International Journal of Pharmaceutics, 101 (1994) 227–236

IJP 03329

Interactions between liposomes and chitosan

Ingrid Henriksen, Gro Smistad and Jan Karlsen

Department of Pharmaceutics, Faculty of Science, The University of Oslo, Oslo (Norway)

(Received 20 April 1993)
(Modified version received 7 May 1993)
(Accepted 28 May 1993)

Key words: Liposome; Chitosan; Interaction; Electrophoretic light scattering; Zeta potential; Cryo-electron microscopy

Summary

Negative and neutral liposomes were coated with the cationic polysaccharide chitosan, and the interaction was studied by photon correlation spectroscopy (PCS), electrophoretic light scattering (ELS) and cryo-electron microscopy. As shown with ELS, both types of liposomes had a reproducible change in zeta potential after coating. Electron micrographs showed no visible change in individual liposomes, but a limited degree of aggregation for neutral and a more extensive formation of liposomal clusters for negative liposomes. After centrifugation and washing, the liposomes were still coated and appeared unchanged on the electron micrographs.

Introduction

The interest in studying interactions between liposomes and different groups of polysaccharides has in many cases derived from new knowledge about the existence of polysaccharides on cell surfaces (Hughes, 1975). Sunamoto et al. (1980a,b) and Iwamoto and Sunamoto (1982) studied polysaccharide-induced aggregation of egg lecithin liposomes using pullulan, dextran, hydroxyethylstarch and amylopectin. The aggregation was observed for various polysaccharide structures, temperatures, ionic strength and liposomal surface charge.

Correspondence to: I. Henriksen, Department of Pharmaceutics, Faculty of Science, The University of Oslo, Oslo, Norway.

Polysaccharides have also been combined with liposomes in an attempt to stabilize the liposomes as drug delivery vehicles (Alamelu and Panduranga Rao, 1991) or for targeting purposes (Sunamoto et al., 1984, 1987, 1988) or artificial red blood cells (ARBCs) (Kato and Kondo, 1987). Several of these studies utilized chitin-derived compounds in place of the polysaccharide. Kato and Kondo (1987) used carboxymethyl-chitin (CM-chitin) to produce their ARBCs, encapsulating hemolysate in liposomes and coating them with this negatively charged polysaccharide. Alamelu and Panduranga Rao (1991) used carboxymethyl-chitosan as a coating on liposomes containing dapsone. Both groups found an increased stability against release of selected markers compared to uncoated liposomes.

Chitosan is commonly used to refer to deacetylated chitin where most of the *N*-acetyl groups

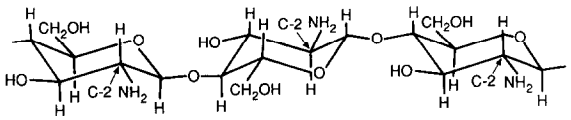

Fig. 1. Structure of chitosan.

have been removed. In contrast to its parent substance, it dissolves in dilute acids to produce a linear polyelectrolyte with a high positive charge density (see Fig. 1). Chitosan has been shown to bind aggressively to a variety of mammalian and microbial cells (Olsen et al., 1989). This cell membrane binding property appeared to be directly related to its cationic polyelectrolyte structure. Several workers have for this reason utilized chitosan for flocculating cell suspensions (Eriksson and Härdin, 1987; Baran, 1988) or selectively flocculating cell disintegrates with high product recoveries (Agerkvist et al., 1990).

Lehr et al. (1992) have studied the mucoadhesive properties of chitosan as a detachment force for polymer films from pig intestinal mucosa. The results were promising. Bioadhesive liposomes have been suggested as topical drug delivery systems for the treatment of wounds and burns or for administration to eye or to tumours (Margalit et al., 1992). Chitosan meets the requirements of being biocompatible and biodegradable (Hirano et al., 1988) and therefore seems to be a good candidate for the production of stable, bioadhesive liposomes. This report presents the results of coating liposomes with this cationic polymer. The results are then discussed in relation to selected literature in the area.

Materials and Methods

Chitosan preparation and characterisation

The chitosan tested in this study was Sea Cure +210 C® from Pronova Biopolymer a/s (lot no. 012-422-01). This sample is in the form of a hydrochloride salt, spray-dried and sieved through a 60 mesh sieve. The production method has been described by Skaugrud (1989).

We redissolved this chitosan sample in water to remove the surplus chloride by dialysis against distilled water. The resulting solution was frozen at −70°C and freeze-dried. The number average molecular weight was estimated to 2.2×10^5 from the intrinsic viscosity and the Mark Houwinks equation (see below).

The degree of deacetylation was found to be 91.6% by proton NMR (100 MHz, JEOL NMR Spectrometer) (Vårum et al., 1991).

The moisture content of the sample was below 10%, and the concentrations of chitosan solutions given here are based on the total weight including moisture. An extensively degraded chitosan sample was used in some preliminary experiments ('low-molecular weight chitosan'). The degradation was performed as described by Vårum et al. (1991) and this gave a sample of only about 15 monomer units.

The molecular weight of polymers can be related to the intrinsic viscosity according to application of the Mark Houwinks equation (Roberts and Domszy, 1982; Wang et al., 1991):

$$[\eta] = K \cdot M^{\alpha}$$

The intrinsic viscosity was measured using an Ubbelohde viscometer with automatic dilution and a thermostated 20°C water bath. The intrinsic viscosity was found to be 8.66 dl/g (SD = 2.06%) in 0.02 M acetate buffer + 0.1 M NaCl. We used the values of $K = 5.85 \times 10^{-4}$ dl/g and $\alpha = 0.78$, as determined by Anthonsen et al. (1993) and so estimated the average number molecular weight to 2.2×10^5 g/mol (the parameters are actually found for a 85% deacetylated chitosan in the same medium, and the molecular weight therefore represents an estimate only).

Preparation of the liposomes

Liposomes were produced using egg L-α-phosphatidylcholine (PC, approx. 99%) only, or with 10% L-α-phosphatidyl-DL-glycerol (PG, approx. 99%) to give a net negative charge. The phospholipids were supplied by Sigma Chemical Co. Liposomes were prepared by the film method as follows: PC and PG chloroform solutions were mixed and rotary evaporated to dryness in a 250 ml

flask. The resulting film was further dried under vacuum for 15 min. The aqueous medium was then added to give a concentration of phospholipids of 5.5 mg/ml (= 7 μmol/ml). The mixture was gently shaken for 10 min at 40°C and then allowed to swell for 2 h under an N_2 atmosphere at room temperature. This suspension of hand-shaken liposomes was extruded 10 times through 100 nm polycarbonate membrane filters (Nuclepore) with a Lipex extruder.

All the lipid films were suspended in 30 mM NaCl with pH adjusted to 5 ± 0.1 using HCl. The chitosan was dissolved in the same medium. This ionic strength was chosen since it was found to be suitable when measuring the zeta potential.

Coating of the liposomes

Coating of the liposomes was attempted by dropwise addition of 0.5 ml of the liposome suspension to 2 ml of the chitosan solution (filtered 0.8 μm) during continuous magnetic stirring and leaving the mixture in a refrigerator overnight. The resulting particles will for simplicity be referred to as 'chitosomes'. The PC/PG chitosome suspension was then diluted to 10 ml and centrifuged on a Sorvall RC5C centrifuge at 47000 $\times g$ for 1 h. The supernatant was removed, and the pellet was resuspended in 10 ml of the medium. The same procedure was repeated twice, and the last pellet was resuspended in 1 ml medium. The PC chitosome suspensions were centrifuged after dilution to 8 ml on a Beckman ultracentrifuge at $214000 \times g_{max}$ for 1 h. The pellet was resuspended and centrifuged twice at $77000 \times g$.

Size measurements

Determination of the size of liposomes was carried out via photon correlation spectroscopy (PCS) using a Coulter N4 MD at a 90° angle. This model has two available programs for analysis of the PCS data: the unimodal analysis and the size distribution processor analysis (SDP). The unimodal analysis provides a measure of the particle mean size of the sample and a measure of the polydispersity or breadth of the particle size distribution. The analysis interprets the size distribution as a log Gaussian. The SDP analysis gives the particle size distribution without this assumption by mathematically separating the decay times in the composite autocorrelation function. This is a complex process, and in our experience it provides reproducible results when the polydispersity is low, or when each fraction of a multimodal sample has a narrow size distribution.

Cryo-electron microscopy

By this method the liposomes can be observed without the addition of any chemicals or stains. The method is suitable for the in-process and product control during formulation of liposomal drug delivery systems (Smistad et al., 1991). The liposome and chitosome suspensions were vitrified in thin films on 300 mesh copper grids coated with a perforated carbon film. The method has been extensively described by Dubochet et al. (1988). Liquid ethane was used as cryogen. Specimen preparation was carried out in a flow of humid air in order to reduce the evaporation and drying effects (Cyrklaff et al., 1990). The specimens were observed at -170°C in a Philips CM12 electron microscope using a Gatan 626 cryo-transfer system. Micrographs were recorded under low dose conditions.

Zeta potential

The zeta potential of the particles was measured with a Coulter DELSA 440® (Doppler-Electrophoretic Light Scattering Analyzer). DELSA combines the principles of electrophoresis and laser Doppler velocimetry, and so provides measurements of the electrophoretic mobility of suspended particles. The system analyses these particles by making independent laser Doppler measurements at four different angles simultaneously, and this facilitates separation of real mobility peaks from artefacts. A more detailed validation of the method is given by Woodle et al. (1992).

Because of the electro-osmotic flow profile in the DELSA sample chamber, the laser beam must be located at the zero electro-osmotic velocity plane (designated the 'stationary layer') during measurements. The liposomes were measured several times in both stationary layers of the sample chamber, to confirm that the measuring

position was correct. Usually, three measurements were performed in each stationary layer for every parallel. Short on-times were used to avoid Joule heating. The zeta potentials (ζ) were deduced from the mobility (U) based on the equation: $\zeta = 4\pi\eta U/\epsilon$, where η is the viscosity of the suspending medium and ϵ denotes the dielectric constant. The diffusive motion of the particles gives a broadening of the peaks in the mobility spectrum, and this can be utilized to give an indication of particle size (small particles give broader peaks).

The pH of the test solutions was checked before and after measurements, and no change was seen.

The viscosities used when computing the zeta potential of chitosomes with excess chitosan still present were determined using a capillary viscometer. These diluted solutions were considered to be Newtonian.

Results

Selection of the coating system

Two methods was initially compared: the addition of chitosan solutions to liposomes, and the opposite order of addition. Two chitosans were tested: a low- (LMW) and a high-molecular weight (HMW) sample. The results are shown in Table 1.

TABLE 1

The effect of order of addition, chitosan concentration and chitosan molecular weight on the resultant chitosan / liposome mixture

Chitosan concentration (ppm)	HMW CHITOSAN		LMW CHITOSAN	
	Ch. added	Lip. added	Ch. added	Lip. added
0.01	*	–	–	–
0.1	*	–	–	–
1.0	*	*	–	–
10	***	***	–	–
20	***	–	–	–
50	****	–	–	–
100	****	**	****	****
250	***	–	–	–
500	**	**	****	****
1000	**	**	****	****
2000	**	**	****	****

2 ml of the given chitosan solution were mixed with 0.5 ml PC/PG liposomes (5.5 mg/ml) *, no change; **, slightly more turbid; ***, visible particles; ****, extensive aggregation. Ch added, chitosan solution added to liposome suspension; Lip. added, liposome suspension added to chitosan solution; –, not tested.

With the addition of 250 ppm HMW chitosan solution, immediate aggregation was seen, but the mixture restabilized within a few minutes to give a turbid suspension. 500, 1000 and 2000 ppm solutions gave stable, slightly turbid suspensions.

Addition of negative liposomes to 100, 500, 1000 and 2000 ppm LMW chitosan solutions and also the opposite order of mixing all led to extensive aggregation.

To avoid extensive aggregation, it appears to be necessary to have a certain excess of chitosan in the solution during coating, and the molecular weight should not be too low. Likewise, the order of mixing of the two solutions seems to affect the coating kinetics, as restabilization of the liposomes occurs at a lower chitosan concentration when liposomes are added dropwise to it. The procedure for the addition of liposomes to a 2000 ppm chitosan solution was chosen on the basis of the above factors for further investigations (Kato et al. (1985) used the same concentration of CM-chitin).

Size measurements with PCS

Table 2 lists the results obtained by both unimodal and SDP analyses of liposome and chitosome size.

Typically, the SDP analysis gave a mean size of the extruded liposomes of 102 nm for those composed of PC/PG, while PC liposomes were somewhat larger. After addition to chitosan solution, the size distribution apparently become too great for the use of SDP analysis. However, it is clear from the unimodal mean that the negative liposomes aggregated to a greater extent than the neutral ones, as indicated on the electron micrographs (Figs 4 and 5).

After centrifugation as described above, the unimodal sizes did not show a significant change. The PCS measurements after centrifugation show a more narrow distribution for the PC/PG chito-

TABLE 2

Size and zeta potential results of liposomes, chitosomes and centrifuged / washed chitosomes

Type	Size (nm) (±SD) (unimodal)	Size (nm) (±SD) (SDP)	Zeta potential (mV) (±SD)
PC/PG liposomes	118± 31	102± 28	−26.9±2.3
			−25.5±2.3
			−24.5±1.5
PC/PG chitosomes	580, broad	–	+88.1±2.1
			+88.5±2.5
Centrifuged	593±220	780±190	+32.4±1.0
			+32.8±0.9
			+35.7±1.5
PC liposomes	130± 35	119± 31	−1.3±1.7
			−3.4±1.3
			−0.8±0.7
PC chitosomes	226, broad	–	+84.7±2.6
			+85.6±2.9
Centrifuged	221, broad	–	+29.6±2.7
			+33.9±2.3
			+28.2±1.0

The size is given as diameter and standard deviation (SD) of the size distribution, while the zeta potential is given as a mean of two parallels with six measurements at four angles (= 48 independent measurements)±SD. Values are given here for two or three different liposome batches.

somes than for the PC chitosomes. This can be explained by a more selective centrifugation method in the first case, since the speed is much lower. Single chitosomes that are present will not be pelleted to the same extent.

The standard deviations given in Table 2 represent the width of the size distribution.

Zeta potential

Doppler electrophoretic light scattering gives a reproducible electrophoretic mobility for small liposomes (Woodle et al., 1992).

As seen from Table 2, DELSA analyses showed that the negative liposomes had an initial zeta potential of about −25 mV. The zeta potentials of the coated particles were about +88 mV. After centrifugation the zeta potential was about +32 mV. Fig. 2 depicts a run comparison of the zeta potential peaks before and after coating and after centrifugation.

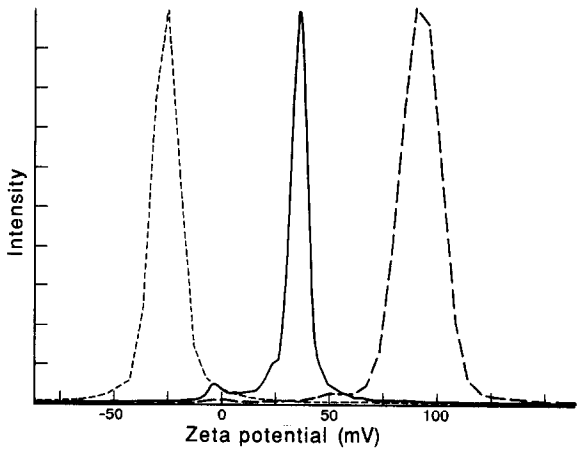

Fig. 2. PC/PG liposomes and chitosomes: DELSA outprints comparing zeta potential peaks at 15° angle for liposomes (- - - - -), chitosomes with chitosan present in solution (— — —) and chitosomes after centrifugation/washing (———).

The PC liposomes showed a small negative zeta potential, in agreement with the observations of others (e.g., Plank et al., 1985; Klein et al., 1987; Law et al., 1988; and Makino et al., 1991). The measurements of zeta potentials showed a change from the initial −1 to +85 mV after coating and +29 mV after centrifugation and washing (Fig. 3). This suggests that even the 'neutral' liposomes are permanently coated. Due

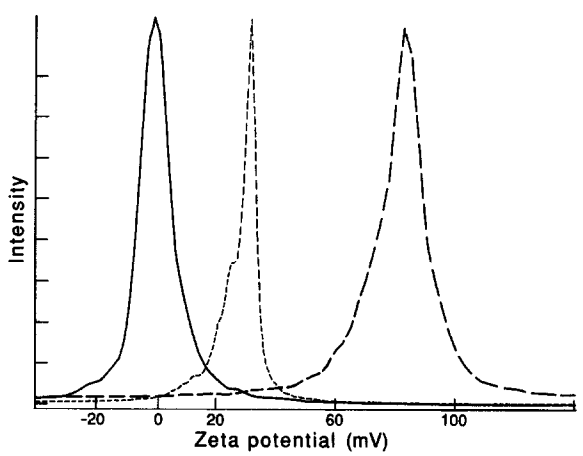

Fig. 3. PC liposomes and chitosomes: DELSA outprints comparing zeta potential peaks at 15° angle for liposomes (———), chitosomes with chitosan present in solution (— — —) and chitosomes after centrifugation/washing (- - - - -).

to the smaller particles (no large clusters) and the high initial viscosity of the solutions, an ultracentrifugation procedure was necessary to collect the chitosomes. This was still less effective compared to the PC/PG chitosomes, and so gave sub-optimal concentrations of the suspension and accordingly a poorer quality of the DELSA measurements.

The reduction in zeta potential seen after centrifugation may result from the removal of excess chitosan in the solution. During measurements, such polymer chains may be loosely associated with the particles and increase their electrophoretic mobility. Ageing of liposomes also influences the zeta potential, and this is now under study in our laboratory.

It is probable that the standard deviations within parallels will be smaller if measurements in only one position are used. We still chose to use both stationary layers, in order to obtain additional confirmation that there is no contribution to the mobility from electro-osmosis. The between-batch variation shown in Fig. 2 can be attributed to small variations in PG content and slight mispositioning of the stationary layer.

Cryo-electron microscopy

Fig. 4a shows the negative PC/PG liposomes after extrusion and before coating. The micrographs generally show a relatively uniform population of mainly unilamellar vesicles. After treatment with chitosan solution, the vesicles seem to remain unchanged in size (Fig. 4b). However, they now appear in clusters. The clusters generally ranged in size from a few liposomes to huge aggregates, but single vesicles were also seen.

Fig. 4c shows the chitosomes after 5 min sonication in a bath sonicator. This treatment does not appear to change the clusters, indicating that they have a certain stability. After centrifugation

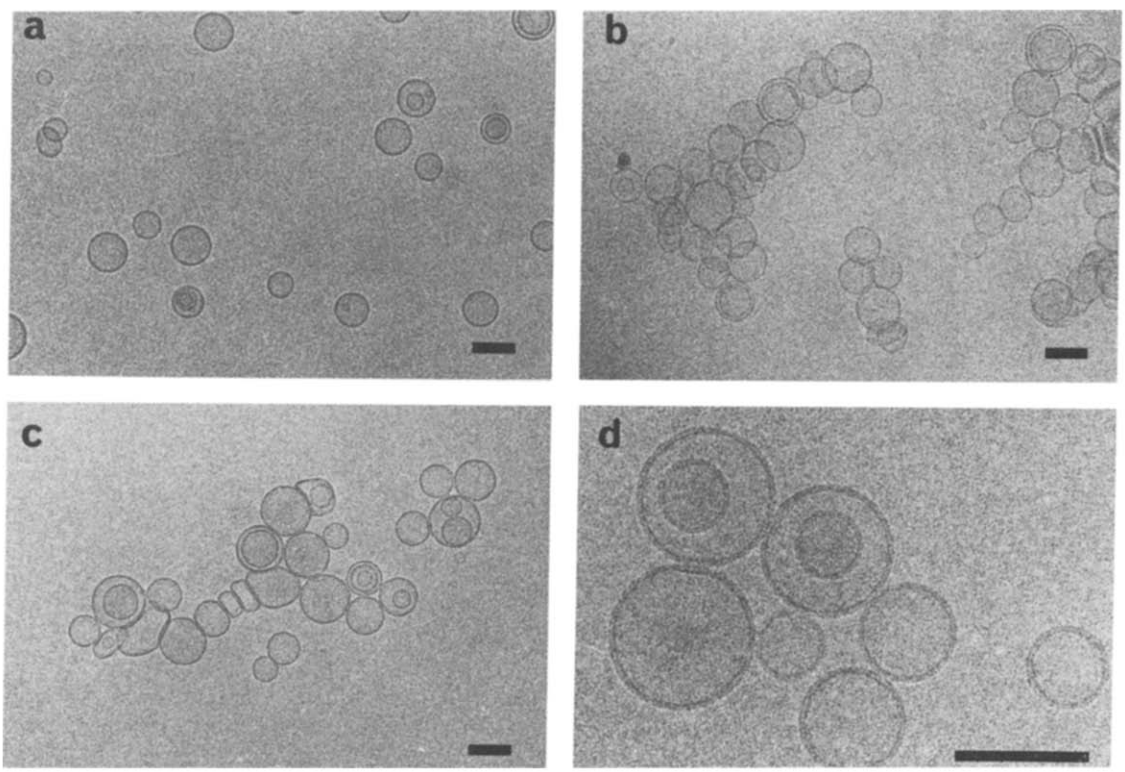

Fig. 4. Cryo-electron micrographs of PC/PG liposome and chitosome suspensions vitrified in thin films. Acceleration voltage 80 kV. Bar represents 100 nm. (a) PC/PG liposomes, (b,d) PC/PG chitosomes, (c) PC/PG chitosomes after 5 min sonication.

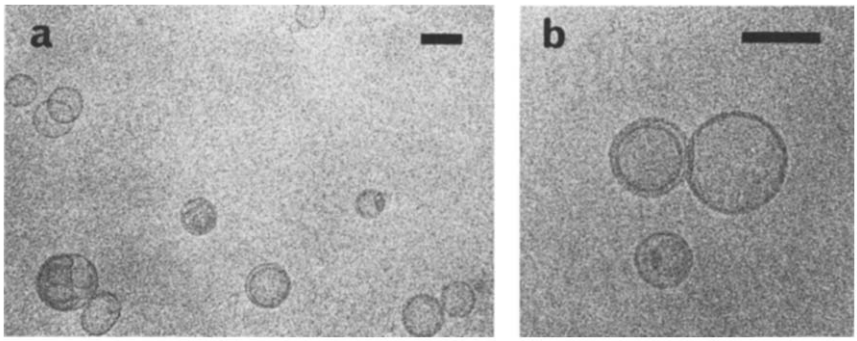

Fig. 5. Cryo-electron micrographs of PC chitosomes vitrified in a thin film. Acceleration voltage 80 kV. Bar represents 100 nm.

and washing as described, the same type of clusters were seen, without any apparent increase in the size of individual liposomes. A chitosome cluster is also shown in Fig. 4d. The double membranes of the liposomes are clearly evident in this picture. The resolution in the optical Fourier transform based on the phase contrast granulation in the EM negative is calculated to 1.5 nm.

The same analyses were also applied to the chitosomes prepared from PC liposomes (Fig. 5). These micrographs showed some signs of aggregates, but they were obviously present to a much lesser extent and were much smaller in size. Centrifuged chitosomes also appeared unchanged.

Discussion

Kato and Kondo (1987) studied the interactions between PC liposomes and a negatively charged polymer (CM-chitin) at ionic strength 0.154. Their micrographs (thin sections of freeze substituted and embedded samples were photographed (Wehrli et al., 1984)) and enzyme disintegration tests (Kato et al., 1985) were concluded to indicate 'granulated structures' in a mesh-like pattern on the surface of the liposomes. We are unable to see any such structures on our micrographs (Fig. 4d). However, it seems reasonable that the polymer is more extended in our experiments as a result of the lower ionic strength. The different polymer used and the production method (Kato et al. (1985) produced and coated the liposomes in one operation) may also explain the difference in observations.

The interaction between our PC/PG liposomes and chitosan may be compared with those between chitosan and negatively charged mica surfaces, which were studied by Claesson and Ninham (1992). They concluded that 0.01% chitosan (Mol. Wt $= 7 \times 10^5$, 90–95% degree of deacetylation) in 0.01% acetic acid was strongly adsorbed in a flat manner (pH 3–5 and low ionic strength). A few hours were needed for adsorption to reach equilibrium. Also, according to Blaakmeer et al. (1990), the adsorption of a strong polyelectrolyte results in a flat configuration. This may make it difficult to observe the polymer at the liposome surface.

Treweek and Morgan (1977) described the destabilization of negatively charged bacterial cells by a cationic polymer (polyethyleneimine, PEI). They proposed that such a flocculation may be caused by one of three mechanisms, namely: double-layer coagulation, adsorption coagulation or polymer bridging. They concluded that high molecular weight PEI produced rapid flocculation at low doses due to adsorption coagulation. Kasper (1971) described this mechanism as a 'charge-mosaic' model, with an alignment of positive surface patches with negative patches on approaching cells. Our electron micrographs show that the connection points between liposomes in the clusters often constitute a flat region (see Fig. 4c) between the spheres, appearing to be consistent fit with a charge-mosaic model of aggregation. Treweek and Morgan (1977) also described

the restabilization of cells when excess polymer was added; HMW polymer was shown to restabilize the *E. coli* cells at positive values of electrophoretic mobility. This is possible because the larger polymer molecules adsorbed through a few segments, leaving an unbalanced positive charge on other segments extending away from the surface. However, for LMW polymer, the flocculation is concluded to result from reduced double-layer interaction energies via adsorption. Furthermore, adsorption of excess polymer reduces the electrophoretic mobility to near zero, but a lack of negative surface sites prevents the adsorption of sufficient LMW species to cause mobility reversal.

Our observations fit well with this theory. At low doses of HMW chitosan the suspension is flocculated (Table 1). A restabilization is observed when the dose is high enough to cause a charge reversal to positive. According to the experiments of Claesson and Ninham (1992), it takes some time until adsorption is complete, and this may explain why it is difficult to avoid some aggregation. Furthermore, for coating purposes, it appears to be most favourable to add liposomes to chitosan solution (and not the opposite order of addition) to make the total excess of polymer available instantaneously.

The effect of the LMW chitosan is also as predicted by the results of Treweek and Morgan (1977): no restabilization of the liposomes is seen in this case.

It should be noted that the samples used here are characterized only by means of mean molecular weight, and the molecular weight distribution is unknown. For a closer study of the effect of chitosan with molecular weights between these extremes, it would probably be necessary to prepare narrow weight distribution samples.

The mechanism of coating neutral PC liposomes with polymer appears to be different from that in the case of PC/PG. Several studies have been performed with PC liposomes and CM-chitin/-chitosan (Kato et al., 1985; Kato and Kondo, 1987; Alamelu and Panduranga Rao, 1991). However, direct comparisons cannot be made, due to the different polymer samples (carboxymethylated samples, polyanion, molecular weight not described) and preparation methods (w/o/w method, coupling, etc.). The polyanion CM-chitin has even been coated onto negative PC/PG liposomes (Mobed et al., 1992) which should indicate that there exist clearly other interactions than the electrostatic ones, at least in this case.

Conclusions

When small unilamellar liposomes are added to a solution with an excess of high molecular weight chitosan polymer, the chitosan will adhere to the liposomal surface and produce a polymer-coated vesicle. This coated layer does not desorb during washing. The interaction leads to the formation of liposomal clusters with negatively charged liposomes, while egg-PC liposomes form clusters to a much lesser extent. The aggregation of PC/PG liposomes may be explained by an initial adsorption coagulation, while for PC liposomes a polymer bridging mechanism is more probable considering their low initial surface charge. Both systems can be utilized in further investigations of a bioadhesive liposomal preparation.

Acknowledgements

The authors wish to thank the NOBIPOL laboratory (The Norwegian Institute of Technology, Trondheim, Norway) for recording the NMR and intrinsic viscosity data on chitosan, and K.M. Vårum (NOBIPOL) for valuable discussion and advice, M. Cyrklaff (European Molecular Biology Laboratory, Heidelberg, Germany) for valuable help and advice regarding the electron microscopy, and B.V. Johansen (National Institute of Public Health, Oslo, Norway) for determining the electron micrograph resolution. We also thank The Electronmicroscopical Unit for Biological Sciences, University of Oslo, for use of facilities, and Pronova Biopolymer a/s (Drammen, Norway) for supplying chitosan samples and for financial support.

References

Agerkvist, I., Eriksson, L. and Enfors, S., Selective flocculation with chitosan in *Escherichia coli* disintegrates: effects of pH and nuclease treatment. *Enzyme Microb. Technol.*, 12 (1990) 584–590.

Alamelu, S. and Panduranga Rao, K., Studies on the carboxymethyl chitosan-containing liposomes for their stability and controlled release of Dapsone. *J. Microencapsulation*, 8 (1991) 505–519

Anthonsen, M.W., Vårum, K.M. and Smidsrød, O., Solution properties of chitosan: Conformation and chain stiffness of chitosans with different degrees of *N*-acetylation. *Carbohydr. Polym.*, (1993) in press.

Baran, A.A., Flocculation of cellular suspensions by polyelectrolytes. *Colloids Surf.*, 31 (1988) 259–264.

Blakmeer, J., Bøhmer, M.R., Cohen Stuart, M.A. and Fleer, G.J., Adsorption of weak polyelectrolytes on highly charged surfaces. Poly(acrylic acid) on polystyrene latex with strong cationic groups. *Macromolecules*, 23 (1990) 2301–2309.

Claesson, P.M. and Ninham, B.W., pH-dependent interactions between adsorbed chitosan layers. *Langmuir*, 8 (1992) 1406–1412.

Cyrklaff, M., Adrian, M. and Dubochet, J., Evaporation during preparation of unsupported thin vitrified aqueous layers for cryo-electron microscopy. *J. Electron Microsc. Tech.*, 16 (1990) 351–355.

Dubochet, J., Adrian, M., Chang, J., Homo, J., Lepault, J., McDowall, A. and Schultz, P., Cryo-electron microscopy of vitrified specimens. *Q. Rev. Biophys.*, 21 (1988) 129–228.

Eriksson, L.B. and Härdin, A.M., Flocculation of coli bacteria with cationic flocculants. In Attia, Y.A. (Ed.), *Flocculation in Biotechnology and Separation Systems*, Elsevier, Amsterdam, 1987, pp. 441–455.

Hirano, S., Seino, H., Akiyama, Y. and Nonaka, I., Bio-compatibility of chitosan by oral and intravenous administrations. *Polym. Eng. Sci.*, 59 (1988) 897–901.

Hughes, R.C, The complex carbohydrates of mammalian cell surfaces and their biological roles. *Essays Biochem.*, 11 (1975) 1–36

Iwamoto, K. and Sunamoto, J., Liposomal membranes: XII. Adsorption of polysaccharides on liposomal membranes as monitored by fluorescence depolarization. *J. Biochem.*, 91 (1982) 975–979.

Kasper, D.R., Theoretical and experimental investigations of the flocculation of charged particles in aqueous solution by polyelectrolytes of opposite charge. Ph.D. Thesis, California Institute of Technology, 1971.

Kato, A., Isao, T., Arakawa, M. and Kondo, T., Liposome-type artificial red blood cells stabilized with carboxymethylchitin. *Biomater. Med. Devices Artif. Organs*, 13 (1985) 61–82.

Kato, A. and Kondo, T., A study of liposome-type artificial red blood cells stabilized with carboxymethyl chitin. In Gebelein, C.G. (Ed.), *Advances in Biomedical Polymers*, Plenum, New York, 1987, pp. 299–310.

Klein, J.W., Ware, B.R., Barclay, G. and Petty, H.R., Phospholipid dependence of calcium ion effects on electrophoretic mobilities of liposomes. *Chem. Phys. Lipids*, 43 (1987) 13–23.

Law, S.L., Lo, W.Y., Pai, S.H. and Teh, G.W., The electrokinetic behavior of liposomes adsorbed with bovine serum albumin. *Int. J. Pharm.*, 43 (1988) 257–260.

Lehr, C., Bouwstra, J.A., Schacht, E.H. and Junginger, H.E., In vitro evaluation of mucoadhesive properties of chitosan and some other natural polymers. *Int. J. Pharm.*, 78 (1992) 43–48.

Makino, K., Yamada, T., Kimura, M., Oka, T., Ohshima, H. and Kondo, T., Temperature- and ionic strength-induced conformational changes in the lipid head group region of liposomes as suggested by zeta potential data. *Biophys. Chem.*, 41 (1991) 175–183.

Margalit, R., Okon, M., Yerushalmi, N. and Avidor, E., Bioadhesive liposomes as topical drug delivery systems: molecular and cellular studies. *J. Controlled Release*, 19 (1992) 275–288.

Mobed, M., Nishiya, T. and Chang, T.M.S., Preparation of carboxymethylchitin-incorporated submicron bilayer lipid membrane artificial cells (liposomes) encapsulating hemoglobin. *Biomater. Art. Cells Immob. Biotech.*, 20 (1992) 365–368.

Olsen, R., Schwartzmiller, D., Weppner, W. and Winandy, R., Biomedical applications of chitin and its derivatives. In Skjåk-Bræk, G., Antonsen, T.,and Sandford, P. (Eds), *Chitin and Chitosan – Sources, Chemistry, Biochemistry, Physical Properties and Applications*, Elsevier, London, 1989, pp. 813–828.

Plank, L., Dahl, C.E. and Ware, B.R., Effect of sterol incorporation on head group separation in liposomes. *Chem. Phys. Lipids*, 36 (1985) 319–328.

Roberts, G.A.F. and Domszy, J.G., Determination of the viscometric constants for chitosan. *Int. J. Macromol.*, 4 (1982) 374–377.

Skaugrud, Ø., Chitosan makes the grade. *Manuf. Chem.*, 60 (1989) 31–35.

Smistad, G., Rolfsen, T., Ågren, T., Tønnesen, H.H., Roos, N. and Karlsen, J., Cryo-electron microscopy of fully-hydrated liposomes as a quality control method. *Micron Microsc. Acta*, 22 (1991) 85–86.

Sunamoto, J., Iwamoto, K. and Kondo, H., Liposomal membranes: VII. Fusion and aggregation of egg lechitin liposomes as promoted by polysaccharides. *Biochem. Biophys. Res. Commun.*, 94 (1980b) 1367–1373.

Sunamoto, J., Iwamoto, K., Kondo, H. and Shinkai, S., Liposomal membranes: VI. Polysaccharide-induced aggregation of multilamellar liposomes of egg lecithin. *J. Biochem.*, 88 (1980a) 1219–1226.

Sunamoto, J., Iwamoto, K., Takada, M., Yuzuriha, T. and Katayama, K., Improved drug delivery to target spesific organs using liposomes as coated with polysaccharides. In Chiellini, E. and Giusti, P. (Eds), *Polymers in Medicine*, Plenum, New York, 1984, pp. 157–168.

Sunamoto, J., Sakai, K., Sato, K. and Kondo, H., Molecular recognition of polysaccharide-coated liposomes. Impor-

tance of sialic acid moiety on liposomal surface. *Chem. Lett.*, 10 (1988) 1781–1784.

Sunamoto, J., Sato, T., Hirota, M., Fukushima, K., Hiratani, K. and Hara, K., A newly developed immunoliposome – an egg phosphatidylcholine liposome coated with pullulan bearing both a cholesterol moiety and an IgMs fragment. *Biochim. Biophys. Acta*, 898 (1987) 323–330.

Treweek, G.P. and Morgan, J.J., Polymer flocculation of bacteria. The mechanism of *E. coli* aggregation by polyethyleneimine. *J. Colloid Interface Sci.*, 60 (1977) 258–273.

Vårum, K.M., Anthonsen, M.W., Grasdalen, H. and Smidsrød, O., Determination of the degree of *N*-acetylation and the distribution of *N*-acetyl groups in partially *N*-deacetylated chitins (chitosans) by high-field n.m.r. spectroscopy. *Carbohydr. Res.*, 211 (1991) 17–23.

Wang, W., Bo, S., Li, S. and Qin, W., Determination of the Mark-Houwink equation for chitosans with different degrees of deacetylation. *Int. J. Macromol.*, 13 (1991) 281–285.

Wehrli, E., Kondo, T., Kato, A., Izawa, H. and Kreuters, J., Electron microscopical study of the structure of liposome-type artificial red blood cells stabilized with carboxymethylchitin. *J. Microencapsulation*, 1 (1984) 329–333.

Woodle, M.C., Collins, L.R., Sponsler, E., Kossovsky, N., Papahadjopoulos, D. and Martin, F.J., Sterically stabilized liposomes. *Biophys. J.*, 61 (1992) 902–910.