

PREPARATION, CHARACTERIZATION AND MUCOADHESIVE EVALUATION OF CHITOSAN COATED LIPOSOMES CONTAINING CYCLOSPORINE A

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تهدف هذه الدراسة إلى تحضير وتمييز ليبوزومات (جسيمات شحمية) ملبسة بمادة كيتوزان ومحتوية على دواء سيكلوسبورين A. ولهذا الغرض، تم تحضير جسيمات شحمية مشحونة بشحنة سالبة ومحتوية على سيكلوسبورين بواسطة طريقة تبخير المذيب. بعد ذلك أضيفت الليبوزومات نقطة نقطة إلى محلول كيتوزان (0.25% حجم/حجم) لتلييسها. وتم تقييم الشكل الخارجي، ومتوسط الحجم، وكفاءة تحفظ الليبوزومات الملبسة بمادة كيتوزان. ولتقييم الخواص المخاطية اللاصقة لهذا النوع من أنواع توصيل الدواء، فقد تم تقدير نسبة إدمصاص مادة ميوسين على سطح الليبوزومات الملبسة. كما أن التأثير المثبط للمناعة معاملياً لمادة سيكلوسبورين المتمحفظ في الليبوزومات قد تم تقديره على خلايا T البشرية بواسطة اختبار MTT [3- (4, 5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]. ولقد كانت الليبوزومات عبارة عن حويصلات متعددة الصفائح. وكان متوسط قطر الليبوزومات الملبسة بمادة كيتوزان 2.76 ميكرومتر وفرق جهد زيتا لها كان موجباً (45.3 ميلي فولت). أما كفاءة التحفظ لليبوزومات فكانت 2.36 ± 10^{-2} % وكانت ثابتة لمدة شهرين. أما متوسط IC_{50} أو نصف أقصى تركيز مثبط لليبوزومات المتحوصلة متعددة الصفائح فكان 3.08 ميلي مولار. وطبقاً لنتائج ادمصاص مادة ميوسين، أظهر هذا النظام الجسيماتي خواصاً مخاطية لاصقة مناسبة. من هذه النتائج تم الاستنتاج بأن تحوير سطح الليبوزومات بالتلييس بمادة كيتوزان قد يزيد من إمكانية الاستفادة منها كنظام للتوصيل الفموي لدواء سيكلوسبورين.

The aim of this study was to prepare and characterize chitosan coated liposomes containing cyclosporine A (CyA). For this purpose, negatively charged liposomes containing CyA were prepared by solvent evaporation method. Liposomes were then added dropwise to chitosan solution (0.25% w/w) for coating. Morphology, mean size and encapsulation efficiency of chitosan coated liposomes were evaluated. To assess the mucoadhesive properties of this drug delivery system, percent of mucin adsorption onto the surface of coated liposomes was determined. The *in vitro* immunosuppressive effects of CyA encapsulated in the formulated liposomes were also determined on human T-cells by MTT [3- (4, 5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] test. Liposomes were multilamellar vesicles (MLVs). Mean diameter of chitosan coated liposomes was 2.76 μ m and zeta potential of them was positive (45.3

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mV). Encapsulation efficiency of coated liposomes was $86.11\% \pm 2.36$ and they were stable during two months. The average of IC₅₀ or the half maximal inhibitory concentration for MLV liposomes was 3.08×10^{-2} mM. According to the mucin adsorption results, this particulate system showed suitable mucoadhesive properties. From these results, it was concluded that the surface modification of liposomes by chitosan coating could increase the prospects of their usefulness as oral drug delivery systems for CyA.

Key words: Cyclosporine A, liposome, chitosan coating, oral delivery.

Introduction

Cyclosporine A (CyA) is a neutral lipophilic cyclic undecapeptide purified from two strains of fungi imperfecti, *Tolypocladium inflatum* Games and *Cylindrocapon lucidum* Booth (1-3). This drug as a unique immunosuppressant is used to prevent the rejection of transplanted organs like kidney, liver, skin, pancreas and bone marrow, and is also used in the treatment of selected autoimmune disorders such as uveitis, rheumatoid arthritis, and early treatment of type I diabetes (4, 5). CyA is absorbed poorly after oral administration, due to its relatively high molecular weight, high lipophilicity and poor solubility in aqueous media (6, 7). With conventional delivery systems, bioavailability after oral intake varies from 20 to 50% (8). To solve these problems using carrier systems like liposomes and microspheres were suggested; however, the residence time of the drug carrier system in the gastrointestinal tract is a determining factor which controls the bioavailability of the drug (9). Recently, to increase the residence time of dosage form at the absorption site, the mucoadhesive carrier systems have been used (9-12).

Pervious studies showed that delayed gastrointestinal (GI) transit induced by bioadhesive polymers could lead to increased oral bioavailability of drug (13). Various mucoadhesive polymers such as HPMC (14), poly acrylic acid (15), carbopol (9) and poly vinyl alcohol (16) have been used in different studies. Indeed, in this situation, improved drug absorption is expected with a combination of mucoadhesiveness and controlled drug release from devices (17). Application of mucoadhesive carrier to the mucosal tissues of ocular cavity, gastric and colonic epithelium could be used for the administration of drugs for localized action. Also, they undergo selective uptake by the M cells of Peyer's patches in GI mucosa (18). These carriers are expected to remain in the GI tract, protecting the entrapped drugs from aggressive conditions in GI so

that they may be absorbed as a released or intact particulate form (17). Enhanced enteral absorption of different drugs such as insulin (15) and calcitonin (9) by chitosan coated liposomes has been reported.

Chitosan (poly[b-(1-4)-2-amino-2-deoxy-D-glucopyranose]) is a natural, biocompatible and biodegradable polymer with low toxicity (10, 12, 20, 21). This water-soluble polymer swells indefinitely in contact with water and eventually undergoes complete dissolution (18). Because of its bioadhesive and permeation enhancing properties, chitosan has received substantial attention in the development of novel bioadhesive drug delivery systems (10, 12). More specifically, chitosan has been used as a delivery vehicle for the nasal and peroral delivery of peptide drugs, in order to improve drug absorption. Also, it seems that chitosan is a good candidate for the production of stable, bioadhesive liposomes (11, 22). It is suggested that the absorption-enhancing mechanism of chitosan might be due to opening of the intercellular tight junctions of the epithelia of membranes (23).

Several authors have used chitosan-related polymers for the coating of liposomes in order to improve the bioavailability of drugs through prolonging their residence time at the site of absorption (9, 10). Chitosan coated liposomes containing superoxide dismutase were prepared by different methods in order to increase its releasing time and to facilitate its cellular penetration (10). The effect of chitosan concentration and lipid type on the characteristics of chitosan coated liposomes and their interactions with leuprolide were investigated (24). Enteral absorption of insulin in rats from chitosan coated liposomes was evaluated. The results showed a decrease in the blood glucose level of rat after the administration of these liposomes (15). The improvement in physical stability of polymer coated liposomes was evaluated by Takeuchi *et al.* They showed an improvement of liposome stability and decreased aggregation through coating liposomes by polyvinyl alcohol and modified polyvinyl alcohol (16). The effect of coating

with water soluble chitosans of different molecular weights and concentrations on vesicle size, size distribution, stability, and apparent viscosity of liposomes were determined. It has been showed that chitosan coated liposomes were more stable than those without a coating (11). It has also been reported that some polymers like chitosan are able to inhibit the activities of the proteolytic enzymes (9, 19).

To solve the problem associated with the poor absorption of drugs after oral administration, in this study, chitosan coated liposomes containing CyA were prepared and converted to liposomes with different sizes of 1000, 400 and 100 nm. All of the preparations were characterized for their size and encapsulation efficiency. Then the immunosuppressive activity of the liposomal preparations containing CyA was evaluated *in vitro* using human T-cell culture.

Materials & Methods

Materials:

Cyclosporine A (CyA) was purchased from LC laboratories. Dipalmitoylphosphatidylcholine (DPPC) and dicetylphosphate (DCP) were from Avanti Polar Lipids (USA). Chitosan (low molecular weight) and stearylamine were ordered from Fluka (Switzerland). Cholesterol, Ficoll, RPMI-1640 growth medium and MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] (tissue culture grade) were from Sigma (USA). Chloroform, methanol and dimethyl sulfoxide (DMSO) were purchased from Merck (Germany). Phytohemagglutinin (PHA) was from Gibco (USA) and fetal calf serum was from Biochrome (Germany). All other materials were of analytical grade unless otherwise stated.

Preparation of chitosan coated liposomes containing CyA:

Solvent evaporation method was used for the preparation of liposomes containing CyA. First, the lipid phase consisting of DPPC (32 $\mu\text{mol/ml}$), DCP, cholesterol and CyA at molar ratios of 1:0.25:0.5:0.1 (negatively charged liposome formulation) was dissolved in chloroform:methanol (2:1, v/v) in a round-bottom flask. The solvent was removed by rotary evaporation (Heidolph, Germany) resulting in the deposition of a thin lipid film on the walls. This lipid film was freeze-dried (Heto Drywinner, Denmark) overnight to ensure the total removal of the solvent.

The lipid film was then hydrated and dispersed in phosphate buffer (0.1 mM, pH 7.2) using vortex at 45°C. The resulting MLVs were kept at room temperature for 1 hour and then transferred to a refrigerator. Liposomes with the size of 1000, 400 and 100 nm were also prepared using Mini Extruder (Avestin, Canada). Also, positively charged and neutral liposomes were prepared as same as negatively charged liposomes but lipid phase was consisted of DPPC:CHOL:Stearylamine:CyA (1:0.5:0.5:0.1 molar ratios), DPPC:CHOL:Stearylamine:CyA (1:0.5:0.1 molar ratios), respectively.

To prepare the chitosan coated liposomes, chitosan were dissolved in acetate buffer (pH 4.4) to form a 0.25% (w/v) solution. Chitosan solution then filtered to remove any undissolved chitosan. Negatively charged liposomes were added dropwise into the respective chitosan solution in a volume ratio of 1:1. The suspension was left overnight at 4 °C to be stabilized. Chitosan coated liposomes were harvested from the mixture by centrifugation (Beckman, USA) at 100,000 g for 30 min at 4°C and resuspended in a phosphate buffer (15).

Morphology, zeta potential and size analysis of liposomes:

Optical microscope (OLYMPUS, Germany) was used for studying the morphological features of liposomes. The mean diameter of liposomes was calculated by a particle size analyzer (Klotz, Germany). The zeta potential was determined using Zetasizer (3000HSA, Malvern, UK) in the basis of laser Doppler spectroscopy.

Determination of encapsulation efficiency:

To determine the encapsulation efficiency of liposomes, a known amount of liposomes was centrifuged at 100,000 g for 30 min at 4°C and the free CyA in the supernatant was quantitated by UV spectrophotometer (Shimadzu, Japan) at 214 nm. The amount of entrapped CyA was determined by subtracting the amount of free drug in the supernatant from the total. Results were reported as Mean \pm SD of three measurements.

Evaluation of liposome stability:

Liposome preparations were stored for two months at 4 °C. At one month interval they were first checked microscopically for the presence of lipid vesicles and then aliquots of the liposomes were removed and encapsulation efficiency of CyA was

determined according to the method described above.

Determination of mucin adsorption on the surface of liposomes:

To study the adsorption of mucin on the surface of chitosan coated MLV liposomes, the procedure described by Filipovic-Grcic *et al* was applied with some modification (10). Briefly, equal volumes of chitosan-coated liposomes and an aqueous solution of mucin (0.5 mg/ml) were mixed, vortexed and shaken at room temperature for 60 min. The suspension was then centrifuged at 12000 rpm for 20 min and the supernatant was used to determine the free mucin content. To determine the mucin concentration, Bradford assay was used.

Bradford protein assay:

The protein reagent was made by dissolving 100 mg of Coomassie Blue G250 in 50 ml of 95% ethanol and then mixed with 100 ml of 85% phosphoric acid and made up to 1 L with distilled water. The protein reagent (2 ml) was added to samples and mixed gently. Finally, after addition of protein reagent the absorbance of each sample was read at 595 nm against the reagent blank (25). γ -globulin (Green Cross Plasma, South Korea) was used as a standard.

In vitro immunosuppressive activity:

Five ml heparinized whole blood from volunteers was mixed with an equal volume of normal saline. This mixture was slowly poured over five ml Ficoll solution and tubes were then centrifuged at 2500 rpm (750 g) (Pars Azma, Iran) for 20 min at room temperature. The middle layer containing the mononuclear cells was removed and placed in clean tube, washed with normal saline and centrifuged at 1500 rpm (250 g) for 10 min. The washing step was repeated twice.

The remaining pellet of cells after centrifugation was resuspended in 2 ml growth medium (RPMI 1640; fetal calf serum, 10% v/v; L-glutamine, 5 Mm; penicillin, 50 IU/ml; and streptomycin, 50 μ g/ml). Viability of cells was tested by trypan blue dye exclusion method at the beginning of the experiment and was found to be greater than 98%. Cells were counted with a hemocytometer. An aliquot of cell suspension was seeded in 96-well flat bottomed tissue culture plate that each well was contained 50000 cells and 20 μ l PHA solution (5 μ g/ml) was

added to each well for induction of T-cell proliferation. Fifty μ l liposomal formulations containing different amount of CyA were added to each well. The final CyA concentration in the wells was adjusted between 10^{-5} to 10^{-2} mM. Some wells containing only cell medium or cell suspension in the presence of PHA or cell suspension without PHA were used as a control. The plates were incubated at 37°C in 5% CO₂ / 95% O₂ for 4 days. After incubation period, 20 μ l MTT dye (5 mg/ml) was added and they were incubated at dark for 4 hours. The plates were centrifuged at 2500 rpm for 15 min, the growth medium was removed and 200 μ l DMSO and 20 μ l glycine buffer were added and the mixtures were shaken for 5 min. Absorbance of each well was measured by an ELISA reader (Statfax–2100, Awareness Technology, USA) at 570 nm and the percent inhibition of T-cell proliferation was calculated for each formulation. In all cases, percentage of T-cell proliferation inhibition was expressed in comparison with the cells treated with PHA which was taken as 100%. IC50 was determined according to the Litchfield and Wilcoxon method using PCS software (26).

Statistical Analysis:

One-way Analysis of variance (ANOVA) was used with InStat software (GraphPad Software, Inc.) to assess the significance of differences among various groups. In the case of significant F value multiple comparison Tukey test was used to compare the means of different treatment groups. Results with $p < 0.05$ were considered to be statistically significant.

Results

Observation of liposomes under optical microscope showed that liposomes were morphologically multilamellae vesicles (MLVs). Mean diameter of chitosan coated liposomes was 2.76 μ m. As it was expected the zeta potential of chitosan coated liposomes was positive (45.3 mV).

Table 1 shows the results of encapsulation efficiency of chitosan coated liposomes with different sizes at the beginning of study and during two months storage at 4 °C. Liposomes with high encapsulation efficiency were prepared and they were fairly stable during 2 months. Encapsulation efficiency for chitosan coated MLV liposomes was 86.11% \pm 2.36. These loading amounts were

respectively reduced to $84.20\% \pm 1.58$ and $84.54\% \pm 2.10$ after one and two months storage at $4\text{ }^{\circ}\text{C}$. Statistical analysis showed that encapsulation efficiency is independent of liposome size and differences between groups (excepted 100 nm liposomes after 2 months storage) is not significant ($p>0.05$).

Table 1: Encapsulation efficiency of chitosan coated MLV and extruded liposomes containing CyA at the beginning of study and during two months storage at $4\text{ }^{\circ}\text{C}$. (Mean \pm SD, $n=3$).

Liposomal formulation	Encapsulation efficiency (%)		
	After preparation	After one month	After two months
MLV liposome	86.11 ± 2.36	84.20 ± 1.58	84.54 ± 2.10
1000 nm liposome	87.84 ± 3.31	84.20 ± 2.58	78.12 ± 4.36
400 nm liposome	85.93 ± 2.48	80.90 ± 3.22	76.21 ± 5.22
100 nm liposome	87.50 ± 1.78	78.99 ± 2.98	70.13 ± 5.58

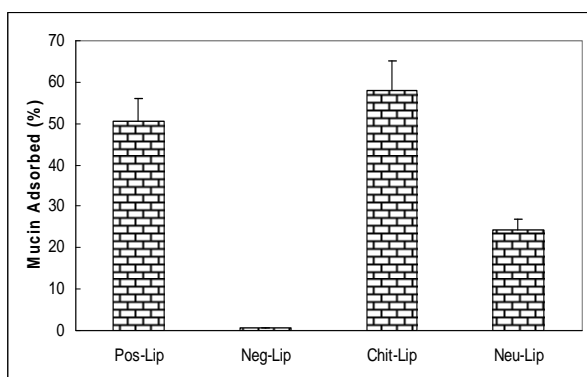


Figure 1. Percent of mucin adsorption on positively charged, negatively charged, chitosan coated and neutral liposomes. The results are expressed as Mean \pm SD ($n=3$).

Mucoadhesiveness can be related to the amount of mucin adhered to the liposomes. The amount of mucin adsorbed on the surface of liposomes was showed in Figure 1. For chitosan coated, positively charged and neutral liposomes, $58.17\% \pm 6.85$, $50.41\% \pm 5.81$ and $24.38\% \pm 2.45$ of mucin were adsorbed to the surface of liposomes, respectively but adsorption for negatively charged liposomes was almost none.

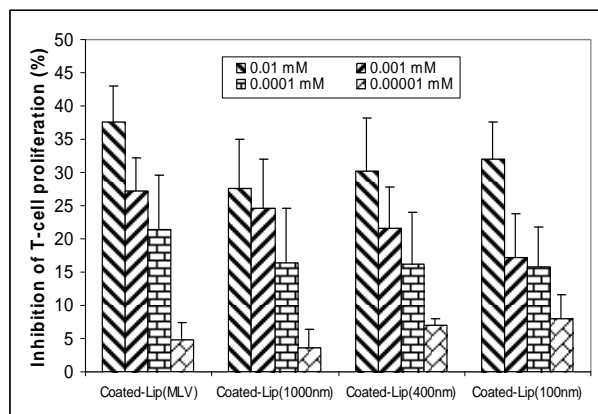


Figure 2. T-cell proliferation inhibition induced by various concentrations of chitosan coated liposomes containing CyA (Mean \pm SD, $n=8$). T-cells treated with PHA as a control considered 100%.

The result of T-cell proliferation inhibition assay of chitosan coated liposomes containing CyA was showed in Figure 2. The averages of IC_{50} for different sized liposomes were 3.08×10^{-2} , 1.23×10^{-1} , 3.54×10^{-1} and 6.86×10^{-1} mM for MLV, 1000 nm, 400 nm and 100 nm liposomes, respectively. IC_{50} results showed that MLV liposomes had the best inhibitory effects on T-cell proliferation. Statistical evaluation of the T-cell proliferation inhibition activity of different sized formulations showed that the effect of size of liposomes on the inhibition of T-cell proliferation is not significant ($p>0.05$).

Discussion

As it was mentioned earlier, to increase the residence time of a dosage form at the absorption site, the mucoadhesive carrier systems have been used recently (9, 11, 15). These mucoadhesive dosage forms are the attractive means to improve the bioavailability of drugs (15). For this reason, in this study, liposomes coated by chitosan were prepared.

Chitosan coated liposomes were considered to be formed via ionic interaction between the positively charged chitosan and negatively charged DCP on the surface of liposomes (9, 11, 15, 22). The formation of chitosan layer on the surface of liposomes is confirmed by zeta potential determination of the liposomes after coating. While some of the positively charged groups of chitosan might be used for complex formation, the residual ones contribute to the positive surface potential of liposomes and the

positive zeta potential support the formation of chitosan layer on the surface of liposomes (10, 22).

In this study, chitosan solution with concentration of 0.25% w/w was used for liposome coating. Guo *et al* study evaluated the effect of chitosan concentration on coating efficiency and reported that the coating efficiency increased up to 0.1% w/w and reached a saturated state at concentration of 0.2% w/w and above (24). They also found that the excess of polymer solution can react with liposome bilayer and cause aggregation (27). In our study, due to the mixing of polymer solution (0.25 %w/w) with the same volume of liposomes, the final polymer concentration in liposome suspension was 0.125 % w/w. In pervious studies, several concentrations (0.2-1%) of chitosan of different molecular weights were tested for liposome coating and it was shown that mucoadhesiveness increased with increased molecular weight and concentration of chitosan applied (10). For coating, it is favorable to add the liposome suspension to chitosan solution (and not the opposite order of addition). According to Henriksen *et al.* study, the excess of polymer solution volume can react instantaneously with liposome bilayer, while the concentration of chitosan itself should not be very high in order to avoid the aggregation and formation of polymer filaments (22).

Mucoadhesiveness was calculated as the amount of mucin adhered on the surface of liposomes. Positively charged liposomes adsorb mucin to a greater extent than negatively charged liposomes. This effect confirms the electrostatic occurrence of interaction of mucin and liposome bilayers but mucin adsorption to neutral liposomes showed that some other mechanisms, beside electrostatic interaction, regulate the process (10).

Taking into account the fact that most of liposomes were smaller than 5 μm , these particles can be suitable for oral delivery due to the possibility of being uptaken by the lymphoid system M cells in peyer's patches (28, 29). Based on the mechanism of drug effect (T-cell inhibition), direct uptake by the lymphoid system will improve the therapeutic effect of CyA. Also, to improve the stability of liposomes in the GI medium, different methods like using polymerized liposomes, PEG, GM1 coated liposomes and chitosan coated liposomes are suggested (30). For this reason, chitosan coating can help to increase the stability of liposomes in aggressive conditions of GI tract, improve liposome or drug transportation across intestine and can protect liposomes against

the digestion of pepsin, trypsin or GI lipases (10, 31).

Furthermore, the polycationic nature of chitosan could provide a strong electrostatic interaction with mucus or the negatively charged mucosal surface. The negative charge of mucin is due to the ionization of sialic acid (20). The flexible structure of chitosan on the surface might also participate in the physical entanglement between chitosan molecular chains and the mucus component on the surfaces, leading to prolonging the residence time of delivery systems in different membranes as well (31). It is expected that the drug molecule may be released upon interaction with mucin or degradation of liposome structure in the mucous layer (9, 15).

Evaluation of *in-vitro* T-Cell proliferation inhibition activity of different chitosan coated liposomes containing CyA revealed that these liposomes have less inhibitory effect compared to CyA aqueous solutions, fusogenic, and negatively charged liposomes that described in our pervious study (32). Chitosan coated liposomes (with positive charge) showed better inhibitory effects compared to positively charged liposomes. This effect shows that other mechanisms beside electrostatic interaction of liposome and lymphocyte contribute in this process.

In conclusion, the loading of CyA into chitosan coated liposomes was performed. They showed suitable stability and mucoadhesive properties. They are utilizable as candidate to improve the oral absorption of CyA.

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