

Liposome Encapsulation of Fluorescent Nanoparticles: Quantum Dots and Silica Nanoparticles

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ABSTRACT

Quantum dots (QDs) and silica nanoparticles (SNs) are new classes of fluorescent probes that overcome the limitations encountered by organic fluorophores in bioassay and biological imaging applications. We encapsulated QDs and SNs into liposomes and separated nanoparticle-loaded liposomes from unencapsulated nanoparticles by size exclusion chromatography. Fluorescence correlation spectroscopy was used to measure the average number of nanoparticles inside each liposome. Results indicated that nanoparticle-loaded liposomes were formed and separated from unencapsulated nanoparticles by using Sepharose gel. As expected, fluorescence self-quenching of nanoparticles inside liposomes was not observed. Each liposome encapsulated an average of three QDs. Our work demonstrated that nanoparticles could be successfully encapsulated into liposomes and provided a methodology to measure the nanoparticle quantity inside each liposome with fluorescence correlation spectroscopy.

Keywords: liposomes, nanoencapsulation, quantum dots, silica nanoparticles

1 INTRODUCTION

Fluorescent probes for biomolecular recognition have been widely used in bioassays and biological imaging [1,2]. However, organic fluorophores have characteristics that limit their effectiveness for these applications, including poor photostability, low brightness, and limited ability for multiplexed analysis [3,4]. QDs and SNs are new classes of fluorescent probes that have the potential to overcome these limitations. QDs (fluorescent semiconductor nanocrystals) have broad excitation and size-dependent, tunable, narrow-emission spectra that allow simultaneous excitation of several different-colored QDs at a single wavelength with little spectral emission overlap for multianalyte analysis [5-7]. Also, QDs are about 20 times brighter and 100 times more photostable in comparison with organic dyes such as rhodamine [8]. Fluorescent SNs are synthesized with a sol-gel technique in which organic dyes are covalently attached to the silica precursor [9,10]. They are brighter and more photostable than free organic dyes [10].

Liposomes are spherical vesicles consisting of phospholipid bilayers surrounding an aqueous cavity. Because dye-loaded liposomes provide greatly enhanced signals and their bilayer membrane can protect the dye from potentially quenching species [11], liposomes have been successfully used as reporter particles in bioassays [12-14]. Several researchers reported that encapsulation of solid particles into liposomes enhanced the overall mechanical strength of the liposome structure [11,15,16]. Therefore, fluorescent nanoparticle-loaded liposomes have the potential to provide not only better fluorescent characteristics but also better mechanical stability than organic dye-loaded liposomes. In addition, the biomimetic lipid bilayers of liposomes provide high biocompatibility [17-19], thereby enhancing the effectiveness of fluorescent nanoparticles on biological detection *in vitro* and *in vivo*.

In this study, we encapsulated QDs and SNs into liposomes by the reverse-phase evaporation method. Nanoparticle-loaded liposomes were separated from unencapsulated nanoparticles by size-exclusion chromatography and their characteristics were investigated. Dual-color, two-photon fluorescence correlation spectroscopy was used to determine the number of nanoparticles inside each liposome.

2 EXPERIMENTAL SECTION

2.1 Reagents

Common laboratory reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). Sepharose CL-2B and Sepharose CL-4B were purchased from Sigma-Aldrich Co. (St. Louis, MO). Dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylglycerol (DPPG), dipalmitoylphosphatidylethanolamine-N-(biotin) (N-biotinyl-DPPE), lissamine rhodamine B-DPPE, nitrobenzoxadiazol-DPPE and polycarbonate syringe filters of 0.4 and 0.2 μm pore sizes were purchased from Avanti Polar Lipids (Alabaster, AL). Quantum dots (EviTags) were purchased from Evident Technologies (Troy, NY). Silica nanoparticles were kindly provided by Hooisweng Ow and Ulrich Wiesner, Cornell University, Ithaca, NY.

2.2 Encapsulant Preparation

A 0.8 μ M QD solution and a 122 nM SN solution were each prepared in HEPES buffer (0.01 M, pH 7.5, 0.2 M NaCl, 0.01% sodium azide). Sucrose was used to adjust the osmolality to 445 mOsmol/kg.

2.3 Preparation of Nanoparticle-Loaded Liposomes

Liposomes were prepared using a modified version of the reverse-phase evaporation method described by Siebert et al. [20]. 45 μ moles DPPC, 5 μ moles DPPG, 5 μ moles N-biotinyl-DPPE, and 45 μ moles cholesterol were dissolved in 3 ml of a chloroform/methanol solution (volume ratio, 5:1). While sonicating the suspension at 45 $^{\circ}$ C, 0.6 ml of encapsulant was added. Using a vacuum rotary evaporator, the organic solvent was removed. Then, an additional 0.4 ml of encapsulant was added and the vacuum rotary evaporator was applied again. After the liposomes were formed, they were incubated for 30 min at 45 $^{\circ}$ C and, finally, extruded 30 times through polycarbonate syringe filters with 0.4 μ m pore size. Samples for fluorescence correlation spectroscopy were extruded with 0.4 and 0.2 μ m pore size in series.

2.4 Size Exclusion Chromatography of Liposomes

Unencapsulated QDs were separated from the liposomes by size exclusion chromatography using Sepharose CL-2B column (25 x 1.5 cm). HEPES buffer (0.01 M, pH 7.5) was used as the eluent containing 0.2 M NaCl and 0.01% sodium azide. Sucrose was used to adjust the osmolality to 515 mOsmol/kg. The flow rate was controlled at 25.1 ml/h. The eluted liposomes were collected at 1 ml/tube by a Retriever 500 fraction collector (ISCO, Lincoln, NE), followed by fluorescence measurement using a RF-551 spectrofluorometric detector (Shimadzu, Kyoto, Japan). Unencapsulated SNs were separated from the liposomes by size exclusion chromatography using Sepharose CL-4B column (25 x 1.5 cm). HEPES buffer (0.01 M, pH 7.5) was used as the eluent containing 0.2 M NaCl and 0.01% sodium azide. Sucrose was used to adjust the osmolality to 515 mOsmol/kg. The flow rate was controlled at 45.2 ml/h. The eluted liposomes were collected at 1.5 ml/tube by a Retriever 500 fraction collector, followed by fluorescence measurement using a RF-551 spectrofluorometric detector.

2.5 Measurement of the Average Number of Nanoparticles inside Each Liposome

We used two-photon fluorescence correlation spectroscopy (FCS, DRBIO Webb Research Group, Cornell University, Ithaca) to measure the number of fluorescent particles in the focal volume, thereby calculating the sample

concentration [21]. Dual-color, cross-correlation experiments were done to measure the intact liposome concentration and nanoparticle concentration released by the liposome lysis, enabling measurements of the nanoparticle quantity inside each liposome. We incorporated 0.4 mol% red lissamine rhodamine B (LRB)-DPPE into the liposome bilayers for green QD encapsulation and 0.4 mol% green nitrobenzoxadiazol-DPPE for red SN encapsulation in the preparation of nanoparticle-loaded liposomes described above. We lysed liposomes by adding 495 μ L of 30 mM n-OG to 5 μ L liposome solution.

3 RESULTS AND DISCUSSION

3.1 Size Exclusion Chromatography (SEC) of QD-Loaded Liposomes

SEC is mainly affected by particle sizes and shapes. The sizes of QDs and liposomes are approximately 50 nm and 300 nm, respectively. We separated unencapsulated QDs from the liposomes by SEC using Sepharose CL-2B. Figure 1 shows that QD-loaded liposomes were formed and separated by Sepharose CL-2B. Because QD-loaded liposomes contained fluorescent QDs, they produced higher fluorescence intensity than the empty (buffer-loaded) liposomes. Empty liposomes were detectable by fluorescence based on the property of large liposomes to scatter light, the degree of which can be detected and measured. The recovery of QDs from the column was poor due to the adsorption of some QDs to the top of the column matrix. Reynolds et al. also reported this problem while separating polystyrene beads using a size-exclusion column[22].

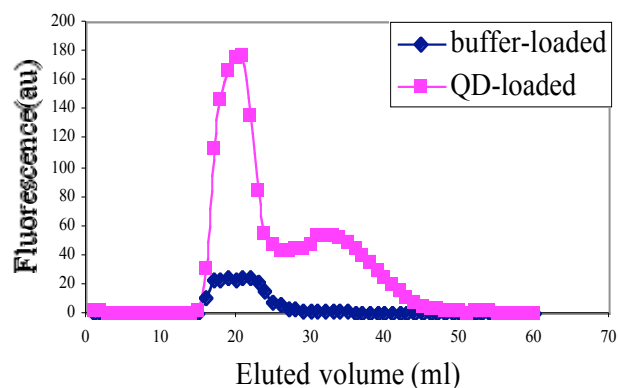


Figure 1. Elution profiles on Sepharose CL-2B of buffer-loaded liposomes and QD-loaded liposomes detected by fluorescence. The first peak at 20 ml corresponds to the eluted liposomes and the second broad peak is the unencapsulated QDs.

3.2 Size Exclusion Chromatography of SN-Loaded Liposomes

Because of the size difference between SNs (~20 nm) and liposomes (~575 nm), we also used SEC to separate unencapsulated SNs from SN-loaded liposomes. Instead of Sepharose CL-2B, we used Sepharose CL-4B, which has smaller pore sizes because SNs are smaller than QDs. The elution profile of SN-loaded liposomes plotted in Figure 2 exhibits two distinct peaks at respective elution volumes of 16 and 32 ml, which correspond to liposome-entrapped and free SNs. This also demonstrated that SN-loaded liposomes were formed and separated well by Sepharose CL-4B. Like QDs, the recovery of untrapped SNs from the column was also poor, thereby producing little fluorescence in Figure 2.

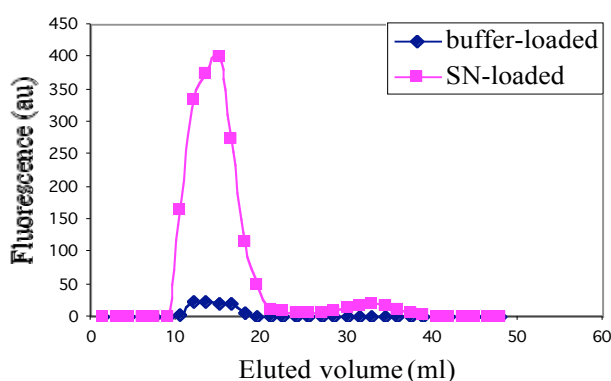


Figure 2. Elution profiles on Sepharose CL-4B of buffer-loaded and SN-loaded liposomes detected by fluorescence.

3.3 Characterization of Nanoparticle-Loaded Liposomes

Although both QD-loaded and SN-loaded liposomes were extruded through polycarbonate syringe filters with 0.4 μm pore size, the mean diameter of QD-loaded liposomes was measured by a particle size analyzer to be 291 nm, while that of SN-loaded liposomes was 575 nm. It seems that the size of nanoparticle-loaded liposomes cannot be controlled as precisely as organic dye-loaded liposomes by the extrusion method. It is possible that because nanoparticles are much larger than organic dyes, they exert a greater effect on the liposome size during the extrusion.

The absorption and emission spectra of both types of nanoparticle-loaded liposomes were measured by a spectrophotometer and spectrofluorometer, respectively. Since liposomes scatter light, their absorption spectrum is just a measure of scattered light. The shorter the wavelength, the higher the intensity. The absorption intensity of liposomes, therefore, increased with decreasing wavelength. Diederichs also reported similar properties of the absorption spectrum of liposomes [23]. On the other hand, liposome-entrapped nanoparticles retained similar fluorescent emission spectra to free nanoparticles.

While the fluorescence of organic dyes in the liposomes is self-quenched, liposome-encapsulated nanoparticles produced a relatively high signal. We were interested in comparing the fluorescence of intact vs. lysed nanoparticle-loaded liposomes. Our results showed no significant fluorescence difference between them (data not shown). This is a great benefit for bioassays since liposome lysis will not be needed for fluorescence measurement. Also, fluorescent signals can be detected when liposome lysis is not feasible, such as in a test strip assay. Conventionally, the signal of organic dye-loaded liposomes on the test strip can only be read by color intensity [13,14]. Due to the absence of self-quenching in nanoparticle-loaded liposomes, the fluorescence intensity in a test strip assay can be detected, thereby providing much higher sensitivity.

3.4 Number of Nanoparticles inside Each Liposome Based on FCS

FCS is a technique that analyzes fluorescence intensity fluctuations arising from molecules diffusing in and out of a microscopic detection volume of about 10^{-15} liter defined by a tightly focused laser beam [24,25]. In dual-color, cross-correlation FCS, the fluorescence signals from the two fluorophores are recorded simultaneously, and the fluctuations in the fluorescence signal of one fluorophore are correlated with those of the other fluorophore [26]. If fluorescent nanoparticles are encapsulated inside dye-labeled liposomes, they will pass through the FCS detection volume together, resulting in coincident fluctuations in both detector channels, which can be detected by cross-correlation analysis of fluctuations in the two channels.

The green curve in Figure 3 shows the cross-correlation between QDs and liposomes in the intact liposome sample, which indicates that QDs were encapsulated inside the liposomes. After liposome lysis, cross-correlation amplitude was observed to be zero due to the dissociation between QDs and lysed liposomes.

For measuring the quantity of QD-loaded liposomes and released QDs after liposome lysis, autocorrelation analysis was used. The amplitude of the autocorrelation curve at $\tau=0$ is inversely proportional to the average number of fluorescent molecules in the detection volume. This provides a direct measure of the concentration [27]. Figure 4 shows the autocorrelation curves of QD-loaded liposomes and released QDs in the same concentration of liposomes. The concentrations of QD-loaded liposomes and released QDs were 0.32 and 0.94 nM, respectively. Hence, we calculated that each liposome contained an average of three QDs. Unfortunately, we could not measure the SN quantity inside each liposome due to the spectral overlap between SNs and nitrobenzoxadiazol-liposomes.

Although liposomes did not provide much signal amplification due to the small number of QDs encapsulated, they still provided protection and biocompatibility for QDs during biological detection in vitro and in vivo. In addition, the low number of encapsulated QDs can be explained by

the large size (~50 nm diameter) and low concentration (0.8 μ M) of QDs solution (encapsulant) for liposome preparation.

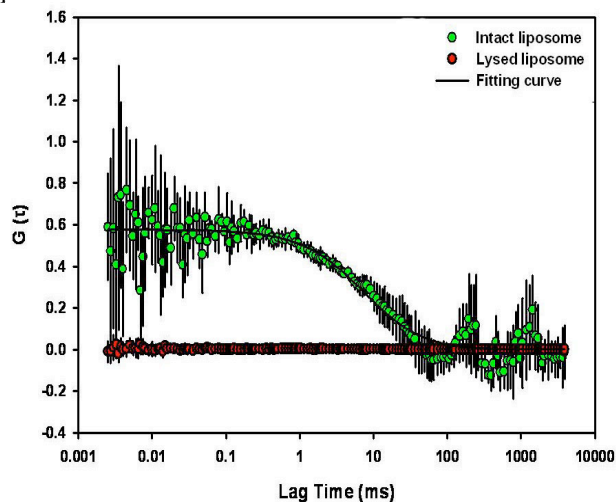


Figure 3. Cross-correlation curves of intact and lysed QD-loaded liposomes.

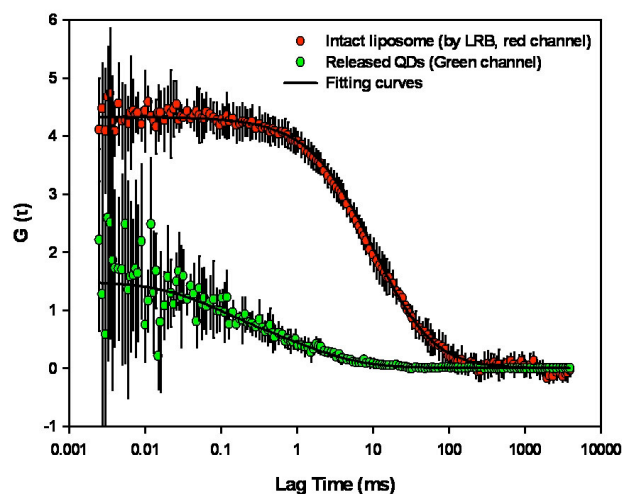


Figure 4. Autocorrelation curves of intact QD-loaded liposomes and released QDs after liposome lysis.

4 CONCLUSION

We successfully encapsulated QDs and SNs into liposomes. No self-quenching effect was observed for either QD-loaded or SN-loaded liposomes. When a 0.8 μ M QD solution was used for liposome preparation, each QD-loaded liposome contained an average of three QDs based on dual-color fluorescence correlation spectroscopy. In the future, smaller and more highly concentrated QDs will be used for liposome encapsulation to increase the quantity of quantum dots inside each liposome.

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