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Variables Influencing Interactions of Untargeted Quantum Dot Nanoparticles with Skin Cells and Identification of Biochemical Modulators

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ABSTRACT

Skin cells (NHEK) take up untargeted quantum dots (QD) with surface polyethylene glycol (PEG), amines, and carboxylic acids, but the mechanisms are unknown. Time courses of QD–NHEK interactions were determined and effects of QD surface coating, temperature, culture medium supplements and inhibitors of the cell cycle and endocytosis identified. The magnitude of QD–NHEK interactions was coating dependent. Low-temperature or unsupplemented medium decreased QD–NHEK interactions. Biochemical inhibitors were identified that attenuate and potentiate QD–NHEK interactions. These results are important for understanding and controlling interactions of untargeted QD with cells.

Quantum dots (QD) are a diverse class of engineered nanomaterials that have biological applications in nanomedicine as drug delivery and diagnostic agents.[1] Interactions of QD with biological systems have been best described in the context of preclinical studies using QD for diagnostic imaging[2−4] and in cellular assays investigating QD cytotoxicity.[5] Nanotoxicology is a branch of toxicology that has emerged to address routes of exposure and hazards of nanoscale materials, which may have unique toxicological properties due to size.[6] The nanotoxicology of QD is of interest due to the potential for use in biomedical applications. Additionally, QD may be valuable models for the interactions of nanoscale materials with biological systems due to ease of detection and availability of QD in different core sizes and shapes and with different surface coatings.

Intravenous injection is a major route by which diagnostic and therapeutic agents are administered. There is experimental evidence that intravenously administered QD can accumulate in unintended tissues.[2−4] Skin may be a route of both QD administration and distribution. Recently, we reported that QD of different core/shell sizes and surface coatings are able to penetrate intact skin and localize in the viable layers of the epidermis and dermis by 24 h.[7] This indicates that topical application of QD for diagnostic applications may also be possible and that unintended exposure to QD could occur by the dermal route.

Accumulating in vitro evidence suggests that some QD may cause cellular toxicity.[5] Hypothesized mechanisms of cytotoxicity include leaching of core metals into the culture medium,[8] oxidative stress as a result of QD-mediated ROS production,[9] and coating-related mechanisms.[10] These studies indicate that exposure to some QD may be hazardous depending upon the physicochemical composition of the QD, the cell types involved, and environmental factors.[5]

Primary neonatal human epidermal keratinocytes (NHEK) are an established in vitro model of skin toxicity. Recently, we used TEM and laser scanning confocal microscopy in live cells to show that low (2 nM), noncytotoxic concentrations of untargeted QD 565 and 655 with surface polyethylene glycol (PEG), amines, and carboxylic acids localize in the nucleus and cytoplasm of NHEK by 24 h.[10] Cytotoxicity and pro-inflammatory cytokine release were observed at higher (20 nM) concentration for NHEK treated with QD 565 and QD 655 with surface amines and carboxylic acids, but not surface PEG.[10] Cellular uptake of untargeted QD has been observed in other, nonphagocytic cell types.[11−14] The pathways of QD uptake and endocytic trafficking in cells may be an important, mechanistic component in cytotoxic potential and effects.[9,10]

The mechanisms of uptake of nontargeted QD by NHEK and other cell types not regarded as professional phagocytes are unknown, but are of interest for understanding and preventing cytotoxicity as well as for minimizing nonselective uptake in applications requiring targeted cellular delivery of QD. The present study investigated nonselective interac-

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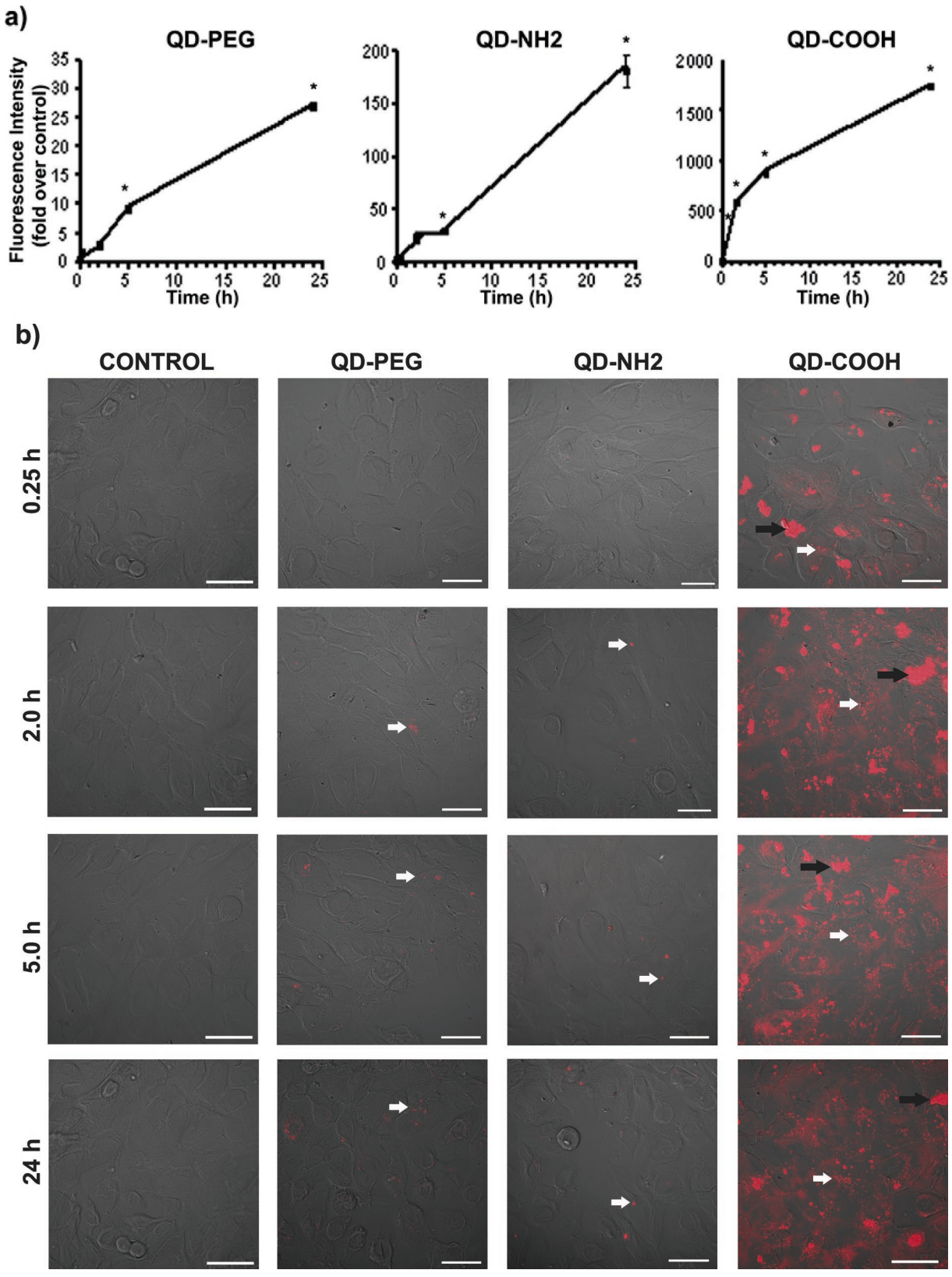

Figure 1. Time courses of QD 655-NHEK interactions at 10 nM. (a) Fluorescence microplate assay. Data are expressed as fold over background. Data are representative of two similar, independent experiments and are the mean and SEM of 5 replicates. (*) significant compared to $t = 0$ ($p < 0.05$). (b) Localization of QD 655 in NHEK by laser scanning confocal microscopy. Data are representative of two similar, independent experiments conducted in duplicate. The cellular interior is shown. Black arrows indicate agglomerated QD. White arrows indicate intracellularly localized QD. All scale bars are 25 μm.

tions of NHEK cells with untargeted, ellipsoid QD 655 with a core/shell diameter of approximately 12 nm (major axis) by 6 nm (minor axis).[7] The QD were coated with PEG (QD−PEG), amines (QD−NH2), or carboxylic acids (QD−

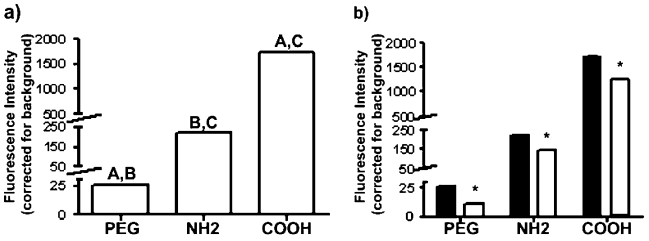

Figure 2. (a) Effect of coating on QD−NHEK interactions. Data are expressed as fluorescence intensity units after subtracting background. Significance was set a $p < 0.001$. (A) compared to QD−NH2; (B) compared to QD−COOH; (C) compared to QD−PEG. Data are representative of four independent experiments and are the mean and SEM of 4−5 replicates per data point. (b) Effect of media supplements on QD−NHEK interactions. Solid bars: medium with supplements (KGM-2). Open bars: medium without supplements. Data are expressed as the percentage of fluorescence intensity units in KGM-2 (supplemented) culture medium, after subtracting for background. (*) significant compared to QD of the same coating in KGM-2 culture medium. Significance was set at $p < 0.05$. Data are the mean and SEM of four independent experiments of 4−5 replicates each. Treatment concentration for all QD was 10 nM for 24 h.

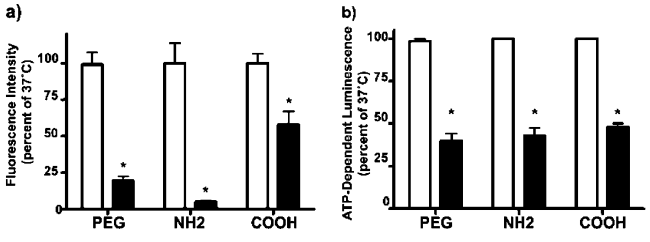

Figure 3. Effect of temperature. Open bars are 37 °C and closed bars are 4 °C. (a) Effect of temperature on QD−NHEK interactions. Data are expressed as the percentage of fluorescence intensity units at 37 °C after subtracting background. (b) Cellular ATP levels as determined by an ATP-dependent bioluminescence assay. Data are expressed as the percentage of ATP produced at 37 °C after subtracting background. (*) significant compared to QD of the same coating at 37 °C. Significance was set a $p < 0.001$. All data are the mean and SEM of four independent experiments of 4−5 replicates each. Treatment concentration for all QD was 10 nM for 24 h.

COOH). The time course of QD−NHEK interactions and parameters affecting the magnitude of these interactions (e.g., QD surface groups, temperature, and culture medium supplements) were investigated. Biochemical agents were also identified that modify cellular interactions with QD.

The time course of uptake of QD 655 in NHEK skin cells for QD−PEG, QD−NH2, and QD−COOH was first determined in order to establish incubation times for subsequent experiments (Figure 1). A QD treatment concentration of 10 nM was chosen because it is the highest concentration at which all QD could be compared over 24 h without causing significant cytotoxicity to NHEK (data not shown). A fluorescence microplate assay revealed significant increases in the signal/noise ratio by 5 h for all QD, indicating significant QD−NHEK interactions by this time (Figure 1a). The ratios of QD−PEG and QD−NH2 at 5 h were 9.0 ± 0.8 and 29 ± 4.1, respectively. By 24 h, the ratio for QD−PEG was 27 ± 3.7 and 180 ± 27 for QD−NH2. A significant increase for QD−COOH was observed as early as 15 min (170 ± 8.9). This increase continued and was significant at 2 h (650 ± 64), 5 h (880 ± 53), and 24 h (1700 ± 92). These results were in agreement with confocal micrographs, which showed intracellular localization of all QD in NHEK (Figure 1b). QD−COOH were visible on the interior of NHEK at 15 min, whereas QD−PEG and QD−NH2 were similar to control (row 1). By 2 h, QD 655 of all coatings were visible on the interior of NHEK (row 2), with more visible at 5 h (row 3) and 24 h (row 4). Large agglomerates were observable for carboxylic acid-coated QD 655 at all time points.

The role of QD surface groups on the magnitude of QD−NHEK interactions was also investigated, and significant differences were observed, with QD−COOH > QD−NH2 > QD−PEG (Figure 2a). The average fluorescence intensity of QD−PEG was 19.4 ± 4.78. This value was 10-fold lower than QD−NH2 (222 ± 34.3) and 100-fold lower than QD−COOH (1960 ± 172). The 5-fold difference in interaction between QD−NH2 and QD−COOH was also significant. These results were obtained in complete culture medium (KGM-2) that has both protein (bovine pituitary extract, insulin, transferrin, and human epidermal growth factor) and small-molecule drug supplements (epinephrine, glucocorticoids, and penicillin/streptomycin). Omitting supplements significantly decreased all QD−NHEK interactions (Figure 2b). Interaction of QD−PEG with NHEK was 2-fold less in the absence of supplements than in complete medium (10.8 ± 3.33 versus 19.4 ± 4.78). Similarly, QD−NH2 and QD−COOH were about 35% less in unsupplemented medium than in KGM-2 (146 ± 10.0 versus 222 ± 14.1 for QD−NH2 and 1250 ± 55.5 versus 1720 ± 16.0 versus for COOH−QD).

The effect of temperature on QD−NHEK interactions was also examined (Figure 3). A significant decrease in QD−NHEK interactions at 4 °C versus 37 °C was observed for QD of all three coatings (Figure 3a). QD−PEG at 4 °C was 19.2 ± 4.52% of that observed at 37 °C, while QD−NH2 was only 4.92 ± 1.20%. Interestingly, QD 655−COOH at 4 °C was 57.7 ± 13.4% of that observed at 37 °C. We verified that decreasing the NHEK incubation temperature from 37 to 4 °C was accompanied by a decrease in ATP levels (Figure 3b). This was achieved by measuring intracellular stores of ATP via a commercially available, ATP-dependent bioluminescence assay. Intracellular ATP levels were significantly decreased (greater than 50%) at 4 °C compared to 37 °C for QD of all coatings.

Three inhibitors of endocytosis and the cell cycle that modulate QD−NHEK interactions were identified: genistein, nocodazole, and cytochalasin D (Figure 4). The phytoestrogen, genistein, is a soy protein isoflavone that causes cell cycle arrest at the G2/M cell cycle checkpoint[15] and inhibits tyrosine kinases.[16] In keratinocytes specifically, genistein inhibits endocytosis[17] and differentiation.[18] Addition of 50 μM genistein to the culture medium attenuated interaction of both QD−NH2 and QD−COOH, but not QD−PEG, with NHEK (Figure 4a). Nocodazole disrupts microtubules, can inhibit endocytosis,[19] and arrest keratinocytes at the G2/M checkpoint.[20] Nocodazole at 10 μg/mL also attenuated

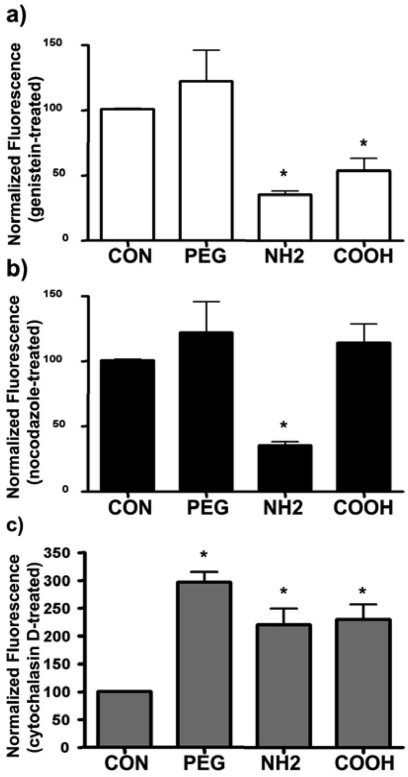

Figure 4. Biochemical modulation of QD 655−NHEK interactions. (a) Attenuation by 50 μM genistein. (b) Attenuation by 10 μg/mL nocodazole. (c) Potentiation by 10 μg/mL cytochalasin D. All cells were treated with 10 nM QD 655 with or without inhibitors (controls) for 24 h. Data are expressed as the percentage of fluorescence intensity units in controls and are normalized to MTT absorbance. (*) significant compared to control. Significance was set at $p < 0.05$. Data are the mean and SEM of 3−4 independent experiments of 4−5 replicates each.

interaction of QD−NH2 with NHEK, but no inhibition was observed for QD−PEG or QD−COOH (Figure 4b). Cytochalasin D inhibits F-actin polymerization, which is required for macropinocytosis[21] and can arrest cells in the G1 phase.[22] Cytochalasin D at 10 μg/mL potentiated QD−NHEK interactions for QD of all three coatings (Figure 4c).

This study identified the time course and effects of QD surface groups, culture medium supplements, and incubation temperature on the interactions of untargeted quantum dots (QD) with keratinocytes (NHEK cells), the dominant cell type in human skin. Biochemical inhibitors of endocytosis and the cell cycle were then identified that modulated QD−NHEK interactions.

Time course studies using a quantitative endpoint that measured QD−NHEK interactions show that cellular interactions increased with time for all QD. Confocal microscopy confirmed cellular uptake of all QD. These results agree with those of others reporting increased QD uptake with time.[12]

There were significant effects of coating on QD−NHEK interactions at 24 h. The least interaction was observed for QD−PEG. QD−NH2 showed greater interaction, while QD−COOH showed the most. This result is interesting in light of a previous cytotoxicity study we conducted at a 2-fold higher concentration.[10] In that study, only QD−COOH exhibited significant cytotoxicity at 24 h, whereas QD−NH2 did not exhibit cytotoxicity until 48 h, and no appreciable cytotoxicity was observed for PEG-coated QD. The coating-related differences in QD cytotoxicity of that study are consistent with the coating-related differences in QD−cellular interactions of the present study, indicating that greater cellular accessibility results in increased cytotoxicity. Others have reported coating-related differences in QD uptake.[12−14] Uptake of uncoated QD 621 into SK-BR-3 breast cancer cells was much greater than PEG 750- or PEG 6000-coated QD 621.[12] In a subsequent study, this same group also noted a positive correlation between increased uptake and cytotoxicity.[13]

QD−NHEK interactions were greater in culture medium with supplements (KGM-2) than without. This was consistent for QD of all three coatings. The reasons for enhanced QD−NHEK interaction in the presence of supplements are not clear, but could result from direct or indirect mechanisms. Media supplements could increase QD−cellular interactions directly by adsorbing to QD or causing agglomeration[23] or indirectly by stimulating NHEK to increase operative cellular uptake pathways such as macropinocytosis or micropinocytosis. Adsorption or agglomeration of QD with supplement proteins that are also cell surface receptor ligands, such as transferrin, insulin, and human epidermal growth factor, could stimulate receptor-mediated endocytosis. This has been observed with gold nanoparticles with adsorbed transferrin.[24] Alternatively, small-molecule media supplements, such as norepinepherine and glucocorticoids and/or supplement proteins, may enhance QD uptake indirectly by stimulating NHEK. The result that media supplements increased QD−cellular interactions for QD with three different surface coatings is most consistent with indirect mechanisms.

The energy dependence of QD−NHEK interactions was assessed by decreasing the incubation temperature from 37 to 4 °C, which decreased cellular ATP levels by over 50%. Other methods of decreasing intracellular ATP levels, such as the use of cyanide or other inhibitors of oxidative phosphorylation, were not feasible for the present study, which required an extended incubation time of 24 h. The magnitude of QD−NHEK interactions decreased as temperature and ATP levels decreased for QD of all coatings, indicating that QD−cellular interactions are energy dependent and require active, and not passive, mechanisms. This result agrees with those of others also reporting decreased cellular uptake of QD with decreasing temperature.[13]

Biochemical modulators of QD−NHEK interactions were also identified. Three compounds were chosen that are known to inhibit endocytosis and the cell cycle: genistein, nocodazole, and cytochalasin D. Both genistein and nocodazole attenuated QD−NHEK interactions in a coating-dependent manner. Genistein attenuated interactions of QD−NH2 and QD−COOH with NHEK by about 50% but had no effect on QD−PEG. Nocodazole attenuated interaction of QD−NH2 by about 50% but had no effect on QD−PEG or QD−COOH. Cytochalasin D potentiated QD−NHEK interactions by over 200% for QD of all coatings. This result indicates regulation of NHEK−QD activity by cell cycle or endocytic mechanisms other than macropinocytosis. Together, these

findings indicate that the cell cycle and endocytic mechanisms can mediate QD−NHEK interactions and that QD surface groups can play a modulatory role.

This study showed that untargeted QD can interact with NHEK skin cells and identified variables influencing these interactions. These variables were time, QD surface groups, supplements in the culture medium, and temperature. The modulation of QD−NHEK interactions by genistein, nocodazole, and cytochalasin D indicates a role for endocytic and cell cycle-based mechanisms. Also, the ability to biochemically modulate QD−NHEK interactions via these inhibitors may provide a means of controlling interactions of untargeted QD with NHEK and other cell types.

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Supporting Information Available: Materials and methods for procurement and dilution of QD, NHEK culture and dosing with QD, fluorescence microplate assay for QD−NHEK interactions, time course studies, temperature studies (including ATP-dependent bioluminescence), treatment with genistein, nocodazole, and cytochalasin D, confocal microscopy, data and statistical analysis. Table of toxicity of inhibitors in NHEK. This material is available free of charge via the Internet at http://pubs.acs.org.

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