A new perspective on *in vitro* assessment method for evaluating quantum dot toxicity by using microfluidics technology

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A new perspective on *in vitro* assessment method for evaluating quantum dot toxicity by using microfluidics technology

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In this study, we demonstrate a new perspective on *in vitro* assessment method for evaluating quantum dot (QD) toxicity by using microfluidics technology. A new biomimetic approach, based on the flow exposure condition, was applied in order to characterize the cytotoxic potential of QD. In addition, the outcomes obtained from the flow exposure condition were compared to those of the static exposure condition. An *in vitro* cell array system was established that used an integrated multicompartmented microfluidic device to develop a sensitive flow exposure condition. QDs modified with cetyltrimethyl ammonium bromide/trioctylphosphine oxide were used for the cytotoxicity assessment. The results suggested noticeable differences in the number of detached and deformed cells and the viability percentages between two different exposure conditions. The intracellular production of reactive oxygen species and release of cadmium were found to be the possible causes of QD-induced cytotoxicity, irrespective of the types of exposure condition. In contrast to the static exposure, the flow exposure apparently avoided the gravitational settling of particles and probably assisted in the homogeneous distribution of nanoparticles in the culture medium during exposure time. Moreover, the flow exposure condition resembled *in vivo* physiological conditions very closely, and thus, the flow exposure condition can offer potential advantages for nanotoxicity research.
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I. INTRODUCTION

Nanotoxicology, a new field of toxicology, has been proposed to address the gaps in knowledge, as well as the special problems that were likely to have been caused by nanomaterials.[1] Therefore, it is recommended that toxicological evaluation of nanomaterials must be carried out in order to assess the potential hazards of nanomaterials. On being asked for a scientific opinion on the "appropriateness of methodologies that would assess the potential risks of nanotechnologies" by the European Commission on the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), it was revealed that the current risk assessment methodologies require some modification in order to deal with the hazards associated with nanotechnology. In particular, they noted that existing toxicological and ecotoxicological methods may not be sufficient to address all of the issues associated with nanoparticles.[2] In addition, they reported that existing exposure assessment methods may not be appropriate for determining the environmental fate of

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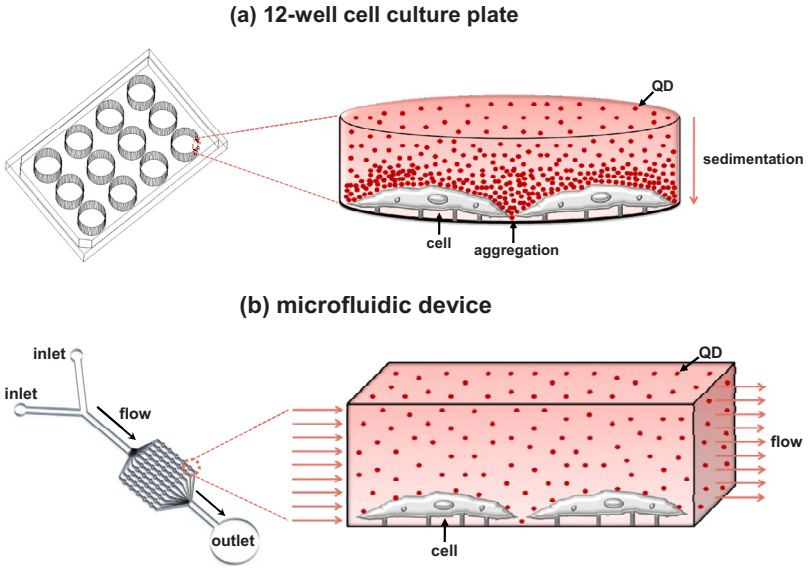

FIG. 1. (a) Schematic illustration of the static exposure condition, which shows the possibility of the sedimentation phenomenon in a conventional well-plate system, which can eventually lead to the nonhomogeneous distribution of nanoparticles, and consequently, the development of physicochemical stress on cells. (b) Pictorial representation of the flow exposure condition in a microfluidic compartment that shows the homogeneous distribution of nanoparticles.

nanoparticles. Also, the Organisation for Economic Co-operation and Development (OECD) initiated several further steps to develop appropriate methods and strategies that would help ensure the health of humans and environmental safety.[3]

Compared to animal studies, cell-based testing is easier to perform, reproduce, and control the experimental conditions, is less ethically ambiguous, and is less expensive. Currently, the methods that are being employed for the cytotoxicity assessment of nanoparticles are entirely based on the static exposure condition, i.e., conventional well-plate system. However, during the static exposure condition, the gravitational settling of nanoparticles on the cell surface could eventually be a major concern[4] because it can directly create physicochemical stress on the cell, leading to death, as depicted in Fig. 1(a). Also, the appropriate cellular dosage of nanoparticles could vary due to the gravitational settling or aggregation of nanoparticles during the static exposure condition. In consequence, developing a new testing strategy that can fulfill the growing demand of an assessment method that would characterize the hazard potential of nanoparticles is one of the most challenging tasks for the researchers and regulatory bodies. Therefore, we have applied a microfluidic technology that would establish a new flow method for the exposure of cells to nanoparticles (specifically quantum dots). This method has the ability to overcome the limitations of the static exposure condition, as depicted in Fig. 1(b).

Quantum dots (QDs), i.e., semiconductor inorganic nanocrystals, have been found to be extensively useful for biological and medical applications due to their unique size-tunable optical and electronic properties.[5,6] Several researchers have argued against the extensive uses of QDs because they may also pose risks and toxicity to human health and environment under certain conditions.[7,8] Cadmium selenide (CdSe) QDs coated with cetyltrimethyl ammonium bromide (CTAB) and sodium dodecyl sulfate have been found to be cytotoxic to the HepG2 cell model.[9] In contrast, when HeLa and *Dictyostelium discoideum* cells were treated with 400–600 nM concentrations of CdSe/ZnS QDs capped with dihydroxylipoic acid, there were no detectable effects observed on the cell morphology or physiology.[10] Recently, microfluidic devices were employed as an advanced alternative platform for assessing biological mechanisms, cellular analysis, and toxicological evaluations of stimulants. For instance, a multilayer elastomeric microfluidic array platform was developed for the high-throughput cell cytotoxicity screening of mammalian cell lines.[11] In addition, several experts have developed microfluidic systems for the generation of

chemical gradients and the monitoring of the cellular responses to these chemical gradients.[12,13] Recently, our group also showed the efficient utilization of a multicompartmented microfluidic platform for the characterization of dose-dependent cadmium cytotoxicity in BALB/3T3 fibroblast cells.[14]

In this study, we demonstrate a new perspective on *in vitro* assessment method for evaluating QD toxicity by using microfluidics technology. A new biomimetic approach, based on the flow exposure condition, was applied in order to characterize the cytotoxic potential of QD. Thereafter, the outcomes obtained from the flow exposure condition were compared to those of the static exposure condition (conventional assessment method). An *in vitro* cell array system was established by using an integrated multicompartmented microfluidic device in order to develop a flow exposure condition. The static exposure condition represents the experimental condition performed on the conventional well-plate system. In order to elucidate the cytotoxicity profiles of QD, it was surface-modified with CTAB/trioctylphosphine oxide (TOPO). The results indicated a significant difference in the cell viability between two different exposure conditions.

II. EXPERIMENTAL

A. Surface modification and characterization of QD

Quantum dot (core/shell; CdSe/ZnSe) was dispersed in aqueous media by using CTAB/TOPO based on the reported protocol.[15]

B. Cell culture conditions and exposure to QD

The microfluidic culture condition and treatments were performed according to the previously reported procedures.[14] A low glucose Dulbecco's modified Eagles medium (Invitrogen, Oregon, USA), supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin-streptomycin (Sigma, Montana, USA), was used to culture the mouse embryonic fibroblast cell line BALB/3T3 (Korean Collection for Type Cultures) in a humidified incubator (Sanyo Electric, Japan) at 37 °C with 5% CO_2 atmosphere. The preliminary cytotoxicity experiment was performed in conventional 12-well tissue culture plates in order to find the most optimum concentration of QD that is cytotoxic to cells. Cell suspension was seeded at a density of 4×10^4 cells/ml into 12-well tissue culture plates. After attaining 60–70% growth of cells in each well, they were exposed to a culture medium that contained variable concentrations of QD ranging from 8 to 80 pM inside a CO_2 incubator for 12 h. In the case of the flow exposure condition, a cell suspension of 4×10^6 cells/ml (200 μl) was introduced into the device outlet. A continuous gradient flow (flow rate: 0.5 μl/min) of culture media that contained 0 and 40 pM CTAB/TOPO-QD through two inlets of a device was established for 12 h by using a customized incubated microscope stage that was maintained at 37 °C with 5% CO_2 atmosphere. In the case of the static exposure condition, all cytotoxicity experiments were performed in conventional 6- or 12-well tissue culture plates. In both experimental conditions, the cells were treated with QD when areal density was estimated to be similar.

C. Cytotoxicity assays

Cytotoxicity assays were performed according to the previously reported procedures.[14] A live/dead cytotoxicity kit (Molecular Probes, Oregon, USA) was used for the assessment of cell viability after the cells were exposed to QD for 12 h. QD-induced intracellular reactive oxygen species (ROS) were estimated using a fluorescent probe, which was 2′,7′-dichlorofluorescein diacetate (Sigma) after 12 h of incubation. The intracellular detection of cadmium was performed using Leadmium™ green AM dye (Molecular Probes) after 6 h of incubation. The Leadmium™ green AM dye that was dissolved in 0.85% saline was introduced into an outlet of the device in order to incubate the cells after being exposed to QD. The device was kept at 37 °C for 30 min. After incubation, the intracellular concentration of cadmium was estimated qualitatively by fluorescence microscopy.

D. Microscopic analyzing system

A perfectly balanced temperature- and gas-controlled environmental device was set up on an inverted microscope (Olympus IX71, Japan) that was equipped with a DP71 CCD camera and motorized microscope stage (Prior Scientific Inc., Cambridge, UK) in order to perform microfluidic experiments. Polyethylene tubing was inserted into two inlets at the top of the chamber in order to inject the media with and without QD into the chamber by using a syringe pump with an inflow rate of 0.5 μl/min. The differential interference contrast (DIC) microscopy was utilized to observe the morphological changes in the cells.

III. RESULTS AND DISCUSSION

A. Basis for the flow exposure condition as an improved method for *in vitro* assessment of QD toxicity

The issue related to the cytotoxicity of QDs has been a subject of concern for many researchers who have been trying to develop various forms of QDs for their efficient utilization in biological and medical sciences. As a result, several studies have been reported so far that have revealed the cytotoxicity induced by QDs.[7,8] The studies reported so far that have revealed the cytotoxicity of QDs are entirely based on the static exposure condition. The suitability of the current assessment method, i.e., static exposure condition, to evaluate the nanoparticle toxicity has been a subject of intensive discussion and is undergoing critical examinations as well.[4] Chemicals that can dissolve in solvents and form solutions can maintain their physicochemical properties even in the solution. However, nanoparticles possess macroscale physicochemical properties, such as size, shape, reactive core/shell metals, and surface chemistry, which can be significantly altered in the solution. Depending on these properties, nanoparticles can settle, diffuse, and aggregate eventually in the solution [Fig. 1(a)]. These processes can thus affect the nature of the nanoparticles and can also lead to the development of physicochemical stress over the cells during the static exposure condition. Also, the appropriate cellular dosage of nanoparticles could vary due to the settling down or aggregation of nanoparticles during the static exposure condition. In contrast to the static exposure, the flow exposure condition can apparently avoid the gravitational settling of particles and can possibly assist in the homogeneous distribution of nanoparticles in solution during the exposure time [Fig. 1(b)]. Furthermore, the flow exposure method presented here provides a biomimetic approach for characterizing the hazardous potential of QDs. The fluidic control and continuous perfusion provided by microfluidic system simulate the physiological characteristics of the circulatory system and create a cellular microenvironment that is typically found in the biological systems.[12,16–18] The microscale dimension of the culture chambers produces a cell-volume-to fluid ratio that more closely simulates physiologic values. Unlike the static exposure condition, the flow exposure provides shear stress and the consistent flow of nutrients present in the physiological environment. Also, the flow exposure maintains a constant temperature and a physiological pH due to high turnover of medium. Since the flow exposure condition resembles *in vivo* physiological conditions very closely, the outcome of the study based on such a condition can provide better insights into the cytotoxicity profile of QD. Therefore, we have attempted to investigate the cytotoxic effects of QD under the flow exposure condition by using microfluidic technology. In addition, the outcomes obtained from the flow exposure condition were compared to those of the static exposure condition.

B. Generation of concentration gradient of QD solutions

The multicompartmented microfluidic device previously reported by our group has been utilized for the assessment of QD cytotoxicity.[14] In order to estimate the concentration profile of QD inside microfluidic multicompartments, a modified multicompartmented device that has a separate outlet for each series of ten parallel microchannels was used for estimating the final concentration of QD entering the downstream cell culture compartments. The phosphate buffered saline solutions that contained both QD and no QD were infused into the modified microfluidic device through two inlets at the same flow rate (0.5 μl/min). Thereafter, the solutions that contained QD,

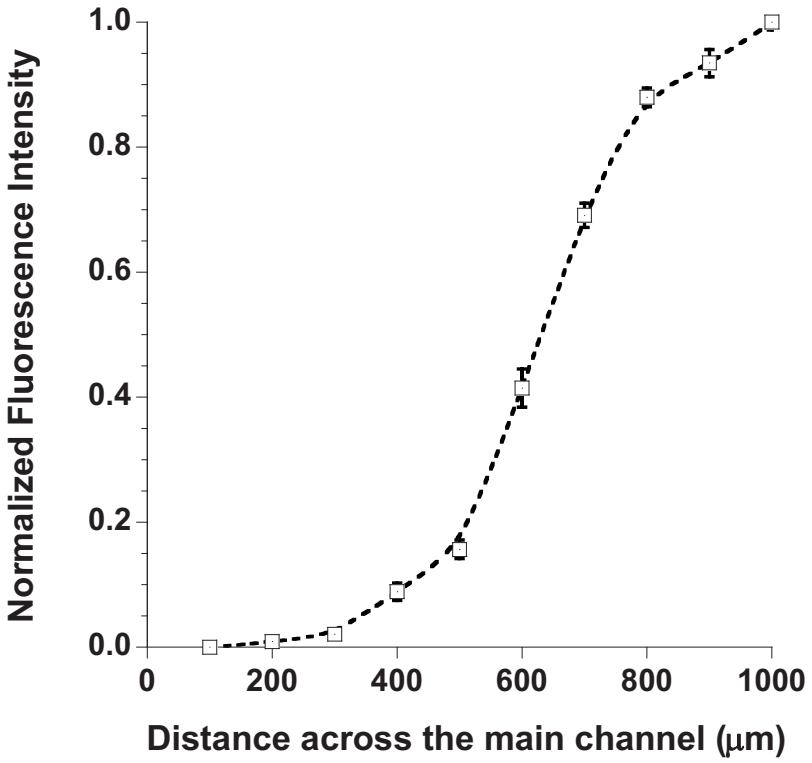

FIG. 2. Fluorescence intensity profile of quantum dots distributed inside a multicompartmented microfluidic device. In order to estimate the gradient profiles of QD, a modified multicompartmented device that has a separate outlet for each series of ten parallel microchannels was used. The quantum dots distributed in each separate outlet were collected and their fluorescent spectra were then recorded. The fluorescence intensities measured at an emission wavelength of QD were normalized in order to estimate the gradient profiles of QD inside microfluidic compartments.

which were distributed in each separate outlet, were collected at regular intervals for 12 h, and their fluorescent spectra were then recorded. The fluorescence intensities measured at the emission wavelength of QD were normalized in order to estimate the gradient profiles of QD inside microfluidic compartments (Fig. 2). The flow rate of both input fluids was maintained at the same rate (0.5 μl/min). The data were captured at a distance of 9 mm downstream across the main wide channel of a microfluidic diffusion diluter of the device. The results suggest that the device can be used to generate a concentration profile of QD. Furthermore, the device can be utilized in the dose-dependent cytotoxicity analysis of QD under a flow exposure condition.

C. Noticeable differences in the number of detached cells and viability percentages between static and flow exposure conditions

The cellular behavior in the presence of QD was assessed through DIC microscopy. The primary experiments started with the degree of morphological changes and the number of detached cells between two exposure conditions. Although the cells showed similar morphological changes in both cases of exposure conditions, i.e., static and flow, the number of detached cells was significantly higher in the static condition [Fig. 3(a)] than in the flow exposure condition [Fig. 3(b)] after being incubated with QD for 12 h. Also, the number of deformed cells was remarkably higher in the static condition compared to the flow condition, as observed through DIC images. A dose-dependent morphological change was observed in the cells exposed to gradient concentrations of QD when they were observed in the microfluidic compartments [Fig. 3(b)]. The cells exposed to QD, under both exposure conditions, exhibited characteristic features of apoptosis, such as cell detachment, oval-shaped appearance, cell shrinkage, and the formation of apoptotic bodies, which were examined by DIC microscopy. Similarly, there was a significant difference

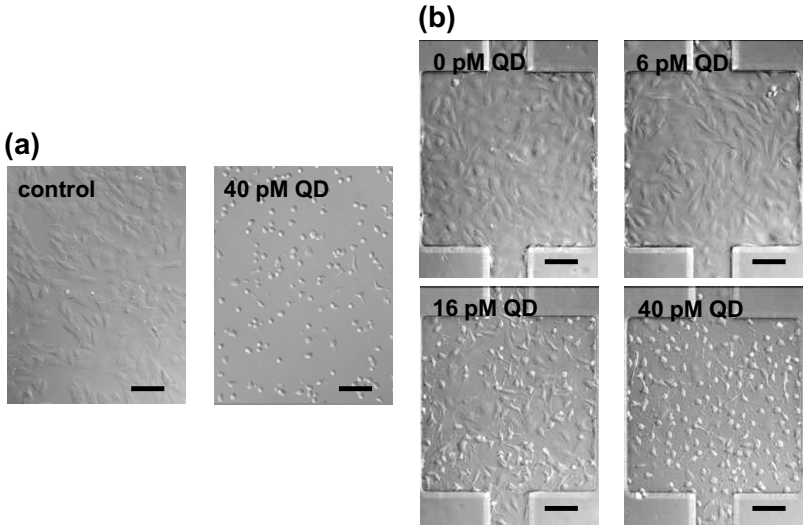

FIG. 3. Morphological effects of QD in fibroblast cells under (a) static and (b) flow exposure conditions. Scale bars: 100 μm.

observed in the percentage of live cells between two different exposure conditions at equal concentrations (40 pM) of QD. The value was estimated to be around 30% and 75% in the cases of static and flow exposure conditions, respectively [Figs. 4(a) and 4(b)]. In addition, QD affected the cell viability in a dose-dependent manner, which was observed in both exposure conditions. The static exposure of cells to QD resulted in a higher percentage of cell death and an increased number of detached and deformed cells. This was probably due to the additional effects of physicochemical stress created from the settling down of QD over the cell surface. These results strongly suggest that the cytotoxicity profile of QD could vary depending on the method of exposure of cells to QD.

D. Production of intracellular ROS and release of cadmium are possible causes of QD-induced cytotoxicity, irrespective of the types of exposure condition

The cells exposed to QD for 12 h showed a dose-dependent increase in the amount of intracellular ROS formation for both cases (Fig. 5). In this analysis, the fluorescent intensity of exposed cells was compared to their respective controls in both exposure conditions. Therefore,

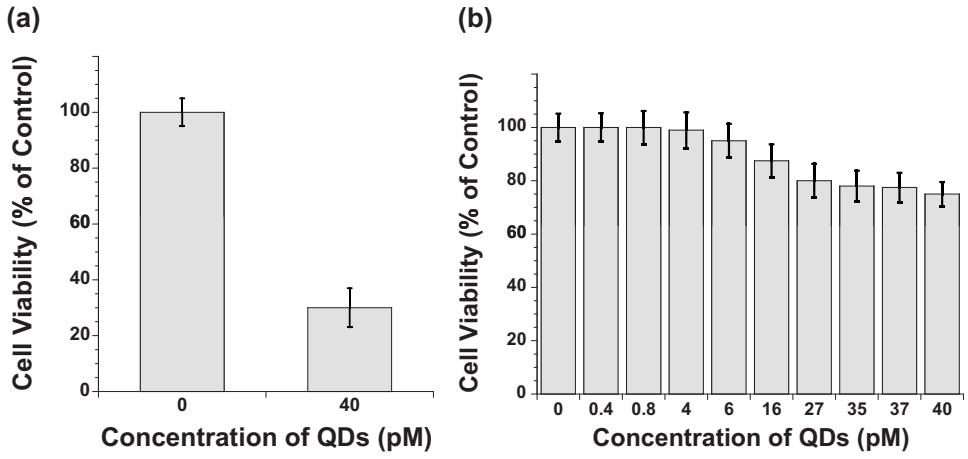

FIG. 4. Effects of QD on cell viability under (a) static and (b) flow exposure conditions.

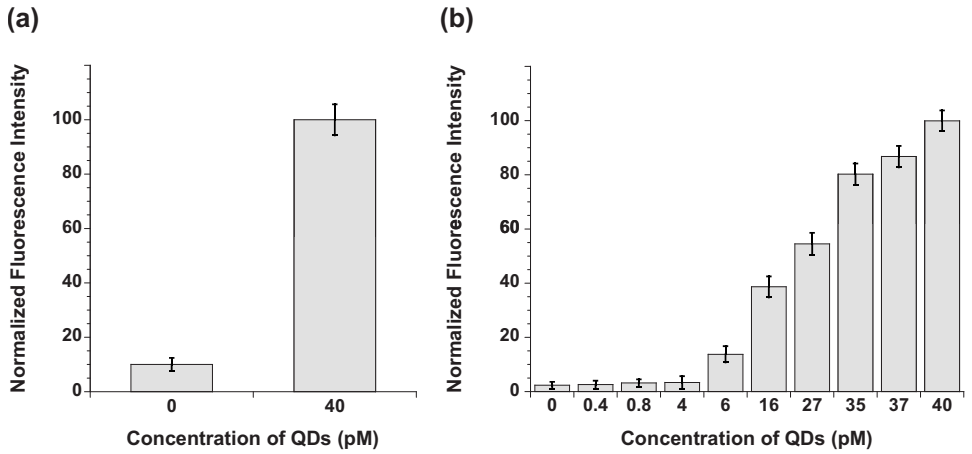

FIG. 5. Quantification of fluorescent intensity that shows relative amount of intracellular ROS formation in BALB/3T3 fibroblast cells under (a) static and (b) flow exposure conditions.

the data only represent the relative amount of ROS formation in both exposure conditions separately and are not based on a comparative study between two exposure conditions (Fig. 5). These results suggest that the reactive oxygen species were significantly involved in QD-induced cytotoxicity, as observed in both exposure conditions. Previous studies have also suggested the involvement of ROS formation in the cytotoxicity of QDs.[19,20] Similarly, the amount of intracellular cadmium was estimated qualitatively by using fluorescent-based methods after QDs were treated for 6 h under both exposure conditions. The relative fluorescent intensity signified the concentration of cadmium estimated inside the cells. Compared to their respective controls [Figs. 6(a) and 6(b)], the cells treated with 40 pM QD [Figs. 6(a) and 6(b)] showed very bright fluorescent intensity in both exposure conditions. The results indicate that CTAB/TOPO-QD released free cadmium ions from their core into the cells, irrespective of the types of exposure condition. Other researchers have also suggested that the Cd release over time could be a leading cause of QD cytotoxicity.[21]

Thus, this study demonstrated the efficient utilization of microfluidic technology in nanotoxicity research and also provided the comparative cytotoxicity profiles of QD based on the static and flow exposure conditions. The flow exposure condition can avoid the issue of nanoparticle settling on cell membranes to a significant extent which is a major concern related to the static exposure condition. Furthermore, cytotoxicity screening based on microfluidics offers several other advantages over conventional systems, such as high-throughput, low cost, and a small amount of reagent consumption and biological targets. Although the study provided significant differences in the outcomes of cytotoxicity assays performed under two different exposure conditions, i.e., static and flow exposures, the possibility of adsorption of QDs by PDMS cannot be

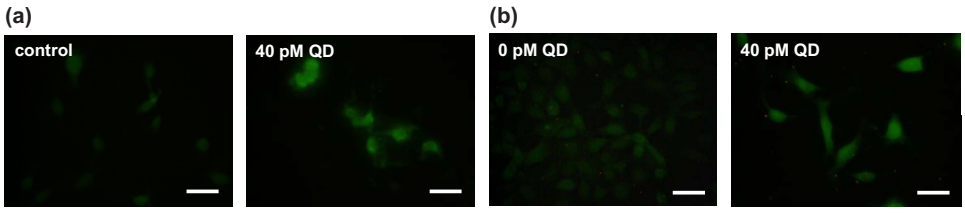

FIG. 6. Qualitative estimation of intracellular cadmium ions in BALB/3T3 fibroblast cells under (a) static and (b) flow exposure conditions. Scale bars: 50 μm.

completely ignored because it can hinder the expected concentrations of QD distributed inside microfluidic devices. As such, further examinations are needed in order to precisely understand the adsorption behavior of QDs in the PDMS structure.

IV. CONCLUSIONS

In conclusion, this study was aimed at developing an improved *in vitro* assessment method for evaluating QD toxicity. A new biomimetic approach operated by microfluidics technology was applied in order to characterize the cytotoxic potential of QD. A multicompartmented microfluidic device integrated with a syringe pump was utilized in order to establish a sensitive flow exposure system capable of exposing cultured cells to variable concentrations of QD. Furthermore, the outcomes obtained from the flow exposure condition were compared to those of the static exposure condition in order to observe the relative differences in the cytotoxicity profiles of QD. The results suggested noticeable differences in the number of detached and deformed cells, as well as the viability percentages between two different exposure conditions. Moreover, the flow exposure condition resembled *in vivo* physiological conditions very closely. Thus, this approach can offer potential advantages for nanotoxicity research.

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