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PREPARATION AND PROPERTIES OF LIPOSOMES COATED WITH *N*-ACYLATED LOW-MOLECULAR-WEIGHT CHITOSAN

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Amphiphilic low-molecular-weight chitosan N-acylated with 3-hydroxytetradecanoic acid was synthesized. It was shown that addition of the acylated chitosan changes the surface potential and size of negatively charged liposomal particles, the magnitudes of which depend on the chitosan concentration. Charge neutralization and aggregation of the liposomes occurs at low chitosan concentration. Increasing its concentration produces positive charge on the surface of the liposomes and decreases their size. This increases the stability of the liposomal particles in solution. Liposomes coated with chitosan may be of interest as carriers for genes, vaccines, and drugs.

Keywords: low-molecular-weight chitosan, acylated chitosan, liposomes, stability of liposomes.

Chitosan is a polycationic linear polysaccharide, the polymer chain of which consists of β-1,4-bonded D-glucosamine and *N*-acetyl-D-glucosamine units. Owing to its biocompatibility, biodegradability, and low toxicity, this biopolymer has been widely used in biotechnology, pharmaceutics, and medicine [1, 2]. Important pharmacological properties of chitosan are its ability to bind to the mucinous layer and epithelial cell membranes. It was found during a study of the interaction of chitosans and their derivatives with model membranes, spherical bilayer liposomes, that chitosan destroys the structure of the lipid matrix [3] but does not form stable pores in the lipid bilayer of the liposomes [4].

At present much attention is directed toward the production of chitosan derivatives that interact more effectively with cell membranes than high-molecular-weight chitosans. A promising area for modifying chitosan is its partial depolymerization. This can increase its solubility at neutral and slightly basic pH values.

A method for changing the membraneotropic properties of chitosan is selective acylation of some of the amines. This increases the hydrophobicity of the chitosan and retains its solubility in aqueous solutions. Acylated derivatives of high-molecular-weight chitosan bind to liposomes to a larger extent than unacylated chitosan [5]. Addition of chitosan to liposomes prevents their aggregation and increases significantly the stability of the liposomes upon administration to an organism [6, 7].

Liposomal forms of chitosan were examined as promising carriers of drugs and vaccines for prolonged release in an organism [5–8]. The interaction of chitosans with liposomes was studied mainly using high-molecular-weight chitosans and their derivatives that were highly substituted with fatty acids [5–7]. It seems interesting to produce liposomes coated with mono-acylated low-molecular-weight chitosans. It is assumed that decreasing the chain length of the polycation bound to the liposome surface reduces its interaction with the epithelial mucinous layer and increases the penetration of liposomal particles into epithelial cells [9].

Low-molecular-weight chitosan (C-LM) and its water-soluble derivative mono-*N*-3-hydroxytetradecanoylchitosan (acyl-C-LM) were produced earlier by us. Certain of its properties have been studied [10]. The goal of the present work was to prepare liposomal forms of an *N*-acylated derivative of chitosan, to determine the physicochemical properties of complexes of acyl-C-LM with liposomes of various composition, and to study the influence of chitosan and its amphiphilic derivative on the size and stability of the liposomes in solution.

Chitosan of molecular weight about 80 kDa and with a low (1.57%) degree of *N*-acetylation was prepared by de-acetylation of commercial chitosan using a mixture of aqueous NaOH in isopropanol. Depolymerization of high-molecular-

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weight chitosan by hydrogen peroxide produced C-LM that had an average molecular weight of 4.5 kDa according to analysis of the terminal reducing groups. C-LM was *N*-acylated using *N*-hydroxytetradecanoic acid. Acyl-C-LM was isolated using C18 reversed-phase chromatography.

The acyl-C-LM contained one 3-hydroxytetradecanoic acid that acylated the glucosamine amine of the reducing terminal polysaccharide according to GC analysis and PMR and ^{13}C NMR spectroscopy. The composition of the resulting chitosan derivative was confirmed by MALDI-TOF MS. The spectrum of acyl-C-LM showed peaks corresponding to the molecular-weight distribution $[M + H]^+$ from 1544.1 to 3929.5 *m*/*z*, corresponding to derivatives containing 8–23 glucosamine units and one 3-hydroxytetradecanoic acid.

Acylated chitosans, even those with a relatively low degree of fatty-acid substitution, are known to aggregate in solution to form small micelles [11]. The critical micelle-forming concentration of acyl-C-LM was 350 μg/mL according to a fluorescence probe (8-anilino-1-naphthalenesulfonic acid) [12]. This is greater than the concentration of the chitosan derivative (5–200 μg/mL) used in the present work. Therefore, it can be assumed that acyl-C-LM in the monomolecular form was complexed to the liposomes.

Derivatives of C-LM and acyl-C-LM with fluorescein (F-C-LM and F-acyl-C-LM), the molecules of which averaged one amine substituted by the fluorescent marker, were synthesized in order to study their binding to the liposomes. The study of the interaction of C-LM and acyl-C-LM with the liposomes was also carried out using their derivatives labeled with biotin (B-C-LM and B-acyl-C-LM). The label was introduced using the *N*-hydroxysuccinimide ester of biotinamidohexanoic acid.

Large unilamellar liposomes that were used in the binding experiments with the chitosans were prepared by the published reversed-phase method [13]. Three phospholipid samples, egg lecithin and synthetic ditetradecanoyl- and dioctadecanoylphosphatidylcholine, were used to prepare the liposomes. Both neutral and positively charged liposomes were prepared by adding dicetylphosphate to the lipid mixture.

The binding of F-C-LM and F-acyl-C-LM with neutral liposomes prepared from dioctadecanoyl phosphatidylcholine and cholesterol was carried out by incubating a mixture of the components at 37°C. The incorporation of F-acyl-C-LM was nine times greater than that of non-acylated F-C-LM and reached 1 mole of F-acyl-C-LM per 230 moles of phospholipid for the maximum binding of the chitosan derivative with the liposomes. Thus, increasing the hydrophobicity of the chitosan by introducing the fatty acid increased the effectiveness of its interaction with the neutral liposomes. This results agrees with the literature, according to which high-molecular-weight chitosan (150 kDa) with 3.5% acylation by dodecyl units bound better to neutral lipids than the non-acylated chitosan [5].

A method based on the interaction of biotinylated chitosan with the liposomes was used to determine the binding effectiveness of chitosan and its acylated derivative with negatively charged liposomes of various phospholipid composition. It is known that a negative charge on the membrane prevents spontaneous fusion of liposomes and increases their binding with positively charged chitosan and its derivatives [5]. The maximum binding was determined by adding various amounts of B-C-LM and B-acyl-C-LM to identical amounts of liposomes. The maximum binding of acylated chitosan depended on the liposome phospholipid composition. The binding effectiveness dropped in the order liposomes containing egg lecithin > ditetradecanoyl- > dioctadecanoylphosphatidylcholine. The resulting trend corresponds with the apparent binding constants of B-acyl-C-LM to liposomes of the different compositions that were calculated from a Scatchard plot, 0.73×10^6 M^{-1}, 0.45×10^6 M^{-1}, and 0.32×10^6 M^{-1}, respectively. An analysis of the data showed that liposomes containing lecithin contain 1 mole of B-acyl-C-LM per 60 moles of phospholipid. Non-acylated B-C-LM was incorporated into negatively charged lecithin liposomes three times more than acylated chitosan.

Natural lecithin is well known to contain a significant amount of unsaturated fatty acids. This lowers the phase-transition point of the phospholipids and decreases the rigidity of the liposomal membrane. In fact, a significant factor affecting B-acyl-C-LM binding is the temperature at which the chitosan interacts with the liposomes. Thus, liposomes formed from dioctadecanoylphosphatidylcholine at 20°C bound only 15% of the amount of B-acyl-C-LM bound at 37°C. This phospholipid exists at 20°C in the gel phase [14]. Apparently this prevents incorporation of the fatty-acid chains of acylated chitosan into the liposome bilayer.

An important step in the production technology of liposomes and liposomal drugs is the control of their size and structure. The influence on the liposome size of the concentration (5–500 μg/mL) of incorporated chitosans was studied using a turbodimetric method for titrating neutral or negatively charged liposomes with various concentrations of C-LM and acyl-C-LM. The optical density decreased slightly upon adding C-LM to a liposomal suspension. This was consistent with an insignificant change in the size of the liposomes [15]. Adding acylated chitosan at low (5–30 μg/mL) concentrations increased significantly the absorption and visible aggregation of neutral and negatively charged liposomes (Fig. 1).

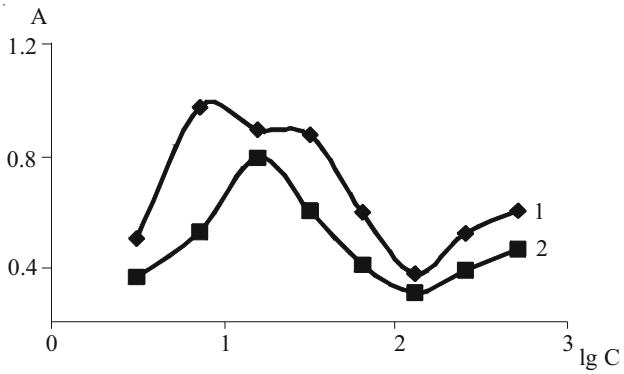

Fig. 1

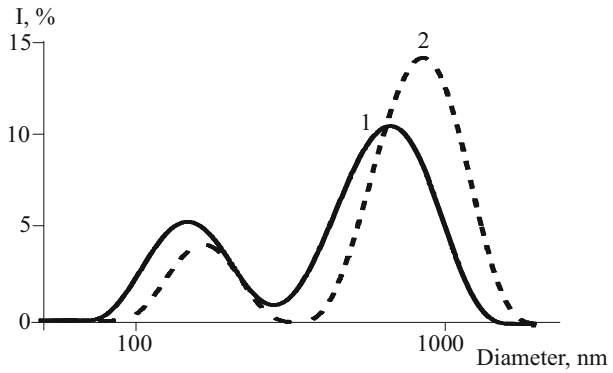

Fig. 2

Fig. 1. Optical density of neutral (1) and negatively charged (2) liposomes (500 μg/mL of phospholipid) as a function of logarithm of acyl-C-LM concentration (5-500 μg/mL). Measurements were made at 450 nm.

Fig. 2. Size-distribution of negatively charged lecithin liposomes coated with *N*-acyl-C-LM passively (1) and actively (2). *I* (%) is the light-scattering intensity.

Increasing the concentration of acyl-C-LM further (60–150 μg/mL) decreased the optical density and; therefore, the size of the liposomes compared with the initial size. The size of the liposomes increased slightly upon increasing the chitosan concentration to 250–500 μg/mL. According to the literature, such size changes of liposomes were observed during their interaction with high-molecular-weight chitosan (205 kDa) with a rather high (10%) degree of acetylation [16].

It can be assumed that the surface ζ-potential of the liposomal particles can be changed by binding of positively charged chitosan to the liposomes. This affects their size or their stability. The ζ-potentials of negatively charged liposomes were measured by laser Doppler electrophoresis methods before and after binding to acyl-C-LM. Concentrations of acylated chitosan (125 μg/mL) at which liposomes were the smallest as determined by the turbodimetric titration method were used in the experiments. Chitosan was incorporated into the liposomes in two ways: actively by adding acyl-C-LM at the liposome production step or passively by using spontaneous incorporation of the chitosan derivative into the liposome membrane.

Measurements of the ζ-potentials indicated that interaction of the liposomes with the polycation acyl-chitosan resulted in compensation of the negative surface charge of the liposomal particles and the appearance of a high positive charge:

Sample	*ζ- Potential, mV*
Liposomes	–20
Liposomes loaded with acyl-C-LM:	
actively	+33
passively	+51

The negative charge was compensated less for active incorporation of acyl-C-LM into the liposomes than for passive loading. Apparently this was due to incorporation of part of the chitosan into the liposome inner volume.

Photon-correlation spectroscopy showed that large initial liposomes greater than 1000 nm in size dissociated under the influence of acyl-chitosan. As a result, a bimodal size distribution of the liposomes was observed for passive and active loading of *N*-acyl-C-LM. Figure 2 shows that average-sized liposomes with particles of diameters 712 and 825 nm, respectively, were the most common with smaller liposomal particles of diameters 141 and 164 nm, respectively, forming a second peak.

The literature contains contradictory information regarding the influence of chitosan on the size of liposomes. According to Fang et al. [3], high-molecular-weight chitosan (213 kDa) disrupts the lipid bilayer, which leads to fusion of multi-lamellar liposomes and their aggregation. It was shown [16] that the concentration of incorporated chitosan has a substantial influence on the size and stability of liposomes upon binding of high-molecular-weight chitosan (205 kDa). Aggregated particles of diameter up to 8,000 nm were formed from starting liposomes of diameter 98 nm at low (0.01%) chitosan concentrations. Increasing the chitosan concentration further up to 0.07% reduced the size of the liposomes to 300 nm and increased the ζ-potential to +50 mV. It is assumed that the appearance of the positive charge produces a mutual repulsion of the similarly charged liposomes and dispersion of the aggregates. This may further stabilize the liposomes in solution [6, 16]. The chitosan-coated liposome preparations obtained in our experiments were stable and did not aggregate over 10 days.

Thus, a liposomal form of *N*-acylated C-LM containing one 3-hydroxytetradecanoic acid unit per chitosan molecule was prepared. The *N*-acylated chitosan interacts effectively with liposomes of various composition. The amount of bound chitosan decreases in the order of liposomes containing egg lecithin > ditetradecanoyl- > dioctadecanoylphosphatidylcholine. Acylated chitosan at low concentrations (5–30 μg/mL) initiates aggregation of the liposomes. At higher concentrations (60–150 μg/mL) is causes enlargement of the liposomes and a change of the surface potential. The shrinkage and appearance of positive charge on the liposomal particles stabilizes suspensions of the liposomes in solution. The results indicate that the selection of the optimum phospholipid composition of the liposomes and the concentrations of chitosans reacted with the liposomes are important. Liposomes coated with C-LM may be of interest as carriers of genes, vaccines, and drugs.

EXPERIMENTAL

PMR and ^{13}C NMR spectra were recorded on a DPX-300 instrument (Bruker, USA) at 300 MHz. The solvent was D_2O with acetone internal standard (chemical shift δ_H 2.225 ppm; δ_C 29.2 ppm). Mass spectrometry used a Biflex III MALDI-TOF spectrometer (Bruker, USA) with linear detection of positive ions.

Preparation of C-LM, *N*-3-hydroxytetradecanoylchitosan and their biotin-labeled derivatives was reported earlier [10]. Chitosans labeled with fluorescein were prepared by reaction with fluroesceinisothiocyanate by the literature method [17].

Preparation of Liposomes Labeled with C-LM and Acyl-C-LM. Large unilamellar liposomes were prepared by a reversed-phase method [13] with some changes. The lipid film obtained from phospholipids, cholesterol, and dicetylphosphate (mole ratio 2:1.5:0.2) was shaken with a mixture of ester and physiological solution, sonicated for 15 min at 4°C, and evaporated in vacuo. The liposome suspension was diluted with acetate buffer (0.01 M, pH 5.5). The fine liposomes and undispersed lipids were removed by centrifugation at 20,000 g, washing the liposomes several times with acetate buffer. The resulting liposome precipitate was suspended in acetate buffer and treated with an equal volume of a solution of chitosan or its derivative (final concentration 2 mg/mL). The mixture was incubated overnight at 37°C. Non-bonded chitosan was separated by centrifugation at 20,000 g. The size of the liposomes (500 μg/mL of phospholipids) after reaction with C-LM and acyl-C-LM added at various concentrations (5–500 μg/mL) was monitored by measuring the optical density of the liposome suspension at 450 nm [15].

Determination of the Amount of C-LM and Acyl-C-LM Incorporated into the Liposomes. An aliquot of liposomes (20 μg of phospholipids) was treated with various concentrations (5–200 μg/mL) of chitosans or their acyl derivatives labeled with fluorescein or biotin. Chitosans not bonded to liposomes were removed by centrifugation at 20,000g, washing liposomes with acetate buffer (pH 5.5). The fluorescence of bound F-C-LM or F-acyl-C-LM was determined in solution after lysis of the liposomes by triton X-100 (2%). The content of chitosans was calculated from a calibration curve. The amount of B-C-LM and B-acyl-C-LM bound to liposomes was determined using the reaction of purified liposomes labeled with the conjugate streptavidin–horseradish peroxidase and *o*-phenylenediamine as a substrate. The maximum number of chitosans bound to the liposomes was determined graphically from a saturation curve.

Determination of the Size and Electrokinetic Properties of Liposomes Coated with Chitosan or Its *N*-Acylated Derivative. The sizes and ζ-potentials of liposomes and their complexes with the chitosans were determined using a ZetaSizer Nano ZS intrument (Malvern, Great Britain) at 25°C, fixed scattering angle 173°, and laser wavelength 633 nm.

REFERENCES

1. A. K. Singla and M. J. Chawla, *Pharm. Pharmacol.*, **53**, 1047 (2001).
2. L. A. Nud′ga, *Chitin and Chitosan* [in Russian], Nauka, Moscow, 2002.
3. N. Fang, V. H.-Q. Chan, and K. W. Mao, *Biomacromolecules*, **2**, 1161 (2001).
4. D. Raafat, K. Bargen, and A. Haas, *Appl. Environ. Microbiol.*, **74**, 3764 (2008).
5. J. Thongborisute, H. Takeuchi, H. Yamamoto, and Y. Kawashima, *Pharmazie*, **61**, 106 (2006).
6. J. Filipovic-Grcic, N. Skalko-Basnet, and I. Jalsenjak, *J. Microencapsulation*, **18**, 3 (2001).
7. G. Liang, Z. Jia-Bi, X. Fei, and Ni Bin, *Pharm. Pharmacol.*, **59**, 661 (2007).

8. V. Shihorkar and S. P. Vyas, *Pharm. Pharmaceut. Sci.*, **4**, 138 (2001).
9. J. A. Hubbell, N. T. Susan, and A. S. Melody, *Nature*, **462**, 449 (2009).
10. G. A. Naberezhnykh, V. I. Gorbach, G. N. Likhatskaya, V. N. Davydova, and T. F. Solov′eva, *Biokhimiya (Moscow)*, **73**, 530 (2008).
11. G.-B. Jiang, D. Quan, K. Liao, and H. Wang, *Carbohydr. Polym.*, **66**, 514 (2006).
12. V. I. Gorbach and E. B. Shentsova, *Khim.-farm. Zh.*, **11**, 6 (1999).
13. E. Ralston, *Biochim. Biophys. Acta*, **641**, 133 (1981).
14. J. T. Mason, C. Huang, L. Rodney, and M. Biltonen, *Biochemistry*, **22**, 2013 (1983).
15. Yu. L. Sebyakin, U. A. Budanova, and L. Yu. Gur′eva, *Biol. Membr.*, **24**, 259 (2007).
16. C. Laye, D. J. McClements, and J. Weiss, *J. Food Sci.*, **73**, 7 (2008).
17. R. B. Qaqish and M. M. Amiji, *Carbohydr. Polym.*, **38**, 99 (1999).