Quantum Dots, Torch Bearers Into Cells

Dual Labeled Liposomes for EGFR-mediated Cellular Delivery

An efficient receptor-mediated delivery system has been developed using preformed complexes of Quantum Dots (QDs) and the Epidermal Growth Factor (EGF) ligand. Liposomes are either loaded or surface modified combining two colors of QDs probes. The dual labeling strategies involve only biotin-streptavidin interactions and do not require further purification from free QDs-EGF. Specific uptake of QD-EGF-labeled liposomes is detected in live cells as colocalization of both dots.

Liposomes Tagged with QD-ligand Complexes: Flexible Applications

Ligand-functionalized submicron sized particles such as liposomes, micelles and polymeric capsules have shown great promise for achieving high sensitivity and specificity in biomedical applications, notably the controlled delivery of drugs to target organs [1, 2]. Quantum dots (QDs) conjugated with ligands have enabled the recognition of multiple targets and elucidation of cellular dynamic processes [3, 4]. Their exceptional photostability is the essential feature for long term fluorescence imaging [5, 6]. The epidermal growth factor receptor (EGFR, erbB1, HER1) is overexpressed in a broad spectrum of malignant tumors and represents a target for delivery of therapeutic liposomes [7]. It was previously demonstrated by our group that biotinylated EGF ligand conjugated to streptavidin coated QDs (QD-EGF) binds to its surface receptor in target cells, leading to activation, retrograde transport on filopodia and endocytosis [3, 8].

Two Colors and a Dual Role for QDs

We explored the simultaneous use of green and red emitting QDs for different roles in the same lipid nanoparticle, first as a ligand carrier for receptor-mediated endocytosis and second as encapsulated cargo in these liposomes for targeted delivery to cells overexpressing the EGFR.

As monocolor complexes of QDs-EGF-liposomes would not be distinguishable from free QDs-EGF upon incubation with cells, we designed the two color tagging
approaches shown in figure 1 to clearly identify the tagged particles.

The simple procedures avoid tedious purification steps and take advantage of the ability to excite both QDs with the same wavelength of light. In Approach A (top panel), the biotinylated liposomes are surface labeled with streptavidin functionalized QD655 and preformed complexes of green emitting QD525-EGF. Free QDs do not internalize into cells in the absence of ligand [3] and thus the rationale behind this strategy is that only QD655 particles bound to EGF-QD525 - liposomes will be endocytosed.

In Approach B (bottom panel), the biotinylated liposomes with encapsulated QD655 are tagged with QD525-EGF. Colocalization of both QDs is indicative of the receptor mediated uptake of liposomes, showing their intracellular distribution clearly distinguished from that of free QDs-EGF.

**QDs Loaded Liposomes**

In the present work biotinylated liposomes (~60 nm) stabilized with polyethyleneglycol (PEG) were prepared using a procedure initially described for plasmid encapsulation in small unilamellar vesicles [9]. For Approach B the method was adapted for the encapsulation of QDs functionalized with carboxyl groups (~ 10 nm size).

Liposomes with encapsulated QDs were purified from non-encapsulated nanocrystals by ultracentrifugation in sucrose density gradients (fig. 2a). The brightest top fraction contained QDs interacting with free lipids and was discarded. We focused our analysis on the particles recovered from the second fluorescent band. This fraction was analysed by Transmission Electron Microscopy (fig. 2b), revealing that an average of 2 to 5 QDs were successfully encapsulated without being aggregated. A size variation in the liposome diameter from 100 to 130 nm was observed, depending on the number of loaded QDs, while empty liposomes or
those containing only one QD were generally smaller than 100 nm. Dynamic light scattering measurements led to similar size estimations for loaded liposomes (fig. 2c). The average mean hydrodynamic diameter for QDs loaded liposomes was 140 nm, compared to 60 nm in control samples containing empty liposomes.

**Imaging the Cellular Binding and Uptake of QD-EGF-liposomes**

The targeted liposomes were incubated with A431 cells that overexpress the EGF receptor for up to 2 h at 37 °C. The intracellular distribution of nanocarriers was analysed in the live cells by confocal fluorescence microscopy using the 488 nm laser line for excitation. During the first 30 minutes incubation, QD-EGF targeted liposomes are mainly accumulated at the cell surface (fig. 3). After 2 h and with both approaches a substantial internalization of QDs-tagged EGF targeted liposomes was observed (fig. 4a-b), whereas QDs-tagged but untargeted liposomes were not taken up (fig. 4c). A punctuate pattern consistent with endosomal entrapment was observed. There was virtually no colocalization of the QDs in the absence of ligand.

In Approach A, QD655 were only detected when they were bound to QD525-EGF tagged-liposomes. The colocalization of QD655 and QD525 suggests that at least two biotins were available on the surface of each liposome.

In approach B, the colocalization of the two QDs in what was probably the late endosome compartment indicated that QD655 remained encapsulated in the liposomes for at least 2 h.

The variation in the red-green fluorescence intensity levels of colocalized QDs is shown in Figure 5, revealed a distribution in the number of QD655 and QD525-EGF associated with individual liposomes as well as the presence of more than one liposome per particle. The colocalization of the QDs in both approaches allowed a clear distinction between the liposomes-associated nanoparticles (yellow-orange pseudocolor) and independently internalized green QD525-EGF complexes.

**Applications**

We have investigated two different approaches for dual fluorescent labeling and targeting of biotinylated lipid particles. The tagging strategies combine i) the flexibility of the biotin-streptavidin system for ligand coupling [10] ii) the photostability and bright fluorescence of QDs, and iii) the pharmacokinetic and drug delivery capabilities of sterically stabilized liposomes [2].

In the surface tagging strategy proposed as Approach A, the internal liposomal space is available for drug loading. In the second approach the encapsulated QDs
can be used to monitor endosomal escape mechanisms at the single molecule level. For delivery purposes the cargo must escape from the endosomes. We are exploring conditions promoting endosomal escape, such as extended incubation times and the use of pH sensitive lipids in the liposome formulation. The bicolor labeling strategies presented here implement two aims: the targeted delivery into cells expressing a specific receptor, and the unambiguous distinction by colocalization between QDs-liposomes from individual QD-EGF complexes. A purification procedure, otherwise unavoidable in monocolor labeling, is thereby bypassed. The flexibility of the strategy involving the biotin-streptavidin interaction can be extended to any preformed ligand-QD complex, limited only by the availability of spectrally distinct QDs probes.

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