Nanomedicine Journal

Received: Apr. 25, 2013; Accepted: Jun. 12, 2013
Vol. 1, No. 3, Spring 2014, page 147-154

Online ISSN 2322-5904
http://nmj.mums.ac.ir

Original Research

# In vivo evaluation of mucoadhesive properties of nanoliposomal formulations upon coating with trimethylchitosan polymer

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## Abstract

***Objective(s):*** Drug delivery via mucosal routes has been confirmed to be effective in inducing strong immune responses. Liposomes could enhance immune responses and mucoadhesive potentials, make them useful mucosal drug delivery systems. Coating of liposomes by mucoadhesive polymers succeeded in enhancing immune responses. Our studies aim at preparation and characterization of trimethylchitosan-coated nanoliposomes for nasal delivery of a model antigen, tetanus toxoid (TT).

***Materials and Methods:*** Anionic liposomes were prepared by dehydration-rehydration method with an average size of 100 nm and were coated with 0.01% (w/v) solution of trimethyulchitosan (TMC) with 50±10% of quaternization. Surface properties and zeta potential were evaluated by DLS. Antigen stability and integrity were studied by SDS-PAGE electrophoresis. Nasal clearance rate and mucoadhesive properties of liposomes were studied by gamma scintigraphy method using $^{99m}$Tc-labelled liposomes.

***Results:*** The zeta potential of non-coated and TMC-coated liposomes was -40 and +38.8, respectively. Encapsulation rate of tetanus toxoid was 77 ± 5.5%. SDS-PAGE revealed that the antigens remained intact during formulation procedure. Gamma scintigraphy confirmed that both types of liposomes could remain in nasal cavity up to ten folds over the normal residence time for conventional nasal formulations.

***Conclusion:*** TMC-coated nanoliposomes have several positive potentials including good mucoadhesive properties, preserved integrity of loaded antigen and presence of TMC as a mucoadhesive polymer with innate immunoadjuvant potential which make them suitable for efficient adjuvant/delivery system.

**Keywords**: Gamma scintigraphy, Nanoliposomes, Tetanus toxoid, Trimethylchitosan

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## Introduction

Antigenic materials can be administrated by various routes such as mucosal surfaces (1-2). Nasal route as a mucosal barrier has been shown many advantages. It has relatively large surface area, low proteolytic activity and can elicit both systemic and mucosal immunoresponses (3). Due to these advantages, the use of the nasal cavity as a route for antigen delivery has been confirmed to be effective in inducing strong immune responses (4-5). Liposomes as novel drug delivery systems represent promising immunization systems (6). Adhering to the mucosal barrier, prolonging the residence time on the adsorption site and reducing mucociliary clearance are the main advantages of liposomal formulations in inducing immune responses (7-8). Incorporation of mucoadhesive polymers such as chitosan to the delivery system can enhance absorption of antigen across the mucosal barrier by increasing their residence time on the nasal cavity (9-10). Several studies have reported that chitosan and its derivates have bioadhesive and permeation enhancing properties (10-11). It has been suggested that positively charged chitosan interact with negatively charged apical cell membrane by direct charge interaction mechanism and leads to transient opening of tight junctions, consequently increasing particle permeability (12). Additionally being mucoadhesive, chitosan and its derivates have also been shown to have adjuvant properties that enhance immune responses (13-14). They can open the intercellular tight junctions, and enhance transport of antigens (14-15). Combination of mucoadhesive properties and transient opening of tight junctions which enables the interaction of the chitosan-antigen form-ulation to interact with the lymph nodes of the nasal cavity resulted in improving immunological responses (16). In previous study, the adjuvant properties of chitosan formulations were demonstrated (14). At physiological pH, chitosan suffers from some limitations such as lack of solubility and positive charge (17). Due to these limitations, using improved derivatives of chitosan with enhanced solubility and high positive charge at neutral or basic pH, such as quaternized derivatives of chitosan, seems suitable. Trimethylchitosan (TMC) is soluble over the wide pH range and it has mucoadhesive properties and excellent absorption enhancing effects even at neutral or weak acid or base pH. These unique properties have sparked particular interest in their use in drug delivery applications (18-19). Several studies have been examined their usage as coating agents for particle formulations such as liposomes (20-21). The aim of the present study is to prepare TMC-coated nanoliposomes and evaluation of their mucoadhesion potential by gamma scinitigraphy method.

## Materials and Methods

### *Materials*

Phosphatidylcholine (PC), chitosan, Chole-sterol (Chol) purchased from Merck (Darmstadt, Germany). Dicethylphosphate (DCP) was from AvantiPolar Lipids, Inc., USA. Tetanus toxoid (TT) solution (1700 Lf/ml) was donated by Razi Inc. (Hesarak, Iran). $^{99m}$Tc-pertechnetate provided by Atomic Energy Organization of Iran (AEOI).

### *Preparation and characterization of lipo-somes encapsulated with TT*

Dehydration and Rehydration (DRV) method was used for preparation of liposomes. This procedure yield multi-lamellar vesicles (MLV) with a lipid com-position in molar ratio of 7:1:7 for PC/DCP/Chol. In brief, liposomes were prepared by solvent evaporation method. The dried lipid film was hydrated by TT solution in PBS. Liposomal suspension was sonicated to obtain small and uniform liposomes. The liposomes were freeze-dried and rehydrated by distilled water followed by vortexing to form liposome suspension. Non-entrapped TT was

separated by centri-fugation (Hettich, Germany) at 14000 rpm for 30 min and liposomes were washed twice.

The final liposomes were extruded repeatedly through 1000, 400 and 200 nm polycarbonate membrane filters at 45 $^0$C. Formulations were passed at least 11 times through the polycarbonate membrane to produce uniformly sized nanoliposomes.

The two-step method was selected to prepare TMC polymer. In the first stage, chitosan was partially methylated with methyl iodide (as the methylation agent). The synthesized derivative was precipitated with addition of ethanol, centrifuged, washed with acetone on a sintered glass filter and dried. The purified powder was methylated in second stage with methyl iodide. The prepared powder was purified similar to the first stage to obtain powder. The TMC.HCl powder was obtained by exchanging the counter ion with chloride.

To determine the degree of quaternization (% DQ), 1H NMR spectrum of TMC was obtained in $D_2O$, using a 600 MHz spectrometer (Bruker-Biospin, Rheinstetten, Germany) at 80 ºC. Coating procedure was employed using prepared TMC powder (22).

0.1% W/V solution of TMC was prepared in distillated water. Liposomal suspension was added dropwise into the trimethylchitosan solution under stirring (125 rpm at room temperature) in a volume ratio of 1:4.

The suspension was left overnight at 4 $^0$C. TMC- coated liposomes were separated by ultra-centrifugation at 9800 g for 30 min at 4 $^0$C and resuspended in distillated water. The washing step was performed twice.

The average particle size and charge of the liposomes were determined before and after coating procedure with a particle size analyzer (PSA) (Malvern, UK) at 25 ± 1°C. Encapsulation rate was determined by an indirect method.

Determination of the TT in supernatants was performed by Bradford protein assay method.

***Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE)***

The fragmentation and aggregation of loaded TT was evaluated by sodium dodecylsulfate-polyacrylamide gel electro-phoresis (SDS-PAGE) method. TT-loaded liposomes were run on a 7.5% SDS-polyacrylamide gel. The protein bands were visualized by silver nitrate staining.

***In vivo nasal clearance studies***

Liposome suspension (1 ml) was perpetrated in the labeling medium containing 1.5 ml of normal saline, 1 ml $SnCl_2 \cdot 2H_2O$ (5 mg/ml) and 0.7 ml technetium-99m pertechnetate (6 µCi activity). After stirring the mixture for 10 min, liposomes were separated by centrifugation at 13,000 **g** for 15 min. Hydroxyl groups as functional groups in trimethylchitosan and phospholipids are electron donating groups and can accept the technetium in active form, 5+ oxidation states (19). About 100 µl of $^{99m}$Tc-labelled liposomes were nasally administered to four rabbits. Nasal clearance of liposomes was determined by gamma-scintigraphy method using a single head SMV Gamma Camera (SMV, France). Data were recorded during 3 hours with 45 minutes intervals. Head position was fixed on the scintillation bed. Quantification of the data from the rabbit involved defining regions of interest (ROIs) around the desirable areas. The selected ROI was drawn around the site of deposition of the particles in the nasal cavity. The count rate from ROI, corrected for radioactive decay and background, was then expressed as a proportion of the highest 1 min count rate, typically the image recorded in the nasal cavity ROI immediately after dosing.

The highest count rate was assigned a 100% value, which was then used to calculate the percentage remaining for the other time points. In this way, the clearance of the formulations from the

nasal cavity was evaluated as a decrease in percentage activity against time for each rabbit.

## Results and Discussion

### *Liposomes characterization*

Zeta Potential and mean diameter of non-coated liposomes were illustrated in table 1. Non-coated liposomes exhibited a unimodal size distribution, but liposome suspensions after coating displayed a bimodal size distribution. Z-potential of non-coated and coated liposomes had negative and positive values, respectively. As seen in table 1, the encapsulation rate for both formulations was the same.

Bimodal size distribution of coated liposomes may be related to Masayukl Hara's theory (23). According to this theory, ratio of polymer coating was responsible for difference between sizes of liposomes. Liposomes with smaller size were partially coated, whereas larger sizes were fully coated by TMC. Electrostatic repulsion between TMC chains is another reason for size increase. TMC chains make them standing away from each other, resulting in size increase of coated liposomes. These findings were consistent with pervious findings (19). In this study, the minimum required concentration of polymer was used for coating procedure. High concentrations of polymer might lead to liposomal agg-regation. Liposomal aggregation upon coating procedure was mentioned earlier (19). As can be deduced from data presented in table 1, significant difference between the sizes of coated and non-coated liposomes was evident indicating the quality of coating procedure. Beside the effect of TMC on the liposomal size, this procedure affected the zeta potential. Shifting of zeta potential to positive values is an indicator of successful coating process. Higher values of zeta potential can prevent from flocculation and coagulation of nanoparticles and improve the liposome stability (24). Shifting of zeta potential to positive values and increasing the liposomal size were indicating that the quality of coating procedure was satisfactory.

It was well-established that structure of TMC had pronounced effects on coated particles (25). Due to this fact, seeking the TMC properties would be logical and reasonable. TMC has acetyl and partially quaternized amine groups. TMC polymers is a polycationic agent because of existence of trimethylated amines. Percent of trimethylated amine groups in TMC structure was determined by degree of quaternization. The quaternization degree of the TMC used in this study was approximately 50± 10%.

In fact, it was well recognized that solubility and quaternization degree have direct influences on drug delivery properties of TMC (24). These features could be controlled by selecting the preparation method. In present study, the TMC polymer was prepared with a two-step method. In two-step method, the minimum degree of quaternization is 40% and water solubility of polymer is optimal (26). Due to the higher quaternization degree and charge density of synthesized TMC, sufficient electrostatic interaction between anionic liposomes and cationic polymer could be predicted. In previous study, TMC with 65% quaternization degree was used to coat liposomes (19).

The results showed the bimodal size distribution of coated liposomes with sufficient coating. These results were in line with our findings.

Encapsulation efficiency (EE) results demo-nstrated that the EE of the polymer-coated liposomes was nearly the same as before coating procedure. These results revealed that during coating process formulations were not influenced by any stress. These findings were consistent with previous study which showed that EE was not altered upon coating (19).

**Table 1:** Size distribution, Zeta potential and encapsulation rate of liposomal formulations.

| Formulations | Zeta average | PDI | zeta potential | Encapsulation rate |
|---|---|---|---|---|
| **Non coated liposomes** | 153±8 | 0.21±0.02 | -40.1±2.5 | 72±5% |
| **Coated liposomes** | 376±79 | 0.47±0.07 | 34.1±6.6 | 70±3% |

***Stability of antigens***

SDS-PAGE result shows that during preparation and coating processes, TT is intact. These results were in agreement with earlier findings (27).

***In vivo nasal clearance studies***

The average nasal clearance in the nasopharynx ROI during three hours was shown in Fig 1. According to these results, the half-life of both formulations was more than 3 hours.

It should be mentioned that there were no significant differences between coated and non-coated liposomes ($P > 0.05$). It has been shown that for non-mucoadhesive particles, the clearance half-life is about 15-20 minutes (28).

Half-lives of both liposomes were about 200 minutes. In previous study, the half life of clearance for starch microspheres was found to be in the order of 240 min (27). These findings supported that both coated and non-coated liposomes have mucoadhesion potential.

The mucoadhesive properties of non-coated liposomes could be related to their nano size. It has been shown that by reducing the particle size to nano range, mucoadhesion property will increa-sed significantly (29). Jubeh et al showed that by reducing the particle size from 800 nm to 100 nm, the mucoadhesion property increased significantly (30). Although both anionic liposomes and mucosa have negative charges, muco-adhesion of particles was not influenced by the repulsion forces between them. These observations supported the effect of size on mucoadhesive particles. Charge of particles is another important factor for mucoadhesion properties.

The mucoadhesive property depends on the charge density. Jubeh et al showed that mucoadhesion potential of positively-charged liposomes was three times greater than neutral or anionic ones (29). As mentioned before, because of the polycationic character and mucoadhesion property of TMC, the TMC-coated liposomes exhibited sufficient mucoadhesive properties, despite of their larger size.

Mechanistically, the mucoadhesion of the TMC-coated liposomes appeared to be, in part, due to electrostatic attraction forces between the positively-charged amine groups in polymer structure and negatively- charged phosphate groups of sialic acid in mucin (31).

The slight reduction of muco-adhesion properties of coated liposomes might be related to polymer conformation. The conformation of polymer and degree of quaternization of the TMC polymers play an important role in interaction between polymer and mucin. A decrease in muco-adhesivity with an increase in the degree of quaternization of the TMC polymers was found.

Decreasing the flexibility of the polymer molecules was led to decrease in the mucoadhesion of TMC compared to the chitosan salts. This circumstance was explained by a change in the conformation of the TMC polymers due to interactions between the fixed positive charges on the quaternary amine groups, which possibly decreases the flexibility of the polymer molecules (32). Based on Hagenaars et al. study, TMC always does not change the antigen retention time in the nasal cavity (33). They suggested that, the possible mechanism for deceasing adjuvant effect of TMC formulations might be related to

the TMC structure. According to their findings, the TMC structure had direct effects on mucoadhesive and adjuvant properties. TMC with different quaternization degree has different properties. Based on presented results, the prepared TMC with quarter-nization degree 50%, did not exhibit better mucoadhesive propreties than uncoated particles. These results were supported by pervious findings. It was demonstrated that by increasing the quaternization degree, the mucoadhesive properties were slightly decreased (33).

Other mechanism which is concerned with the lack of mucoadhesive properties of coated particles might be due to enhanced enzymatic degradation by lysozyme. Lysozyme is a strong antibacterial cationic protein that is excreted in high conce-ntrations in the nasal cavity and can degrade TMC polymer (34-35).

Results of this study were also in agreement with findings of Khatri et al. They showed that both types of liposome, coated and non-coated one, adhered to the mucosal tissue.

It was suggested that non electrostatic interactions might be involved in muco-adhesive properties of liposomes (36-37).

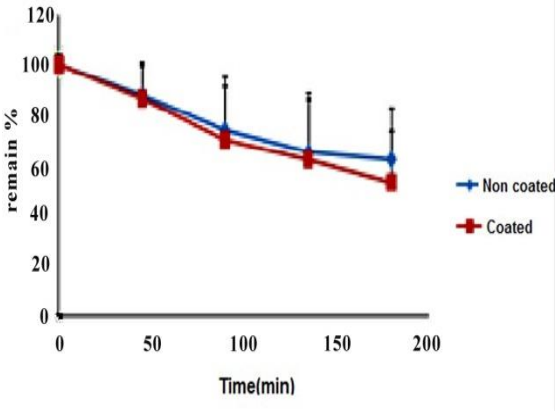


**Figure 1**. The percentage of remained liposomal formulations in the nasopharynx region.

## Conclusion

TMC-coated nanoliposomes showed several advantages which make them useful for an efficient adjuvant delivery system. The advantages include good mucoadhesive properties, preserved integrity of loaded antigen and presence of TMC as a mucoadhesive polymer with innate immune-adjuvant potential.

## Acknowledgements

This project was financially supported by a grant from Vice Chancellor for Research, Mashhad University of Medical Sciences. This manuscript is part of a Pharm.D. thesis.

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