffer concentrations. In acidic citrate/NaOH buffer (pH 5.0), the fluorescence of red TGA-capped QDs drops down to 5% of the original PL after a few minutes, while, for the green and blue species, the intensity remaining was about 25%. The reason for this behavior could be the clusterlike, more defined structure of very small nanocrystals.[11−14] The surface structure of very small particles and hence the occurrence of charge carrier traps strongly depends on the size and the symmetry of the nanoobjects.

The TGA 6 fraction, for example, remains stable in PBS for 1 week, while the emission of fraction TGA 1 decreases notably over the same time span (Figure 2). These results can be applied to other buffers. Figure 3 shows the observed temporal evolution of the relative intensity maxima of the TGA-capped fractions in PBS. The maximum at 0 h after adding the buffers was set to 100%. For the larger nanocrystals, a decrease in intensity over time is evident. The tendency to degrade diminishes with decreasing particle size, until the size has reached 3.06 nm beyond which no further changes in intensity are observed. This behavior can also be seen in most of the other buffers. The slope of the intensity change with time, $\partial I/\partial t$ (cf. Figure 4), and the critical size, D_k, below which no change in intensity can be determined, depend on the buffer and, in case of the slope, on the particle size. With these two values, namely, $\partial I/\partial t$ which is proportional to the particles' rate of decay and the critical size, D_k, a means to characterize a specific particle/buffer mixture is found.

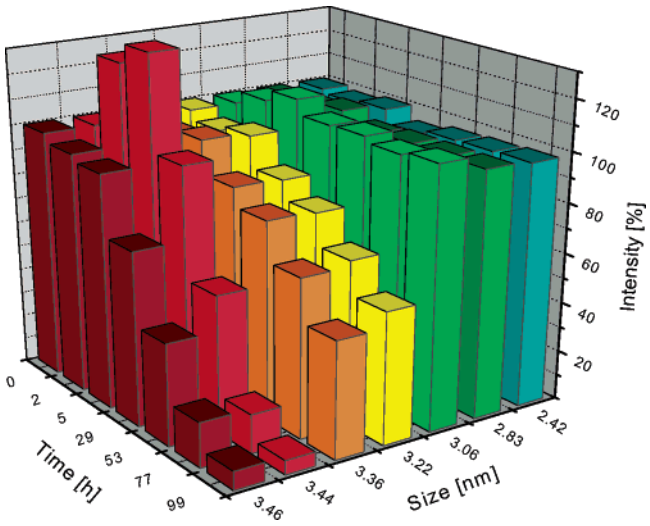

Figure 3. Relative intensity of all TGA-capped fractions in PBS (pH 7.4) with respect to time. The intensity at $t = 0$ h was set to 100%.

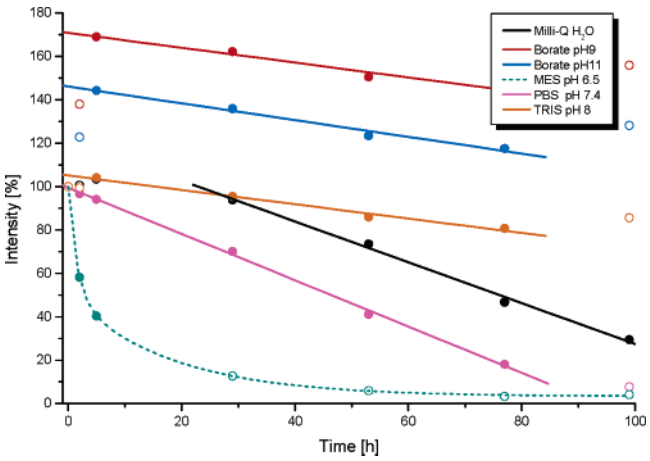

Figure 4. Temporal decrease in intensity for fraction TGA 1. Zero-order kinetics is recognized in many cases.

TABLE 1: Size and OD of the Particles Used for the Test[a]

name	size (nm)	color (PL UV)	V_{3mL} (μL)	OD (a.u.)	$V_{100\mu L}$ (μL)
TGA 1	3.46	red	20	0.05	2.4
TGA 2	3.44	red	20	0.16	0.75
TGA 3	3.40	red	20	0.12	0.99
TGA 4	3.36	orange	20	0.08	1.5
TGA 5	3.22	yellow	30	0.05	3.6
TGA 6	3.06	green	40	0.03	8.0
TGA 7	2.83	green	50	0.02	15
TGA 8	2.42	light blue	60	0.01	36
MPA A	~3.6	red	90	0.09	6.14
MPA B	~3.6	red	30	0.10	1.82
MPA C	~3.6	red	100	0.03	21.43
MPA D	~3.6	red	60	0.05	7.35
MPA E	~3.6	red	120	0.05	14.4
MPA F	3.57	red	200	0.04	30.8
MPA G	3.53	red	200	0.05	25.0

[a] Due to the large full width at half-maximum of the first absorption maximum, the size of the MPA-capped QDs could only be approximately determined.

The slope (Figure 4) is linear in buffers which do not aggressively destroy the QDs. This is not trivial, since one could expect more complex kinetics for this reaction, which includes desorption of ligands from the particle surface, dissolving or aggregation of QDs, and adsorption of other molecules. Table 2 summarizes the experimental results including $\partial I/\partial t$, D_k, and

TABLE 2: Buffers Employed in the Studies

buffer	pH value	slope of intensity,[a] $\partial I/\partial t$ (%/h)	critical size,[b] D_k (nm)	red shift,[c] $\Delta\lambda$ (nm)	comment
Millipore Milli-Q H_2O	7.0	−0.94	2.42	−2.1	
citrate/NaOH	5.0	*e*		*d*	destroyed all samples at once
$B(OH)_3$/KCl/NaOH	9.0	−0.39	3.44	−2.1	
$B(OH)_3$/KCl/NaOH	11.0	−0.39	3.36	0.0	
MES	6.5	*e*	<2.42	19.9	no linear $\partial I/\partial t$
MOPS	6.5	*e*	2.42	*d*	red shift of ∼24 nm
PIPES	7.0	*e*	<2.42	*d*	red shift of ∼24 nm
500 mM Tris/HCl	7.4	−0.72	2.83	*d*	
1 M Tris/HCl	7.4	*e*	2.42	*d*	destroyed most of the samples
PBS	7.4	−1.07	3.06	0.0	
PBS/glycine/saponin	7.4	*e*	2.42	0.0	saponin is an extract from the bark of the South American soaptree
2% BSA in PBS/Gly/saponin	7.4	*e*	<2.42	−4.0	
4% PFA in PBS	7.4	*e*	3.06	18.0	low intensity, QDs > D_k destroyed
MOWIOL		*e*		22.0	nonaqeous medium
medium	7.1	*e*	<2.42	32.9	complex mixture
glycine in H_2O	5.9	−0.72	<2.42	4.0	7.5 mg/mL Gly
Tris	8.0	−0.33	3.44	0.0	200 mM

[a] Taken from fraction TGA 1. [b] For TGA-capped QDs. [c] For fraction TGA 6 after 2 h of incubation. [d] No data. [e] Decay not of pseudo-zero-order kinetics.

$\Delta\lambda$, the observed red shift of the emission (see below) in all of the buffers used in this study.

To derive a model for a reaction of pseudo-zero order, strongly bound ligands on the particle surface are assumed. Desorption is slow, and adsorption practically does not occur, since the concentration of free ligands in solution is very low. On the contrary, the concentrations of buffer ions are high, namely, between 50 mM and 1 M. Adsorption and desorption of ions at the surface are fast processes due to the weak, unspecific bonding between ions and QDs. This results in a constant, low concentration of free coordination places at the surface. Quenching of the QD luminescence can be an effect of oxidation by dissolved oxygen. The multiplate was kept under open-air conditions, resulting in a concentration of oxygen in solution which is also assumed to be constant. Following this explanation, the kinetics do not depend on any concentration that was varied in the experiment.

Indeed, the kinetics appear to be more complex in the first hours after the addition of the buffers. However, since the slope is constant over several days, it can be used to quantify the stability.

Peng et al. have proposed zero-order kinetics to explain the photocatalytic oxidation of thiols to disulfides on the surface of CdSe with regard to the concentration of free thiols in solution.[15] They proposed three processes contributing to the decay of the QDs: photocatalytic oxidation of the ligands, photocatalytic oxidation of the CdSe core, and precipitation of the QDs. Water-insoluble disulfides which might be made up from MPA will form micelle-like structures around a nanocrystal until the core has shrunk to the point that it can no longer stabilize the micelle. After a micelle has broken down or when the disulfides are soluble in water, CdTe will precipitate. In contrast to Peng's results, especially the small particles seem to be stable toward oxidation. Keeping the samples in the dark also leads to the conclusion that photooxidation is not necessarily taken into account. Figure 5 summarizes the processes taking place in the degradation mechanism of semiconductor QDs in liquid environments.

It is noted that in alkaline buffers the intensity seems to increase over the first 5 h. A dependency of the PL quantum yield (QY) on the pH value is known from the literature.[2,16,17] The highest PL QY was reported to occur at pH 4.5, which could not be reproduced using the above parameters. At least

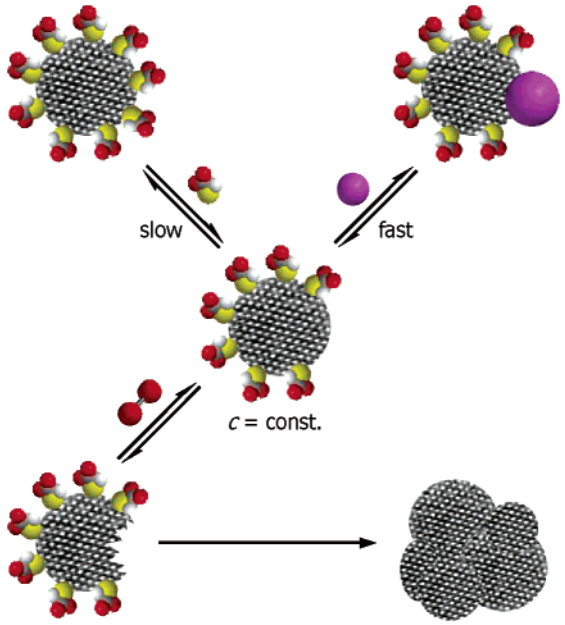

Figure 5. The proposed mechanism for a zero-order reaction is based on a constant, low number of free binding sites on the surface of the particles.

in alkaline borate buffers, the difference in the slope seems to be not or only slightly dependent on the pH value. Henglein et al. reported on an "activation" of the luminescence of CdS nanocrystals at pH 10.0 and an excess of free Cd^{2+} ions, probably by passivation of the surface by cadmium hydroxide.[18] Here, borate was used, in the form of $Na_2B_4O_7$, to set the pH value. This results in conditions which are comparable to the above-mentioned parameters.

Until now, the derived values that characterize the particles' stability ignore the wavelength of the emission maximum. In several buffers, profound red and blue shifts were observed. Since photometric measurement at a constant wavelength can be disturbed by this, the red shift, $\Delta\lambda$, must be regarded as a third characteristic value to describe the stability of QDs in a buffer.

The blue shift ($\Delta\lambda < 0$) can be the result of a partial destruction of the nanocrystal when ions are removed from the surface. In this case, an increase of the concentration of toxic

Cd^{2+} and Te^{2-} ions has to be taken into account for applications involving living cells. This explanation is supported by the fact that a blue shift is registered when particles are diluted with distilled water. This phenomenon has already been described earlier.[3] A blue shift was also measured when proteins were present in the solution (2% BSA in PBS/glycine/saponine). Both cadmium and tellurium ions are known to react with disulfide bridges in proteins.

Two models may be used to explain the red shift: First, it is possible that the particles agglomerate due to the loss of stabilizers. In this case, CdTe bulk material will precipitate and a decrease of the PL intensity and scattering and a larger full width at half-maximum should be observed. The second possibility is the occurrence of Ostwald ripening. This process requires the surface of the QDs to be dynamic and will be strongly dependent on the temperature.

When using MPA instead of TGA as ligands, the results were similar, although slightly larger particles were used in the case of MPA as the stabilizer: buffers that adversely affect TGA-capped QDs also decrease the PL intensity of MPA-capped particles. Conversely, buffers that stabilize TGA-capped QDs also stabilize MPA-capped ones. As with TGA as a ligand, MES, PFA, MOWIOL (a mixture of polyvinyl alcohols), and cell medium also result in a large red shift. Only with MPA-capped particles do the alkaline borate buffers seem to be less stabilizing than PBS and Tris buffer.

Summary and Outlook

The results demonstrate the possibility of the use of water-soluble, thiol-capped QDs for immunofluorescence labeling employing the commonly used buffers. The nanocrystals endure both frequently used buffers PBS and Tris. High pH values and moderate buffer concentrations stabilize the QDs, while acidic media and high dilution extinguish the PL completely. Even under neutral conditions, the particles keep their photoluminescence for days. The only problematic solutions found are aggressive substances such as PFA and the cell medium, the latter being a mixture of so many substances that the reason for the instability of the particles cannot be easily determined.

Measurement with a varying concentration of free ligands to verify the assumptions made to explain the kinetics are in progress. As higher concentrations of free ligands should result in a higher stability of the nanocrystals, experiments are in progress to verify the assumptions made above in order to explain the kinetics observed. Further investigations will also focus on measuring the change in absorption and light scattering as well as the interaction of thiol-capped particles with proteins, regarding the desired application in protein labeling.

By using only three values, $\partial I/\partial t$, D_k, and $\Delta\lambda$, a large number of dependent variables are reduced to a few parameters which may then be used to quantify the stability of a particular particle/buffer combination. The buffer concentration is not a variable that requires a large range, since biological application calls for specific, physiological concentrations.

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