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REVIEW ARTICLE

Toxicity of nanocrystal quantum dots: the relevance of surface modifications

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Abstract With the development of nanotechnology, nanometer-sized products smaller than several 100 nm have been applied for all areas of science and technology. The nanometer-sized products, including carbon nanotubes, fullerene derivatives, and nanocrystals made of various materials, are widely employed as novel tools in various fields, not only in material engineering, electronics, plastics, automobile, aviation, and aerospace industries, but also even in cellular biology, molecular biology, and basic and clinical medical fields. In particular, nanocrystal quantum dots (QDs) have been widely used in biological and medical studies because of their far brighter photoemission and photostability. The physical and chemical properties of QDs have been circumstantially investigated, but little is known about the potential harmful effects of QDs on human health. In addition to the physical and chemical properties of the QDs, their toxicity and biological behavior are generally regulated by three other conditions: (1) the QD core material itself, (2) the surface modifications of the QD, and (3) the external environmental condition of the QDs. We herein report on the in vitro and in vivo toxicity and biological behavior of nanocrystals such as QDs. Accumulating evidence suggests that the QD-capping material, rather than the core metalloid complex, is responsible for the majority of their toxicity and biological activity. For example, molecules covered with a toxic agent showed cytotoxicity, whereas QDs conjugated with biomolecules retained the biological effects of the conjugate.

Keywords Nanoparticle · Toxicity · Surface modification

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Quantum dots as an electronic device

A quantum dot (QD) is a semiconductor nanostructure that confines the motion of conduction-band electrons in three spatial directions, called zero dimensions. Small QDs have 1–10 nm diameters with 100–100,000 atoms, and the confined electrons do not move in the free space, but in the semiconductor host crystal. Due to the confinement of the electrons, the semiconductor host crystal has the capacity to receive photon energy when QDs are excited by visible, ultraviolet and infrared light. The QDs release their excited energy by emitting fluorescence, along its intrinsic potential energy band gap that is regulated by the size of QDs. In the case of QDs, the size range of particles from 2 to 10 nm corresponds to the emission wavelengths from 400 to 1,350 nm (Bruchez et al. 1998). Although such applications were predicted by Professor Ryogo Kubo (the University of Tokyo) in the 1960s (Kubo 1957, 1962a, b), the recent advances in the field of nanotechnology now allow for conventional materials to have additional function(s) based on nanoscale effects (Springholz et al. 1998; Chan et al. 2002). Since the first achievement of the chemical synthesis of nanoparticles in the 1990s, semiconductor nanocrystal QDs have been used as a test bed for fundamental physics on the nanoscale because of their tunable optical properties, which are useful for photonic applications. Due to their emission of far brighter fluorescence owing to their high quantum yield, the QDs are frequently used as the electronic assembled parts for semiconductor devices. Their intrinsic photoelectrical properties allow for the precise measurements of the spin to be made by applying small voltages, thus enabling us to make use of the multiplexed state of spins without interference. Therefore, QD technology is expected to become a widely used tool in solid-state quantum computation for telecommunication applications, such as the

quantum memory effect (Maenosono et al. 2000; Cordero et al. 2000). The QDs that have specific functions based on their size are now widely attracting attention because their intrinsic characteristics are enhanced by their significantly broader surface area for nanomaterials (Chan et al. 2002; Chan and Nie 1998; Jaiswal et al. 2003).

Apart from the fundamental physics studies, QDs have also been investigated by researchers in the biological fields because they have specific photo-emitting functions based on their nanometer size, making them attractive fluorophores for multicolor imaging due to their broad absorption and narrow emission spectra, and their brighter and far more photostable emissions, compared to conventional organic dyes.

The three factors that define the color spectrum of the quantum dots

The physical properties of QDs have well-investigated by material sciences researchers. Many advanced improvements on the modification of QDs are performed to make brighter nanocrystals. Among the various factors that define the activity of QDs, their photophysical properties are defined by three factors: (1) the composition of the QD core itself, (2) surface modifications, and (3) extraparticular conditions. These three factors change the properties and behavior of the entire QD particle.

Core/shell structures define the activity of QDs

The QD core structure is one of the important factors that determine its activity. After the successful demonstration of colloidal synthesis of QDs, an extension from single-material nanostructures to semiconductors has been performed. Hereafter, QDs are referred to as hetero-structures with other semiconductors, as "core" structures and other attachments. A core/shell QD structure, surrounded by a thin second material monolayer, has had a major impact owing to their ability to increase quantum yield and stability (Hines and Guyot-Sionnest 1996; Peng et al. 1997; Dabbousi et al. 1997). The enhancement of the energy band by the core/shell structure can be described by the combination of a spherical square quantum well with the electrons in the conduction band, and the improvement of their properties is attributed to confinement of the excitation states to the core of the QD (Kim et al. 2003). Successful syntheses of core/shell QDs have been demonstrated for various combinations such as CdTe, ZnS, InP, and InAs. A core/shell structure using ZnS shells of up to three monolayers in thickness over the core particle is theoretically estimated to provide the maximum performance and the optimal increase in the photostability and photoluminescence quantum yields of whole QD particles (Cao et al. 2004). In addition, a CdSe core with small amount of some transition metal dopants, such as manganese and titanium, leads to a decreased fluorescence intensity, and a fluorescent emission shift toward red with the same particle size (Yang et al. 2004a, b; Santra et al. 2005; Sapra et al. 2005). Thus, the QD core itself regulates the photoluminescence properties of the complex, and cores are the ultimate ultrafine structure unit of the QDs.

Surface modifications enhance the activity of QDs

The chemical property of the molecules covering the surface of the QD particle is another factor that defines the property of the QD particle. Since colloidally synthesized QDs are highly hydrophobic, their advanced photonic properties are regarded as being similar to organic dyes and several fluorescent proteins. The incompatibility of QDs with aqueous conditions had long limited their use for biological applications. Over the past several years, various strategies to solubilize QDs in aqueous buffers have been designed. For example, molecules with sulfhydryl (–SH) groups were among the first to be attached on QD-surfaces (Chan and Nie 1998), but subsequent studies have also demonstrated the utility of colloidal gold particles, and the hydrophilic carboxyl (–COOH) groups that let QDs dissolve in aqueous solutions.

Various surface modifications to QDs affect their fluorescence intensity (Hoshino et al. 2004). The fluorescence intensity and peak wavelength can be changed by the surface capping molecules of the QDs; QDs with carboxyl groups (QD-COOH) have higher luminescence than the other groups (Fig. 1). Furthermore, the peak wavelength varies according to the type of surface modification. QDs containing amino groups emit a shorter wavelength (513 nm wavelength) than the originally synthesized TOPO-capped QDs, which was synthesised in the micelle of detergents tri-n-octylphosphine oxide (TOPO), with 518-nm emission. In contrast, QDs with hydroxyl groups are slightly red-shifted. We assumed that a longer carbon chain and the addition of the carboxyl groups of mercaptoundecanoic acid (MUA) might contribute to the long lifetime and high quantum yield of QD-COOH. In contrast, the decreasing fluorescence of amino-QD (QD-NH_2) particles may be caused by the oxidation of the QD-metal because of the leakage of electrons from QDs through surface amino groups in aqueous solution. This suggests that the luminescence intensity of QDs may vary according to the molecule structures added to the surface.

Extraparticular conditions: the importance of intermolecular effects

The third factor is the intermolecular effects between the QD particles and the extraparticular conditions surrounding

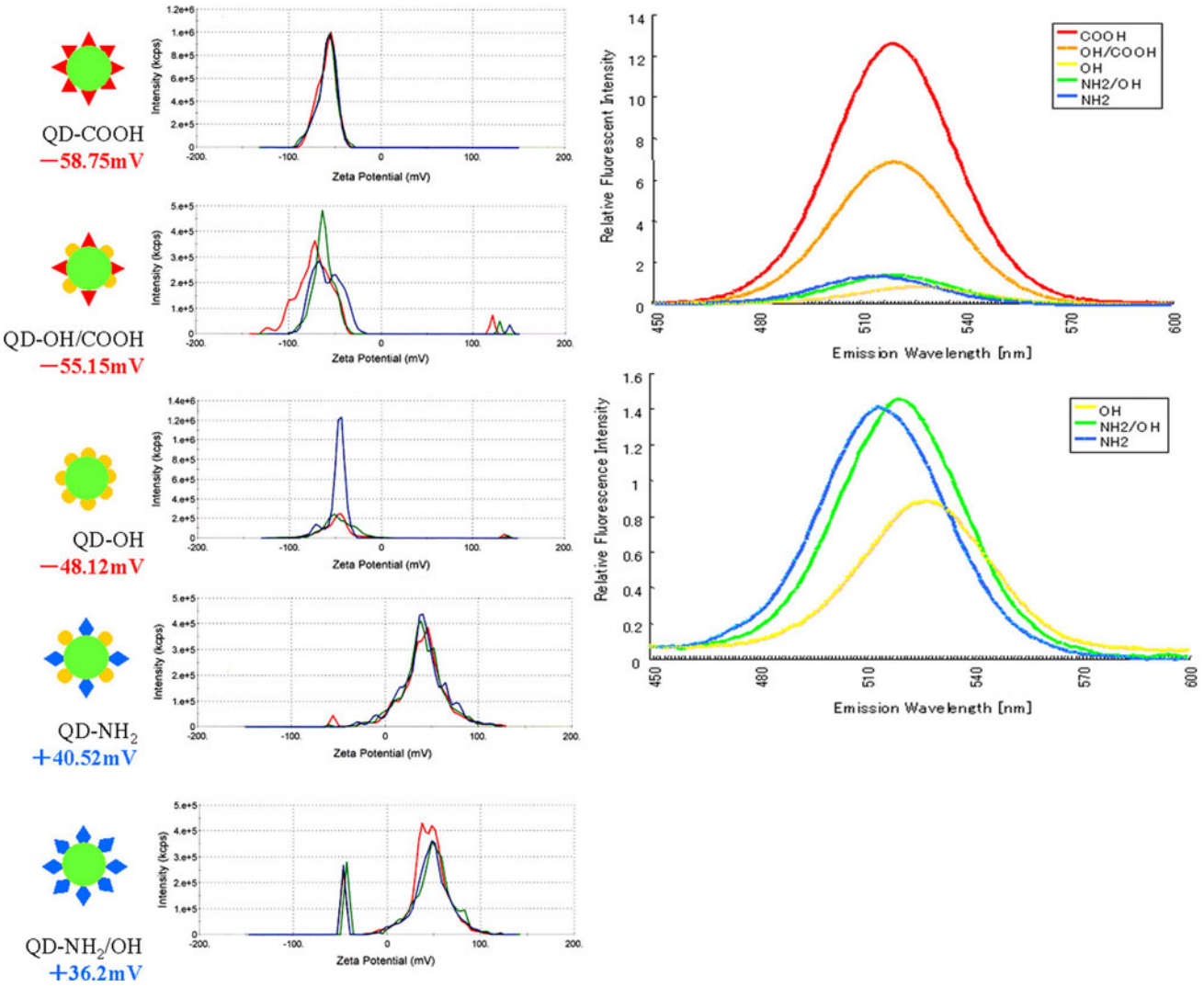

Fig. 1 Surface ζ-potential and fluorescence intensity of QDs varied by their surface modifications. **a** Cartoon (*left*) and ζ-potential (*right*) of the novel-modified QDs. Surface ζ-potential of QDs is measured by electrophoresis. *Each line* shows the electrophoretic mobility of QDs in the stationary layers. The data are the average of 30 assays. **b** Relative fluorescence intensity and peak wavelength of QD-COOH (*red*), QD-COOH/-OH (*orange*), QD-OH (*yellow*), QD-NH2/OH (*green*), and QD-NH2 (*blue*) measured by fluorescence spectrometry. (*Lower*) enlarged panel of QD-OH (*yellow*), QD-NH2/OH (*green*), and QD-NH2 (*blue*) in the *upper panel*. The peak emission wavelengths of QD-COOH, QD-OH/COOH, QD-OH, QD-NH2/OH, and QD-NH2 were observed at 519, 520, 526, 520, and 513 nm, respectively

them. In particular, a specific photophysical feature of QDs, called blinking, affects their activity. The blinking is a fluctuation of photoluminescence which every QD shows intrinsically when QDs are observed under a steady laser illumination, such as evanescent fields (Neuhauser et al. 2000; Eiha et al. 2003; Uematsu et al. 2004; Kimura et al. 2004). Blinking is one of the specific characteristics of single fluorescent nanomaterials and is measured by fluorospectrometry as the bright "on-phase" fraction and on-dark "off-phase" fraction events that occur in the span of several minutes (Fig. 2a) (Tang and Marcus 2005; Yao et al. 2005). In addition, a sudden jump in the enhancement of photoluminescence intensity in the span of millisecond is often observed. This increased photoluminescence continues after transition from the "on-phase" to the "off-phase" of QD (Fig. 2b). Another fluctuation called millisecond oscillation is a different phenomenon from blinking that can also affect the photoluminescence.

The surrounding environmental molecules could affect the emission properties of both the QDs themselves and the blinking. The preservative sodium azide (NaN_3), which is a preservative against bacterial growth in the storage buffer, has the ability to enhance the fluorescence intensity of QD in a dose-dependent manner (Fig. 2c). On the other hand, QD-NH_2 shows weakened fluorescence intensity, whereas NaN_3 enhanced QD-COOH and QD-OH because of their negative charge. These data suggest that the electric charge of the QD was significantly affected by the changes in

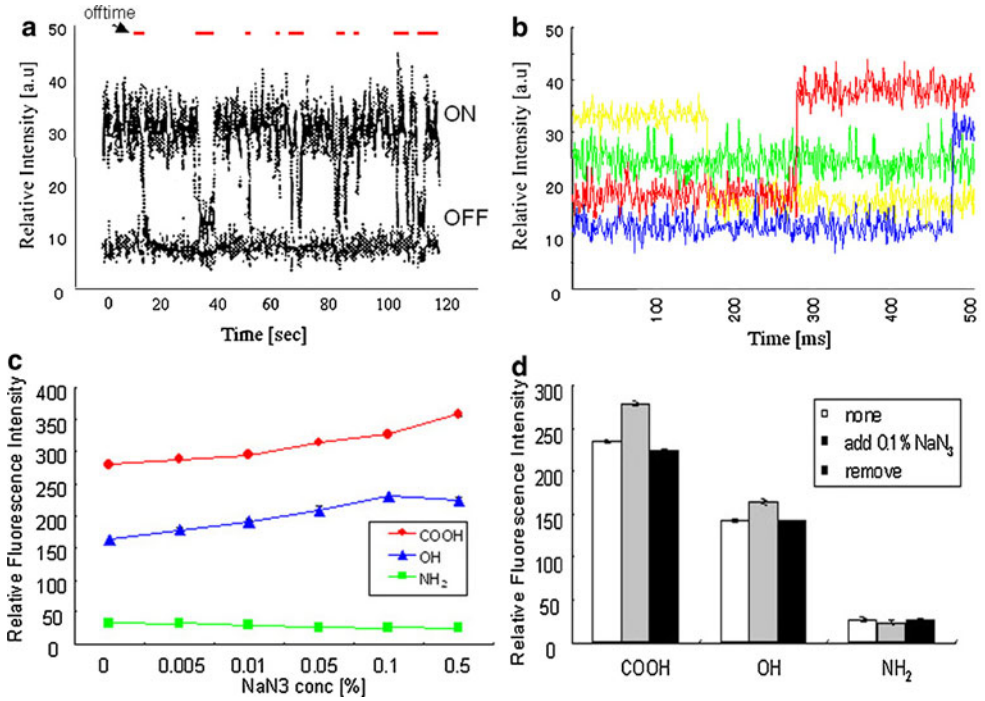

Fig. 2 The mechanism of the millisecond oscillation is different from that of blinking. **a** A blinking change of QD-COOH fluorescence intensity in aqueous solution. Fluorescence intensity was continuously measured for 120 s with a fluorospectrometer. The *red line* in the *upper graph* indicates "off" phase of blinking. **b** The millisecond oscillation of QD-COOH in aqueous solution was observed on the evanescent field with an ultra sensitive high-speed fiber-coupled CCD camera (FASTCAM® MAXI-I^2, Photoron corp., Tokyo, Japan). The *x-axis* indicates the time and *y-axis* indicates the relative fluorescence intensity. *Red, blue*, and *yellow lines* that indicate the emission from individual QD during the on–off switching phase ($n = 3$) were overlaid. The *green line* indicates the photoemission of the "on-phase". **c** The external environment surrounding QDs affects the fluorescence intensity. NaN_3 in aqueous solution enhances the fluorescence intensity of QD. A 100 μM aliquot of QD-COOH (*circle*), QD-OH (*triangle*), or QD-NH_2 (*square*) was diluted with 0.1% NaN_3 solution. **d** Removal of NaN_3 by dialysis recovered the enhanced fluorescence intensity. QD-solutions with 0.1% NaN_3 were dialyzed using an ultrafiltration membrane. The concentration of the collected solution was adjusted with an adequate volume of distilled water. Data are the mean aggregation areas ±SD of triplicate experiments

fluorescence intensity induced by NaN_3. Removal of NaN_3 by dialysis from the solution recovered the QDs' enhanced fluorescence (Fig. 2c), implying that the solutes around the QD-solution influence the fluorescence intensity of the QDs. These results suggest that not only the molecules covering the surface, but also the surrounding external environmental solutes around the QD regulate the fluorescence intensity.

Further supporting the possibility that aqueous solvents are involved in the millisecond oscillation, QD-COOH fixed on the surface of cover slips were filled with different organic solvents, implying that the polarity of the solvents was also acting as an electron donor in the solution. In nonpolar organic solutions, such as toluene, each QD displayed no detectable fluorescence oscillation (Fig. 3). These results suggest that both solutes and solvents that surround the QDs may function as the donor/interceptor of excited electrons on the nanocrystal QDs. The theory that electron transfer processes regulate the power-law distribution for the lifetime of a blinking of a QD has already been proposed. Another examination reported that the stabilization of the surface of QDs by the passivation with thiol moieties could reduce the blinking of QDs (Hohng and Ha 2004), further suggesting the existence of electron leakage. These results demonstrated that the electron status of the core of the QD, the molecules covering the QD, and even the external environment around the QD affect the millisecond oscillation. This implies that the emission of QD depends on at least these factors: the substance covering the surface, the solvent, and the other solutes in solution.

Therefore, the properties of QDs depend on multiple factors derived from both the inherent physicochemical properties of the QDs and various environmental conditions. For example, QD properties like QD size, charge, concentration, outer coating bioactivity (capping material and functional groups), and oxidative, photolytic, and mechanical stability are each factors that, collectively and individually, can determine the toxicity of a QD (Hoshino et al. 2004; Hardman 2006). The fact that the photoluminescence property of QDs also depends on multiple factors supports the possibility that the brightness of the whole QD particle can be improved by extraparticular modifications, including

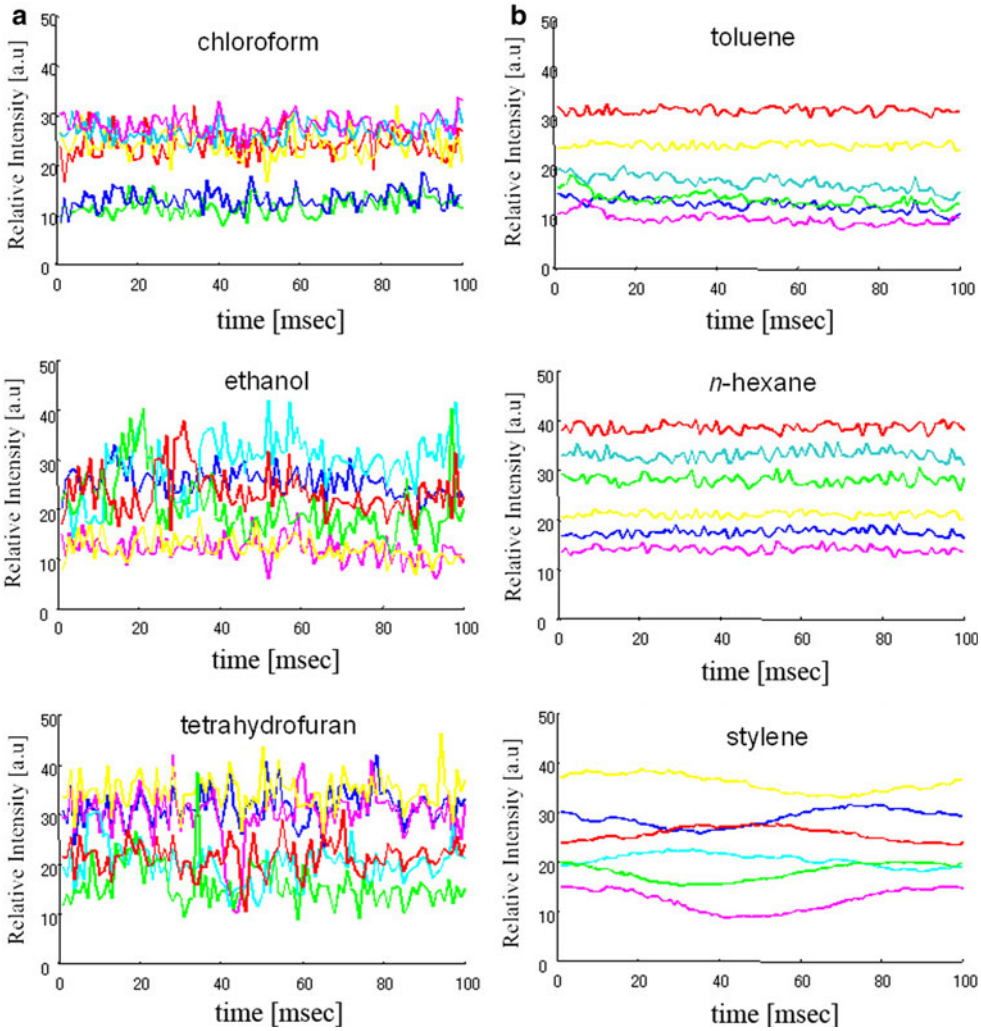

Fig. 3 The polarity of the solvent regulates the frequency of the millisecond oscillation. The millisecond oscillation of QD-COOH in **a** polar organic solvents, chloroform, ethanol, and tetrahydrofuran, and **b** non-polar inorganic solvents, toluene, *n*-hexane, and styrene, were observed on the evanescent field by the total-reflective fluorescent microscopy with a CCD camera unit FASTCAM®. The blinking frequency of each monodispersed QD ($n = 6$) during each millisecond is measured by a 1-ms exposure. The *x-axis* indicates the time [milliseconds] and the *y-axis* indicates the relative fluorescence intensity [a.u.]. *Data* shown are one of three independent experiments that gave the same results

changes to the capping materials, functional bioactive groups, and even in periparticular molecules.

Applications of quantum dots

In modern biological analysis, various organic dyes are used to visualize the objective molecules. At present, many organic fluorophores have been used in various biological applications including fluorescent-labeled antibodies, molecules that are used to stain cells or cellar organs. Experiments using organic dyes are limited to short-time assays such as flow cytometry due to the lifetime of their fluorescence. These conventional dyes are less suitable for extended periods of bio-imaging using fluorescent and confocal microscopy because the organic fluorophores tend to quench rapidly (Bruchez et al. 1998; Akerman et al. 2002). Furthermore, it is sometimes difficult or impossible to record fine fluorescent images, while the organic coloring probes fade in the course of adjusting the focus. Therefore, much brighter fluorophores are required.

Currently, QDs are widely used in biological and even in medical studies because they are attractive fluorophores for multicolor imaging due to their broad absorption and narrow emission spectra, and the fact that they are brighter and far more photostable than organic fluorescent dyes (Bruchez et al. 1998; Chan et al. 2002; Chan and Nie 1998; Jaiswal et al. 2003; Akerman et al. 2002; Hoshino et al. 2004; Gao and Nie 2005; Xu et al. 2003; Dubertret et al. 2002; Hanaki et al. 2003). The most popular and successful use of QDs is their application as a tool for cell-labeling, especially for monitoring living cell trafficking. Several nanoparticles are commercially available for this purpose. A number of researchers have performed a variety of cellular assays using QD fluorescent probes, especially those to stain for cellular macromolecules, such as microtubles, actin (Mansson et al. 2004) and nuclear antigens (Hoshino et al. 2004). Immunohistochemical staining of membrane proteins (Wu et al. 2003) and immune cell trafficking have also been examined (Hoshino et al. 2004, 2007a, b; c; Zheng et al. 2006); QDs are also useful for these applications due to their far brighter fluorescence with extremely

resistant photostability against bleaching over long periods of cellular experiments. However, the intracellular delivery pathways for QDs still remain to be elucidated. In some applications, QDs are easily incorporated into the cytoplasm and reach the nucleus (Hoshino et al. 2004), perhaps as a result of cationic charges concomitant to signal peptides attached to the QDs. Conversely, biotinylated peptide-coated QDs revealed that trafficking of QDs into the cytosol could be done, and these experiments demonstrated the relationship between glycosylphosphatidylinositol-anchored receptors and lipid rafts in the membrane at a single-molecule level (Michalet et al. 2005). Recently Anas et al. (2009) demonstrated that multiple pathways are involved in the intracellular delivery of peptide-conjugated QDs, with clathrin-mediated endocytosis being the most important pathway (accounting for approximately 60% of the delivery) for the intracellular delivery of peptide-conjugated QDs.

Unfortunately, even now QDs will not replace the conventional organic dyes or fluorescent proteins except for cell trafficking applications, although their unique photonic and morphological properties would provide an evolving approach in biology. The lack of enthusiasm for using the QDs stems primarily from concern about their size; frequently used modifications of streptavidin-coated QDs have a relatively large size of ~30 nm diameter, which is larger than an antibody conjugated with organic dyes.

Toxicity induced by quantum dots

Great quantities of "artificial nanomaterials," including carbon nanotubes, fullerenes and QDs, are produced and consumed on the unfounded inference that nanomaterials are biologically and environmentally harmless. However, very few studies have examined the toxicity of these nanomaterials (Hardman 2006; Borm et al. 2006; Bottero et al. 2006; Garnett and Kallinteri 2006; Guzman et al. 2006; Lanone and Boczkowski 2006; Lawson et al. 2006; Moore 2006; Talapin and Murray 2005; Nel et al. 2006; Oberdorster et al. 2005; Robichaud et al. 2005; Service 2005; Wiesner 2006; Worle-Knirsch et al. 2006). Recently, nanomaterial researchers have been dealing with nanomaterial-induced toxicity problems (Hoshino et al. 2004; Adams et al. 2006; Braydich-Stolle et al. 2005; Fortner et al. 2005; Kim et al. 2006; Magrez et al. 2006; Sayes et al. 2005, 2006; Tian et al. 2006; Yamawaki and Iwai 2006; Yang et al. 2006; Zhu et al. 2006; Shiohara et al. 2004). There are several reasons why so few studies of nonomaterial toxicity have so far been done: (1) some research on nanomaterials has been performed under the premise that there is no toxicity, (2) various nanomaterials, which have their own unique properties, have been synthesized, and a variety of QD concentrations have been reported, making it difficult to select an appropriate test substance and dose. (3) The quantity of the nanomaterials is often insufficient to carry out a series of toxicology studies.

Moreover, the toxicity of nanomaterials is highly complicated, because of the diversity of materials. In sharp contrast to conventional hazardous materials, researchers also need to pay attention to the nanoparticle-specific problems, including the fact that surface of nanomaterials is highly active due to the "specific surface area", a ratio of surface to diameter. In addition, it is necessary to exclude the effect of solubility and possible contamination, which also would decrease the validity of any toxicity testing.

Magic enchantments have expired

We and other nanomaterial researchers have reported that the QD behavior in the body (called ADME; the absorption, distribution, metabolism, and excretion) and toxicity are dependent on multiple factors derived from both its individual physicochemical properties and its environmental conditions (Hardman 2006; Robichaud et al. 2005; Kako et al. 2006; Ryman-Rasmussen et al. 2007; Zhang et al. 2006). The toxicity is primarily caused by the inherent physicochemical properties. In addition, environmental conditions including oxidative, photolytic, and mechanical stability often lead to destabilization of the QDs, resulting in another kinds of QD toxicity.

QDs have their own physicochemical properties, which can exert toxic effects dependent on the size, electric charge, concentration, and surface coating materials, including capping material and functional groups, which are present. We assessed the dose and size dependency of QD-mediated cytotoxicity up to a high concentration (approximately 1,000-fold concentration of usual cellular applications). We observed the dose-dependent (Fig. 4a) and the size-dependent (Fig. 4b) cytotoxicity of QDs (Shiohara et al. 2004). The observed cytotoxicity is proportional to the number of QDs that are incorporated into the cells. In addition, we demonstrated that the red QDs are incorporated into cells less frequently than green ones (Shiohara et al. 2004). Other groups have also demonstrated that QD-induced cell death was due to chromatin condensation and that the size of QDs contributed to their subcellular distribution (Lovric et al. 2005a). For example, one report implied that the toxicity and distribution of internalized and accumulated nanoparticles were dependent on the types of cells being examined (Alsharif et al. 2009). Moreover, cadmium and selenium, two of the most widely used constituent metals in our QD core crystalline, are reported to cause acute and chronic toxicities in vertebrates when QDs were irradiated to the UV-mediated oxidative stress (Lovric et al. 2005b). Some other experiments also

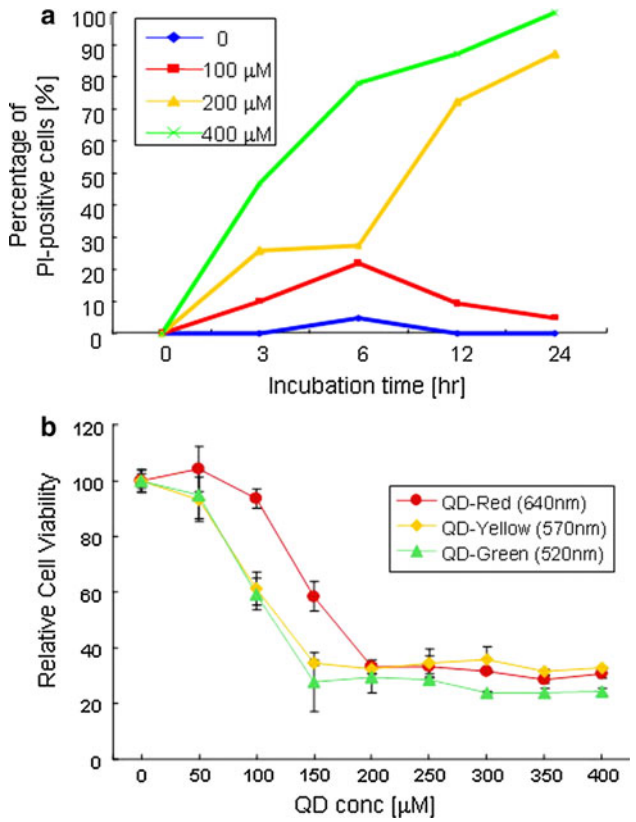

Fig. 4 Dose and particle size-dependent QD toxicity at high concentrations. **a** The cytotoxicity of the QD520 (*green*) solution depends on its concentration. Vero cells were plated at 1×10^5 cells and stimulated with culture media alone or with QD at a concentration of 100, 200, or 400 μM. The cells were incubated for 3, 6, 12, or 24 h at 37°C. The cells were harvested, stained for observation of dead cells with propodium iodide (PI), and measured by flow cytometric analysis. Fluorescence was measured by FACS Calibur (Beckton Dickinson). **b** The relative cell viability following incubation with QDs of different sizes was measured with the MTT assay. Vero cells were plated at 1×10^5 cells on 24-well plates and incubated for 12 h with the indicated concentration of QDs. Results are representative of three separate experiments. $P < 0.05$

support that leakage of heavy metal ions (Mahendra et al. 2008) and their derivatives, such as TeO_2 and CdO (Schneider et al. 2009), are also involved in the toxicity of the molecules. Conversely, another group reported that the stress response caused by QDs is different from and independent of the Cd^{2+} ion (Zhang and Monteiro-Riviere 2009).

Furthermore, the capping materials and biomolecules which cover the QD-surface also define the total biological behavior of the whole nanocrystal QD, including its toxicity (Shiohara et al. 2010). We assessed the genotoxic potential of QDs by comet assay, which could quantitatively evaluate the apoptotic cell damage by the fragmented DNA tail length, among various kinds of surface hydrophilic modifications of QDs: carboxylic-QDs (QD-COOH, covered with hydrophilic 11-mercaptoundecanoic acid sodium salt (MUA), amino-QDs (QD-NH_2, covered with cysteamine hydrochloride), hydroxyl-QDs (QD-OH, covered with thioglycerol), and their mixtures (such as QD-OH/COOH and QD-NH2/OH) (see schematic cartoon, Fig. 5a) (Hoshino et al. 2004). The assay clearly revealed that QD-COOH samples showed stronger DNA damage than other modifications (Fig. 5b). Because we speculated that the genotoxicity of QDs was caused by QD particles themselves, three surface-modification (MUA, cysteamine and thioglycerol) and two possible impurities (tri-n-octylphosphine oxide (TOPO) and zinc sulfide (ZnS)), which are ingredients for QD-synthesis, were also assayed. As a result, some surface-covered toxic molecules caused severe cytotoxicity in a dose-dependent manner, whereas other QDs with less toxic molecules covering their surface caused less cytotoxicity (Fig. 5c). The complete removal of impurities such as TOPO from the QD samples is important in reducing toxicity, because TOPO itself was also well known to be a cytotoxic and genotoxic compound. Next we evaluate whether the harmful cytotoxicity is caused by the QD-surface modification with biological molecules to verify the safety before the use of QDs for biomedical application. Indeed, the cytotoxicity induced by naked QDs (α-lipoic acid coated QD-COOH see bared QD in Fig. 5d) was observed, whereas our advanced surface modification using polyglutamate has achieved to reduce QD-mediated cytotoxicity (Fig. 5d). Other groups have also demonstrated that QD cytotoxicity is dependent upon the properties of the particle as a whole, and not exclusively the metal core material (Stern et al. 2008). This result suggests that the bioactive behavior of QDs in biological systems is not only dependent on the nanocrystal particle itself, but also on the biochemical properties of the molecules on the surface (Kako et al. 2006). These results provided evidence that some hydrophilic compounds which capped on the surface of QDs are responsible for the biological effect of QD-whole particles.

Oxidative damage is a critical cause of cell damage (Fujioka et al. 2008). Thus, a tight coating of the surface of the QDs by synthetic polymers has been believed to be effective and critical for preventing oxidative damage. Notably, a recent report indicated that the radical oxygen species (H_2O_2 and HClO) generated on the surface of QDs could easily penetrate through the polymer-coated modification and finally degrade the core structures of the QDs (Mancini et al. 2008).

Are QDs toxic in vivo?

Quantum dots have potential in biomedical applications, but concerns persist about their safety. Most toxicology data were derived from in vitro studies as described previously and may not reflect in vivo responses. In addition, the

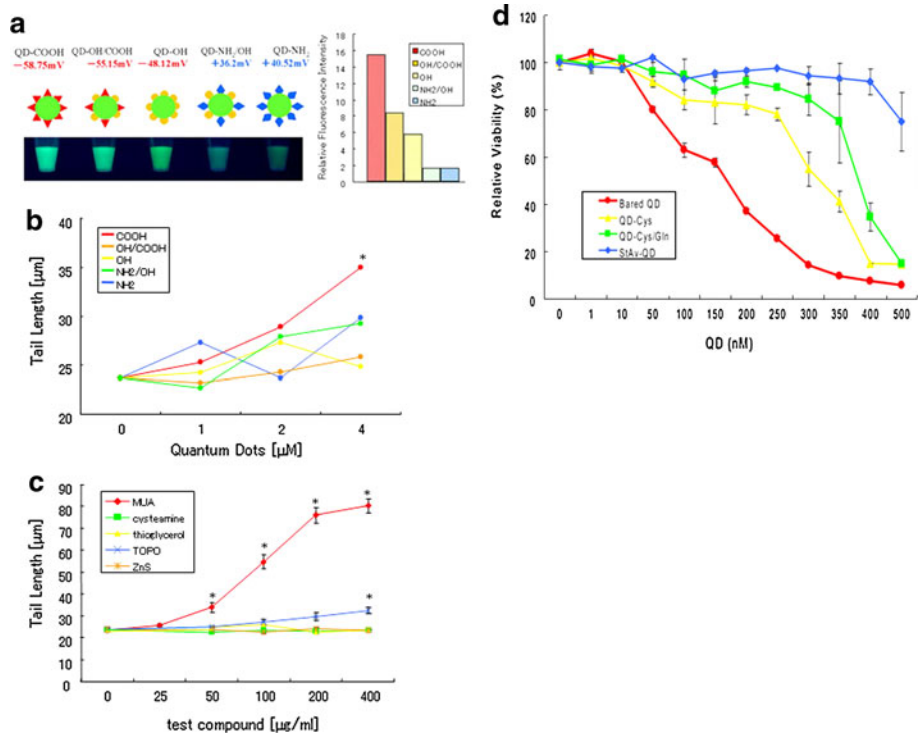

Fig. 5 Surface-capped molecules participate in QD-induced cell damage and lethal toxicity. **a** ZnS-coated CdSe nanocrystal QDs (fluorescence wavelength: approximately 518 nm, emitted *green*) were capped with 11-mercaptoundecanioc acid (QD-COOH), 2-aminoethanethiol (QD-NH_2), and 3-mercapto 1,2-propanediol (QD-OH) by thiol exchange reactions. *Cartoon images* and *solution photos* of QDs capped with various surface molecules are shown in the figure. The snapshots of the QD aqueous solution (final concentration 100 nM) were captured using a digital camera with 1/30 s exposure excited by a 365-nm wavelength (UV-A). The relative fluorescence intensity of each QD (shown at *right*) was measured by fluorescence spectrometry. **b**, **c** DNA-damaging effects of hydrophilic nanocrystalline QDs as determined by comet assay. When WTK-1 cells were incubated with each sample, the fragmented DNA of the apoptotic cells (called a comet tail) was eliminated from the nuclei by electrophoresis. The length of the comet tail stained with ethidium bromide was measured. **b** QD solution induced genotoxic cell death. Cells were treated with QD-COOH (*red*), QD-NH2 (*blue*), QD-OH (*yellow*), QD-OH/COOH (*orange*), or QD-NH2/OH (*green*) for 2 h. **c** Other reagents used for QD-modification also induce cell death without QD condition. Cells were treated with 11-mercaptoundecanoic acid (MUA, indicated by *red line*), cysteamine (*green*), thioglycerol (*yellow*), tri-n-octylphosphine oxide (TOPO, by *blue line*), or ZnS (*orange line*) for 2 h. The difference between the means in the treated and control plates was compared with the Dunnett test after one-way ANOVA. $*P < 0.05$. Results are representative of three separate experiments. **d** Advanced surface coating ameliorates the cytotoxicity of QDs. HEK293 cells were incubated with indicated concentrations of various QD preparations for 24 h at the indicated concentrations. Cytotoxicity was measured by the culture for further 24 h in the presence of the sterile assay reagents. The data are the representative of two separately performed, shown as the mean ± standard deviations of duplicated samples. Bared QD, α-lipoate-coated QD-COOH; QD-Cys, α-lipoate-coated QD-COOH with oligocysteine layers, QD-Cys/Gln, polyglutamate-coated QD-Cys; StAv-QD, streptavidin-conjugated QD-Cys/Gln

in vivo toxicity of QDs remains controversial, due to the multiple parameters that are secondary occurred in targeted organs including change of particle size and shape after metabolism, tissue distribution and concentration, electrical charges, particle-mediated induction of redox activity, characteristics of surface coating, and mechanical stability of the particles.

Early in vivo reports insisted that QDs had no acute or obvious toxicity when they were properly coated by hydrophilic shells (Jaiswal et al. 2003; Dubertret et al. 2002; Ballou 2005; Ballou et al. 2004). However, further studies will bear out whether this is true (Hauck et al. 2009). Tissue kinetics of nanoparticles of QDs is reported by Yang et al. (2007) using an inductively coupled plasma mass spectrometry (ICP-MS) that maximum cadmium concentration in liver, followed by blood, spleen and kidney at 1 day after intravenous administration of QDs, whereas it was increased at 28 days later in liver and kidney by mass balance studies. It was also reported that QD were removed rapidly from the blood to the reticuloendothelial systems, where they persisted for several months (Ballou et al. 2004). Fischer et al. demonstrated that albumin-coated QDs were removed from the circulation within hours of injection and were found in Kupffer cells of the liver (Fischer et al. 2006). However, it has been shown that some surface modifications can cause acute toxicity due to the platelet aggregation (Geys et al. 2008). Another report demonstrated that UV irradiation increases the risk of transdermal penetration

of QDs (Mortensen et al. 2008). Notably, a couple of groups observed the induction of proinflammatory responses following QD administration (Jacobsen et al. 2009; Hoshino et al. 2009). A significant report on fertilization and fetal development has also been reported (Hsieh et al. 2009). Further investigations are required to elucidate the full picture of the toxicity/safety of QDs.

Advanced nanoparticles: targeted activity and minimized toxicity

Our observation that the molecules capped on the surface of QDs are responsible for the biological effect of whole QDs, prompted us to attach medicine or functional molecules to QDs and observe whether the complexes could exert their intrinsic medicinal effects in vivo. First, we conjugated QDs with an anti-hypertension medicine, captopril (Laffan et al. 1978; Rubin et al. 1978). We next measured the effect of QD-conjugated captopril (QD-cap) in vitro, administered them to the spontaneously hypertensive rat, and assessed the effect of QD-medicine in vivo (Manabe et al. 2006). As we expected, we synthesized the functional nanocomposite particles of QD and captopril without losing the fluorescence activity and anti-hypertensive effects (Fig. 6). These results suggest that the surface treatment of nanocrystals (surface capped functional groups and biomolecules covered the surface of QDs) defines the biological behavior of the whole nanocrystal QDs (Bottero et al. 2006; Garnett and Kallinteri 2006; Pison et al. 2006; Bianco et al. 2005; Lin and Datar 2006). However, there is no evidence whether the biological behavior of QD-cap is the same as cap alone, particularly with regard to the ADME (the absorption, distribution, metabolism, and excretion). Indeed, the sizes of the QD-cap (approximately 12 nm diameter) are larger than that of the captopril molecules (0.2 nm).

We thereafter examined whether QDs have the ability to deliver gene fragments into living cells, since several challenging reports on nanomaterial-mediated gene delivery have been reported (Bhakta et al. 2005; Bharali et al. 2005), such as the delivery of antisense oligodeoxynucleotides with carbon nanotubes (Jia et al. 2007; Biju et al. 2007) and plasmid delivery using QDs (Srinivasan et al. 2006). We observed that QDs conjugated with nuclear localizing signal peptides (NLSP) successfully introduced gene fragments with promoter elements, which promoted the expression of the enhanced green fluorescent protein (eGFP) gene in mammalian cells (Hoshino et al. 2008). The QD + NLSP-construct was subsequently used to deliver the gene fragments containing an expression cassette for eGFP to cultured cells. HEK293 cells incubated with either 5′-eGFP/QD640 or 5′-eGFP/QD640 + NLSP for 24 h were observed by confocal

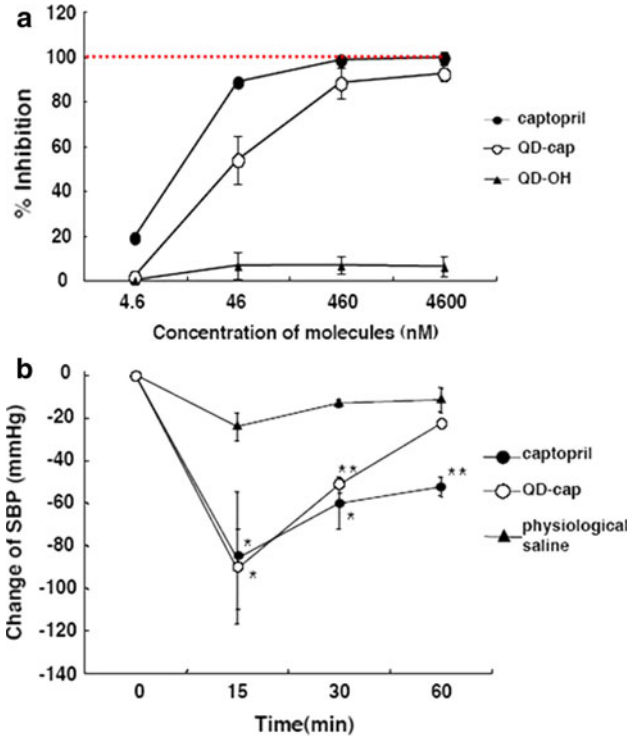

Fig. 6 Captopril-capped QD (QD-cap) exerts its medicinal effect both in vitro and in vivo. Captoprol, an anti-hypertension medicine, is directly conjugated to QDs (QD-cap). Each QD-cap particle has approximately 350 captopril molecules by peptide content assay. **a** Inhibitory activity of angiotensin-1-converting enzyme (ACE, the target molecule of captopril) by QD-cap was measured by the Cushman and Cheung method. The data presented is the mean value ± standard deviation of triplicate samples. The QD-cap shows inhibitory activity at least as effective as capotpril alone. **b** QD-cap decreases the blood pressure in hypertension rats. QD-cap (5 mg/kg) was injected i.v. into the SHR-SP spontaneous hypertension rat via the carotid artery. After injection, systolic blood pressure was measured with the tail-cuff method at indicated times ($n = 3$). The results are representative of three separate experiments

microscopy (Fig. 7a). The flow cytometric analysis revealed that both the 5′-eGFP/QD640-construct and 5′-eGFP/QD640 + NLSP-construct entered into the cell cytoplasm, and $eGFPP^{+P}$ expression was observed in about 10% of cells (Fig. 7b). Therefore, the QD + NLSP-gene fragment construct could be used to deliver gene therapy without using a viral vector. Those in vitro results prompted the delivery of these gene fragments using QD bionanocomplexes in vivo. The direct injection of the solution of QD bionanocomplexes into the peritoneal cavity induced eGFP expression (approximately 16% cells) in peritoneal cells (Fig. 7c). However, the cell population dramatically changed after the injection of the QD bionanocomplex with gene fragments (Fig. 7c), indicating that the QD bionanocomplex induced inflammation in vivo. Notably, QDs + NLSP (without gene fragments) induced no inflammation. Therefore, the inflammation was probably due to the gene fragments. To confirm this, the enhanced levels of TNF-α and CCL3/MIP-1α were produced

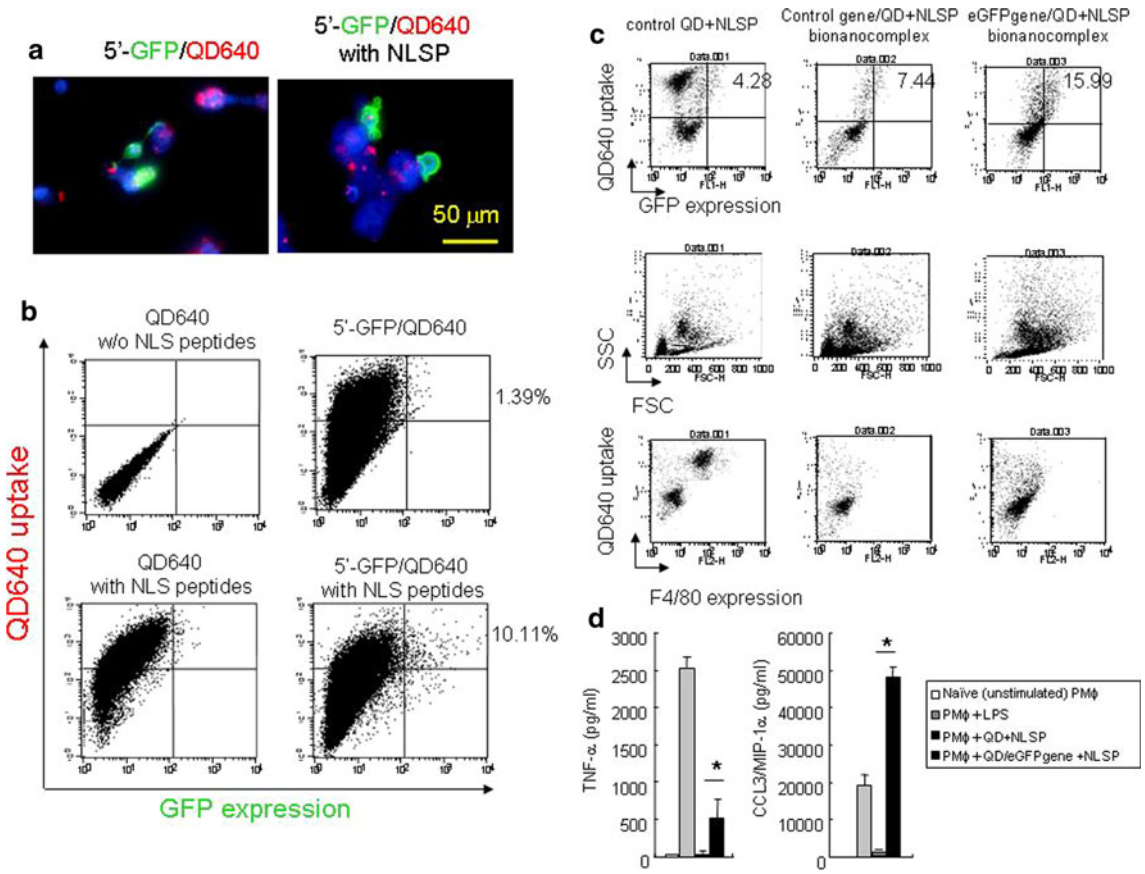

Fig. 7 QD-mediated gene transfection and inflammatory responses. **a** Fluorescent microscopy photograph images of cultured HEK293 cells 3 days after incubation with the eGFP/QD640-constructs, in the presence or absence of NLSP. Approximately 10% of cells could express the eGFP protein. Magnification ×400. The *bar* indicates 50 μm. **b** A flow cytometric analysis of HEK293 cells cultured with eGFP/QD-fragments. The *data* shown are representative of two independently performed experiments. **c** A direct injection of the bionanocomplex of QDs plus gene-encoding fragments induces an inflammatory response in the peritoneal cavity. A representative flow cytometric analysis of peritoneal cavity cells collected from naïve mice (*left*), mice injected with NLSP-attached QDs (*center*), and mice injected with QD + gene-coding fragments (*right*), stained with PE anti-F4/80 Ab (used as macrophage marker), were shown as dot plots. **d** The bionanocomplex of QDs plus gene-encoding fragments activates PMM in vitro. The production of TNF-α and CCL3/MIP-1α by naïve PMM (*open column*), PMM stimulated with 100 ng/ml LPS (*light gray column*), PMM stimulated with QD + NLSP (*dim gray column*), or PMM stimulated with QD/eGFP + NLSP (*filled column*) and cultured for 12 h were measured by ELISA. The data are presented as the means ± standard deviation of 3 samples. * indicates significant differences determined by the post hoc test ($P < 0.05$)

by peritoneal macrophages (PMM) stimulated with the QD/gene bionanocomplex, but not with the QD + NLSP bionanocomplex (without gene fragments) (Fig. 7d). These results indicate that the attached gene fragments on the surface of the QDs activate the PMM to produce proinflammatory cytokines and inflammatory chemokines. The accumulation of eGFP-coding gene fragments with QDs in the endosome facilitates the enhanced inflammatory response of the PMM to the bionanocomplex. Furthermore, it is probable that another immune response might occur in other immune cells and non-immune cells, because non-immune cells also have a series of receptors which are significantly concerned with immune response.

Notably, recent studies also suggested that innate immune system activation, which is a critical step in the initiation of an effective adaptive immune response, could play crucial roles as some kind of modulator system for optimizing vaccine efficacy (Demento et al. 2009). Another report demonstrated that it is possible to use a vault nanoparticle vaccine to induce protective mucosal immunity (Powell et al. 2010; Champion et al. 2009). Further studies will make it possible to apply QDs to clinical applications. In addition, QDs may represent a novel material suitable for the gene delivery in the future.

Conclusions: quantum dots are complex, and their activity and toxicity depends on a large number of factors

We revealed that the toxicity of QDs in biological systems is not only dependent on the nanocrystal "core particle"

itself but also on the surface molecules. We observed no cytotoxicity from the ingredients of the QD core itself, suggesting that surface processing will overcome the toxicity of nanomaterials unless the core structure is broken. Surface modifications using functional molecules combined with nanomaterials can work as novel bio-nanomachines conforming to the functions designated by their surface molecules (Kako et al. 2006; Manabe et al. 2006). On the other hand, our results suggest that the inappropriate treatment and disposal of QDs may still pose risks to the environment and human health under certain conditions. Another concern about cadmium and selenium has been raised, because augments described above could establish unless the core structure is broken. Cadmium and selenium, two of the most widely used constituent metals in QD-core, are well known to cause acute and chronic toxicities in vertebrates and are of considerable human health and environmental systems. For example, ionized cadmium (Cd^{2+}) is well known as a probable carcinogen and has a long biological half-life of 15–20 years in humans. Cd^{2+} could be systemically distributed to all bodily tissues by crossmoving to Ca^{2+}, can penetrate through the blood–brain barrier and placenta, and finally accumulates in brain, liver, kidney and even in bone tissue, which are target organs of toxicity. The most well-known documented case of mass cadmium poisoning is "Itai-itai disease" in Japan. The main effects of cadmium poisoning are osteomalacia and bone deformities caused by the cadmium. Other complications include coughing, anemia, and kidney failure, leading to death. In addition to cadmium, the potential environmental impacts of selenium contamination are well understood from the cases of Kesterson National Wildlife Refuge in California, and Belews Lake of North Carolina, where a marked impact on the local ecosystem resulted from elevated environmental concentrations of selenium. The potential risks posed by QD materials to human health and the environment should be seriously considered.

In summary, research into the potential biomedical applications for QDs is ongoing, despite the uncertainty about whether the agents are toxic. Here, we have discussed the physicochemical, biological and toxicological properties of QDs. Changes to any or all of these factors can result in changes in activity, and as such, are also likely to bring about changes in toxicity. However, as additional studies are completed, they will give new insights into biology and may provide new applications in clinical diagnosis and even treatment. We anticipate that these new QD-mediated agents and methods will be used broadly, allowing various diseases to be diagnosed and understood more easily and fully in the usual clinical setting, thereby promoting a safer and more complete treatment for many medical situations.

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