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Cell-Penetrating Quantum Dots Based on Multivalent and Endosome-Disrupting Surface Coatings

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Abstract: We report the development of cell-penetrating quantum dots (QDs) based on the use of multivalent and endosome-disrupting (endosomolytic) surface coatings. Hyperbranched copolymer ligands such as polyethylene glycol (PEG) grafted polyethylenimine (PEI-*g*-PEG) are found to encapsulate and solubilize luminescent quantum dots through direct ligand-exchange reactions. Because of the positive charges and a "proton sponge effect" associated with multivalent amine groups, this class of ligand-exchanged QDs is able to penetrate cell membranes and is also able to disrupt endosomal organelles in living cells. The grafted PEG segment is essential for reducing the cytotoxicity of PEI as well as for improving the overall nanoparticle stability and biocompatibility. In comparison with previous QDs encapsulated with amphiphilic polymers, the cell-penetrating QDs are smaller in size and are considerably more stable in acidic environments. Cellular uptake and imaging studies reveal that the number of PEG grafts per PEI molecule has a pronounced effect on the intracellular pathways of internalized QDs. In particular, QDs coated with PEI-*g*-PEG_2 are rapidly internalized by endocytosis, and are initially stored in vesicles, followed by slow endosomal escape and release into the cytoplasm. These insights are important toward the design and development of nanoparticle agents for intracellular imaging and therapeutic applications.

Semiconductor quantum dots (QDs) are emerging as a new class of fluorescent labels for molecular, cellular, and in-vivo imaging applications, due to their narrow and size-tunable emission spectra, broad absorption profiles, and superior photostability.[1−3] Recent work by several groups has achieved considerable success in using QDs for labeling fixed cells, tissue specimens, and cell membrane acceptors.[4−9] However, only limited progress has been made in developing QD probes for molecular imaging inside living cells.[1] A major limiting factor is the difficulty in delivering single QDs into the cytoplasm of living cells. Another problem is that QDs tend to aggregate inside living cells and are often trapped or sequestered in organelles such as vesicles, lysosomes, and endosomes. For example, cell-penetrating peptides such as polyarginine and TAT have been used to deliver QDs into living cells, but the delivered dots are trapped in endosomes and are not available for molecular recognition or targeting.[3,10,11]

Here, we report the development of cell-penetrating QD probes based on direct ligand-exchange reactions and the use of endosome-disrupting polymers such as polyethylene glycol (PEG) grafted and hyperbranched polyethylenimine (PEG-*g*-PEI). Used widely as a gene delivery vector, PEI is not only able to move across cell membranes through rapid endocytosis but also able to disrupt intracellular organelles through a "proton sponge effect".[12] This endosomolytic effect is believed to arise from the large number of amine groups on each PEI molecule, leading to proton absorption in acid organelles and an osmotic pressure buildup across the organelle membrane. This osmotic pressure causes a disruption of the acidic endosomes and a release of the trapped materials into the cytoplasm.

Recent work has shown that polyamine dendrimers are able to coat and solubilize hydrophobic QDs, especially when some amine groups are converted into thiol (−SH) functions.[13−16] However, these cationic dendrimers and micelles are unstable (e.g., aggregation and precipitation) in biological buffers or culture media and are highly toxic to living cells.[14,17] In this

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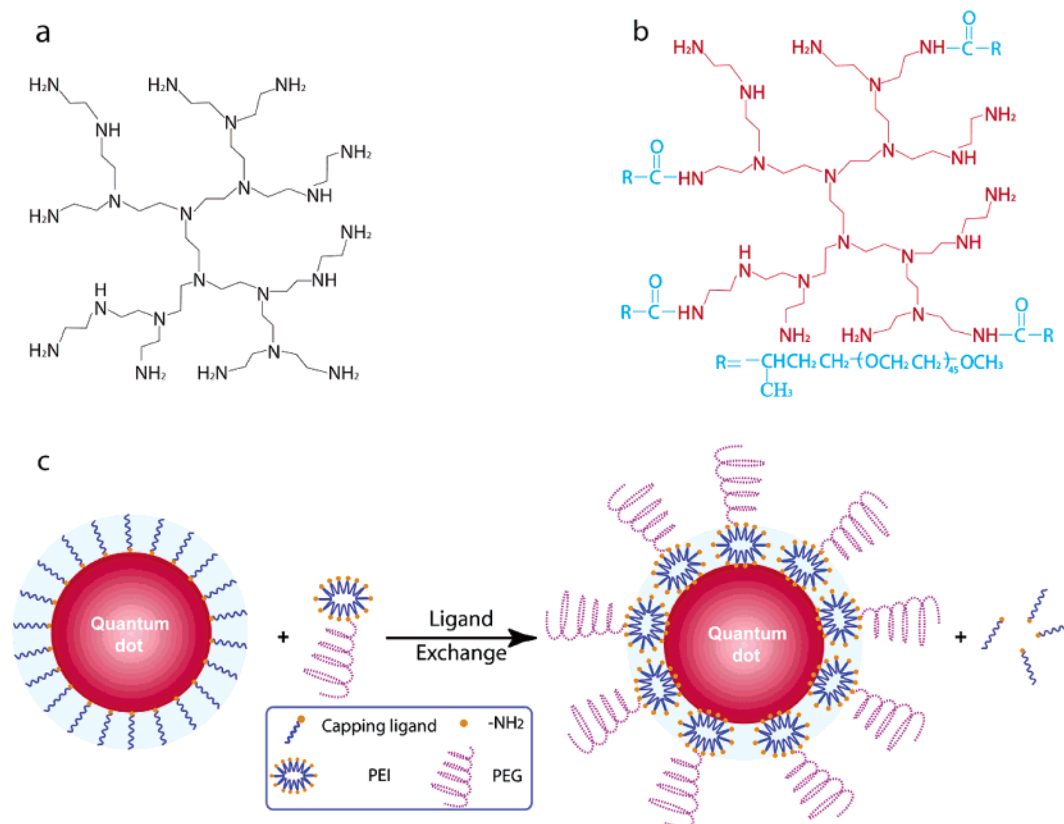

Figure 1. Encapsulation and solubilization of core−shell CdSe/CdS/ZnS quantum dots by using multivalent and hyperbranched copolymer ligands. (a and b) Chemical structures of PEI and polyethylene glycol (PEG) grafted PEI (PEI-*g*-PEG) copolymers consisting of two or four PEG chains per PEI polymer molecule. (c) Schematic diagram showing direct exchange reactions between the monovalent capping ligand octadecylamine and the multivalent copolymer ligands.

work, we have developed polyethylene glycol (PEG) grafted PEI (PEI-*g*-PEG) copolymers to reduce the cytotoxicity of PEI. The PEG segment also improves the solubility and stability of the coated QDs in biological buffer and cell culture media. We show that the PEG/PEI-coated QDs are able to escape from acidic intracellular organelles via the proton sponge effect and that their cellular distribution pattern and transport behavior are dramatically different from that of peptide-delivered QDs. One particularly interesting finding is that the degree of PEG grafting (that is, the number of PEG grafts per PEI molecule) has a significant effect on endosomal escape and intracellular distribution of the internalized QDs, indicating that there is still room in optimizing the intracellular properties of QDs by fine-tuning the surface coating chemistry.

Experimental Section

Synthesis of PEI-*g*-PEG Copolymers. Two different PEI-*g*-PEG copolymers were prepared by reacting methoxy poly(ethylene glycol) succinimidyl α-methylbutanoate (mPEG-SMB, M_n = 2000 Da) with PEI in chloroform. Briefly, 2.5 mL of a chloroform solution of 250 mg of mPEG-SMB was mixed with 500 mg of PEI (M_n = 10k Da) in 2.5 mL of chloroform. The solution was stirred for 5 h. After the solvent was removed under vacuum, the product was dissolved in water and was dialyzed against water (MW cutoff = 3500 Da) to remove unreacted PEG. Lyophilization of the solution led to a white solid product. Given the known M_n of PEI, ^{1}H NMR measurement ($-CH_2$-CH_2O- of PEG at 3.65 ppm and $-CH_2CH_2-$ of PEI at 2.4−2.8 ppm) showed that M_n of PEI-*g*-PEG was about 14 100 Da, which suggested that each PEI-*g*-PEG had approximately two PEG grafts (abbreviated as PEI-*g*-PEG_2). The second copolymer was prepared by changing the ratio of mPEG-SMB:PEI to 1:1, in which each PEI molecule was grafted with four PEG chains (M_n = 18 500 Da, abbreviated as PEI-*g*-PEG_4). DLS measurements showed that both of the copolymers had hydrodynamic sizes of 7−8 nm.

Ligand-Exchange Reaction. CdSe/CdS/ZnS QDs were synthesized following the procedures reported previously.[14,18] Before ligand exchange, the QDs were precipitated with acetone two times to remove free octadecylamine (ODA) in the solution and were redispersed in chloroform. 40 mg of PEI (M_n = 10k Da) or 50 mg of PEI-*g*-PEG was mixed with 0.5 nmol of CdSe/CdS/ZnS QDs in chloroform, and the mixture was shaken for 2 h. Afterward, the solvent was evaporated under argon, and the dried film was dissolved with water; further centrifugation (centrifugation at 6000*g* for 10 min) yielded a clear supernatant with white deposits. Free unbounded PEI molecules were removed by dialyzing (MW cutoff = 50k Da) the supernatant against water or buffer solution.

Cellular Uptake and Imaging. Hela cells (ATCC number CCL-2) were cultured in ATCC-modified Eagle's minimum essential medium (EMEM) with 10% fetal bovine serum (FBS) at 37 °C (5% CO_2) and were grown in 8-well LabTek chambers (Nalgene Nunc) to 20% confluency. Twenty-four hours after seeding, cells were rinsed with the medium, and QD solution (1 or 5 nM) was added. Fluorescence imaging was performed with a spinning disk confocal microscope (Ultraview, Perkin-Elmer) using 488 nm laser excitation, a long pass filter, and a high sensitivity CCD camera (ORCA-ER, Hamamatsu). For colocalization studies of QDs with dyes such as 3,3′-dihexadecyloxacarbocyanine perchlorate (DiO), the dye solution (5.0% (w/v) DiO in DMF) was mixed with the QD solution before adding to the cell culture. QD cytotoxicity effects were evaluated by using standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays.

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Briefly, Hela cells (about 60% confluency) were treated with QDs (1 nM) for 2 h before adding the MTT solution. After an incubation period of 4 h, UV−vis absorption was measured at 490 nm with 670 nm as the reference wavelength.

Results and Discussion

Surface Coating Chemistry. A number of procedures are currently available to encapsulate and solubilize semiconductor quantum dots (QDs) for biological applications.[1] Most of these procedures are based on the use of coordinating ligands, amphiphilic polymers, or amphiphilic lipids.[1,4−8,19,20] Recent work by Smith et al. has examined the effects of surface coating chemistry on the hydrodynamic size, fluorescence quantum yield, chemical stability, and biocompatibility of water-soluble QDs.[14] The results indicate that quantum dots with the smallest hydrodynamic sizes are best prepared by direct ligand exchange with hydrophilic molecules, but the resulting particles are less stable than those encapsulated in amphiphilic polymers. For stability against chemical oxidation, QDs should be protected with a hydrophobic layer. For cellular permeability and high stability under acidic conditions, the best QDs are prepared by using hyperbranched polyethylenimines. For stability in high salt buffers, it is preferable to have uncharged and sterically stabilized QDs, like those coated with polyethylene glycols (PEG) or neutral hydroxyl groups.

On the basis of these insights, we have developed multivalent PEG grafted PEI polymers for coating quantum dots by direct ligand-exchange reactions. The hyperbranched copolymers and their QD surface coating chemistry are shown schematically in Figure 1. This ligand-exchange reaction is based on the strong binding affinity of multiple amine groups to zinc atoms on the QD surface. Assuming that PEI has a close-packed structure with a packing density of 0.64,[21] we estimate that each QD contains about 25 PEI molecules. The PEI-coated QDs are stable over 4−5 weeks under ambient conditions, whereas previous work only yielded unstable QDs that undergo fast photooxidation.[13] A major difference between this work and earlier studies is the nature of coordinating groups in the ligand-exchange reaction. An important finding is that it is essential to use the same coordinating groups for the exchange reaction; that is, one primary amine group in the original capping ligand is exchanged with another primary amine group in the multivalent ligand.

Hydrodynamic Size and Optical Properties. Upon transferring into water, the optical properties of QDs exhibit no major changes. The fluorescence quantum yield (QY) of the PEI-coated QDs is about 60% of the original value (79%) (Figure 2a). The small decrease of QY is a common phenomenon during QD water solubilization, especially by using ligand-exchange methods.[14,15,19,20] Transmission electron microscopy (TEM) (Figure 2b) showed primarily single dots, indicating that the ligand-exchange reactions did not cause QD aggregation. Optical imaging studies also revealed a distinct on−off blinking behavior for the PEI-coated QDs, as reported for single dots spread on a glass surface.[22]

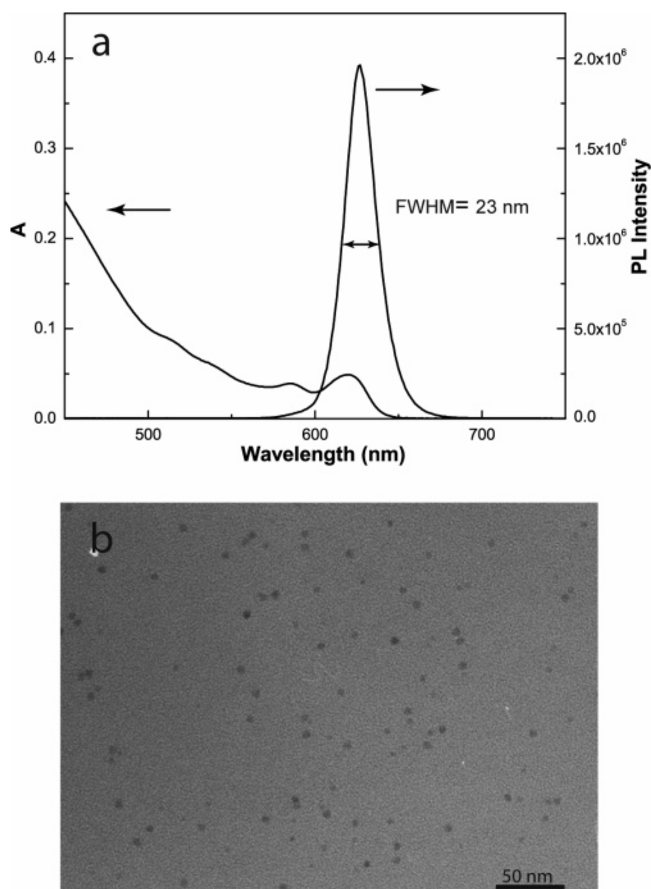

Figure 2. (a) Optical absorption and fluorescence spectra, and (b) transmission electron microscopy (TEM) image of PEI-coated quantum dots in water solution.

The PEI-coated dots are more compact in size than current QDs prepared by amphiphilic polymer encapsulation. For the same core−shell QDs (6.5 nm diameter), dynamic light scattering (DLS) measurements (Figure 3a) showed that the PEI-coated dots have a hydrodynamic size of 15.3 nm, considerably smaller than the hydrodynamic sizes (20−30 nm) of octylamine poly(acrylic acid)-coated QDs. Furthermore, the size of PEI-coated dots is dependent on pH and is further reduced to 13.0 nm in slightly basic (pH 8.5) borate buffer. This behavior is related to the proton buffering capability of PEI, which modulates the electrical charge and the hydration layer on the QD surface. When coated with PEG-grafted PEI polymers, the QD hydrodynamic sizes increase to about 21−22 nm (Figure 3). This is reasonable because an additional layer of PEG further increases the radius of coated QDs by 3−4 nm. More importantly, the PEI-*g*-PEG capped QDs are less positively charged and are considerably less toxic than the PEI capped dots, as judged from zeta potential measurements at neutral pH (Figure 3d) and cellular toxicity studies (see below).

It is worth noting that the PEG/PEI-coated QDs are exceedingly stable in acidic environments. No obvious changes of the emission peak or the optical density at the first exciton peak are observed when the dots are treated in 0.1 M HCl solution for 1 week. In contrast, QDs coated with amphiphilic polycarboxylate are unstable under acidic conditions due to aggregation and precipitation.[14] Also, the optical density at the first exciton peak of the polycarboxylate-coated QDs decreases to about 20% of the original value after 60 h in 0.1 M HCl, indicating that

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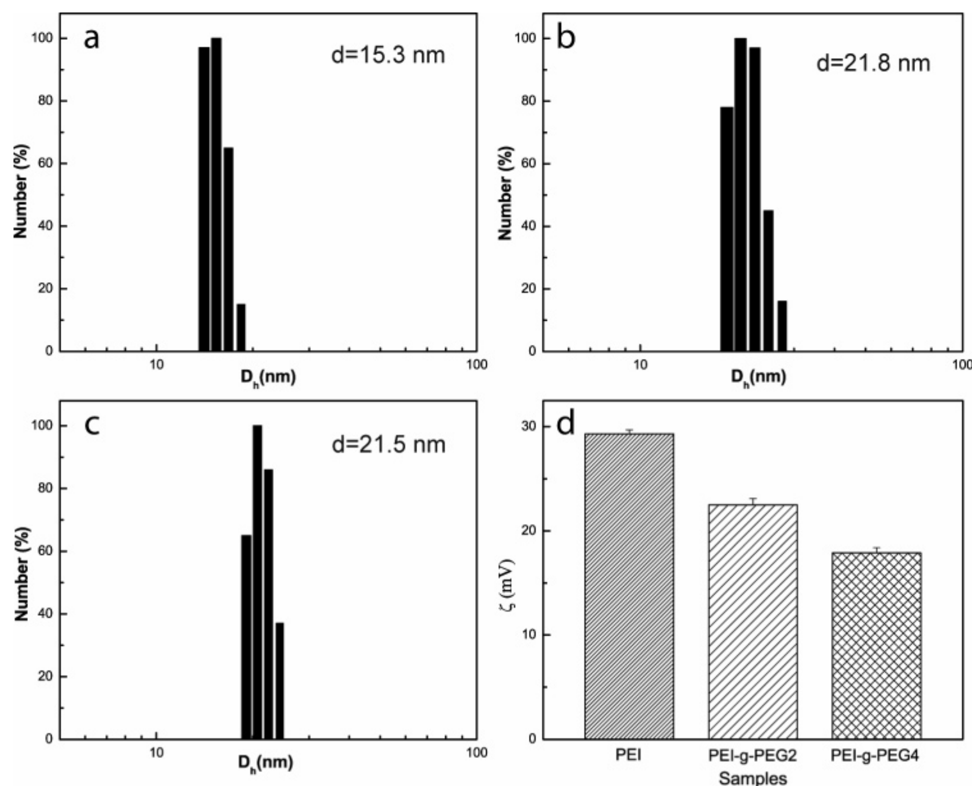

Figure 3. Dynamic light scattering and zeta potential measurements of quantum dots coated with PEI and PEI-*g*-PEG polymer ligands. (a−c) Hydrodynamic sizes (diameter) of PEI, PEI-*g*-PEG_2, and PEI-*g*-PEG_4-coated QDs in water (pH = 7), respectively. (d) Surface charges (zeta potentials) of the corresponding QDs.

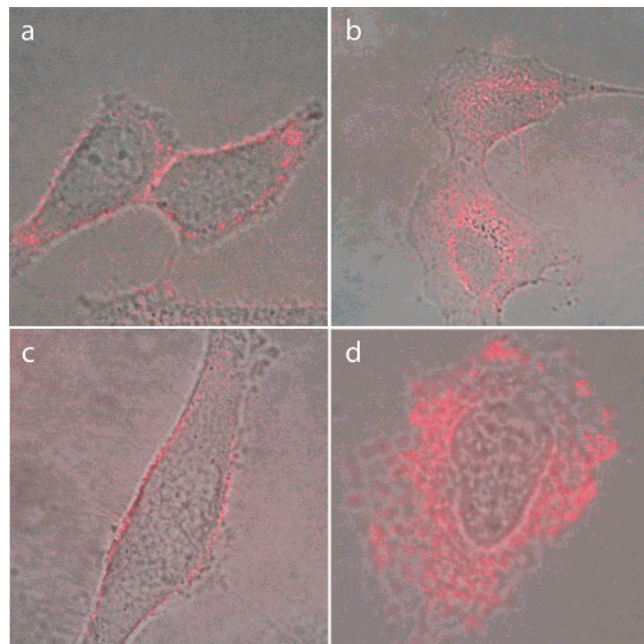

Figure 4. Confocal microscopy images showing spontaneous internalization and intracellular distribution of QDs coated with two different PEI-*g*-PEG copolymers. (a and b) PEI-*g*-PEG_4 and (c and d) PEI-*g*-PEG_2-coated dots in cultured Hela cells. The bright field and fluorescence images were overlaid, and represented time points (a and c) $t = 0$ min, and (b and d) $t = 1-2$ h after QD addition to the cell culture medium.

the hydrophobic layer is not tight enough to prevent H^+ access to the QD surface.[14] The acidic stability of PEI-coated QDs arises from the large buffering capacity of PEI, which generates a protecting barrier around the QDs. This property is of special interest for QD applications as intracellular imaging probes because most intracellular organelles such as endosomes and lysosomes are acidic (pH 4−6).

Cellular Uptake and Endosomal Release. The PEI-*g*-PEG capped QDs are very stable in Eagle's minimum essential medium (EMEM) cell culture medium (supplemented with 10% FBS). Without the PEG groups, however, the QDs are susceptible to aggregation in biological media. Clearly, the grafted PEG chains are crucial to prevent nonspecific interactions in biological systems. To explore their suitability in intracellular imaging, we have examined the cellular uptake of PEI-*g*-PEG QDs in cell culture medium. As shown in Figure 4, the PEG/PEI-coated QDs are immediately attached to the cell surface by electrostatic interactions between the positively charged QDs and the negative charged cell membrane. After an incubation period of 1−2 h, confocal fluorescence imaging showed that the QDs enter cells through endocytosis or macropinocytosis (nonspecific uptake, not mediated by receptors). However, the two QDs coated with different PEI-*g*-PEG copolymers showed different distribution patterns inside the cells. The majority of PEI-*g*-PEG_4-coated QDs are accumulated around the perinuclear region (Figure 4b), suggesting the QDs are trapped in organelles; these organelles are transported to the microtubule organization center (called MTOC, just outside the nucleus)[23] by molecular motors along microtubule tracks.[24] In contrast, the majority of PEI-*g*-PEG_2-coated QDs are distributed in the cytoplasm, indicating that these QDs have escaped from the endosomes and are released into the cytoplasm.

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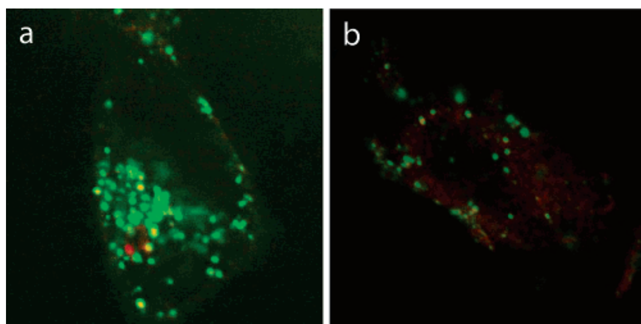

Figure 5. Two-color colocalization studies of PEG/PEI-coated QDs (red) and organelle tracking dye (DiO, green) that are co-delivered into Hela cells. (a) QDs coated with PEI-*g*-PEG_4, and (b) QDs coated with PEI-*g*-PEG_2.

More direct evidence has come from intracellular colocalization studies, in which QDs and organelle tracking dyes are co-delivered into living cells. If the delivered QDs are trapped in organelles, their fluorescence signal will be colocalized with that of the organelle dye. This colocalization can be readily detected because the QDs and the dyes have different fluorescence colors. On the other hand, if the QDs are able to escape from the organelles and are released into the cytoplasm, their fluorescence signal will not be colocalized with the organic dye. Figure 5 shows the two-color colocalization results obtained from red QDs and green DiO (a lipophilic dye that is commonly used to stain vesicles, endosomes, and related organelles in living cells).[25] Significantly, QDs coated with PEI-*g*-PEG_4 are found to colocalize with DiO-stained organelles (left panel), whereas diffuse intracellular distribution is observed for QDs coated with PEI-*g*-PEG_2 (right panel).

As mentioned earlier, PEI is able to facilitate the escape of gene delivery vectors from endosomes through the "proton sponge effect". The ligand-exchange reaction used in the current study is a "grafting" method, by which polymer ligands are attached to the QD surface. In this method, the grafting polymer density is typically dependent on the degree of a "shielding effect" caused by the grafted polymers, because the incoming ligands must overcome an existing polymer barrier to reach the QD surface.[26] This shielding effect is expected to be less pronounced in PEI-*g*-PEG_2 because of the lower content of PEG, giving rise to a higher exchange and grafting density of this polymer on the QD surface than that achieved with PEI-*g*-PEG_4. Thus, the difference observed for PEI-*g*-PEG_4 and PEI-*g*-PEG_2 coatings can be explained by a higher PEI content in the latter case (that is, more PEI-*g*-PEG_2 polymer molecules on the QD surface), which favors endosomal disruption and QD release. This explanation is also consistent with the cellular uptake results of QDs conjugated with polyarginine peptides, which showed that even one residue difference (three and four arginines) could significantly affect the cellular uptake efficiencies.[27]

Cytotoxicity Data. Cationic nanoparticle agents are often associated with significant cytotoxic effects, due to their electrostatic interactions with negatively charged glycocalyx on cell membranes.[28] It is thus important to evaluate the toxicity profiles for the PEG/PEI-coated QDs using standard cytotoxicity assays. Figure 6 shows cell viability data obtained from three types of QD particles at the same molar concentration, together with a control (no QDs) for comparison. The results show that the PEI-coated dots are very toxic to cultured cells, but this toxicity is dramatically reduced when more PEG chains are grafted to the PEI polymer. In this context, it is important to note that the cytotoxic effects of QDs could arise from the release of cadmium ions, photochemical generation of free radicals, and nanoparticle aggregation on the cell surface.[29,30] Recent work by Derfus et al. indicates that CdSe QDs are highly toxic to cells under UV illumination for extended periods of time.[31] This is understandable because UV-irradiation often dissolves the semiconductor particles, releasing toxic cadmium ions into the medium. In the absence of UV irradiation, QDs with a stable polymer coating have been found to be essentially nontoxic (no effect on cellular ATP production or cell replication). For the PEG−PEI-coated QDs, we believe that the observed cytotoxicity is mainly associated with the PEI polymer, not due to the release of toxic cadmium ions. To explore the possibility of reducing the toxicity but maintaining the endosome-escape properties of the PEI-*g*-PEG_2-coated QDs, we are currently working on fine tailoring of the QD surface chemistry. One method is to "glycidylate" the PEI-*g*-PEG_2 coating, which can transfer primary amines to less toxic secondary or tertiary amines.[28]

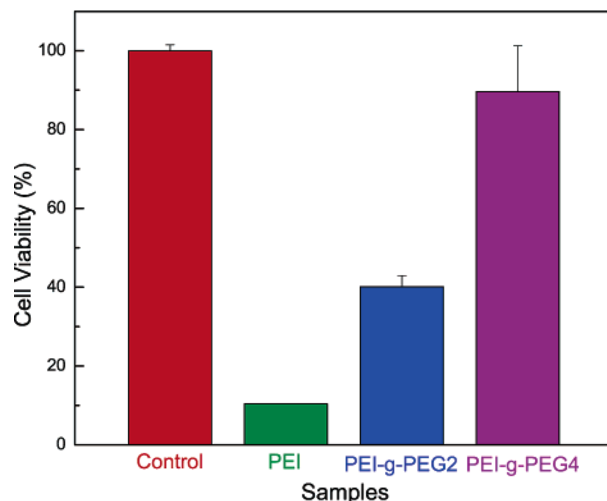

Figure 6. Cell viability data of QDs coated with PEI and PEI-*g*-PEG copolymers obtained from cultured Hela cells using standard MTT colorimetric assays. QD concentration = 1 nM; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide. See text for a detailed discussion.

In conclusion, we have reported a class of cell-penetrating quantum dots (QDs) based on the use of multivalent and endosome-disrupting (endosomolytic) surface coatings such as polyethylene glycol (PEG) grafted polyethylenimine (PEI). Because of the positive charges and a "proton sponge effect" associated with multivalent amine groups, these ligand-exchanged QDs are not only able to penetrate cell membranes but also able to disrupt endosomal organelles in living cells. The grafted PEG segment serves to reduce the cytotoxicity of PEI and to improve the overall probe stability and biocompatibility. Cellular uptake and imaging studies reveal that these

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dots are rapidly internalized by endocytosis, are initially stored in vesicles, and are followed by slow endosomal escape and release into the cytoplasm. The current work demonstrates the use of surface coating chemistry to deliver QD probes across the plasma membrane and to facilitate their release from subcellular organelles. Further improvements are still needed in QD coating chemistry and probe design. Also needed is a transduction mechanism that could signal target binding inside living cells, for which excess and unbound probes cannot be washed away.

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