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Hepatoma Cell Uptake of Cationic Multifluorescent Quantum Dot Liposomes

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Cationic multifluorescent quantum dot liposomes (QD-Ls) have been prepared with both hydrophobic and hydrophilic CdSe/ZnS quantum dots by reverse phase evaporation. QD incorporation was confirmed by fluorescence and confocal microscopy. Incorporation did not affect QD photoactivity or damage bilayer or liposome structure. Cell uptake was examined in human hepatocellular carcinoma cells (HuH-7) using cationic and zwitterionic QD-Ls. Cationic QD-Ls were stable in vitro and exhibited high uptake, while zwitterionic QD-Ls aggregated and exhibited low uptake. Given that liposomes are established and versatile platforms for creating cell-targeting therapeutic agents, multifluorescent QD-Ls may offer advanced techniques for imaging hydrophobic and hydrophilic domains simultaneously. If coupled with an encapsulated drug, QD-Ls could be multifunctional and provide imaging, detection, and drug delivery in a single assembly.

Quantum dots (QDs), such as cadmium selenide/zinc sulfide (CdSe/ZnS) core/shell semiconductor nanocrystals, offer excellent fluorescence properties for cellular imaging and diagnostic applications.[1−5] They are also being examined for photodynamic cancer therapy.[6] Their spectral emission is an order of magnitude higher than conventional fluorescent dyes and can be tuned by changing core/shell diameter and composition. However, in their native state, they are hydrophobic and cannot be dissolved in aqueous medium. Hydrophilic surface coatings such as poly(ethylene glycol) (PEG) can be used to solubilize the QDs in water; however, these aqueous QDs exhibit cytotoxicity that limits their application.[4,7−9] One approach to overcome this challenge and to deliver a sufficiently high concentration for imaging is to incorporate QDs in lipid bilayer vesicles or liposomes.[10−14] Liposomes are nanoscale, spherical structures composed of a self-assembled lipid bilayer shell surrounding an aqueous core.

The preparation of QD liposomes (QD-Ls) has been accomplished by two means: (1) by encapsulating surface-modified hydrophilic QDs within the aqueous liposome core[10,14] and (2) by embedding native hydrophobic QDs within the acyl bilayer region.[11,13] For instance, Al Jamal et al.[10] encapsulated PEG-coated QDs within liposomes, and Gopalakrishnan et al.[13] and Al-Jamal et al.[11] have embedded hydrophobic trioctylphosphine oxide (TOPO)-coated QDs with overall diameters near 4−5 nm (QD core + hydrophobic coating) within the liposome acyl bilayer region. In these studies, QD-Ls were prepared by thin film hydration and cationic lipids, which are known transfection agents that aid cell uptake. When embedded within the bilayer, hydrophobic QDs exhibit greater photostability relative to unencapsulated QDs.[11] Cationic QD-Ls have exhibited cell uptake in human embryonic kidney cells[13] and human lung epithelial cells in vitro and in human cervical carcinoma xenografts (mice) in vivo.[11] Although the size of the hydrophobic QDs is similar to the thickness of a lipid bilayer (∼5 nm depending on the phase state), Wi et al.[15] have shown that the interfacial energy for bilayer embedding is favorable up to a critical diameter of 6.5 nm. Furthermore, Jang et al.[16] have shown that energy gain associated with removing a hydrophobic nanoparticle surface from water into the bilayer overcomes the energy penalty for deforming the bilayer.

Our objective was to design a liposomal system that could deliver both hydrophobic and hydrophilic QD imaging agents into carcinoma cells. Mixed zwitterionic and cationic lipid QD-Ls were prepared by reverse phase evaporation (REV).[17] Through REV, green hydrophobic and red hydrophilic CdSe/ZnS core/shell QDs were incorporated in a single step (Figure 1). The hydrophobic QDs contained a TOPO coating and were received from the manufacturer in decane (Qdot 545 ITK, green 545 nm; Invitrogen, Carlsbad, CA). Fernández-Argüelles et al.[18] calculated a diameter of 3.05 nm for the Qdot nanocrystals. The hydrophilic QDs had carboxyl-terminated surface coatings and were received in deionized water (T1 EviTag, Maple Red-Orange 620 nm; Evident Technologies, Troy, NY). The manufacturer specification for the approximate hydrodynamic diameter was 40 nm, and we measured a core/shell diameter of ∼11 nm using transmission electron microscopy (results not shown). Potential advantages of delivering multiple QDs include the ability to image hydrophobic and hydrophilic domains simultaneously and to exploit the mixed colorimetric properties for monitoring intracellular processes.

Multifluorescent QD-Ls were prepared at 10 mM lipid in deionized water (Direct-3Q, Millipore, Billerica, MA). Zwitterionic liposomes were composed of dipalmitoylphosphatidylcholine (DPPC) or DPPC/cholesterol (8:2 molar basis), and cationic liposomes were composed of DPPC/dipalmitoylethylphosphocholine (ethyl-DPPC)/cholesterol (6:2:2). In the REV process, an organic solvent phase containing lipids and the green

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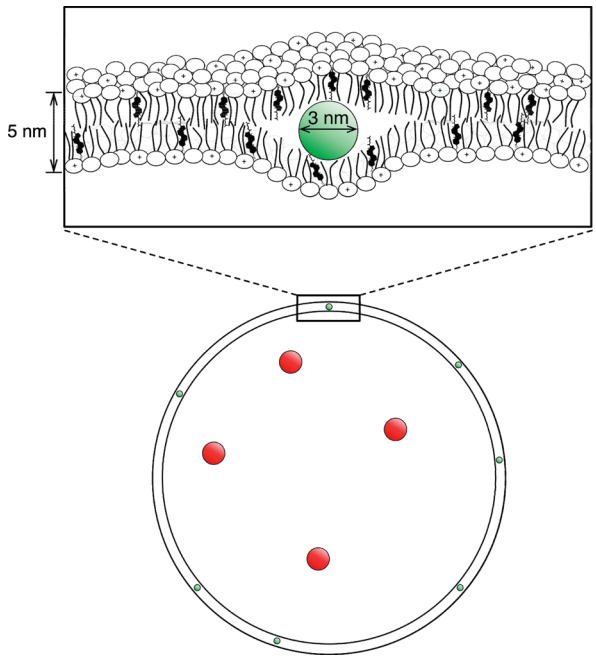


Figure 1. Schematic of a cationic QD-L containing green hydrophobic (~3 nm[18]) and red hydrophilic (~11 nm) CdSe/ZnS core/shell quantum dots. The multifluorescent cationic QD-L bilayers are composed of DPPC/ethyl-DPPC/cholesterol (6:2:2) and are depicted to scale.

hydrophobic QDs was mixed with an aqueous phase containing the red hydrophilic QDs to form an emulsion. The solvent phase was prepared by drying the hydrophobic QDs from decane and redissolving in the desired chloroform/lipid solution.[10] Chloroform was removed by rotary evaporation to collapse the emulsion phase and drive QD incorporation within the liposome. The final concentration of each QD in solution was 1 μM, which yielded a lipid to QD ratio of 10 000 to 1. This ratio was determined on the basis of the work by Al Jamal et al.,[11] who demonstrated that at this ratio there is no noticeable physical deformation of the liposomes by hydrophobic QDs. QD-L samples were purified by size exclusion chromatography (Sephadex G-50, Sigma-Aldrich, St. Louis, MO), which has been used by Banerji and Hayes[19] to separate unencapsulated

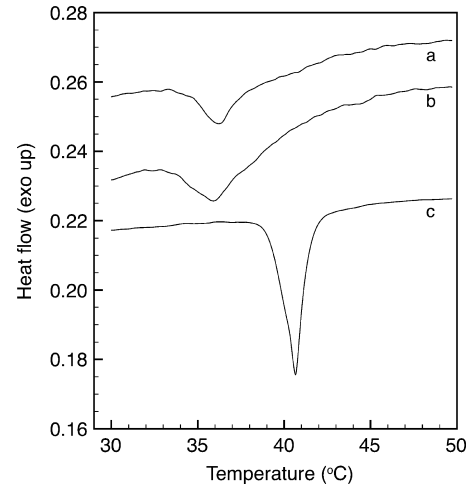
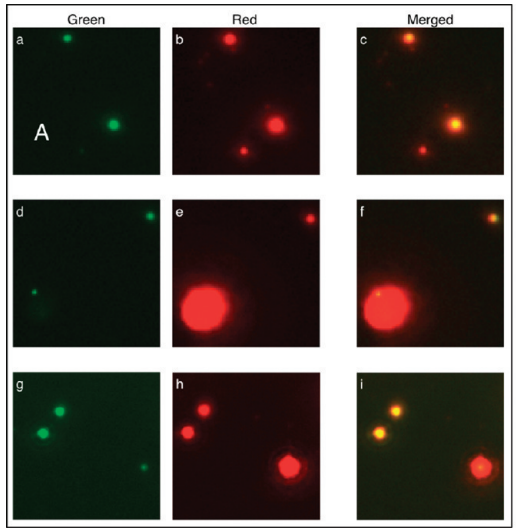


Figure 3. DSC thermograms of QD-L bilayer melting (DPPC/ethyl-DPPC/cholesterol, 6:2:2; 10 mM lipid) at (a) 0.1 μM Qdot 545 nm and 1 μM EviTags 620 nm and (b) 1 μM Qdot 545 nm and EviTags 620 nm. (c) DPPC bilayer melting is shown for comparison.

7, 10, and 15 nm colloidal gold from giant unilamellar vesicles. Cryogenic transmission electron microscopy for DPPC/ethyl-DPPC/cholesterol shows large unilamellar vesicles (LUVs) with an average diameter of 99 ± 46 nm in the absence of QDs (see the Supporting Information). We cannot provide micrographs for the QD-Ls (620 nm EviTags were discontinued by the manufacturer); however, their structure was likely similar to the liposome control. Al Jamal et al.[11] have shown that incorporating hydrophobic QDs did not significantly change the size of zwitterionic dioleoylphosphatidylcholine (DOPC) QD-Ls.

The incorporation of both QDs within the cationic liposomes was confirmed by fluorescence (Eclipse E600, Nikon Instruments Inc., Melville, NY) and laser scanning confocal microscopy (LSM 5 Pascal, Carl Zeiss Inc., Thornwood, NY) (Figure 2). In Figure 2A, the presence of colocalized green and red QDs combined to produce a yellow color. Colocalization was observed in nearly all cases and suggests that most of the unencapsulated red QDs were removed by size exclusion chromatography. The merged images in Figures 2A, f and i



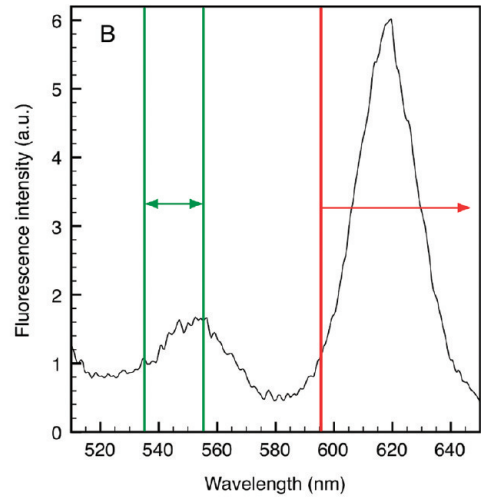


Figure 2. (A) Fluorescence microscopy images using oil immersion lenses at 60× (a−f) and 100× (g−i) magnification of cationic multifluorescent QD-Ls with green (left; FITC filter, 535−555 nm) and red (middle; rhodamine filter, 590+ nm) filters. The merged image (right) shows yellow QD-Ls. (B) Fluorescence spectrum of cationic QD-Ls excited at 490 nm. The green and red regions denoted by vertical lines show the FITC and rhodamine filter ranges, respectively, used for fluorescence microscopy.

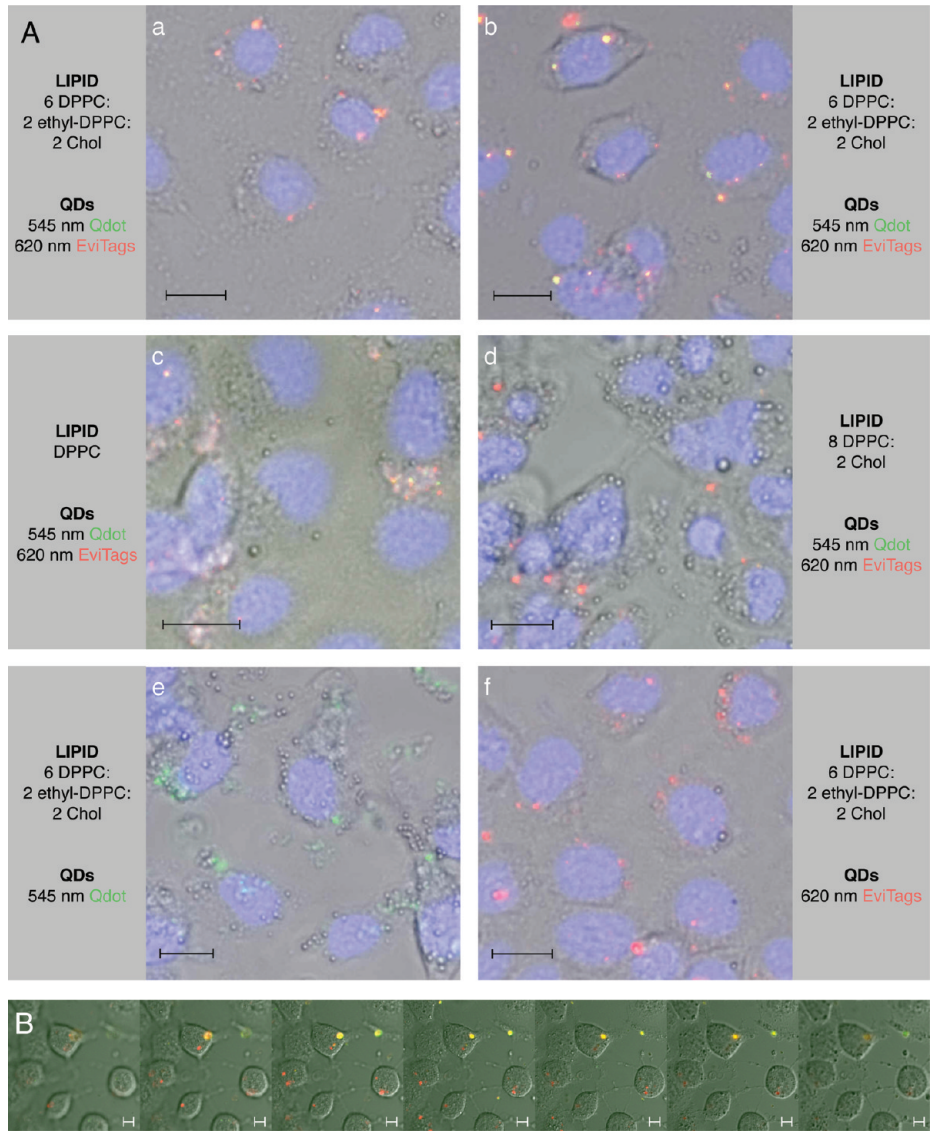


Figure 4. (A) In vitro hepatoma cell uptake of cationic (a, b - multilabeled; e, f - unilabeled) and zwitterionic (c, d - multilabeled) QD-Ls in serum-free media. The nucleus was stained blue with Hoechst 33342 stain to evaluate delivery to the nucleus. (B) Z-plane images at 1 μm increments from top to bottom obtained by confocal microscopy for hepatoma cells in DMEM serum-free media. The scale bars are 5 μm.

appear to show single hydrophobic green QDs embedded within lipid bilayers.

With the presence of both QDs within the liposomes confirmed, the effect of liposomal incorporation on QD fluorescence and the effect of bilayer-embedded hydrophobic QDs on lipid bilayer phase behavior were examined. The fluorescence spectrum for the multifluorescent QD-Ls with an excitation at 490 nm (LS55, Perkin-Elmer, Waltham, MA) was nearly identical to the individual spectra for unencapsulated QDs (Qdots in decane; EviTags in water), confirming the photoactivity of the QD-Ls (Figure 2B). In our studies, the maximum emission was near 553 nm for the Qdot nanocrystals and 620 nm for the EviTags.

A lipid melting temperature (T_m) near 36 °C was measured for the cationic QD-Ls by differential scanning calorimetry (Model Q10, TA Instruments, New Castle, DE) (Figure 3). T_m represents the transition from an ordered rippled-gel bilayer phase to a disordered liquid crystalline or fluid bilayer phase. An increase in the hydrophobic QD concentration from 0.1 to 1 μM led to a slight reduction in T_m and broadening of the transition region, as denoted by the full width at half-maximum peak height. This suggests that increasing the QD concentration within the bilayer may have led to greater bilayer disordering (additional experiments are needed to determine this conclusively). Interestingly, the QD-L melting temperature is near the physiological temperature, which is advantageous for maximizing liposome release at the interface of coexisting gel and fluid domains.

QD-L uptake was examined using HuH-7, human hepatoma cells (ATCC, Manassas, VA). The HuH-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 5% fetal bovine serum, antibiotic/antimycotic solution, and other standard medium additives (GIBCO/Invitrogen), and plated at 5% CO_2 and 37 °C. For QD-L experiments, cells were seeded into 6-well plates and the medium was changed to DMEM without serum after cells had attached overnight. The nuclei were stained blue with 20 mM Hoechst 33342 (AnaSpec, San Jose, CA). After plating, the cells were cultured with QD-Ls at 1.25 mM lipid (medium concentration) for 24 h. The culture medium was removed and replaced with fresh QD-L-free medium prior to imaging. Fluorescence microscopy results show that cationic QD-Ls were taken up by the cells and collected near the nuclei (Figure 4A, a and b). Z-plane stacks further confirmed that the QD-Ls collected near

the nuclear membrane (Figure 4B). Distinct red QD spots are seen in Figure 4B with no clear evidence of colocalized green QDs. These spots could represent unencapsulated hydrophilic QDs or QD-Ls containing only these QDs. Additional experiments are being conducted to determine the effect of different synthesis conditions on encapsulation efficiency and QD-L structure. Not surprisingly, unifluorescent QD-Ls were also effective in delivering hydrophobic or hydrophilic QDs (Figure 4A, e and f).

Gopalakrishnan et al.[13] have shown that hydrophobic QDs embedded within lipid bilayers could be used to label human embryonic kidney cell membranes (HEK293) through liposome fusion when cationic dioleoyloxy-trimethylammonium-propane-methylsulfate (DOTAP) was used at 25 mol %. For our multifluorescent QD-Ls, there was no evidence of cell membrane labeling with green QDs based on the cell type, exposure, and media conditions used. Hence, hydrophobic green QDs remained embedded within the multifluorescent QD-Ls. The preference to fuse or stay intact will depend on the cell membrane composition and liposome construction, and strategies may be developed to label different cell regions or different cell types in a mixed culture.

Zwitterionic multifluorescent QD-Ls were employed to examine the role of the cationic lipid ethyl-DPPC on cell uptake and trafficking. Significant agglomeration and little cell uptake was observed when DPPC was used alone (Figure 4A, c). When 20 mol % cholesterol was added to the DPPC bilayer, agglomeration was reduced, but uptake was still low compared to the cationic QD-Ls, which were stabilized electrostatically through charge repulsion between positively charged bilayers (Figure 4A, d). We attribute cell uptake to the ability of the cationic lipids to prevent QD-L aggregation in conjunction with their transfection properties.

We have shown that it is possible to design a cationic liposome system containing dual hydrophobic and hydrophilic QDs, and we have demonstrated their uptake in human hepatoma cells. Additional studies are being conducted to determine the size and structure of the QD-Ls, their long-term colloidal stability, and the effect of cell media composition on QD-L uptake. This includes serum proteins, which are known to adsorb and aggregate nanoparticulate therapeutic agents in vivo. We envision that the QD-Ls could be modified to selectively target and deliver drugs to cocultures with diseased and healthy cells.

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Supporting Information Available: Description of the cryo-TEM analysis and a figure showing a cryo-TEM micrograph of DPPC/ethyl-DPPC/cholesterol liposomes. This material is available free of charge via the Internet at http://pubs.acs.org.

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