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Properties of liposomes coated with hydrophobically modified chitosan in oral liposomal drug delivery

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Chitosan (CS) has been widely used as an adhesive coating polymer for oral liposomal drug delivery systems because of its adhesive properties on mucous layers. The coating mechanism or interaction of chitosan and liposomes or mucin mainly depends on electrostatic forces. Thus, to enhance the adhesive properties of chitosan, a hydrophobically modified chitosan, i.e., dodecylated chitosan (DC), was synthesized. BIACORE results showed that both CS and DC could interact with mucin. Differences in sensorgram patterns between chitosan-mucin and dodecylated chitosan-mucin were observed and tentatively attributed to differences in binding kinetics. The zeta potential of dodecylated chitosan-coated liposomes (DC-Lip) showed positive values in both liposomal formulations, i.e., negatively charged and neutral-charge liposomes. These results indicated that DC could be considered a more suitable polymer for coating neutral-charge liposomes than CS because the hydrophobic side chain of DC inserts itself into the lipid bilayer of liposomes. Moreover, CS seemed to be less effective in the coating of a neutral-charge liposome because of the low positive values of its zeta potential. CS provided solely electrostatic forces when used for coating liposomes while DC provided electrostatic and hydrophobic forces due to the long alkyl chain in its backbone. Confocal Laser Scanning Microscopy (CLSM) images indicated that both chitosan-coated liposomes (CS-Lip) and DC-Lip could adhere to and penetrate through the small intestine of rats after oral administration. The pharmacological results showed that DC-Lip had a greater effect in decreasing blood calcium concentration during the first 12 h compared with CS-Lip. Therefore, it can be concluded that dodecylated chitosan can be useful in designing oral liposomal drug delivery systems.

1. Introduction

There are many drugs such as peptides or proteins that are poorly absorbed when administered orally. To improve this situation, mucoadhesive polymer-coated liposomes have been used to increase the localized or trapped time at the active sites, presumably resulting in prolonged duration of absorption. Chitosan is adhesive to the intestinal mucosa, and it has been used to enhance drug absorption from microparticulate dosage forms (Kawashima et al. 2000). In a previous work, we examined the mucoadhesive properties of chitosan (CS) and chitosan-coated liposomes (CS-Lip) by labeling chitosan with FITC; our results showed that CS-Lip could adhere and penetrate through rat's small intestine without the separation of chitosan from the surface of liposomes (Thongborisute et al. 2004). Many authors have addressed the fact that, in an acidic medium, chitosan molecules will protonate, resulting in soluble and positively charged molecules (Hejazi et al. 2003; Qaqish et al. 1999; Liu et al. 2002; Agnihotri et al. 2004). In general, each part of the small intestine has a different pH value, ranging from 6 to 7 (Leopold 2001). Thus, the mucoadhesive properties of CS-Lip via electrostatic interaction between the negatively charged sialic acid residue of mucin on the surface of the intestine and the positive charge of chitosan might be inhibited due to the decrement of positive charge.

Dodecylated chitosan (DC), a hydrophobically modified chitosan, is an interesting adhesive polymer because of the long alkyl chain in its backbone molecule. Liu et al. (2001) used DC with DNA to form a polyelectrolyte complex for enhancing the thermal stability of DNA. At present, there is no literature addressing the use of DC for enhancement of the bioavailability of drugs in oral liposomal drug delivery systems.

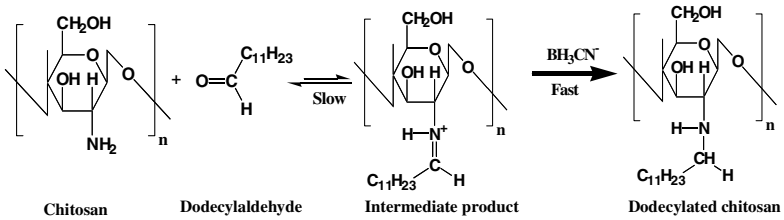
Thus, the objective of this work was to investigate and improve the physico-chemical properties and pharmacological properties of DC and DC-coated liposomes (DC-Lip) such as coating efficiency, interaction of coating polymer and liposomes, and hypocalcemic potency after oral administration to rats.

2. Investigations, results and discussion

2.1. Structural characterization and substitution degree (SD) of dodecylated side chain on chitosan backbone

Dodecylated chitosan (DC) was modified from chitosan (CS) because of the presence in the repeat unit of a $-NH_2$ group in the C-2 position, which may be specifically mod-

Scheme: Synthesis of dodecylated chitosan (DC) by alkylation reaction



ified by controlled chemical reactions. (Auzely et al. 2003). The alkylation reaction proceeded on the C-2 position of the repeated unit of chitosan as shown in the Schema.

The chemical structure of DC after modification with dodecylaldehyde was confirmed by an NMR spectrum as shown in Fig. 1 and can be compared with the NMR spectrum of CS shown in Fig. 2.

The percentage of substitution of dodecylated side chains on amino groups of CS was calculated using Eq. (1) to be around 3.4%. This means that DC still left enough amino groups to show a positive charge under acidic conditions. Moreover, DC could show hydrophobic properties due to the hydrophobicity of the dodecylated side chain.

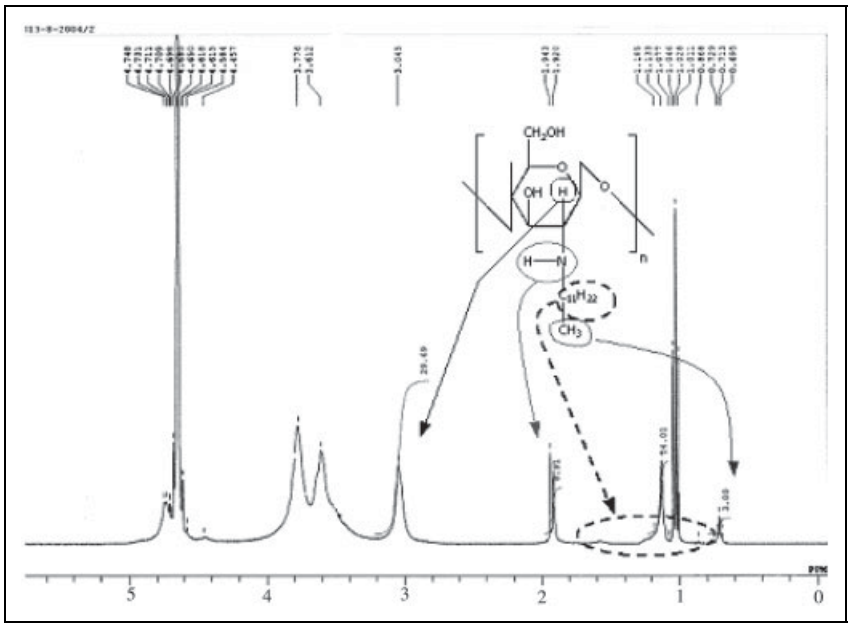


Fig. 1:
NMR spectrum of dodecylated chitosan (DC)

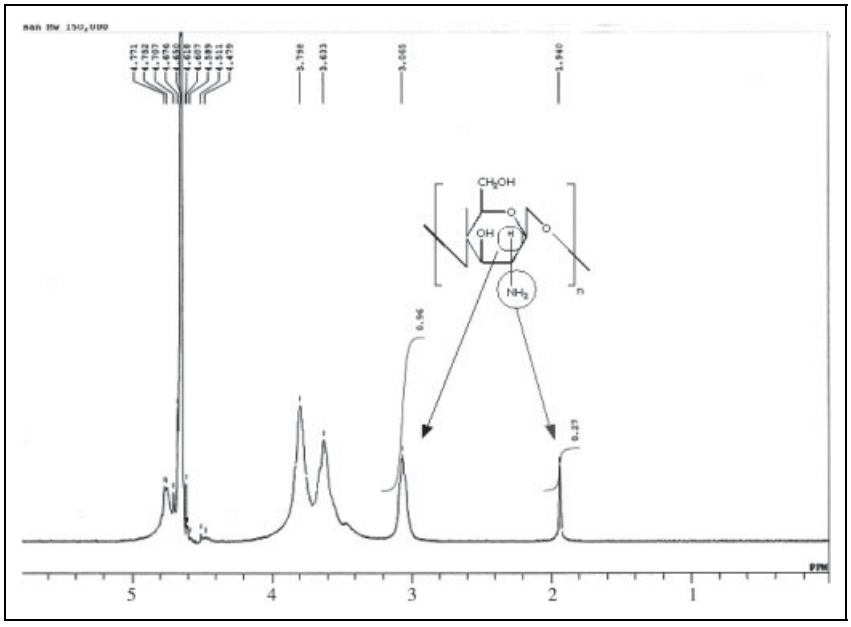


Fig. 2:
NMR spectrum of chitosan (CS)

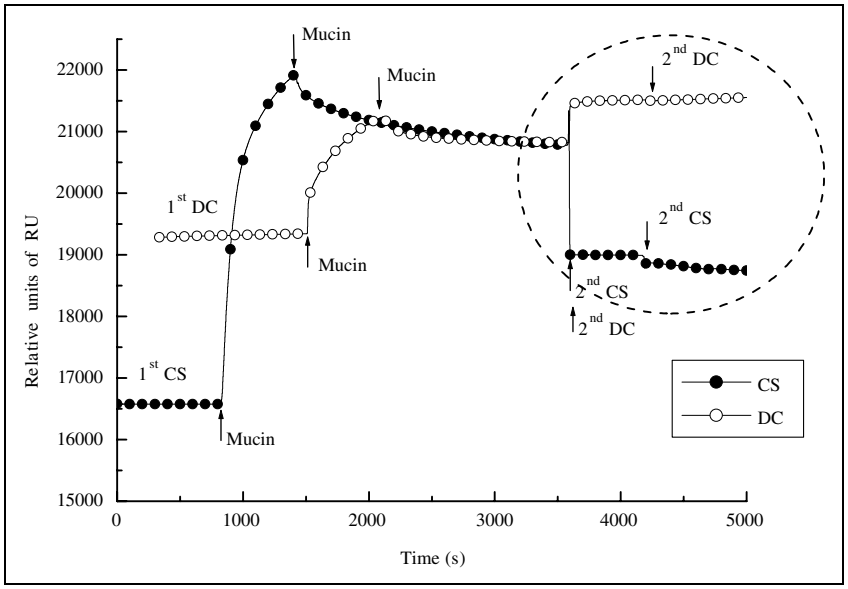


Fig. 3:
Overlay sensorgrams of the interaction of chitosan-mucin-chitosan (CS-mucin-CS) and dodecylated chitosan-mucin-dodecylated chitosan (DC-mucin-DC) on sensor chip CM5, CS: Chitosan, M: Mucin, ↑: Start injection, ↓: Stop injection

2.2. Interaction phenomena between chitosan-mucin-chitosan (CS-mucin-CS) and dodecylated chitosan-mucin-dodecylated chitosan (DC-mucin-DC)

To detect the difference in interaction between chitosan-mucin and dodecylated chitosan-mucin, a BIACORE instrument was used. BIACORE is based on Surface Plasmon Resonance (SPR), an optical phenomenon used at a biospecific surface to measure changes in the solution concentration of molecules that lead to a change in the RU response (resonance unit). The signal depends on the refractive index of solutions in contact with the surface of the sensor chip. The advantages of BIACORE are label-free detection of binding and the ability to monitor binding kinetics in real time. (Sikavitsas et al. 2002).
Fig. 3 shows the differences in interaction of CS and DC on mucin. At the beginning, the RU response after the CS- or DC-immobilized sensor chip surface was passed over mucin increased in both cases. However, after CS or DC was passed over mucin again (2^{nd} time), the RU response of DC further increased or remained elevated whereas that of CS fell. One possible way to explain these results is that CS might catch or hold the mucin particles on the sensor surface, leading to a loosening of the mucin particles from the sensor surface. In the case of DC, as described above, DC possesses a long hydrophobic side chain; thus, the hydrophobic chain of DC molecule might interact with DC (1^{st} time) leading to an increase in RU response (2^{nd} time).

2.3. Physico-chemical properties of zeta potential values of coated liposomes under various pH conditions

In general, the coating mechanism between chitosan molecules and liposomes depends on the positive charge of the amino group of the chitosan molecule under acidic conditions and the negative charge of the liposome surface. In this section, both negatively charged liposomes and neutral charged liposomes were used to compare the efficiencies of the coating ability of CS and DC on liposomes.
The results shown in Fig. 4 indicate that in the case of negatively charged liposomes (Lip1), both CS and DC display effective coating interaction with the liposome surface, leading to a changing of the zeta potential value from negative to positive (see Fig. 4; CS-Lip1 and DC-Lip1). In the case of neutral charged liposomes (Lip2),

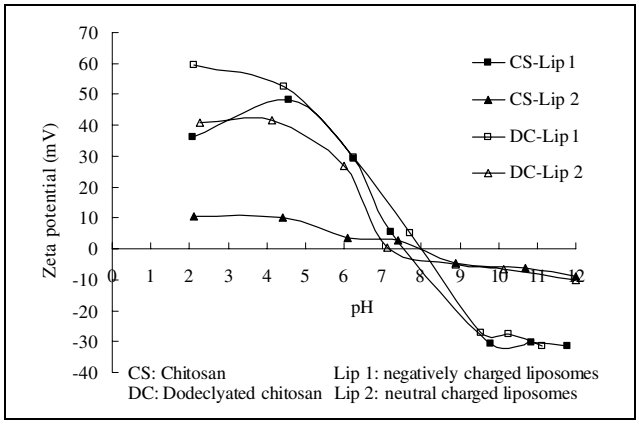


Fig. 4: Zeta potential profiles of coated liposomes in various pH conditions

both CS-Lip2 and DC-Lip2 showed positive-value zeta potentials after coating, but the positive zeta potential value of CS-Lip2 was lower than that of DC-Lip2. Thus, CS seemed to be less effective in coating neutral charged liposomes.
One possible way to explain these phenomena is that in the case of negatively charged liposomes, the positively charged amino group on the chitosan molecule interacted with the negative charge on liposome surface via electrostatic interaction, whereas DC not only showed the electrostatic interaction via positively charged amino groups and the negative charge of the liposome surface, but also exhibited a hydrophobic interaction with the hydrocarbon part of the phospholipid bilayer. In the case of neutral charged liposomes, the hydrophobic side chain of DC was able to closely interact with the phospholipid bilayer, facilitating the coating of the vesicles while CS possessed only a very weak hydrophobic interaction force between its remaining acetyl groups (i.e., after the deacetylation process) and lipid bilayer.

2.4. Muco-penetrative behavior of CS-Lip and DC-Lip in rats small intestine

Following the above results, chitosan and dodecylated chitosan were used as coating materials on liposomes (negatively charged and neutral charged liposomes), and their

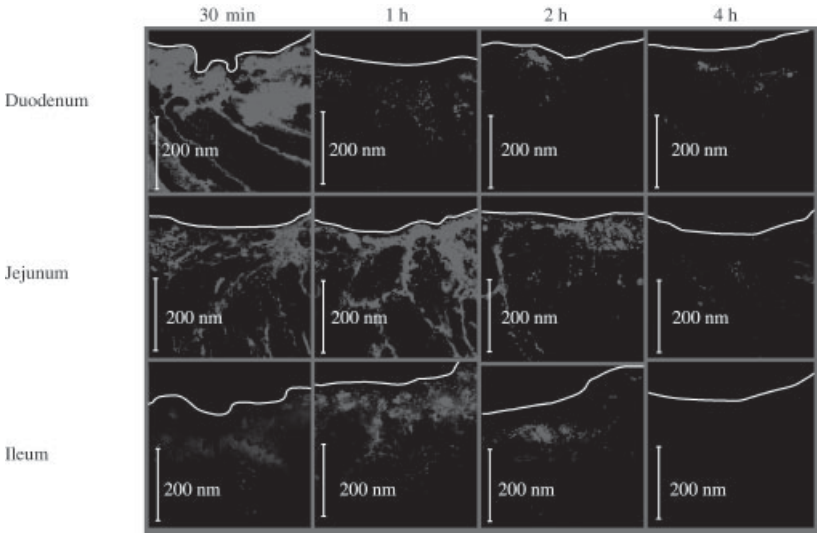


Fig. 5:
CLSM images of CS-Lip1 through rat's small intestine at 0.5, 1, 2 and 4 h after intragastric administration. (CS: Chitosan, Lip1: negatively charged liposomes, particle size of CS-Lip1: 2–5 μm)

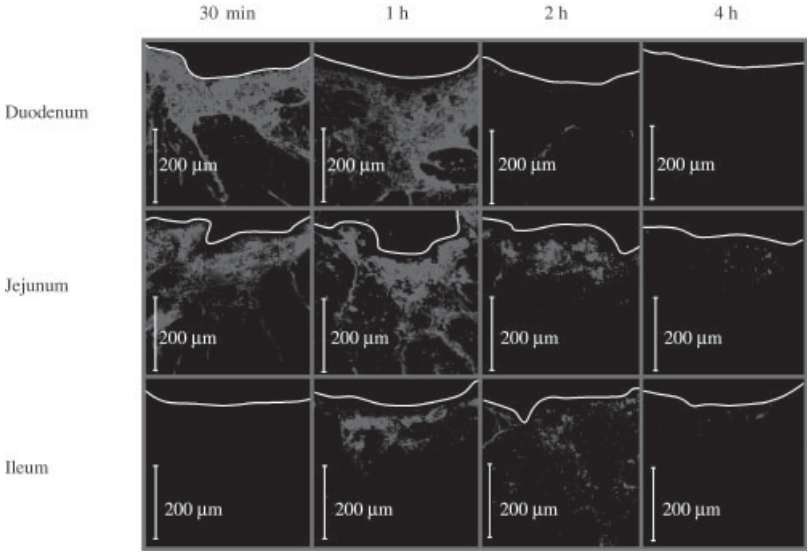


Fig. 6:
CLSM images of DC-Lip1 through rat's small intestine at 0.5, 1, 2 and 4 h after intragastric administration. (DC: Dodecylated chitosan, Lip1: negatively charged liposomes, particle size of DC-Lip1: 330–350 nm)

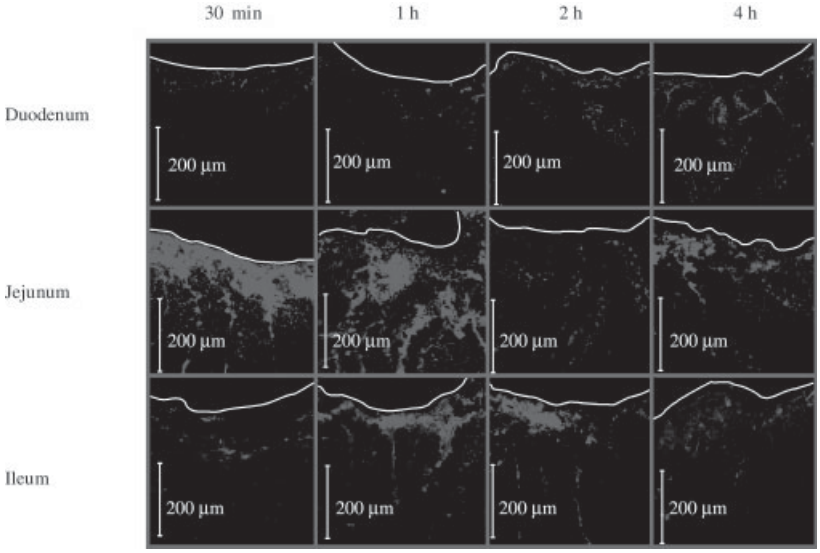


Fig. 7:
CLSM images of DC-Lip2 through rat's small intestine at 0.5, 1, 2 and 4 h after intragastric administration. (DC: Dodecylated chitosan, Lip2: neutral charged liposomes, particle size of DC-Lip2: 260–290 nm)

muco-penetrative behavior at the rat small intestine was determined after intragastric administration. The muco-penetrative behavior of both CS-Lip1 and DC-Lip1 depended on time, as shown in Figs. 5 and 6. The intensity of the fluorescence marker for liposomes indicated that both coated liposomes could adhere at and penetrate across the mucous gel layer through the epithelial cell of the intestine, even 2 h after administration.

The mucoadhesive behavior of DC-Lip2 was a little different from those of CS-Lip1 and DC-Lip1. As shown in Fig. 7, while the muco-penetrative manner of DC-Lip2 depended on time like that of CS-Lip1 and DC-Lip1, the

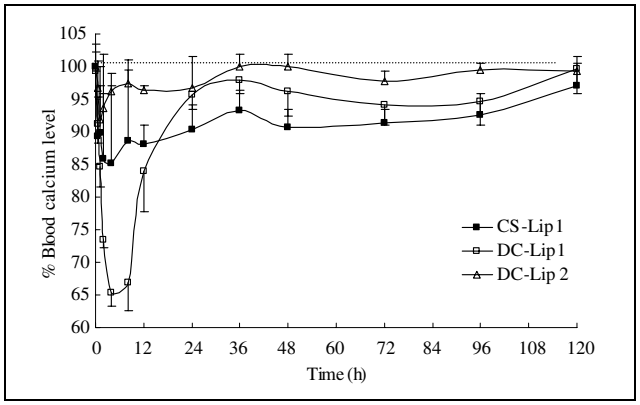


Fig. 8: Blood calcium level profiles of coated liposomes containing elcatonin during 120 h after intragastric administration into rats; n = 3 (Particle size of CS-Lip1, DC-Lip1 and DC-Lip2 are 435, 331 and 300 nm, respectively, % Encapsulation efficiency: Lip1 was 100% and Lip2 was 70%)

fluorescence intensity of DC-Lip2 was mostly located in the jejunum and ileum. In addition, this fluorescence was still located in part of the intestine 4 h after administration, especially in the jejunum part.

These behavior might be explained by the positive-negative charged interaction and hydrophobic interaction between DC and the liposome surface. The long alkyl chain on the backbone of DC interacting with the hydrophobic part of the lipid bilayer meant that the coating of the liposomal particles and that on the DC molecule still possessed the positively charged amino group, which could interact with the negatively charged mucous gel layer in the GI tract. Therefore, DC-Lip2 could adhere to the GI tract even at 4 h after administration.

2.5. Pharmacological effect of CS-Lip and DC-Lip containing elcatonin on blood calcium concentration

Elcatonin, a peptide hormone, was used as a model drug to investigate the efficiency of coated liposomes, CS-Lip and DC-Lip. The hypocalcemic potency of these coated liposomes is shown in Fig. 8.

The pharmacological tests on the coated liposomes revealed that during the first 12 h, DC-Lip1 resulted in a greater decrease in blood calcium concentration compared to CS-Lip. This phenomenon might be the result of the perturbation of the long alkyl side chain on lipid bilayer leading to a rapidly increased release of elcatonin. However, AAC values (Table) indicated that CS-Lip over the duration of the experiment showed a more effective decrease in blood calcium concentration than DC-Lip1. The DC-Lip2 formulation was least effective in decreasing blood calcium concentration although its muco-penetrative ability was similar to that of CS-Lip1 and DC-Lip1.

Table: Area above the percentage of blood calcium concentration-time curves (AAC) of CS-Lip1, DC-Lip1 and DC-Lip2

	$AAC_{0-24\,h}$	$AAC_{0-120\,h}$
CS-Lip1	276.88	1000.83
DC-Lip1	445.76	837.21
DC-Lip2	87.87	180.35

AAC was analyzed with Program Microcal Origin 6.0

The hydrophobic properties of DC and its ability to be protonated facilitate its use for coating both neutral and anionic liposomes. Although the AAC value after oral administration of DC-Lip to rats was slightly lower than that of CS-Lip, the pharmacological effect during the first 12 h of DC-Lip was significantly higher than that of CS-Lip.

3. Experimental

3.1. Drugs and chemicals

Distearoylphosphatidylcholine (DSPC, Nippon Oil and Fats, Japan), dicetyl phosphate (DCP, Sigma, USA.), cholesterol (Chol, Sigma, USA.), chitosan Mw. 150000 (Seelab,% deacetylation > 85%, Germany), dodecylaldehyde (92%, Mw. 184.32, Aldrich), sensor chip CM5 (research grade, BIACORE® Sweden), BIACORE amine coupling kit (containing 400 mM of EDC, 100 mM of NHS, and a solution of 1 M ethanolamine HCl, pH 8.5), 50 mM of sodium hydroxide solution as a regenerating reagent, 10 mM acetate buffer (ABS), pH 4.5 as a running buffer, DiI (1-1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate, LAMBDA, Austria), elcatonin (it was kindly supplied by Asahi Chemical, Japan), Calcium C-test (Wako Pure Chemical, Japan) and all other reagents used were of analytical grade.

3.2. Synthesis of dodecylated chitosan (DC)

DC was synthesized by following the method of Desbrieres et al. (1996) with some modifications. Four grams of chitosan Mw. 150000 were dissolved in 220 ml of 0.2 M CH_3COOH overnight for complete dissolution. 150 ml of EtOH was slowly added into the chitosan solution to allow the aldehyde used for alkylation to be in a solvation medium. Afterwards, the pH was adjusted with 0.2 M NaOH to 5.1 to prevent the precipitation of the macromolecules. (The optimal pH for this reaction is between 4 and 8.) The solution of dodecylaldehyde (92%, Mw. 184.32, Aldrich) in EtOH was added in a way that the ratio of the number of amino groups of chitosan to aldehyde was 1 to 0.05. Then, excess $NaCNBH_3$ was added as a catalyst. The mixture was stirred at room temperature for 24 h, after which the by-product was removed by filtration. Dodecylated chitosan was precipitated with EtOH, and the precipitate was separated by a centrifuge at 20,000 rpm for 5 min and then washed with EtOH/water mixtures with, increasing EtOH content from 70% v/v to 100% v/v. DC was dried in a vacuum at room temperature.

3.3. Structural characterization and substitution degree (SD) determination of dodecylated chitosan

Two milligrams of dried DC were dissolved in 0.75 ml of 2% v/v of deuterated acetic acid (CD_3COOD, 99.94%D, Cambridge Isotope laboratories, Inc., USA) in D_2O. Then the solution was transferred into an NMR tube and observed by 400 MHz ^1H NMR spectrometer (FT-NMR, AL-400, JEOL, Japan). The structure of DC was analyzed by ^1H NMR spectrum and the substitution degree was calculated by the following equation:

$$SD = (\delta_{1.1\,ppm}/3) * (100/\delta_{3.4\,ppm}) \quad (1)$$

where chemical shifts (δ) were expressed in ppm with respect to the CH_3 triplet at 1.1 ppm and a proton at the amino group at 3.4 ppm. (Liu et al. 2003)

3.4. Interaction phenomena between chitosan-mucin-chitosan (CS-mucin-CS) and dodecylated chitosan-mucin-dodecylated chitosan (DC-mucin-DC)

3.4.1. Preparation of mucin suspension

A one percent weight by volume of mucin suspension (from porcine stomach, type II crude, bound sialic acid 1%, Sigma, USA) was prepared by suspending and continuously stirring in 10 mM ABS, pH 4.5 overnight. Then it was incubated at 37 °C for one night and ultrasonicated with a probe sonicator (Branson sonifier 250) until the particle size was smaller than 1 μm. Afterwards, it was centrifuged at 4000 rpm for 20 min to extract the supernatant, and the supernatant portion was diluted to a concentration of 0.01% w/v with 10 mM ABS, pH 4.5. The particle size was determined by a Zetasizer 3000 HS_A (Malvern instruments, UK.).

3.4.2. Interaction of chitosan-mucin-chitosan (CS-mucin-CS)

First, for the immobilization process, 0.02% w/v of CS solution (Mw. 150000), prepared in 10 mM ABS pH 4.5, was passed over the surface of sensor chip CM5 for 10 min, and baseline data were collected for 1 h before starting the experiment. Then, 0.01% w/v of mucin was injected for 10 min, and a sensorgram was collected until equilibrium was reached. Afterwards, 0.02% w/v of chitosan Mw. 150000 was injected again for 10 min to ensure complete equilibrium.

3.4.3. Interaction of DC-mucin-DC

First, for the immobilization process, 0.02% w/v of DC solution prepared in 10 mM ABS pH 4.5 was passed over the surface of sensor chip CM5 for 10 min, and baseline data were collected for 1 h before starting the experiment. Then 0.01% w/v of mucin was injected for 10 min, and a sensorgram was collected until equilibrium was reached. Afterwards, 0.02% w/v of dodecylated chitosan was injected again for 10 min to ensure complete equilibrium. All BIACORE experiments were performed under the following conditions:

Running buffer	: 10 mM acetate buffer pH 4.5
Sensor chip	: CM5 (Carboxymethylated dextran)
Flow rate	: low (~15µl/min)
Temperature	: 25 °C

3.5. Preparation of non-coated liposomes (Non-Lip), chitosan-coated liposomes (CS-Lip) and dodecylated chitosan-coated liposomes (DC-Lip)

3.5.1. Liposomes for physico-chemical properties test

Two liposomal formulations were used in this study. Liposome formulation 1, DSPC : DCP : Chol (8 : 2 : 1 molar ratio), for negatively charged liposomes and formulation 2, DSPC : Chol (8 : 2) for neutral charged liposomes. Briefly, a mixture of DSPC, DCP and cholesterol or DSPC and cholesterol was dissolved in a small amount of chloroform. Then, it was evaporated to dryness in a rotary evaporator. The lipid film was further dried in a room temperature vacuum for one night to completely remove solvent. Afterwards, the liposome suspension was prepared by the hydration method with 66.67 mM PBS pH 7.4 as a loading buffer. The SUV liposomes were prepared by extrusion (LipoFast-Pneumatic, Avestin, Canada) through polycarbonate filters (Nucleopore®) until the particle size was less than 200 nm; then, liposomes were incubated at 10 °C for 30 min.
In order to prepare CS-Lip, the same volume of liposome suspension prepared from the above method and 0.6% w/v of chitosan solution (prepared in 100 mM ABS pH 4.4) were mixed by vortexing. Because the particle size of CS-Lip could not decrease to SUV size, MLV-size particles of CS-Lip were used in this experiment.
In order to prepare DC-Lip, the same volume of liposome suspension prepared as explained above and 0.6% w/v of dodecylated chitosan solution (prepared in 100 mM ABS pH 4.4) were mixed by vortexing to get DC-Lip formulation 1 (DC-Lip1) and DC-Lip formulation 2 (DC-Lip 2). Then they were extruded through polycarbonate filters to obtain SUV-size particles.
The particle size and zeta potential value of Non-Lip, CS-Lip and DC-Lip were determined using a Zetasizer 3000 HS_A (Malvern instrument, UK) and Zetamaster (Malvern instrument, UK), respectively.

3.5.2. Liposomes for muco-penetrative behavior test

Preparation of CS-Lip and DC-Lip for this experiment was the same as that explained above, except that 0.002% w/v of DiI solution, in chloroform, was added into a mixture of lipid composition before evaporation as a marker of liposomes.

3.5.3. Liposomes for pharmacological potential test

Preparation of Non-Lip, CS-Lip and DC-Lip for this experiment was the same as that explained above, except that elcatonin solution, prepared in 66.67 mM PBS pH 6.8, was used instead of 66.67 mM PBS pH 7.4 for the hydration process.

3.6. Physico-chemical properties of zeta potential values of coated liposomes under various pH conditions

Zeta potential values of CS-Lip formulation 1 (CS-Lip1), CS-Lip formulation 2 (CS-Lip2), DC-Lip formulation 1 (DC-Lip1) and DC-Lip formulation 2 (DC-Lip2) were determined in various pH-diluted mediums ranging from 2 to 12 using a Zetamaster (Malvern instrument, UK). The pH of the diluted medium was adjusted with 10 mM HCl and 10 mM KOH.

3.7. Muco-penetrative behavior of CS-Lip and DC-Lip at rat small intestine

Male Wistar rats, 11–13 weeks old, were fasted at least 48 h before starting the experiment; then, 1 ml of coated liposome suspension (CS-Lip 1, DC-Lip1 and DC-Lip 2) was intragastrically administered. Rats were sacrificed 0.5, 1, 2 and 4 h after administration. Their intestines were removed and divided into 5 segments (duodenum, upper-lower jejunum and upper-lower ileum). Each segment was rinsed with normal saline solution and sliced into parts of 10 µm thickness with a Cryostat (Leica CM1850). Then, Confocal Laser Scanning Microscopy (CLSM150, Carl Zeiss Jena, Germany) was performed in order to visualize the penetration of the coated liposomes into the intestinal mucosa.

3.8. Pharmacological effect of CS-Lip and DC-Lip containing elcatonin on blood calcium concentration

Male Wistar rats, 9 weeks old, were fasted 12 h before starting the experiment and were fed 48 h after drug administration. Each elcatonin-loaded liposomal formulation (500 IU/kg rat) was intragastrically administered; then 0.3 ml of blood was collected from the jugular vein at 0, 0.5, 1, 2, 4, 8, 12, 24, 36, 48, 72, 96, and 120 h. The blood sample at each time point was centrifuged at 6,000 rpm for 10 min, and plasma was collected for determining blood calcium concentration. Afterwards, the plasma calcium level was determined with a commercially available kit (Calcium C-test, Wako Pure Chemical, Japan) and AAC (Area Above the blood calcium concentration-time Curve) values were calculated using the program Microcal Origin 6.0.

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