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Improved stability and skin permeability of sodium hyaluronate-chitosan multilayered liposomes by Layer-by-Layer electrostatic deposition for quercetin delivery

Soha Jeon, Cha Young Yoo, Soo Nam Park*

Department of Fine Chemistry, College of Energy and Biotechnology, Seoul National University of Science and Technology, 232, Gongneung-ro, Nowon-gu, Seoul 137-743, South Korea

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ABSTRACT

Layer-by-Layer (LbL) technology, based on the electrostatic interaction of polyelectrolytes, is used to improve the stability of drug delivery systems. In the present study, we developed multilayered liposomes with up to 10 alternating layers based on LbL deposition of hyaluronate-chitosan for transdermal delivery. Dihexadecyl phosphate was used to provide liposomes with a negative charge; the liposomes were subsequently coated with cationic chitosan (CH) followed by anionic sodium hyaluronate (HA). The resulting particles had a cumulative size of 528.28 ± 29.22 nm and an alternative change in zeta potential. Differential scanning calorimetry (DSC) and transmission electron microscopy (TEM) revealed that the multilayered liposomes formed a spherical polyelectrolyte complex (PEC) after deposition. Observations in size distribution after 1 week found that the particles coated with even layers of polyelectrolytes, hyaluronate and chitosan (HA-CH), were more stable than the odd layers. Membrane stability in the presence of the surfactant Triton X-100 increased with an increase in bilayers as compared to uncoated liposomes. An increase in the number of bilayers deposited on the liposomal surface resulted in a sustained release of quercetin, with release kinetics that fit the Korsmeyer–Peppas model. In an *in vitro* skin permeation study, negatively charged (HA-CH)-L and positively charged CH-L were observed to have similar skin permeability, which were superior to uncoated liposomes. These results indicate that multilayered liposomes properly coated with polyelectrolytes of HA and CH by electrostatic interaction improve stability and can also function as potential drug delivery system for the transdermal delivery of the hydrophobic antioxidant quercetin.

1. Introduction

Transdermal drug delivery systems (TDDS) are an alternative route through which an effective amount of drugs can be delivered across the skin. One of the main advantages of these systems is the non-invasive administration, in comparison with parenteral routes. Some of the challenges include low permeability of drugs, due to skin barrier such as the stratum corneum, the outermost layer of epidermis. Thus, the development of a robust TDDS is required to enhance the delivery of active compounds [1–4]. Liposomes, which are spherical vesicles composed of a bilayer of phospholipids, are similar to cell membranes in the body, and are most commonly used in drug delivery systems. Both hydrophilic and hydrophobic drugs can be accommodated in the internal core and lipid membranes [5,6]. However, the poor stability of liposomes can lead to rapid leakage of incorporated drugs [7]. Several researchers have proposed modifying the surface of the liposome in order to overcome this limitation, including using a polymer coating [8,9] or cell-penetrating peptide [10], or conjugating the surface with poly(ethylene glycol) [11].

Layer-by-Layer (LbL) technology is a simple and versatile method used to develop multilayer films by alternating the deposition of oppositely charged polyelectrolytes *via* electrostatic attraction. It improves stability by forming a polyelectrolyte complex (PEC), and has general biomedical or biotechnology applications [12–14]. In particular, drug delivery systems fabricated by LbL deposition of polyelectrolytes could significantly contribute to the delivery of the therapeutic protein by increasing tolerance to extended shelf storage and drug loading [15], as well as to oral administration by protecting drugs against external environments such as low gastric pH due to the functionality of the surface

* Corresponding author. Tel.: +82 2 970 6451; fax: +82 2 972 9585.
E-mail address: snpark@seoultech.ac.kr (S.N. Park).

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[16–18]. They could also enhance *in vivo* biological performance by improving characteristics such as stability and pharmacokinetic profiles [19]. Xavier et al. [20] observed the slow release rate of aloin when incorporated into liposomes immobilized in LbL films, and suggested a possible use in patches for transdermal delivery. However, the functionality of a multilayer of polyelectrolytes deposited on the liposome in transdermal delivery is unknown; further analysis of skin permeability is also needed.

Chitosan is a cationic polysaccharide present in nature that is biocompatible and has low toxicity; hence, it is commonly used as a biomaterial [21], particularly in drug delivery systems [22]. Hyaluronate is a negatively charged polysaccharide, and the main component of the extracellular matrix (ECM). It has versatile properties, including viscoelasticity, biocompatibility, water absorption and water retention, proving that it can be used in transdermal delivery in spite of it being an anionic macromolecule [23–26]. A chitosan-hyaluronate complex assembled using electrostatic attractions based on LbL liposomes has not been investigated yet in transdermal delivery.

In this study, we improved the stability of the liposome by fabricating multilayered liposomes using LbL deposition of polyelectrolytes. We also investigated the potential and roles of alternate deposition of chitosan and hyaluronate on the liposome as carriers for the transdermal delivery of drugs. Quercetin, widely known as a hydrophobic antioxidant, was loaded onto the lipid membranes of liposomes. The physical characteristics of multilayered liposomes were observed using dynamic light scattering (DLS), zeta potentials, differential scanning calorimetry (DSC), and transmission electron microscopy (TEM) analysis. Additionally, the storage stability and membrane stability against an external stimuli factor, Triton X-100, were evaluated. The release kinetics of quercetin from multilayered liposomes was analyzed using several mathematical models. Finally, we performed *in vitro* skin permeation to investigate interactions between the skin and multilayered liposomes.

2. Materials and methods

2.1. Materials

L-α-Phosphatidylcholine (from egg yolk, ≥60%, egg PC), cholesterol (≥99%, Chol), dihexadecyl phosphate (DHP), triton X 100 (BioXtra), quercetin, and chitosan (low molecular weight, MW 50,000 ~ 190,000, CH) were purchased from Sigma–Aldrich (USA). Sodium hyaluronate (MW 490,000, HA) was obtained from Bioland Co., Ltd. (Korea). Solvents such as 1,3-butylene glycol, methanol, ethanol, chloroform, acetic acid, and sodium hydroxide were of analytical grade.

2.2. Preparation of liposomes

Liposomes were prepared using the thin-film hydration method. Egg PC, Chol, DHP (10:2.5:1, molar ratio) and 500 μM quercetin were dissolved in a chloroform-methanol mixture (2:1). The solvent was removed by a rotary evaporator (Buchi, Switzerland), resulting in the formation of a lipid film over the inner surface of a round-bottom flask. The film was hydrated with distilled water (DW), and then the solution was homogenized using a probe sonicator (Branson, USA). It was filtered by passing through 1.20 μm filter (Minisart, CA, 26 mm).

2.3. Preparation of multilayered liposomes

2.3.1. Preparation of polysaccharide solutions

CH solutions (1 mg/ml) were prepared by dissolving chitosan in 1% acetic acid aqueous solution. The final pH was adjusted with 0.05 M NaOH to 4.0. Sodium hyaluronate solutions (1 mg/ml) were prepared in DW. Both the solutions were stirred overnight [27].

2.3.2. Deposition of polysaccharides onto liposomes

Multilayered liposomes were coated with a series of polysaccharides using LbL assembly, mediated by ionic interactions. The first layer of CH solution (1:1, v/v) was deposited onto the negatively charged liposomes and gently mixed for 10 min with stirring (HSD 120-03P, Misung Scientific Co., Ltd., Korea) at 500 rpm. Excess polyelectrolytes were then removed by ultracentrifugation (2236R, Gyrozen Co., Ltd., Korea) at 36,000 × *g* for 90 min followed by a single washing with DW. The resulting CH-coated liposomes were resuspended in DW. The subsequent layer was fabricated by adding HA solutions to the CH-coated liposomes (1:1, v/v) using the same procedure. The resulting HA-CH-coated liposomes were referred to 1 bilayered liposomes (HA-CH-L). To successfully build up each layer, the liposomes were coated with alternating layers of positively charged CH and negatively charged HA until the 10 layers ((HA-CH)5-L) were achieved (Scheme 1). All the formulations were obtained at pH 4.5 ± 0.3 [28].

2.4. Characterization of multilayered liposomes

2.4.1. Particle size and zeta potential measurement

The particle size and distribution of liposomes were determined by DLS (Otsuka ELS-Z2, Otsuka Electronics, Chiba, Japan) at 25 °C, with a scattering angle of 165°, with an Argon laser. The average particle size is indicated by cumulative analysis, and distribution is resolved using the Contin method. The zeta potentials of the liposomes were measured based on electrophoretic mobility under an electric field (Zetasizer, Malvern Instruments, UK).

2.4.2. Differential scanning calorimetry (DSC)

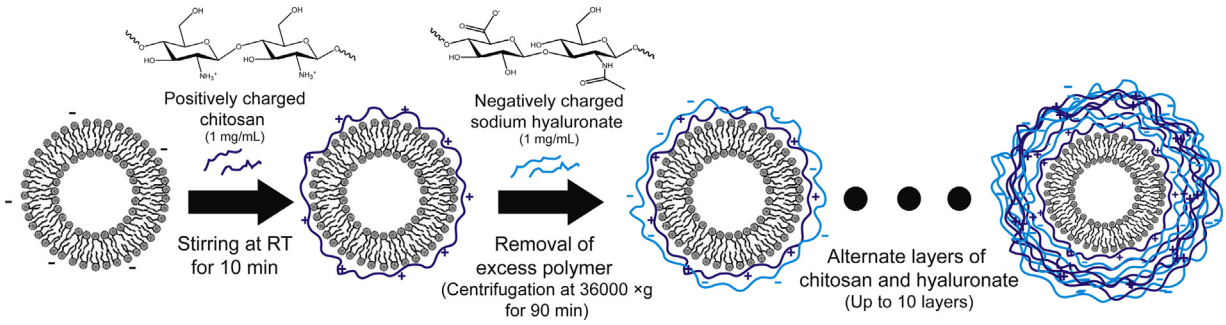
The thermal analysis of the CH solution, HA solution, uncoated liposomes, and (HA-CH)-L were studied by DSC using a JADE DSC (PerkinElmer, USA). A sample was placed in aluminum pan and heated from 10 °C to 150 °C at a heating rate of 1 °C/min with continuous purging of nitrogen at 20 ml/min.

2.4.3. Shape and surface morphology

The morphology of the multilayered liposomes was observed using TEM (JEOL-JEM1010 instrument, JEOL Ltd., Japan). Samples were dropped into a carbon-coated copper grid and dried for 1 min. Excess sample was removed using filter paper. Then, the samples were instantly stained with 0.4% (w/v, %) phosphotungstic acid, allowed to stand for 1 min, and drained. The analysis was performed at an accelerating voltage of 80 kV.

2.5. Membrane stability of multilayered liposomes against Triton X-100

The nonionic surfactant Triton X-100 is generally used as a good solubilization agent of the lipid membrane [29]. So, liposome membranes were destabilized under different Triton X-100 concentrations. To evaluate the stability of multilayered liposomes, we measured optical density (OD) of the liposome suspension at 500 nm using ultraviolet spectrophotometry (Cary 50, Varian, Australia) with a 1 cm path-length cell at room temperature. The % loss in OD after the addition of Triton X-100 can reflect the % solubilization of lipid membrane [30]. Different concentrations of Triton X-100 (10 μL) were added to 1 ml of the liposome suspension. This measure of turbidity was normalized against the control absorbance values (before the addition of Triton X-100) to quantify the effect of Triton X-100 on the liposomes [31].



Scheme 1. Schematic illustration of the fabrication of multilayered liposomes by Layer-by-Layer deposition of chitosan and sodium hyaluronate.

2.6. In vitro *drug release study*

The *in vitro* release study was performed using the dialysis membrane method under two different pH conditions at room temperature: phosphate buffer (pH 7.4) with 30% EtOH, and acetate buffer (pH 5.5) with 30% EtOH. Prior to use, the dialysis membrane (molecular weight cut-off approximately 14,000, Sigma, USA) was pretreated by washing the tubing in running water for 4 h to remove the glycerol. Then, sulfur was removed by treatment with a 0.3% (w/v) solution of sodium sulfide at 80 °C for 1 min, followed by acidification with a 0.2% (w/v) sulfuric acid solution. Finally, it was rinsed with water to remove the excess acid. Different formulations (4 ml) were poured into the dialysis bags, and subsequently placed in flasks containing 120 ml of release medium at 100 rpm. The release medium (1 ml) was withdrawn at each sampling time and was replaced with fresh medium of equal quantity. The release study was carried out up to 24 h [18]. The concentration of drugs in the release medium was quantified by measuring the absorbance at a wavelength of 376 nm using a UV/vis spectrophotometer.

In vitro release data were analyzed by various mathematical models to determine the kinetics and mechanism of release of quercetin from the different formulations. The release data were assessed using zero-order, first-order, Higuchi, Hixson–Crowell, Korsmeyer–Peppas, and Baker–Lonsdale kinetic models. The regression analysis was performed, and the model that best fits the release data was chosen on the basis of the correlation coefficient (R^2)[32].

2.7. In vitro *skin permeation study using Franz diffusion cell*

The *in vitro* skin permeation study with quercetin-loaded different formulations was carried out using Franz diffusion cells (Permegear, USA). Full-thickness skin was removed from the dorsal side of hairless mice (7 weeks, female). Subcutaneous fat and excess tissue were carefully removed from the skin. The receptor phase (40% EtOH in 0.9% NaCl) was filled in the receptor chamber and stirred at 150 rpm for 24 h. The skin was fixed between the donor and the receptor phase, with the stratum corneum side facing the donor compartment. Samples (0.4 ml) were applied to the skin in the donor compartment. The skin area contacting the receptor phase was 0.6362 cm^2, and the receptor medium was kept at 37 °C throughout the experiment using a constant temperature water bath. Receptor phase solution (0.4 ml) was withdrawn through the sampling port at 2, 4, 8, 12, and 24 h. The receptor phase was immediately replenished with an equal volume of fresh receptor phase. The withdrawn sample was analyzed by high performance liquid chromatography (HPLC; Shimadzu, Japan).

The amount of quercetin retained in skin was determined at the end of the *in vitro* permeation experiment (24 h). The skin surface was washed with PBS solution on each side to remove the residual donor sample. The stratum corneum was removed using the tape-stripping method thrice with 3M scotch tape (Korea 3M), and skin was dissolved in 100% ethanol using a sonicator. The concentrations of extracted quercetin were determined by HPLC.

2.8. *Statistical analysis*

All the experiments were performed in triplicate, and statistical data were analyzed by the Student's *t*-test at the level of $P < 0.05$.

3. Results and discussion

3.1. *Particle size and zeta (ζ)-potential surface charge*

We prepared multilayered liposomes based on LbL assembly of chitosan and sodium hyaluronate, and investigated the change in particle size and ζ-potential from uncoated to 10 layered liposomes. As alternate polyelectrolytes were deposited onto the liposomes, there was an increase in particle size (Fig. 1a). The average particle diameter of an uncoated liposome was 121 ± 2.27 nm; for liposomes coated with 10 layers it was 528.28 ± 29.22 nm. The polydispersion index (P.I.) indicates the homogeneity of particle. The values were measured approximately between 0 and 0.3, indicating a homogeneous dispersion (Fig. S1). As shown in Fig. 1a, from three to eight layers, we observed that the deposition of chitosan causes a rapid increase in the average particle diameter. However, the addition of HA slightly decreased their size. This might be because of the formation of a denser network due to the strong electrostatic interaction between chitosan and hyaluronate [15].

Zeta potential is the electrostatic potential in the shear plane of particles, and indicates the degree of attraction or repulsion between adjacent and similarly charged particles. The surface charge of the particle was measured after the deposition of each polyelectrolyte. As indicated in Fig. 1b, the zeta potential of uncoated liposomes with the addition of the anionic lipid, DHP, was −38.27 ± 2.5 mV. A steep change in the charge of the liposome surface was accompanied by adding 10 layers of the alternating polyelectrolytes, chitosan and hyaluronate (>|30 mV|). It also demonstrated by electrostatic interaction of functional groups, $-NH_3^+$ and–COO^-. Therefore, these results provide evidence that adsorption of polyelectrolytes might be generated *via* LbL deposition onto liposomal surface.

3.2. *Differential scanning calorimetry (DSC) analysis*

Table 1 shows the thermodynamic data detected in the DSC thermograms of polyelectrolytes, uncoated liposomes, and HA-CH-coated liposomes ((HA-CH)-L). The data were collected to understand their physicochemical characteristics following polyelectrolyte deposition. All the samples were measured in solution states. The endothermic peak of (HA-CH)-L (141.47 °C) showed a shift to higher temperature than that of the uncoated liposomes

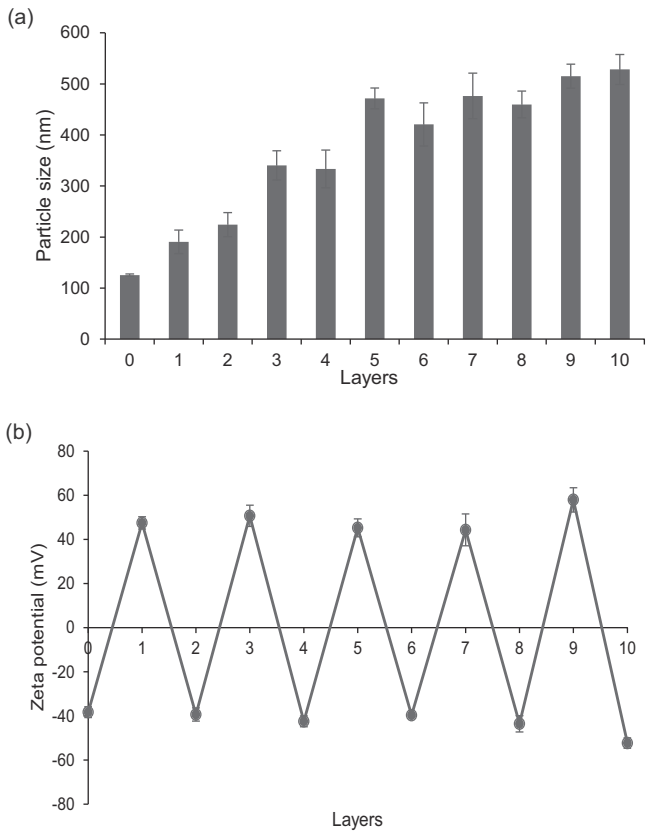


Fig. 1. (a) Average particle size change, (b) zeta-potential surface charge alternation with the deposition of each polyelectrolyte layer.

(127.26 °C). This might be because of an increase in thermal stability due to the electrostatic attraction between the liposome and polyelectrolytes. In addition, this result indicated that the polyelectrolyte complex was formed [33]. The absence of the quercetin melting peak at 322.38 °C in the uncoated liposomes and (HA-CH)-L suggested that quercetin was successfully incorporated into the lipid bilayers of liposomes (data not shown) [19].

3.3. Morphology

Fig. 2 shows TEM images of the structural morphology of uncoated liposomes, chitosan single layered liposomes (CH-L), and the multilayered liposomes (HA-CH)-L and (HA-CH)5-L. The images revealed nanosized, spherical shapes with no rupturing of the membrane wall after coating with polyelectrolytes. The average particle size decreased slightly compared with the results of the DLS analysis, because the liposome had shrunk during the drying procedure. The image of CH-L confirmed the efficient coating as evident from the visible dark layers on the liposomal surface. The (HA-CH)-L was surrounded by a fluffy layer, which was presumably made of hydrophilic HA, similar to the results reported by several authors [34,35]. It was clearly demonstrated that HA-CH (1 bilayers) was successfully coated onto the surface of liposomes. After the further deposition of 5 bilayers, a distinct core and shell was not observed because of the attachment of successive layers. However, a less heavily stained shell that appeared like corona (white arrow) and a rough dark layer (white bar) surrounding the darkest core was visible. Chun et al. mentioned that full saturation of the charged surface using natural polymers was difficult because the distribution of charges is generally heterogeneous [36]. Thus, successive adsorption of polyelectrolytes may cause a heterogeneously rough surface.

Table 1
Thermodynamic data determined by differential scanning calorimetry (DSC).

	Onset (°C)	Endset (°C)	Peak (°C)	ΔH (J/g)
HA	120.04	122.04	121.04	867.72
CH	132.16	141.11	139.11	2787.68
Uncoated liposomes	116.14	129.37	127.26	2422.65
(HA-CH)-L	139.25	144.31	141.47	2585.83

3.4. Stability studies

The average diameter and distribution of particles were observed after 1 week (Table S1). The liposome formulations with an odd number of layers, such as 1, 3, 5, 7, and 9, were unstable; the size increased rapidly or polydisperse particles were formed, leading to flocculation. In contrast, the group with even-numbered layers maintained monodispersity and did not rapidly change in size. This might be assumed that (i) the HA-CH electrostatic interaction is stronger than the CH-(HA-CH), which correlates with DLS results mentioned in Section 3.1, and (ii) hyaluronate can potentially form a hydrated membrane around the surface of the particles because of a stronger affinity to water than the other polysaccharides [25]. Therefore, bilayers of polyelectrolytes, such as hyaluronate and chitosan (HA-CH), play an important role in the stability of multilayered liposomes. The storage stability was observed using a unit of bilayers, and was observed over 3 weeks as shown in Fig. 3. After 3 weeks of storage at room temperature, although the average size of 1 and 2 bilayered liposomes had gradually increased, they were still found to be stable without any rupturing. However, flocculation was observed with 3, 4, and 5 bilayered liposomes over 2∼3 weeks. Zeta potentials, less than approximately −38 mV, were exhibited in all the formulations over 3 weeks. Hence, the flocculation of liposomes could be probably attributed to the fact that their average diameter was larger than 500 nm.

Triton X-100 is a nonionic surfactant that has a hydrophilic polyethylene oxide chain and an aromatic hydrocarbon hydrophobic group. The vesicle-micelle transition involved when Triton X-100 interacts with liposomes usually induces the solubilization of lipid vesicles, thus causing deformation of liposomal membrane structure [29]. The turbidity of a suspension of liposomes was assessed using UV/vis spectrophotometry at 500 nm. As shown in Fig. 4, the turbidity of the suspension of uncoated liposomes was gradually decreased by Triton X-100, indicating deformation of the membrane. However, as the number of bilayers increased, the turbidity curve shifted significantly despite the addition of Triton X-100. The results indicate that the multilayer coating of polyelectrolytes successfully protected the lipid membranes against Triton X-100, as compared with the uncoated liposomes. In particular, the formulations from (HA-CH)3-L to (HA-CH)5-L exhibited a similar turbidity (over 90%). These results suggest that the deposition of HA-CH polymers can enhance membrane stability by forming a compact shield around liposomal surfaces from external stimuli such as surfactants [31]. Therefore, the interaction of HA-CH with the liposomes is important to improve their physical stability.

3.5. In vitro *drug release studies*

The *in vitro* drug release profiles of uncoated L, CH-L, (HA-CH)-L, (HA-CH)3-L, and (HA-CH)5-L are shown in Fig. 5. The release profiles were evaluated at two different pH conditions (pH 5.5 and 7.4). Because the pH of the skin surface is known to be 5.5 (weakly acidic), the pH condition of 5.5 might help us understand the indirect release behavior on the skin surface. The uncoated L exhibited relatively rapid quercetin release, with 62% release at pH 5.5 and

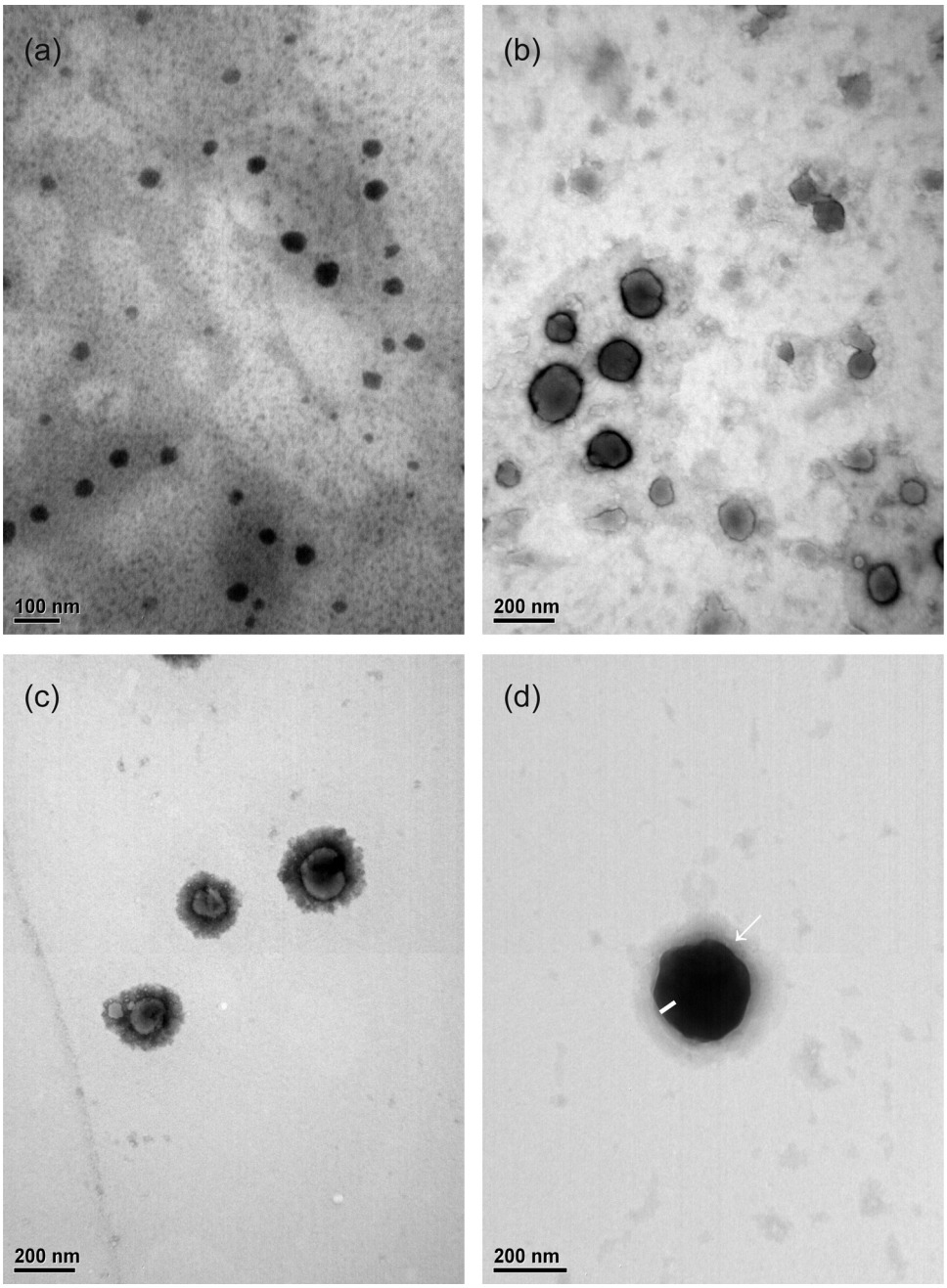


Fig. 2. Transmission electron microscopy images of (a) uncoated liposomes, (b) chitosan single layered liposomes, CH-L; (c) hyaluronate-chitosan 1 bilayered liposomes, (HA-CH)-L; (d) hyaluronate-chitosan 5 bilayered liposomes, (HA-CH)5-L.

50% at pH 7.4 in 24 h, compared with other formulations. Weak protonation on the negatively-charged liposomal surface might occur at weak acidic conditions such as pH 5.5, and the excess hydrogen ions, dissolved in the medium, could diffuse into the core aqueous phase of the liposome [17,37]. This can account for the conformational change of the lipid bilayers of the liposome, leading to a rapid release of quercetin encapsulated in lipid membranes. Release profiles within 24 h from the other formulations (except uncoated L) were similar irrespective of the pH conditions. The amount of quercetin released from CH-L after 24 h was similar to the release from (HA-CH)-L, which was approximately 40%. It is worth noting that the first polymeric coating on the liposome plays an important role in suppressing the rapid release of drugs at both pH conditions. As previously studied, the polymer deposition of polysaccharides could lead to a decrease in the permeability of drugs caused by rupture of the liposome due to lower fluidity of lipid membranes [38]. In addition, the multilayered liposomes, such as (HA-CH)3-L and (HA-CH)5-L, exhibited increased sustained release profiles below 20% at both pH conditions. Some previous studies demonstrated that complexation of alternative polyelectrolyte layers resulted in sustained release by protecting the shell from degradation, due to the formation of a denser shell through the ionic interaction of the two polymers [15,37]. It can be noted from these results that the successive deposition of HA-CH using electrostatic interactions can contribute to control of sustained release of a drug and protect against leakage of encapsulated drugs in different external pH conditions. The cumulative percentage of quercetin released was calculated by considering the encapsulation efficiency of quercetin in uncoated liposomes and (HA-CH)5-L (Fig. S2). As the number of bilayers increased, quercetin encapsulation was

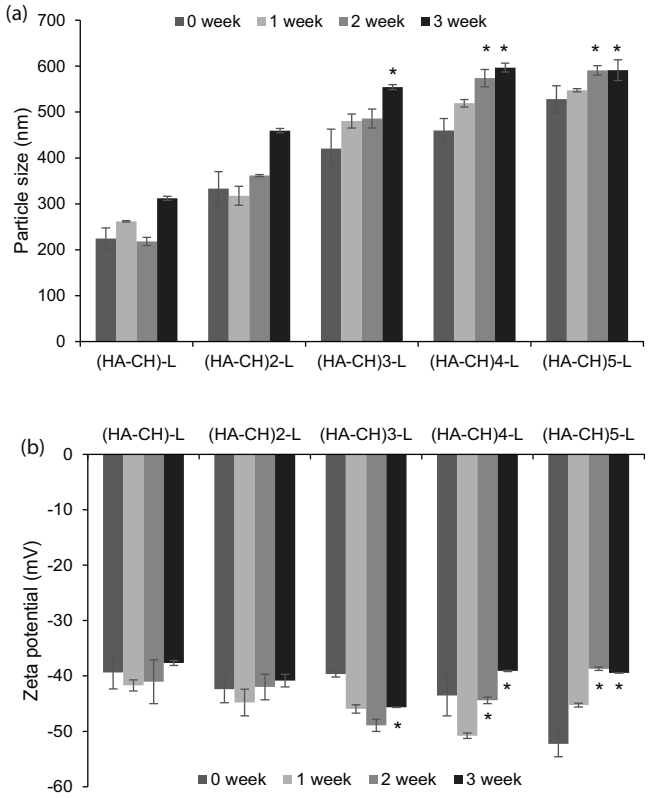


Fig. 3. The storage stability of multilayered liposomes with different numbers of bilayers: (a) particle size distribution, (b) zeta potential over 3 weeks. Values are reported as mean ± standard error of the mean. (* = flocculation).

accompanied by a slight decrease from 70% to 61%. This indicated a loss of drug during the LbL assembly process.

In order to analyze the kinetics and mechanism of quercetin release from multilayered liposomes, several mathematical models of release kinetics were assessed (Table 2). The model that best fits the release data is selected based on its correlation coefficient (R^2) value. The model with the highest value is considered the most optimal model. The drug release kinetics of every formulation thus suggested the Korsmeyer–Peppas model as the optimal model. An increase in the number of bilayers (HA-CH) correlated with an increase in the R^2 value. The Korsmeyer–Peppas model is usually used to analyze the release of the polymeric dosage form in

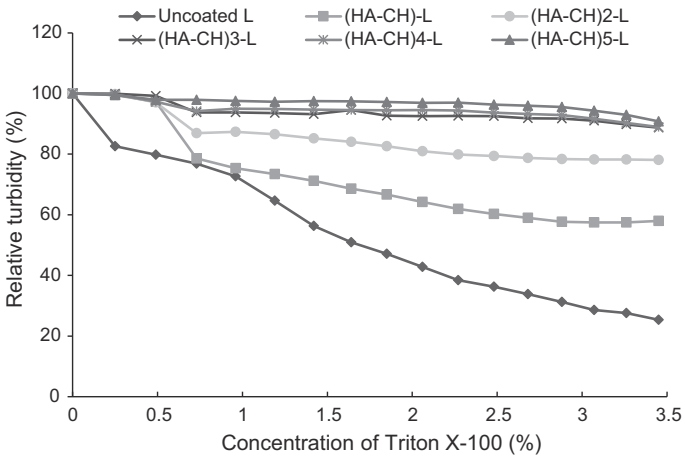


Fig. 4. Relative turbidity of uncoated liposomes and different multilayered liposomes at different concentrations of Triton X-100.

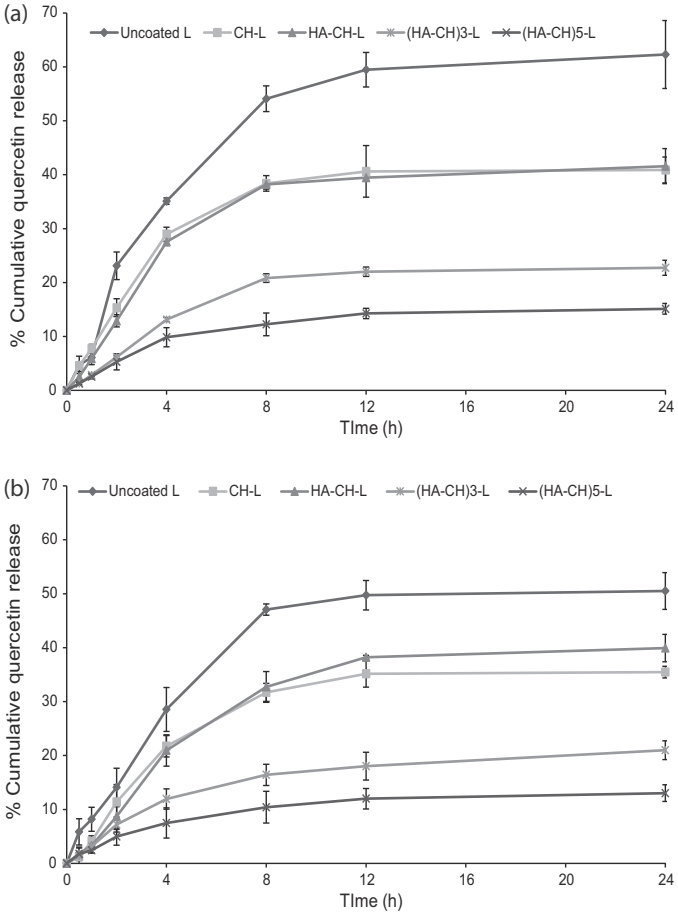


Fig. 5. *In vitro* drug release profiles of different formulations in (a) pH 5.5, (b) pH 7.4. Values are presented as mean ± SD ($n = 3$).

pharmaceuticals or when more than one type of release phenomena could be involved (Eq. (1)).

$$\frac{M_t}{M} = K \times t^n \quad (1)$$

M_t is the cumulative amount of drug released in time "t," M is the total amount of drug, K is a release rate constant, and n is the diffusional exponent indicative of the mechanism of drug release. A value of $n = 0.45$ indicates a Fickian (case I) release, indicating drug release by diffusion. When $0.45 < n < 0.89$, a non-Fickian (anomalous) release is indicated. It generally refers to a combination of both diffusion and erosion of the polymeric chain. When $0.89 \leq n$, a super case II type of release is indicated, and release by erosion of polymeric chain is the major mechanism. In the present study, the multilayered liposomes suggested an anomalous diffusion mechanism, involving both diffusion and erosion of the drug.

3.6. In vitro *skin permeation studies*

The *in vitro* skin permeation profiles of different quercetin-loaded formulations were carried out using Franz diffusion cell (Fig. 6). We prepared a control group using 1,3-Butylene glycol (1,3-BG) and uncoated liposomes; 1,3-BG is widely used as an ingredient for dissolving active components in cosmetics and has skin moisturizing properties. As shown in Fig. 6a, after 24 h, cumulative permeation of quercetin was shown to be the highest for CH-L (7.23 μg/cm^2), followed by (HA-CH)-L (6.57 μg/cm^2). Although CH-(HA-CH)4-L (2.86 μg/cm^2) and (HA-CH)5-L (2.86 μg/cm^2) had lower permeations than uncoated L, there were a statistically significant difference in comparison to the control group of 1,3-BG

Table 2
Correlation coefficient (R^2) of various release kinetic models for different formulations.

Release model	pH 5.5					pH 7.4				
	Uncoated L	CH-L	HA-CH-L	(HA-CH)3-L	(HA-CH)5-L	Uncoated L	CH-L	HA-CH-L	(HA-CH)3-L	(HA-CH)5-L
Zero order	0.7313	0.6507	0.6802	0.7067	0.6296	0.7045	0.6944	0.7551	0.7655	0.7507
First order	0.7875	0.6798	0.6072	0.7197	0.6378	0.7346	0.7197	0.7852	0.7623	0.7865
Higuchi	0.8762	0.8207	0.8394	0.8585	0.7928	0.8900	0.8837	0.9103	0.9369	0.9360
Hixson–Crowell	0.7691	0.6703	0.7015	0.7154	0.6350	0.7251	0.7114	0.7753	0.7795	0.7584
Korsmeyer–Peppas (n^a)	0.9003 (0.7396)	0.9130 (0.6091)	0.9038 (0.7519)	0.9242 (0.7823)	0.9204 (0.6567)	0.9433 (0.6345)	0.8840 (0.8678)	0.9349 (0.8748)	0.9179 (0.7074)	0.9515 (0.5582)
Baker–Lonsdale	0.8416	0.7407	0.7827	0.7824	0.8427	0.7715	0.7860	0.8518	0.9123	0.8763

[a] The diffusional exponent is indicative of the mechanism of drug release. $n = 0.45$, Fickian (case I) diffusion; $0.45 < n < 0.89$, non-Fickian (anomalous) release; $0.89 < n$, super case II type of release.

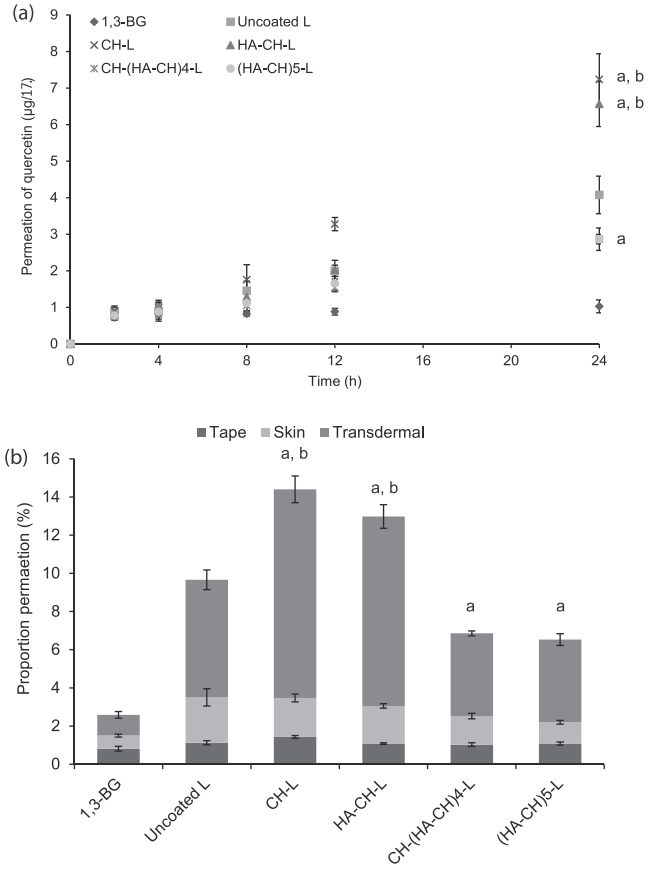


Fig. 6. (a) *In vitro* skin permeation profiles, (b) proportion of permeated quercetin from the 1,3-BG solution and various formulations after 24 h. (Tape: stratum corneum, Skin: epidermis without stratum corneum and dermis, Transdermal: dissolved in receptor phase through skin). a, statistical significance with 1,3-Butylene glycol (1,3-BG); b, statistical significance with uncoated L ($p < 0.05$).

(1.03 μg/cm^2). Fig. 6b shows the amount of drug present in the stratum corneum (tape), in epidermis and dermis except for stratum corneum (skin), and amount of drug in the receptor phase that had penetrated the skin (transdermal) by quantitative analysis. Even though the total skin permeation ratio of CH-L (14.41%) was superior to other formulations, it was similar to that of (HA-CH)-L (12.98%) when the error range was taken into consideration, which is consistent with the results of Fig. 6a. In addition, the amount of quercetin that permeated through the skin from CH-L and (HA-CH)-L were distinctly higher than uncoated L, but the skin and tape data of the uncoated L were not clearly different from both. These results indicate that the deposition of polysaccharides could contribute to an improvement in the skin permeability of the drug. As reported in many previous studies, coating liposomes with chitosan was well known as a transdermal delivery system that improved the permeation of the compound because the positively-charged chitosan-coated liposomes could interact with the skin surface *via* ionic interaction [8]. Polysaccharides are generally used as moisture-control agents [39]. Of all the polysaccharides, HA is considered to have a high skin hydration ability due to its tremendous water uptake properties [23,25]. Despite a negatively charged surface on (HA-CH)-L, HA may result in increased hydration of the stratum corneum, leading to temporary confusion of the skin barrier. Thus, the (HA-CH)-L can improve permeation of drugs through the skin in comparison with uncoated L. An almost similar skin permeation profile was observed in the case of CH-(HA-CH)4-L and (HA-CH)5-L, which have similar particle sizes but a different charge on the outer layer, suggesting that permeability is not

dependent on the surface charge. The interaction between these formulations and the skin surface becomes difficult due to the deposition of more polymer layers (9 and 10 layers, respectively) on the liposomes. So, the low skin permeation of these formulations probably might be related to properties caused by the slow release behavior of drug. In support of our hypothesis, there is a limit to the successive deposition of polymer layers on the liposomes for skin permeation of the drug. However, the liposomes coated with HA-CH *via* strong electrostatic interactions could increase liposome stability and improve skin permeability. These results suggest that the interaction of HA with CH on the liposomal surface plays an important role in terms of transdermal delivery.

4. Conclusion

Multilayered liposomes were successfully developed using LbL deposition of alternating cationic chitosan and anionic sodium hyaluronate on liposomes. The fabrication of monodispersed nano-sized particles was observed using DLS (~528 nm) and zeta potentials. The multilayered liposomes had significantly improved membrane stability against the surfactant Triton X-100, as compared to uncoated liposomes. *In vitro* release studies demonstrated that quercetin exhibited a sustained release as the number of bilayers deposited on liposomes increased. Similar skin permeability of the drug was observed in the case of (HA-CH)-L and CH-L, which were superior to uncoated liposomes. Despite a negatively charged surface, HA presented as the outer layer of HA-CH *via* electrostatic interaction, temporarily loosened the skin barrier through skin hydration; hence, it improved the skin permeation of active compounds and simultaneously may show an increased stability in comparison with a single layer of chitosan. The results of this study suggest that the system we developed has potential as a promising transdermal delivery system for the delivery of hydrophobic antioxidants such as quercetin.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.colsurfb.2015.03.018.

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