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Surfactant-stable and pH-sensitive liposomes coated with *N*-succinyl-chitosan and chitooligosaccharide for delivery of quercetin

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ABSTRACT

Layer-by-layer (LbL) self-assembly of multilayered liposomes is used to improve the stability of conventional liposomes. In this study, the LbL technology was used to prepare novel multilayered liposomes from chitooligosaccharide and *N*-succinyl-chitosan. We propose that this preparation can be used as a transdermal drug delivery system (TDDS) to enhance stability against surfactants and control drug release. Particle size increased with the number of layers in the multilayer and the zeta potential varied between positive and negative values with alternate deposition of polyelectrolytes. Finally, approximately 300–400 nm-thick four-layered liposomes were prepared. These liposomes were more stable against surfactants and showed a relatively high release of quercetin at pH 5.5 than the uncoated liposomes as assessed via *in vitro* drug release and skin permeation studies. In summary, the multilayered liposomes showed potential for use as a surfactant-stable TDDS that effectively enhanced the permeation of quercetin, a poorly soluble drug, into the skin.

1. Introduction

The skin is the largest organ and is the first line of defense against external stimuli and dryness. It largely comprises the epidermis, dermis, and subcutaneous fat, and the stratum corneum of the epidermis forms its outermost part. The stratum corneum consists of keratinocytes and desmosomes connecting the keratinocytes to keratinocyte lipids, and acts as a barrier to external factors (Michaels, Chandrasekaran, & Shaw, 1975). However, the barrier function of the skin limits the entry of active substances inside the body, such as those with antioxidant and anti-aging activity. Therefore, studies on transdermal drug delivery system (TDDS) have been conducted with the goal of achieving efficient penetration of the skin barrier. Among these, liposomes are biocompatible spherical carriers with amphiphilic phospholipid bilayers that are capable of harboring hydrophobic and hydrophilic materials and can be produced at the nanoscale. Hence, liposomes are widely used in medicine and cosmetics (Elsayed, Abdallah, Naggar, & Khalafallah, 2007; Touitou, Dayan, Bergelson, Godin, & Eliaz, 2000). However, the surfactants present in cosmetics can disrupt and solubilize lipid membranes, thereby affecting the stability of the products (Cho, 2011). Several studies have attempted to circumvent these difficulties. The LbL technology, a method that enhances liposome stability, involves coating liposome surfaces with multiple layers on the basis of the electrostatic attraction between polymer electrolytes of opposite charge. For example, liposomes have been coated with chitosan and alginic acid (Liu, Liu, Liu, Li, & Liu, 2013) and albumin and chitosan oligosaccharide lactate (Liao, Lu, Lin, Chen, Sytwu, & Wang, 2016), and multilayer liposomes have been generated using poly(ethylene glycol)-block-poly (L-aspartic acid) and poly-L-lysine (Ramasamy et al., 2014) or poly(acrylic acid) and poly(allylamine hydrochloride) (Jain, Patil, Swarnakar, & Agrawal, 2012). We conducted studies using resveratrol-loaded chitosan-coated liposomes in 2014 (Park, Jo, & Jeon, 2014), quercetin-loaded multi-layered liposomes of hyaluronic acid and chitosan in 2015 (Jeon, Yoo, & Park, 2015), and a novel capsosome with multilayered liposomes of hyaluronic acid and chitosan in 2016 (Yoo, Seong, & Park, 2016). However, multilayered liposomes using chitosan and derivatives of chitosan have not yet been investigated, and studies on multilayered liposomes of higher stability and better skin permeability have not yet been conducted. In the present study, we intended to use *N*-succinyl-chitosan (NSC), a well-known pH-sensitive polymer electrolyte as a coating around liposomes to study the formulation's pH-sensitive drug release under skin pH conditions. NSC is synthesized by attaching a succinyl group to the amine group of chitosan, which improves the otherwise low solubility of chitosan in water. NSC is a biocompatible and safe material for the human body, and is made available to a pH-sensitive polymer by controlling the degree of succinylation (Bashir, Teo, Ramesh, Ramesh, & Khan, 2015). Studies on NSC include preparation of siRNA–loaded micelles using copolymers of NSC, poly L-

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lysine, and palmitic acid (Zhang et al., 2016), pH-sensitive gels (Mukhopadhyay, Sarkar, Bhattacharya, Bhattacharyya, Mishra, & Kundu, 2014), resin combined with Pb(II) (Sun & Wang, 2006), and coated films of chitosan derivatives containing NSC on a flat surface (Graisuwan et al., 2012). However, NSC-coated liposomes have not yet been studied. The NSC synthesized by Yan et al. in 2006 had zero water solubility at pH 5–6 and its zeta potential value is close to 0 mV (Yan, Chen, Gu, Hu, Zhao, & Qiao, 2006). However, NSC dissolves in water and has a negative zeta potential under neutral or basic conditions. In the present study, we used NSC as an anionic polymer electrolyte for liposome coating, and chitooligosaccharide (COS), which has a low molecular weight because of acid or enzyme-mediated decomposition and was used as a cationic polymer electrolyte in previous studies, was used to improve the water solubility of chitosan. In this study, the adsorption of polyelectrolytes and liposomes on the 2D plane and the viscoelastic properties of the prepared films were analyzed using quartz crystal microbalance with dissipation monitoring (QCM-D). Multilayered liposomes carrying quercetin, a hydrophobic flavonoid, were prepared and their physical properties were determined by analyzing particle size and zeta potential. We prepared multilayered liposomes for improving liposome stability, which was evaluated using Triton X-100 as an external stimulus. The release behaviors of quercetin from multilayered liposomes in neutral condition (pH 7.4) and at a normal skin pH (pH 5.5) were assessed. Additionally, *in vitro* skin permeability using Franz diffusion cells was analyzed to determine the possibility of using multilayer liposomes as a transdermal delivery system.

2. Materials and methods

2.1. Materials

L-α-phosphatidylcholine (from egg yolk, ≥60%, egg PC), Triton X-100 (BioXtra, USA), quercetin, chitosan (low molecular weight, 75–85% deacetylated, MW 50,000–190,000 Da, CH), and succinic anhydride were purchased from Sigma-Aldrich (USA). Chitooligosaccharide (90% deacetylated, MW 800–3000 Da, COS) was obtained from Bioland Co. Ltd. (Korea). Chemicals 1, 3-butylene glycol (1, 3-BG), acetone, dimethyl sulfoxide (DMSO), methanol, ethanol, chloroform, and sodium hydroxide were of analytical grade.

2.2. Synthesis and analysis of N-succinyl-chitosan

2.2.1. Synthesis of N-succinyl-chitosan

One gram of chitosan was dissolved in 20 mL DMSO, and 1 g of succinic anhydride was added thereto, followed by stirring at 500 rpm for 4 h while maintaining the temperature at 60 °C; the pH of the mixture was adjusted to 5 with 5% (w/v) NaOH. The resulting precipitate was collected and dispersed in 50 mL H_2O. The pH was adjusted to 10–12 with 5% (w/v) NaOH to form a yellow solution. Subsequently, this solution was recrystallized from acetone to form NSC, washed thrice with acetone, and then lyophilized to obtain 1.26 g pale yellow NSC powder (Aiping, Tian, Lanhua, Hao, & Ping, 2006; Yan et al., 2006).

2.2.2. Analysis of N-succinyl-chitosan

Infrared (IR) spectra were obtained using an Agilent Cary 630 FTIR (Agilent Technologies, USA) in the 4000–400 cm^{-1} range. ^1H nuclear magnetic resonance (NMR) spectra were recorded by dissolving NSC powder in D_2O on a Mercury 400 MHz/CP-MAS (Varian, USA).

2.3. Formation of multilayered liposomes

2.3.1. Formation of core liposome

The core liposome was prepared using a thin-film hydration method. Egg PC (0.2 g) and 500 μM quercetin were dissolved in a chloroform-methanol mixture (2:1). The solvent was removed by a rotary evaporator (Buchi, Switzerland), forming a lipid film over the inner surface of a round-bottom flask. The film was hydrated with 20 mL distilled water (DW) using a probe sonicator (Branson, USA), which formed multi-lamellar vesicles. After centrifugation at 4000 rpm for 5 min, the solution was passed through a 1.20-μm filter (26 mm; Minisart, USA) to remove impurities. Finally, homogeneous, small-sized liposomes of approximately 100 nm were obtained. The average pH of the prepared core liposomes was 4.14 ± 0.06.

2.3.2. Formation of polyelectrolyte solutions

NSC (10 mg/mL) and COS stock solutions (10 mg/mL) were prepared by dissolving NSC and COS in DW, respectively. Both solutions were stirred until clear, and then the solutions were passed through a 5.00-μm filter (26 mm; Minisart) to remove impurities. NSC stock solution was diluted to 1.0 and 1.5 mg/mL with DW, and the COS stock solution was diluted to 0.3, 0.6, and 1.0 mg/mL with DW for further use.

2.3.3. Adsorption of polyelectrolytes on liposome surface

Negatively charged liposomes prepared using egg PC with the LbL technology were alternately coated with a positively charged COS solution and a negatively charged NSC solution. The method used was similar to that used in the previous study (Yoo et al., 2016). First, 0.3 mg/mL COS solution (1:1, v/v) was added dropwise into the core solution for 20 min at room temperature with a stirring speed of 500 rpm, followed by 10 min of incubation without stirring. A centrifugal filter (Amicon Ultra-15 PLHK Ultracel-PL Membrane, 100 kDa; Sigma-Aldrich) was used for concentrating the diluted solution in the coating process to the same concentration as the initial liposome solution to minimize the effects of high-speed centrifugation and liposome loss during centrifugation. Excessive water and salts were eliminated by centrifugation at 4000 rpm for 60 min, and the COS-coated liposomes were then re-dispersed with an equal amount of DW. In the same manner, the COS-coated liposomes were coated with 0.6 mg/mL NSC solution in a 1:1 vol ratio. In polyelectrolyte solutions, the succinyl groups of NSC and amine groups of COS ionize to negatively charged COO^- and positively charged NH^{3+}, respectively. Positively charged COS is spontaneously coated onto the anionic surface of the core liposome by electrostatic attractions, and the negatively charged NSC is attached spontaneously onto the COS-coated surface of the liposomes. This was followed by one more round of coating alternately with 1.0 mg/mL COS solution and 1.5 mg/mL NSC solution to finally produce four-layered liposomes surrounded by COS-NSC-COS-NSC. The mean pH values of the multilayered liposomes were 4.41 ± 0.10, 6.05 ± 0.04, 4.77 ± 0.03, and 6.62 ± 0.10 for one, two, three and four-layered liposomes, respectively.

2.4. Characterization of multilayered liposomes

2.4.1. Quartz crystal microbalance with dissipation monitoring

Adsorption kinetics of the polyelectrolytes on the planar surface was measured using QCM-D (Q-SENSE E4, Q-Sense, Sweden). In QCM-D analysis, a decrease in frequency (*f*) indicates an increase in mass, and an increase in energy dissipation (*D*) indicates increase in viscoelastic properties (Liu, Zhang et al., 2013). All measurements were performed at 25 °C with a flow rate of 100 μL/min. Gold electrodes of 14 mm diameter at 5 MHz were used as the sensor crystals. As an experimental method, the baseline was first set with DW; then, the negatively charged core liposome solution was flown on the surface of the Au chip for approximately 2 h for adsorption, followed by rinsing with DW for 15 min. The positively charged COS solution was then allowed to flow for 30 min and rinsed for 10 min. Next, the negatively charged NSC solution was allowed to flow for 35 min and rinsed for 10 min. Then, the COS solution was allowed to flow for 20 min and rinsed for 10 min, and the same procedure was repeated with the NSC solution. The concentrations of all solutions were 1 mg/mL. Data was analyzed using