Preparation of Gel-Liposome Nanoparticles for Drug Delivery Applications*

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***Abstract*— Liposomes are amongst the most effective delivery vehicles developed to date. Despite many advantages including biocompatibility, biodegradability, and the ability to carry both hydrophilic and lipophilic compounds, liposomes suffer from low physical stability. This limitation can be effectively addressed by inclusion of a polymeric scaffold within the core of liposomes. Given the versatility of poly(ethylene glycol) (PEG) hydrogels, these polymers have a great potential for the use in liposomal core. As a step towards the development of a robust liposomal delivery platform, here we aim to develop a simple and reliable technique for the fabrication of liposomes with PEG gel cores. We assess the resultant nanoparticles using scanning electron microscopy and dynamic light scattering and demonstrate that the presented approach can successfully produce gel-liposome nanoparticles with spherical shape and 150-200 nm size. These nanoparticles are further evaluated for colloidal stability in physiological solution. Moreover, we demonstrate the versatility of this method by studying the effect of changing (A) the membrane composition in liposomes, and (B) the hydrogel concentration in liposomal core, on the formation of gel-liposome particles.**

I. Introduction

Liposomes are one of the most promising nano-carriers used for delivery of therapeutics to date. Liposome is a spherical vesicle consisting of a thin and flexible lipid bilayer [1]. These lipid vesicles can serve as versatile platforms for drug and gene delivery, as they can encapsulate both hydrophilic and hydrophobic substances [1, 2]. The hydrophilic compounds fit in the liposomal aqueous core while the hydrophobic ones fit within their lipid membrane. In addition, these vesicles are nontoxic, biocompatible, and biodegradable, which has made them particularly popular for delivery purposes [1, 3].

Despite the numerous advantages of liposomes as delivery vehicles, their low physical stability limits their success [2, 4]. One of the most promising approaches used to address this limitation is to include a polymeric scaffold in the liposomal core to provide mechanical support for the lipid membrane and thus, enhance its physical stability. Examples of polymeric materials used for this purpose include poly(lactic-co-glycolic) acid (PLGA) [5], poly(acrylic acid) (PAA) [6], and alginate [7]. A versatile synthetic polymer with great potential for this application is poly(ethylene glycol) (PEG). PEG hydrogels are widely applied for biomedical applications such as tissue engineering and drug delivery as they have tunable physical and chemical properties [8]. For instance, nanoparticles of a photo-crosslinkable PEG hydrogel, PEG-diacrylate (PEG-DA), has been utilized to study the significance of particles' mechanical stiffness for their delivery performance [9]. In this hydrogel, the presence of diacrylate groups on PEG chains results in crosslinking of the polymer chains in the presence of a photoinitiator upon exposure to UV [10]. PEG-DA hydrogel can thus, serve as an effective scaffold within the liposomes, creating an attractive platform for delivery purposes.

The aim of this study was to develop a facile and robust method for fabrication of liposomes with PEG-DA hydrogel in their cores. To this end, we designed and optimized a simple method for the preparation of gel-filled liposomes and assessed this method's reliability and versatility by examining the effect of variations in liposomal membrane composition and the PEG-DA core on the formation of gel-liposome nanoparticles.

II. Materials and Methods

A. Materials

L-α-phosphatidylcholine (Egg, Chicken) and 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) were purchased from Avanti Polar Lipids, Inc (Alabaster, AL). Cholesterol, poly(ethylene glycol) diacrylate (PEG-DA) with an average molecular weight of 700 Da, 2-hydroxy-2-methylpropiophenone (photoinitiator), potassium chloride, sodium phosphate dibasic, and potassium phosphate monobasic were purchased from Sigma-Aldrich (St. Louis, MO). Chloroform and centrifugal filter tubes (MWCO 10K) were purchased from Millipore Sigma (Burlington, MA). Triton X-100 and sodium chloride were purchased from VWR chemicals (Solon, OH). Dialysis membrane (MWCO 3.5-5 kD) was purchased from Spectrum laboratories, Inc (ancho Dominguez, CA).

B. Preparation of Gel-Liposome Nanoparticles

In this study, we attempted to fabricate PEG-DA gel-liposomes via two different methods. In the first method, a mixture of POPC and cholesterol solution in a molar ratio of 7:3 was poured into a round-bottom flask and dried under vacuum using rotary evaporator for 2 hr. Then, the dried lipid

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film was hydrated by the hydrogel precursor solution including PEG-DA, 2-hydroxy-2-methylpropiophenone (photoinitiator), and distilled (DI) water, at a volume ratio of 30:1:69. Upon bath sonication for 10 min, the solution was extruded 25 times through a polycarbonate porous membrane with 100 nm pore size using an Avanti miniextruder (Avanti Polar Lipids). This process results in the formation of small unilamellar liposomes. In order to prevent macrogelation outside of the liposomes, the solution was either dialyzed through a porous membrane with 3.5-5 kDa cut off for 6 hr, or diluted to eliminate the non-encapsulated PEG-DA and photoinitiators. At the end, half of the solution was exposed to UV to form crosslinked PEG-DA gel inside the liposomes and the rest was used as the control sample. To confirm the formation of gel core within liposomes, Triton X-100 was added to the sample to solubilize the liposomal lipid membrane and the samples were characterized by DLS (before and after treatment).

In the other method, a mixture of POPC and cholesterol solution at a molar ratio of 9:1 was used. In this method, the uniform lipid film was hydrated by PEG-DA solution with a concentration of 30% or 20% (v/v) without the photoinitiator. After extrusion, the sample was washed in filter tube via centrifugation to remove the non-encapsulated PEG-DA molecules. Next, the photoinitiator (2-hydroxy-2-methylpropiophenone) with a total concentration of 1% (v/v) was added to the solution. The sample then went through five freeze-thaw cycles between zero and 37 °C. This process was used to enhance the inward transport of the photoinitiator across the liposomal membrane. At the end, the non-encapsulated photoinitiator molecules were removed via centrifugation in filter tubes and the obtained liposomes were re-suspended in DI water. After characterization of the particles, Triton X-100 was added to the solutions to confirm the formation of gel in the liposome core.

C. Size Distribution

Particle size distribution was measured by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) at 20 °C. For each measurement, at least three replicates were used to calculate the average of the particle sizes.

D. Scanning Electron Microscopy

Nanoparticles were spread on a clean silicon wafer using a spin coater (Brewer Cee 200 Spin Coater) and dried on a hot plate for ~30 min. After gold sputter coating of the sample, scanning electron microscopy (SEM) (FEI 235 Dual-Beam Focused Ion-beam) at 3 kV was used to visualize and evaluate the prepared gel-liposome nanoparticles. The nanoparticle formulation used for microscopy was composed of a POPC/cholesterol (at ratio of 9:1) membrane and a 30% (v/v) PEG-DA gel core treated by Triton X-100.

E. Colloidal Stability

To examine the stability of the produced gel-liposome particles, the liposomes containing 30% (v/v) PEG-DA hydrogel (UV-exposed) was re-suspended in PBS (phosphate-buffered saline) after the last centrifugal washing step and incubated at 37 °C. To monitor the particle size changes by time, the size distribution of the particles was measured by DLS for three days.

III. Result and Discussion

A. Method Optimization and Characterization of Gel-Liposome Nanoparticles

To prepare gel-liposome particles, we modified a simple method of dehydration-rehydration of lipid film that is commonly applied for the preparation of liposomes [11]. In this modified protocol, first a thin dried film of desired lipid composition is formed and is rehydrated using an aqueous solution of hydrogel precursors. The resultant mixture is then extruded through a membrane with nano-scale pores, resulting in the formation of nano-sized liposomes that contain gel precursors. Upon removal of the non-trapped hydrogel precursors (to prevent gel formation outside of liposomes), the liposomes are exposed to UV to crosslink the hydrogel in their core. As described below, we optimized this protocol to enable production of gel-liposome nanoparticles in a reproducible manner.

At first, a POPC/cholesterol lipid film was prepared and hydrated with a solution of hydrogel precursors comprising PEG-DA polymer, photoinitiator, and DI water at a volume ratio of 30:1:69. After the formation of nano-liposomes by extrusion, we removed the non-encapsulated PEG-DA and photoinitiator molecules using dialysis. Then, half of the sample was exposed to UV light to crosslink the polymers encapsulated in the liposomes, while the other half was used as the control (i.e. liposomes with non-crosslinked gel core). In order to confirm the formation of hydrogel inside the liposomes, we treated the gel-liposome particles with Triton X-100 to solubilize the lipid bilayers and utilized DLS to compare the size of these particles before and after the treatment. We anticipated that upon Triton X-100 treatment, in the UV-exposed sample, the DLS peak corresponding to the gel-liposome particles would only have a slight shift to left (as the bare gel is expected to have a smaller size than membrane-coated gel) and that a new peak corresponding to the detergent micelles (~10 nm) [12] will appear. In contrast, we expected that in the control sample, the DLS peak of gel-liposome particles would only be present before Triton X-100 treatment (as the entrapped polymer chains are not cross-linked) and that the only peak present after the detergent treatment would be that of the detergent micelles. However, as shown in Figure 1A-D, DLS results before and after the Triton X-100 treatment showed no evidence for the presence of crosslinked gel core in liposomes that were prepared with the dialysis-containing protocol. Considering that the molecular weight of the photoinitiator (164 Da) is much lower than the PEG-DA molecules (700 Da), we attributed this result to the escape of the encapsulated photoinitiator molecules from the liposome interior during several hours of dialysis. Under such condition, the PEG-DA molecules in liposomes could not be crosslinked upon UV irradiation.

Therefore, we hypothesized that an alternative method for removing the non-trapped hydrogel precursors from outside of liposomes would minimize the escape of photoinitiator from the liposomal core and will, thus enable proper gel formation inside the liposomes. To test this hypothesis, we first explored the dilution technique to prevent macrogelation

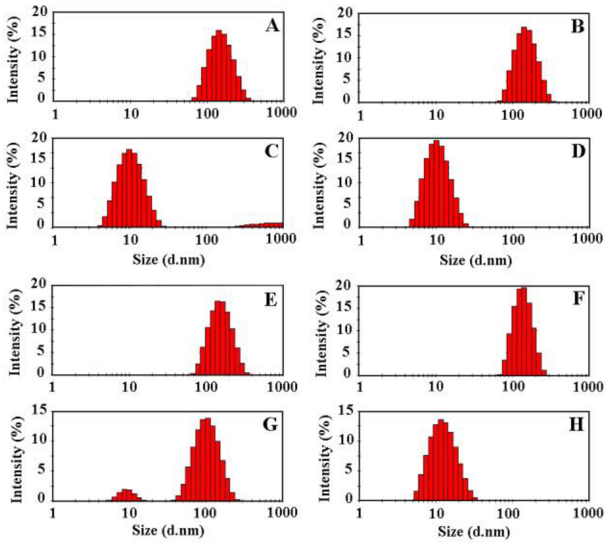

Figure 1. Size distribution of nanoparticles prepared via two described methods. (A-D) DLS data showing the particle size of UV-exposed and control samples prepared by the dialysis-containing protocol, before adding Triton-X 100 (A, B), and after Triton-X 100 treatment (C, D). (E-H) DLS data showing the particle size of UV-exposed and control samples prepared via centrifugation-containing protocol, before adding Triton-X 100 (E, F), and after Triton-X 100 treatment (G, H).

outside of liposomes. While this approach led to a higher probability of gel formation within the liposomes, its results were inconsistent (data not shown). Next, we utilized centrifugation to wash the samples as an alternative for dialysis and slightly modified the fabrication protocol as outlined below. In this approach, the lipid film was hydrated with a PEG-DA solution without the photoinitiator, and upon extrusion, the sample was washed through filter tubes via centrifugation to eliminate the non-trapped PEG-DA molecules (to prevent gelation outside of liposomes). We then added the photoinitiator to the sample followed by freeze-thaw cycling to facilitate transport of the photoinitiator into the liposomes. Next, the sample was exposed to UV to crosslink the polymer chains encapsulated in the liposomes, forming a gel core. Lastly, the sample was washed by centrifugation to remove the photoinitiator molecules remaining outside of the liposomes. As illustrated in Figure 1E-H, DLS results confirmed that this approach was indeed successful in preparing gel-liposome nanoparticles. As depicted in Figure 1G, in the UV-exposed sample, after Triton X-100 treatment DLS showed two peaks; a large peak of ~150 nm corresponding to the PEG-DA nano-gels and another peak of ~10 nm corresponding to the micelles. However, after detergent treatment of the control sample (not exposed to UV), the DLS peak corresponding to liposomes (~150 nm) disappeared and only one peak of ~10 nm was observed (Figure 1H), which was attributed to the micelle formation of the Triton X-100 and the solubilized lipids [13]. These results confirmed the formation of a hydrogel within the core of these liposomes, demonstrating that the presented method can effectively create gel-liposome nanoparticles.

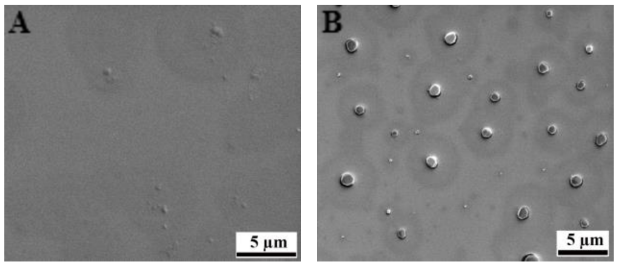

Figure 2. Representative SEM images of gel-liposome nanoparticles after Triton X-100 treatment in (A) control (non UV–exposed), and (B) UV–exposed samples.

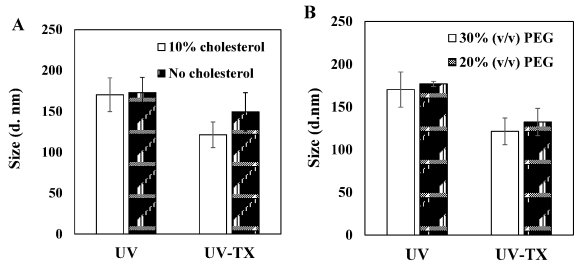

Figure 3. Particle size in UV-exposed and control samples for evaluation of (A) lipid membrane composition and (B) PEG-DA concentration. Bars represent the mean and error bars show the standard deviation.

B. Scanning Electron Microscopy

To further confirm the formation of gel-liposome nanoparticles, we applied SEM to visualize both control (i.e. not exposed to UV) and UV-exposed samples after the treatment with Triton X-100. As illustrated in Figure 2A, no nanoparticle was observed in the control sample while the UV-exposed sample (Figure 2B) contained a number of particles, presumably the hydrogel core of liposomes. These images provided direct evidence for the gel formation within the liposomal core.

C. Effect of Lipid Membrane Composition

In order to assess the versatility of the present method for fabrication of gel-liposome nanoparticles, we next examined the effect of membrane lipid composition on the formation of these structures. To this end, we prepared samples with and without cholesterol and compared the produced gel-liposome nanoparticles. Cholesterol is an important part of natural membranes and is thus, often used in the preparation of liposomes. The relatively rigid sterol ring structure of the cholesterol enhances the stability and rigidity of the lipid bilayer [14]. As shown in Figure 3A, gel formation was evident in both 0 and 10% cholesterol-containing liposomes as the Triton X-100 treatment led to a relatively small reduction in size of these nanoparticles, presumably due to the elimination of lipid membrane. Given the known effect of cholesterol in improving the membrane integrity [14], liposomes with 0% cholesterol were probably more leaky, which could result in the escape of hydrogel precursors, particularly the photoinitiator molecules, from these liposomes. However, in the present method, the photoinitiator is added to the sample after the washing process and shortly

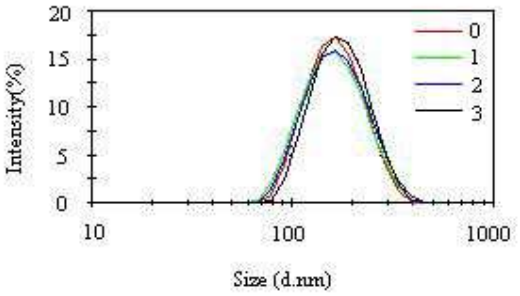

Figure 4. Size distribution of the gel-liposome nanoparticles measured by time (after 0, 1, 2, and 3 days).

before the UV exposure and thus, the leakiness of the liposomes does not have a significant effect on the gel formation in liposomal core. This result demonstrated the versatility and reliability of the present approach for the preparation of gel-liposome particles for drug delivery applications.

D. Effect of PEG-DA Concentration

To further evaluate the flexibility of the present fabrication technique, we next tested the effect of PEG-DA concentration used in the preparation protocol. Varying the PEG-DA concentration is an easy way to tune the properties of this hydrogel [9]. For these experiments, we prepared the gel-liposome nanoparticles with two different concentrations of PEG-DA, 20 and 30% (v/v) and measured size of the particles using DLS. As presented in Figure 3B, evaluating these samples using DLS showed that gel formation occurred in both formulations upon UV irradiation.

E. Colloidal Stability

Stability of nanoparticles is one of the most critical factors which determines their shelf life, safety, and efficiency especially for drug-loaded particles [13]. In order to evaluate the colloidal stability of gel-liposome nanoparticles, we prepared a sample formulated with 10% cholesterol and 30% (v/v) PEG-DA in PBS incubated at 37^{o} C and monitored the particles' size distribution over few days. As depicted in Figure 4, the mean size of the particles did not change significantly within three days. Therefore, the prepared gel-liposome particles exhibited a good colloidal stability, which is appealing for their future use as drug carriers.

IV. CONCLUSION

This study describes a simple and reliable method for the preparation of liposomes with PEG hydrogel cores, towards the development of a robust and versatile liposomal delivery platform. Using this method, we fabricated gel-liposome nanoparticles of 150-200 nm size. Through removal of the lipid membrane surrounding the nano-gels, we were able to provide direct evidence for the formation of a gel within the liposomes using DLS analysis and SEM imaging. Furthermore, we demonstrated the versatility of this method by showing its ability to form gel-liposome nanoparticles of various lipid membrane compositions and hydrogel concentrations. The resultant gel-liposome nanoparticles exhibited good colloidal stability in physiological solution, further showing their great potential for the use as delivery vehicles.

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