Optimizing Quantum Dot-Conjugated Immunoliposomes for Cancer Diagnostics and Targeted Therapeutics

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ABSTRACT

A multifunctional nanoparticle that combines the optical properties of luminescent quantum dots (QDs) and therapeutic modality of immunoliposomes (ILs) has been developed [1]. Carboxyl QDs cross-linked with amino-functionalized liposomes were modified with anti-human epidermal growth factor receptor 2 (anti-HER2) scFv, resulting in quantum dot-conjugated immunoliposomes (QD-ILs). By varying the conjugation conditions, QD-ILs of average diameter from ~ 140 nm to ~ 360 nm were synthesized with QD conjugation efficiency ranging from ~ 25% to ~ 96%. Internalization of these QD-ILs by HER2-overexpressing human breast carcinoma cells SK-BR-3 and MCF7-C18 were investigated using low HER2-expressing MCF-7 as negative control. We demonstrated that the uptake of QD-ILs was efficient and specific. We also found that the QD to cross-linking agent ratio affects the QD-ILs cellular internalization efficiency. The results serve as guidelines for improving QD-ILs nanoparticles as well as nanomedicine systems in general.

Keywords: quantum dots, immunoliposomes, nanomedicine, anti-HER2, targeted drug delivery systems

1 INTRODUCTION

Nanomedicines offer the promise to simultaneously image diseased areas, track the drugs, and exert therapeutic activity by well-defined macromolecular constructs. Recently, an effort has been taken to incorporate fluorescent semiconductor nanocrystals (quantum dots, QDs) into nanoscale immunoliposomes to combine the functional modalities into a single nanoparticle system. Our attempt led to the development of quantum dot-conjugated immunoliposomes (QD-ILs) as illustrated in Figure 1. Compared to conventional dyes, QDs are superior in optical and chemical properties such as photostability, wide adsorption/narrow emission, and resistance to biological degradation [2-4]. Therefore, QDs have emerged as promising agents to improve imaging capability in biological systems. QD-ILs could be loaded with chemotherapeutic agents or other functional molecules for ad hoc purposes. QD-ILs equipped with anti-HER2 scFv [5] exhibited extremely efficient and specific internalization by the HER2-overexpressing cells SK-BR-3 and MCF7-C18 [1] but not low HER2-expressing MCF-7. Thus, QD-ILs could be used for immunoassays as well as *in vivo* tracking of immunoliposomes and for determining the intracellular fate of liposomal drug delivery systems. Deciphering the traffic of nanoscale anticancer agents through the intracellular matrix could provide useful information for the design of drug delivery systems [6, 7].

To preserve the aqueous interior for drug loading, QDs were chemically attached to the functionalized surface of ILs by zero-length cross-linking reactions, leading to various degrees of aggregation. It has been reported that cellular endocytosis is highly size dependent [8], and the dimensions of the nanoparticles could hinder potential *in vivo* applications if not optimized. For example, to fully take advantage of enhanced permeability and retention (EPR) effect [9], nanoparticles at around 100 nm in diameter typically reach the maximum penetration, thus serving as general guidelines for assembling nanoparticles for tumor-targeting and drug delivery *in vivo*. Understanding and controlling the particle size during formulation is required for optimizing constructs for systemic application.

To investigate the dependence of cellular interactions on the physical properties of QD-ILs, four different QD-ILs were synthesized by varying the stoichiometric ratio of cross-linking reagent, reactive amino groups, and QDs. Besides measuring QD fluorescence, lipidic dye ($DiIC_{18}(5)$ oil) was included in the lipid bilayers of QD-ILs to simultaneously quantitate the amount of immunoliposome delivered intracellularly. The amount of immunoliposome may not correspond to the amount of QDs endocytosed due to both the inhomogeneous conjugation between QDs and liposomes, and preferential uptake of nanoparticles by cells. In this study we address how conjugation conditions affect the physical properties of QD-ILs nanoparticles and subsequently their cellular interactions. Further results obtained will direct optimization of the QD-ILs system for practical applications.

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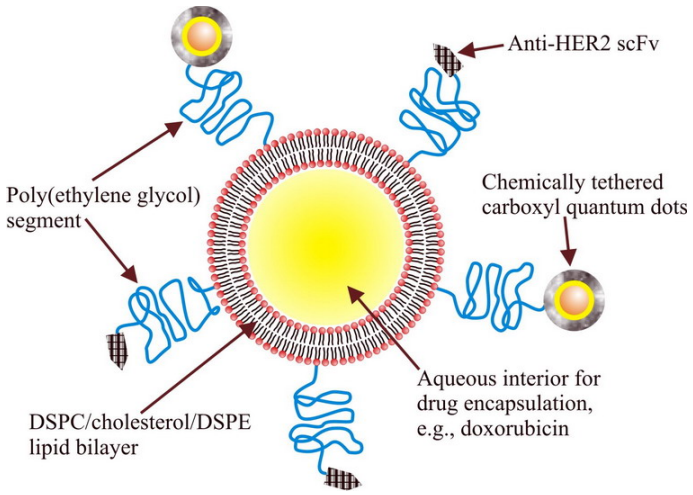

Figure 1. Schematic of an anti-HER2 quantum dot-conjugated immunoliposome

2 EXPERIMENTAL

2.1 Nanoparticle Synthesis and Characterization

To synthesize QD-ILs, CdSe/ZnS core/shell QDs with a carboxyl-derivatized surface (Qdot 605 ITK, Invitrogen, Eugene, OR, or QDs obtained from Life Sciences Division, Lawrence Berkeley National Laboratory) were used to provide the functionality for chemical conjugation with primary amine groups. The liposomes consist of 3 mol% 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)2000] (amine-PEG$_{2000}$-DSPE) along with 60 mol% 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) (Avanti Polar Lipids, Alabaster, AL) and 36.75 mol% cholesterol (EMD Biosciences, La Jolla, CA) were prepared by extrusion through nominal 100 nm polycarbonate membranes (Whatman, Florham Park, NJ) in 5 mM HEPES, 135 mM NaCl buffer (pH ~ 7.4). The lipid composition was selected to ensure a high phase transition temperature with increased rigidity compared to cholesterol-free DSPC liposomes. 0.25 mol% of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate (DiD, DiIC$_{18}$(5) oil) (Invitrogen, Eugene, OR) was also included in the mixture to serve as the secondary, lipidic fluorescent probe. Carboxyl QDs and liposomes were covalently linked by zero-length crosslinker 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (Pierce, Rockford, IL) at room temperature for 2 hours by four stoichimotric ratios: EDC : Amine : QD = ~ 100 : 1000 : 1 for QD-ILs^{A}, ~ 1000 : 1000 : 1 for QD-ILs^{B}, ~ 10000 : 1000 : 1 for QD-ILs^{C}, and ~ 50000 : 10000 : 1 for QD-ILs^{D}. The products were purified by home-made dialyzers with 50 or 80 nm polycarbonate membranes at ~ 4°C up to three days. The enabling of liposomes for target selectivity was achieved by one-step post-insertion of an anti-HER2 scFv lipid conjugate, F5-PEG-DSPE, at 60°C for 30 minutes [10]. Purified nanoparticles were characterized by fluorescence spectrophotometry (Fluorolog, ISA Jobin Yvon-SPEX Instruments S.A., Inc.) and dynamic light scattering (Beckman Coulter PCS Submicron Particle Size Analyzer N4 Plus). Autocorrelation functions were analyzed by multi-modal analysis to yield average volume-weighted hydrodynamic diameters and standard deviations.

2.2 Cell Cultures and Treatment

120,000–150,000 cells were cultured in 12-well sterile polystyrene plates (Corning, Corning, NY) and incubated in humidified isotemp tank at 37°C with constant 5% carbon dioxide flow for 2 days before treated by various liposomal formulations at 37°C for one hour. The media McCoy's 5A was used for SK-BR-3 and Dulbecco's Modified Eagle Medium, D-MEM High Glucose (DME H-21) was used for MCF-7 and MCF7-C18 except that 0.4% of gentamicin was also added in the media for MCF7-C18. All media were supplemented by 10% fetal bovine serum and penicillin/streptomycin in cultures. All materials were acquired from UCSF Cell Culture Facility.

2.3 Quantitative Flow Cytometry

Cells were trypsinized after treatment and washed 2 times by sterile, filtered phosphate buffer saline containing Ca^{2+} and Mg^{2+} (UCSF Cell Culture Facility). 0.5–0.6 ml of cell suspensions was analyzed by a multi-channel flow cytometer (BD Biosciences FACSCalibur System) for detection of QD and DiD fluorescence. To quantitatively compare the results, we calibrated the fluorescence emission at the readout channels, i.e., 585 nm and 661 nm, of the flow cytometer by phycoerythin-tagged fluorescent microbeads and Cy5-tagged microbeads (Bangs Laboratories, Fishers, IN), respectively, for each measurement. The intensities of fluorescence collected from 10,000 cells were converted to units in molecules of equivalent soluble fluorochrome (MESF) to eliminate dependence on instrument and settings. In addition, integrated intensities from the histograms are calculated and presented instead of peak intensities typically reported in literature.

3 RESULTS

Liposomes without modifications are unimodally distributed with a hydrodynamic diameter of ~ 135 ± 19 nm, typical for unilamellar vesicles manually extruded through nominal 100 nm polycarbonate membranes. As expected, when the amount of EDC increased, the extent of cross-linking increased as well as the average particles size and size distribution. As shown in Table 1, QD-ILs$^{A, B, C, D}$ showed an increase of ~ 3.7%, ~ 8.9%, ~ 81.5%, and ~ 165.2% in diameter, ranging from ~ 140 to 358 nm, compared to liposomes without modifications. Higher EDC concentration also led to higher conjugation efficiency. The numbers of QD per liposome could be calculated from fluorescence spectrophotometry results and were also summarized in Table 1.

Quantitative flow cytometry results are shown in Figure 2 and 3. In all data shown the cell autofluorescence has been subtracted. SK-BR-3, MCF7-C18, and MCF-7 were treated by 1 nM (Figure 2, concentration designated for QDs) and 30 μM (Figure 3, concentration designated for phospholipid) of QD-ILs$^{A, B, C, D}$. MCF-7 showed minimal non-specific uptake, in accordance to previous observations. Interestingly, SK-BR-3 and MCF7-C18 internalized similar amounts of QDs regardless of the particle size and amount of immunoliposome present. Although the amount of immunoliposome administered has a difference of 3.84 times for QD-ILs^{A} and QD-ILs^{D}, the difference in QD uptake is essentially the same for SK-BR-3 and only 1.4 times for MCF7-C18. QD-ILs^{C} showed slightly higher QD uptake of ~ 1.13× and 1.41× for SK-BR-3 and MCF7-C18, respectively, and appeared to be the most efficient QD-ILs.

Comparing the 661 nm fluorescence signals for immunoliposome uptake in Figure 2, one would find that uptake of QD-ILs^{D} is ~ 2.8 times less than that for QD-ILs^{A}. This indicates more efficient uptake of ODs by larger nanoparticles. However, when QD-ILs were administered at an equivilent phospholipid concentration, as shown in Figure 3, QD-ILs^{B} showed the most uptake and QD-ILs^{C} yielded the highest QD internalization.

4 DISCUSSIONS

Higher cross-linking of QD and immunoliposomes did not seem to significantly impede the uptake by targeted cells. In our study, QD-ILs^{B}, which has an average size of ~ 147 nm, was internalized with slightly higher efficiency compared to QD-ILs^{A}. However, for the same amount of immunoliposome administered, QD-ILs^{C} with an average size of ~ 245 nm delivered the most QD intracellularly. Therefore, an excess EDC to make EDC : amine : QD ~ 10000 : 1000 : 1 could make the most efficient QD-IL nanoparticle without suffering from size limitations. QD-ILs will be exploited for practical applications such as quantitation for internalizable receptors and *in vivo* imaging using the optimized formulations. These results also indicated the low concentrations applied could be well below saturation and the time and incubation conditions allowed were not able to fully distinguish the difference in cellular interactions with the four QD-ILs tested here.

5 ACKNOWLEDGEMENTS

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QD-IL	EDC : Amine : QD ratio	Hydrodynamic diameter (nm)	QD conjugation efficiency	QD per liposome
Liposome without modification	N/A	135 ± 19	N/A	N/A
QD-IL^{A}	~ 100 : 1000 : 1	140 ± 24	~ 25%	~ 1.2
QD-IL^{B}	~ 1000 : 1000 : 1	147 ± 31	~ 40%	~ 2.1
QD-IL^{C}	~ 10000 : 1000 : 1	245 ± 97	~ 70%	~ 10.1
QD-IL^{D}	~ 50000 : 1000 : 1	358 ± 112	~ 96%	~ 29.5

Table 1. Physical properties of QD-ILs prepared by various conjugation conditions.

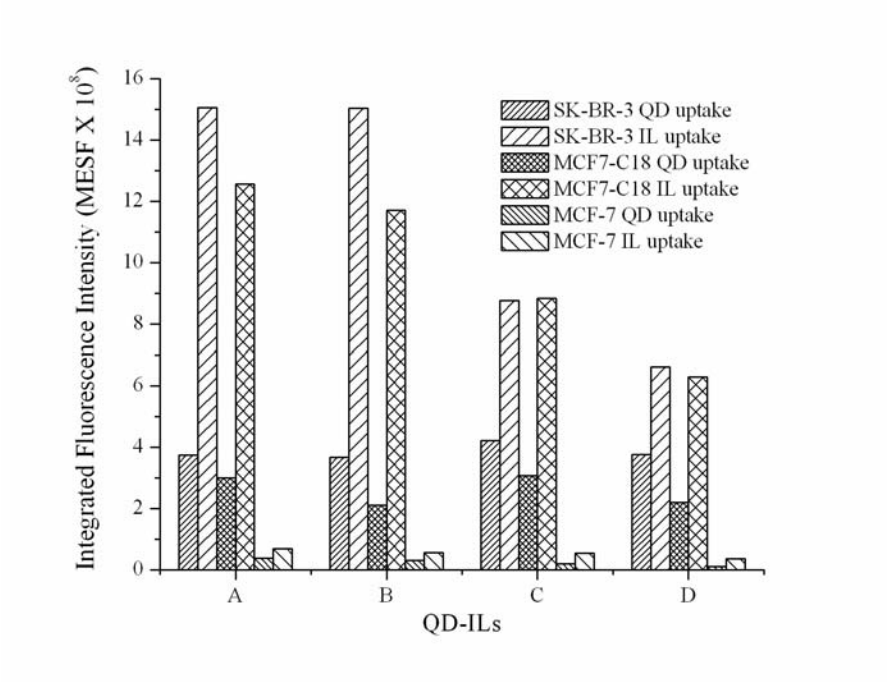

Figure 2. Integrated fluorescence intensities of SK-BR-3, MCF7-C17, and MCF-7 treated by 1 nM QD-IL^{A}, QD-IL^{B}, QD-IL^{C}, and QD-IL^{D} where concentration is designated for QDs. Each group of bar data is for one type of QD-IL. QD uptake and DiD uptake (immunoliposome) are presented for each cell line. Fluorescence intensities are expressed in the units of molecules of equivalent soluble fluorochrome (MESF) by calibrating the flow cytometer with fluorescent standards using phycoerythrin-tagged microbeads for QD detection at Channel 585 nm and Cy5-tagged microbeads for DiD detection at Channel 661 nm. Autofluorescence was subtracted from all results shown. QD = quantum dot; IL = immunoliposome. MESF values for IL uptake (661 nm) has been divided by a factor of 10 for easy comparison with QD uptake (585 nm).

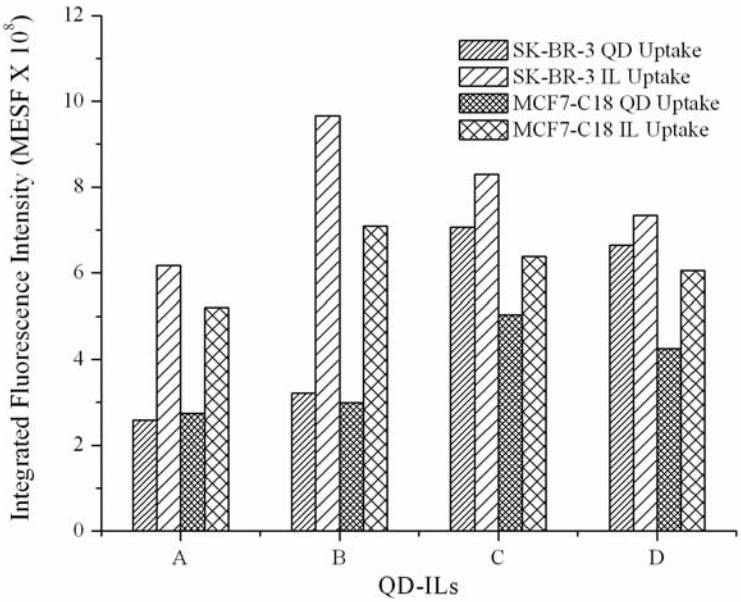

Figure 3. Integrated fluorescence intensities of SK-BR-3 and MCF7-C18 treated by 30 μM QD-IL^{A}, QD-IL^{B}, QD-IL^{C}, and QD-IL^{D} where concentration is designated for phospholipid.