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On the Cyto-Toxicity Caused by Quantum Dots

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Abstract: Quantum dots (QDs) such as CdSe QDs have been introduced as new fluorophores. The QDs conjugated with antibody are starting to be widely used for immunostaining. However there is still not sufficient analysis of the toxicity of QDs in the literature. Therefore we evaluated the cell damage caused by the quantum dots for biological applications. We performed cell viability assay to determine the difference in cell damage depending on the sizes and colors of mercapto-undecanoic acid (MUA) QDs and the cell types. The results showed that the cell viability decreased with increasing concentration of MUA-QDs. But in the case of Vero cell (African green monkey’s kidney cell) with red fluorescence QD (QD640), the cell damage was less than for the others. Furthermore through the flow cytometry assay we found that this cell damage caused by MUA-QD turned out to be cell death after 4–6-hr incubation. From the two assays described above, we found that there is a range of concentration of MUA-QDs where the cell viability decreased without cell death occurring and thus we conclude that attention should be given when MUA-QDs are applied to living organisms even in low concentrations.

Key words: Cell damage, MUA-QD, Cell death

Quantum dots (QDs) such as CdSe QDs are nano-sized metal clusters. QDs have specific characteristics such as the quantum effect, which is a special photo quality caused by the widening of the band gap when the spatial dimension is reduced. Kubo et al. predicted the specific character of the quantum dot theoretically in 1962 (14–16). Since then, research concerning the applications of QDs has gained a great amount of interest. For example, in the field of Information Technology and optical-engineering (3, 10, 21, 29, 30), QDs have been proposed for use as a new material for memory, and as miniature laser-beam emitting devices. Furthermore, the biological applications of QDs conjugated with antibody have started to attract much attention, especially in immunostaining, separating cells, and diagnostics, because of their advantages such as longer lifetime and higher fluorescence over conventional organic fluorophores (1, 2, 8, 27). The first synthesized QDs are insoluble in biological solvents because non-polar groups of organic molecules are exposed on the surface of QDs. However the water-soluble QDs covered with mercapto-undecanoic acid (MUA) have been reported (2). In addition, the MUA-QD covered with sheep serum albumin (SSA) is well dispersed in water (2, 9). The advantages of MUA-QDs described above make it possible to consider the application of MUA-QDs to drug delivery systems (6, 20, 25, 28) as a drug-carrier and cell delivery system. Quantum dots have a longer lifetime compared to conventional organic fluorophores and thus make it easier to trace the drug delivered in living organisms. To make sure the application is feasible, an in-depth evaluation using MUA-QD in living organisms is needed. In fact cadmium (13) and selenium (24) are known to be toxic. Though the use of MUA-QDs for organisms has been known and some other studies about the actual injections into organisms have been conducted, the toxicity of MUA-QDs has not been reported in detail yet. Published works regarding

Abbreviations: DMEM, Dulbecco’s Modified Eagle’s Medium; FCS, fetal calf serum; HC, human primary hepatocyte; MUA-QD, mercapto-undecanoic acid quantum dot; PBS, phosphate-buffered saline; PI, propidium iodide; SSA, sheep serum albumin; TOPO, tri-n-octylphosphine oxide.
QDs have so far only effectively assumed that QDs are safe. In this paper, we proceeded one step further by investigating the cell damage caused by MUA-QDs through an extensive and comprehensive experiment. We chose CdSe QDs because they are one of the QDs that have the strongest emission and they are used the most in many fields. In order to analyze the cell damage caused by MUA-QDs, a cell viability assay, which assesses the mechanism of glycolytic pathways, was conducted (12, 17, 26). Then in order to figure out whether the cell damage was cell death or not, we examined cell death using the flow cytometry assay.

Materials and Methods

**Preparation of MUA-QDs.** CdSe/ZnS QDs were synthesized in tri-n-octylphosphine oxide (TOPO) in accordance with the standard method (5, 11, 18, 19). For these experiments, three MUA-QDs were prepared; QD520, QD570 and QD640 which emitted green, yellow, and red, respectively.

**Preparation of MUA-QDs solution with sheep serum albumin.** The same volumes of 10 mg/ml MUA-QD and 10 mg/ml sheep serum albumin (SSA) were mixed as described in Hanaki et al. (9). Then we centrifuged this solution with a 0.45 µm filter at 5,000×g for 5 min at room temperature. The MUA-QD/SSA solution for all the cells was diluted with DMEM into several concentrations.

**Cell viability assay.** The cell viability was measured after the exposure of cells with MUA-QD to 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt to generate hormazan. The number of the living cells is known to be proportional to the concentration of the generated hormazan (12, 26). Cell viability was measured for the following two cell lines and a primary cell culture; Vero cells (African green monkey kidney cells), HeLa cells, and human primary hepatocyte (HC). The above-mentioned cell types were cultured at 37°C, in 5% CO₂ in DMEM, supplemented with 5% heat-inactivated fetal calf serum (FCS). All the cells were suspended in DMEM, supplemented with 5% FCS and 50 µg/ml gentamicin after they had been treated with trypsin and centrifuged at 1,800 rpm for 5 min at room temperature. The cell count was performed for the three types of cells respectively. Each cell was plated into a 96-well plate (Iwaki Co., Tokyo) at 3×10⁴ cells/well (100 µl/well). After a 24-hr incubation, the DMEM was removed and the prepared MUA-QD/SSA solution diluted to several different concentrations was poured into the wells. After another 24-hr incubation period, a Cell Counting Kit8 (Dojindo Laboratories Co., Kumamoto, Japan) was added into the 110 µl/well. The Cell Counting Kit8 was diluted with DMEM (Cell Counting Kit8:DMEM=1:10). Then the absorbances were measured at 450 nm by an absorptiometer (Molecular Devices Co.).

**Flow cytometry assay.** For the flow cytometry assay (23), in all the experiments, each cell was plated into a 12-well plate (Iwaki Co., Tokyo) at 10⁶ cells/well (1,000 µl/well).

The cells were incubated for 24 hr. The culture medium was removed, and then the prepared MUA-QD/SSA solution diluted to several different concentrations was poured into the wells. After incubation, the cells were washed with PBS and the dead cells were stained with propidium iodide (PI) (4, 7) (0.1 mg/ml) for 5 min at room temperature, followed by treatment with Puck’s EDTA solution (4 mM, NaHCO₃; 136 mM, NaCl; 4 mM, KCl; 1 mM, EDTA; 1 mg/ml, glucose), which will do less damage to cells than trypsin. The cells were suspended in PBS after they were fixed with 3% formaldehyde. Then, the fluorescence intensity of PI and QD520 was measured using the flow cytometry (Cyto Ace 300 JASCO, Tokyo) assay.

Results and Discussion

**Cell Viability Assay**

We conducted the cell viability assay to confirm whether the MUA-QDs do damage to the cells or not (12, 26). We used three cell types; Vero cell, HeLa cell, and primary human hepatocyte for three MUA-QDs (QD520, QD570, and QD640). Their spectrums are shown in Fig. 1. The result showed that MUA-QDs affect the cell viability even in rather low concentrations (Fig. 2). The tendencies of the cell viability with QD570 and QD520 were almost the same. However only in the case of QD640 with Vero cells does the result show a difference in cell viability of less than 0.4 mg/ml. The cell damage was less than for the others only in this experiment though the tendency was the same.

**Flow Cytometry Assay**

Cell viability assay is easy to handle and quantitatively good as well. However, if the intracellular activity is affected; for example, that of NADH-Dehydrogenase, the results will not reflect the true number of cells. Therefore fluorescence intensity of PI was measured using flow cytometry; another method of counting living cells based on a different principle. Figure 3 shows the result of the experiment incubated for 24 hr with QD520 (23).

Collins et al. reported that living cells do not take in
propidium iodide (PI), which has 610 nm fluorescent (4). Only QD520 was used for the flow cytometry assay because the emission peaks of QD570 and QD640 could not be distinguished from that of PI. The top two Figs. (without MUA-QD) show that the emission intensity obtained with a PI filter and that obtained with a QD filter were both quite low. The emission intensity obtained from the PI filter, however, increased gradually, according to the concentration of the MUA-QDs. At more than 0.15 mg/ml concentration of MUA-QDs, the emission intensity of PI was split into two peaks; the left peak shows the living cells, and the right peak shows the dead cells. On the other hand, in the right lane (with the QD filter), in the cases where the concentration of MUA-QD was more than 0.15 mg/ml, the emission intensity of MUA-QD increased, depending on the concentration of MUA-QDs. The higher emission peak contains both the damaged cells and the undamaged cells in the left panel. At the same time, however, the intensity of PI also increased steadily, which means that the population of dead cells increased from 0.15 mg/ml upward. The results showed the cell damage caused by MUA-QD is cell death.

To analyze the dependence on the incubation time, we measured the ratio of the damaged cells (PI stained cells) against the total number of the cells chronologically (Fig. 4). The ratio of damaged cells increased sharply from 4-hr incubation in 0.2 mg/ml concentration of MUA-QD, and slowly in 0.1 mg/ml. On the other hand, we cannot observe any difference between the result obtained from the concentration of 0.05 mg/ml and that from the control. The result from the flow cytometry assay is compatible with that from the cell viability assay in the view of the concentration of MUA-QD causing cell damage. Cell damage caused by MUA-QD probably occurs because the connection of SSA that covers MUA-QD is not a chemical bond; it just attaches to the surface of the MUA-QD (9). Therefore SSA is easy to remove from the surface of MUA-QD and MUA comes out to the surface. To solve this problem, the surface-processing should be reexamined. Safer materials should be used to coat the surface of QDs or new safer QDs, such as silicon-QD, etc., can be considered for use for the DDS. As for its application for the DDS, the coating with peptide is effective because the tagging of target-molecules will be necessary: Peptide is more easily applicable for pharmaceutical biology and it is much safer. What is more, we have seen the difference in the extent of the cell damage only in the case of the combination of Vero cells and

Fig. 1. Photoluminescent properties of three different MUA-QDs. (A) QD520, (B) QD570, and (C) QD640. Three different MUA-QDs were dissolved in DW, and their photoluminescent properties were measured with FP-6500. Emission spectra of QD520 excited at 300 nm, QD570 excited at 350 nm, and QD640 excited at 360 nm, represented as black lines. Excitation spectra represented as gray lines collected with detection at the respective peak spectra.

Fig. 4. Flow cytometry assay for the effect of the incubation time difference and concentration of QD. Vero cell and QD520 were used for this experiment. The vertical axis stands for the damaged cell % (the ratio of the number of the PI stained cell against the total number of cells). The intensity of PI is measured between 565 and 605 nm, and the intensity of QD520 is measured between 515 and 545 nm. The horizontal axis stands for the incubation time of the cell. The bold line stands for a concentration of QD520 at 0 mg/ml, the broken line stands for a concentration of 0.05 mg/ml, the solid line, 0.1 mg/ml, and the dotted line, 0.2 mg/ml. The vertical lines are the error bars.
Fig. 2. Cell viability for the different sizes of the QDs and different cell types. Three of the different sizes of QDs are tested on the cell viability for each cell type (MTT assay, \( n = 5 \)). The top three panels stand for the cell viabilities of Vero cells, those in the middle for HeLa cells, and those in the bottom for primary human hepatocyte. The three panels in the left lane stand for QD640, those in the middle lane for QD570, and those in the right lane for QD520. In each panel, the horizontal axis stands for the concentration of QD, and the vertical axis stands for the absorbance at 450 nm. The columns in all the panels stand for the amount of hormazan, which reflect the cell viability, and I is standard deviation. A T-test was performed; * stands for the significance level <0.01, and ** stands for the significance level <0.001.
Fig. 3. Flow cytometry assay with the different concentrations of QD. Vero cell and QD520 are used for the flow cytometry assay. The horizontal axis, in the left lane, is the fluorescent intensity of propidium iodide with the filter (565 nm–605 nm), and in the right lane, the fluorescent intensity of QD520 with the filter (515 nm–545 nm). The vertical axes, in both the columns, stand for the cell count. Each row, from the top to the bottom, is given with respect to the concentration of QD520. In each row, the left panel and the right panel show the result with the same sample measured with a PI filter (left) and a QD filter (right), respectively.
QD640. It has been strongly suggested that the mobility of the MUA-QDs inside the cell depends on the size of the MUA-QDs (22). This might also explain the difference in the cell damage in our study.

In order to utilize quantum dots for humans, further study should be done on the relationship between the cell type and MUA-QD cell damage, an estimate of the cell damage in our study. We are grateful to Dr. Ohta of Tokyo University of Pharmacy and Life Science for his help with data collection, and proofreading. This work was supported by Grant ‘H14-nano-004’ of the Ministry of Health, Labour and Welfare of Japan.

References

