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Delivery of cationic quantum dots using fusogenic liposomes in living cells

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ABSTRACT

Quantum dots (QDs) are fluorescent nanocrystals that present unique optical properties, especially a high photostability. However, their use for intracellular studies is still limited since their passage through the living cell membranes does not occur passively. In this work, we adapted the ethanol injection method to encapsulate cationic hydrophilic QDs into fusogenic liposomes, to deliver them in living cells. Liposomes were characterized using zeta potential, dynamic light scattering (DLS), fluorescence microscopy and transmission electron microscopy (TEM). Red blood cells (RBCs) were applied as models in this study to probe the liposome fusion with the cell membrane since RBCs do not present endocytic activity. Therefore, HeLa cells were also applied to test the QDs delivery by the liposomes. The TEM and the fluorescence microscopy confirmed the QDs encapsulation, with an efficiency of 43%, determined by UV–vis spectroscopy. Zeta potential showed that the QDs-loaded fusogenic liposomes were positively charged and presented an average size of 343 nm, determined by DLS. Furthermore, fluorescence microscopy analyses of RBCs and HeLa cells confirmed the liposomes fusion with the cell membrane and suggested the release of QDs into cells. Thus, we expect that this work will contribute to improve the use of QDs as fluorescent probes to intracellular studies.

1. Introduction

The comprehension of how many intracellular processes occur is a major aim in life sciences. One way to elucidate processes such as, how healthy cells become tumor cells and how they further grow into tumor tissues, or how stem cells perform their roles in therapies, is by using fluorescence assays. Fluorescence-based techniques present high sensitivity and specificity, and also allow to monitor biological events in real time [1]. Emerging materials, which have arisen as new fluorescent probes for cell labeling and biomedical research, are the semiconductor nanocrystals, known as quantum dots (QDs). These nanocrystals present unique optical properties, such as bright fluorescence, low photobleaching rate, wide absorption spectra and narrow emission profile tuned according to their size. Another important characteristic of these materials is their active surface, which allows the binding of QDs with biomolecules or other nanoparticles [2,3].

To take full advantage of these fluorescent probes in intracellular studies, QDs have to be delivered freely into the cytosol, since their passage through the living cell membranes does not occur passively. Their dimension (2–10 nm) is larger than the membrane pores and when cells uptake QDs, they become trapped in endocytic vesicles. Among the methods more applied for QDs intracellular delivery, we can highlight electroporation, microinjection and cell fixation [4,5]. Nevertheless, these techniques are either laborious or can cause considerable cell damage during the experiments.

In this context, the use of fusogenic liposomes has emerged as a promising method to overcome these drawbacks, being vesicles of lipid bilayers that can fuse with cells releasing their contents into the cytosol [6]. Csiszár and collaborators [7] reported liposome formulations that were able to fuse with living cells. They showed that there was required a combination of a lipid associated with a dye, the DOTAP (1,2-dioleoyl-3-trimethylammonium-propane, a positive lipid) and the DOPE (1,2-dielaidoyl-*sn*-glycero-3-phosphoethanolamine, a neutral lipid) to destabilize the membrane and trigger the liposomes fusion with the cells [8].

In the literature, there are few reported methods related to the encapsulation of hydrophilic QDs in liposomes, which use thin film hydration [8–10], reverse evaporation method [11] and detergent dialysis [12]. However, as far as our knowledge goes, these studies showed QDs labeling at membrane level, not delivering the QDs freely into the cytosol [8,9,11,12].

In a previous work, we encapsulated negatively charged CdTe QDs, stabilized with mercaptopropionic acid (MPA), in fusogenic liposomes, using the freeze and thaw method. We were able to deliver the QDs to

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living cells, but there was no release of free QDs into the cytosol, probably due to electrostatic interactions between the cationic lipids (composing the liposomes) and the anionic QDs [13]. Thus, in this work, we encapsulated positively charged CdTe QDs, stabilized with cysteamine, in liposomes to avoid electrostatic interactions. We adapted the ethanol injection method to load hydrophilic QDs into liposomes and deliver them to living cells. This method is not laborious and presents potential to deliver QDs conjugated with biomolecules, since it avoids the freeze and thaw procedure usually applied to improve the encapsulation efficiency. We used red blood cells (RBCs) and HeLa cells (human epithelial cervical carcinoma) to test the liposome-cell interactions.

To our knowledge, this is the first report about the encapsulation of hydrophilic QDs into liposomes using the ethanol injection method, as well as the first one that uses cationic QDs. We hope that this study will help to expand the use of QDs as fluorescent probes to follow and study biochemical processes that occur not only at membrane level but also inside living cells.

2. Materials and Methods

2.1. Materials

Cysteamine (CyA), cadmium perchlorate ($Cd(ClO_4)_2{\cdot}6H_2O$), sodium borohydride ($NaBH_4$), tellurium (Te^0), Dulbecco's modified Eagle's medium with high glucose content (DMEM), streptomycin, penicillin, poly-L-lysine and routine chemicals were purchased from Sigma-Aldrich. Fetal bovine serum (FBS) and Trypsin-EDTA Dissociation Buffer was obtained from Gibco™. The lipids 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), 1,2-dielaidoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (DPPE-Rh) were obtained from Avanti Polar Lipids, and the soybean phosphatidylcholine (PC) was obtained from Lipoid. Lipid stock solutions were prepared in ethanol. All the chemicals used in all experiments were of analytical grade.

2.2. Synthesis and Characterization of CdTe-CyA

CdTe QDs were synthesized in aqueous colloidal dispersion, according to the previously method reported by our research group with modifications [14]. Te^{-2} ions in aqueous solution were added to a Cd $(ClO_4)_2$ solution at pH 5.8 in the presence of CyA as the stabilizing agent, in a molar ratio of 10:1:12 for Cd:Te:CyA, respectively. The reaction proceeded under constant stirring and heating at T ~ 90 °C for 1 h. The Te^{2-} aqueous solution was prepared by reducing metallic tellurium with $NaBH_4$ at a high pH, under nitrogen saturated atmosphere and continuous heating (T ~ 90 °C).

QDs were characterized by electronic UV–vis absorption spectroscopy (Evolution 600, Thermo Scientific) using ultra-pure water as reference, and by emission spectroscopy (LS 55, PerkinElmer), exciting the sample at 365 nm. The average diameter (d) of the QDs was estimated using Dagtepe' equation (Eq. (1)) and the wavelength at the first absorption maximum (λ) [15].

$$d = \frac{(1.\ 38435 - 0.00066\lambda)}{(1 - 0.00121\lambda)} \quad (1)$$

The molar extinction coefficient (ε) was calculated using the Yu' equation (Eq. (2)) [16]. Applying the Beer-Lambert law, it was determined the concentration of the QD' suspension.

$$\varepsilon = 10043 d^{2.12} \quad (2)$$

2.3. Liposomes and QDs-loaded Liposomes – Preparation and Characterization

Three different liposomes were prepared: PC and DOPE:DOTAP, as the non-fusogenic systems, and DOPE:DOTAP:DPPE-Rh as the fusogenic one [7]. Typical 1.3 $mmol{\cdot}L^{-1}$ final phospholipid concentration was used to prepare the vesicles. Molar ratios of 1:0.3 were used for DOPE:DOTAP. The DOPE:DOTAP:DPPE-Rh liposomes were prepared using different molar ratios depending on the experiment, such as 1:0.3:0.05 and 1:0.7:0.05. The two step injection method [17] was applied to prepare either empty or QDs-loaded liposomes.

Firstly, to obtain 1.5 mL of plain liposomes, the amount of 1.5 mL of NaCl (1 $mmol{\cdot}L^{-1}$) was added to a test-tube and then heated up to 55 °C (± 5 °C). Simultaneously, in a second vial glass, the lipid solution was also heated to 55 °C (± 5 °C). The lipid mixture was injected, under vigorous stirring (vortex), into the NaCl solution and the plain liposomes were formed in *ca.* 2 min. The liposomes were prepared at 50–60 °C, above the lipids' glass transition temperature, to achieve a more homogeneous size distribution [18,19].

Using the injection method, the QDs-loaded liposomes were prepared by mixing an aliquot of NaCl (1 $mmol{\cdot}L^{-1}$) and the hydrophilic QDs suspension, 70% and 30% (v/v) respectively. The procedure followed the same steps used for the preparation of plain liposomes, heating the lipid mixture in a vial glass and injecting them into the NaCl-QDs' tube. For preparing the liposomes, QDs were purified to remove precursor residues by using Vivaspin concentrator devices of 10 MWCO (GE Healthcare) by centrifuging the suspension for 2 min at 335 × *g*. This procedure was performed twice. For removing the non-encapsulated QDs, it was used the Amicon® 100 MWCO filter device (Merck Millipore) to centrifuge the liposomes for 2 min at 524 × *g*.

The encapsulation efficiency was estimated by electronic UV–vis spectroscopy, using the DOPE:DOTAP composition, to avoid any eventual superposition of the CdTe-CyA QDs and the DPPE-Rh absorptions. Firstly, it was recorded the absorption profile of freshly prepared DOPE:DOTAP liposomes-QDs system. Then this sample was added to the Amicon® 100 MWCO tube and centrifuged for 2 min at 524 × *g*, as already described above. After each centrifugation, the pellet was separated and the same volume of ultrapure water was added to the liposomes sample, and the process was repeated until complete removal of non-encapsulated QDs. The final point was confirmed by the loss of fluorescence of the pellet, observed under 365 nm light. After this process, the absorption spectrum of the purified QDs-loaded liposomes was recorded and the encapsulation efficiency (EE%) was estimated using Eq. (3):

$$EE(\%) = \frac{A_{\text{purified liposomes}}}{A_{\text{non-purified liposomes}}} \times 100 \quad (3)$$

Plain and QDs-loaded liposomes with the molar ratio of 1:0.3:0.05 for DOPE, DOTAP and DPPE-Rh respectively, were analyzed by fluorescence microscopy (Leica DMI 4000B) and transmission electron microscopy (FEI TECNAI Spirit BioTwin G2). The systems were also analyzed using dynamic light scattering (DLS) and zeta potential measurements (ZetaSizer Nano ZS90, Malvern). For the acquisition of micrographs by fluorescence microscopy, we used the 480/40 nm band pass (BP) filter for exciting the QDs and their fluorescence was collected with a 527/30 nm BP filter. The red emission of liposomes was excited and detected using the excitation and emission BP filters 560/40 nm and 645/75 nm, respectively. For TEM analysis *ca.* 6 μL of the desired liposome was dropped onto 200-mesh holey carbon-coated copper grids and placed under Osmium steam for 1 min.

2.4. DOTAP Assays

Highly positively charged liposomes can be harmful to live cells. Due to the high charge difference between these vesicles and cell