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Interactions between liposomes and chitosan II: effect of selected parameters on aggregation and leakage

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Abstract

The interaction between liposomes and chitosan has been described in an earlier paper. In the present work, the production of liposome-chitosan complexes (Chitosomes) was further studied using factorial designs. The parameters studied were the stirring rate, rate of addition of liposomes to polymer, ionic strength, chitosan quality, lipid/polymer ratio and pH. Particle size, polydispersity and charge were the measured parameters. Cryo-electron microscopy was used for further study of one of the combinations. It was found that the ionic strength, chitosan quality, lipid/polymer ratio and pH had a significant effect on the resulting aggregate size. The leakage of quinine from Chitosomes was compared to the leakage from liposomes using a fractional dialysis method. The leakage rates were similar, but the Chitosomes produced a higher initial release and showed less scattering of the data. Measurements of zeta potential indicated adsorption of quinine to the liposomal membrane. Chitosomes could be stored in a refrigerator for at least 6 months without significant change in particle size. © 1997 Elsevier Science B.V.

Keywords: Chitosan; Liposome; Factorial design; Zeta potential; Cryo-electron microscopy; Quinine release

1. Introduction

Chitosan is a polysaccharide produced by deacetylation of chitin found in the shells of the Crustaceans. It dissolves in dilute acids to produce a linear polyelectrolyte with a high positive charge density. This property has been shown useful in many applications, such as clarification of waste water, selective flocculation of biological material and more recently, in the field of bioadhesion. Several authors have at present used chitin or chitosan related polymers as a liposome coating, to increase the stability towards drug release (Nishiya and Ahmed, 1990; Alamelu and Panduranga Rao, 1991; Dong and Rogers, 1991), to stabilize haemosomes ('Artificial Red Blood Cells', Kato et al., 1985; Kato and Kondo, 1987) and for targeting purposes (Takeuchi et al., 1994).

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Some aspects of the interaction between chitosan and biological tissues have been addressed in a recent study (Henriksen et al., 1996). Interactions between liposomes and chitosan has also been described previously (Henriksen et al., 1994).

The number of factors that can influence a polymer/colloid system is large and small changes in one factor may seriously alter the stability as well as other characteristics of the system. These mechanisms are well known from the literature on polymer flocculants, but the goals are opposite, since in that case a destabilisation of the colloidal system is desired. In an intended drug delivery system, factors of production need to be standardized to achieve a reproducible result and to be able to predict stability on storage and the stability in a physiological environment. The purpose of the present work is to investigate the effect of some factors on the size, charge and leakage from the chitosan-liposome complex. In parts of the study, simple factorial designs are used to utilize the data optimally and to be able to detect possible interactions between factors (Davies, 1954; Montgomery, 1991).

2. Materials and methods

2.1. Materials

The chitosan chloride samples were kindly provided by Pronova Biopolymer, Norway. The samples were dialysed against distilled water to remove excess of chloride, filtered and freeze-dried. The resulting qualities were characterized with regard to degree of deacetylation (DD%) by ultraviolet spectrophotometry (Muzzarelli and Rochetti, 1986) and molecular weight (MW) estimated from the intrinsic viscosity as described by Anthonsen et al. (1993). The qualities were:

Chitosan chloride 110 (CL110); MW 11 400, DD 92% (used in the 3 × 4 design)

Chitosan chloride 210 (CL210); MW 116 000, DD 93% (used in the 2^3 design, 3 × 4 design and stability on storage)

Chitosan chloride 310 (CL310); MW 369 000, DD 78% (used in the 3 × 4 design)

Table 1
Levels of factors in the 2^3 and the 3 × 4 design

Design	Factors	Levels	
2^3		−1	+1
	A: Speed of addition (ml/min)	0.33	1.09
	B: Mixing speed (rpm)	100	300
	C: Ionic strength (mM NaCl)	10	154
3 × 4			
	Chitosan grade (MW, g/mol):	11 400-116 000-369 000	
	Ionic strength (mM NaCl):	0-10-15-30	

Chitosan chloride 210 (CL210b); MW 178 000, DD 92% (used in the central composite design and the leakage experiment)

Egg L-α-phosphatidylcholine (PC, ca 99%) and L-α-phosphatidyl-DL-glycerol (PG, ca 99%) were from Sigma, while Quinine sulphate was supplied from Norsk Medisinaldepot, Norway. All other chemicals were of analytical grade.

2.2. Factorial designs and statistical analysis

Three different factorial designs were used to investigate the liposome-chitosan interaction in this study. The first part was a screening of the effect of the rate of addition of liposomes to polymer, the stirring rate and the ionic strength of the polymer solution and liposome suspension on

Table 2
Measured response (unimodal size) and relative S.D.'s (%) in the 2^3 design (average of six batches)

Treatment	Size (nm)	SDrel ($n = 6$)
(1)	240	3.17
a	245	1.46
b	230	3.58
ab	229	3.13
c	1204	23.02
ac	963	7.63
bc	1032	25.23
abc	889	14.31

Table 3

(A) Formula	Response			
	Size (nm)	PD	PDest	Zeta potential (mV)
1 (pH 2.63, ratio 1.52)	201	0.51	0.49	50.9
2 (pH 5.67, ratio 1.52)	907	0.60	0.61	36.6
3 (pH 2.63, ratio 6.48)	182	0.47	0.40	50.9
4 (pH 5.67, ratio 6.48)	2253	0.87	0.86	34.8
5 (pH 2.00, ratio 4.00)	186	0.40	0.48	53.8
6 (pH 6.30, ratio 4.00)	1590	0.87	0.89	24.5
7 (pH 4.15, ratio 0.50)	276	0.52	0.44	45.2
8 (pH 4.15, ratio 7.50)	231	0.53	0.56	43.9
9 (pH 4.15, ratio 4.00)	223	0.48	0.50	44.1
10 (pH 4.15, ratio 4.00)	222	0.48	0.50	44.9
11 (pH 4.15, ratio 4.00)	219	0.49	0.50	45.9
12 (pH 4.15, ratio 4.00)	221	0.48	0.50	45.4
13 (pH 4.15, ratio 4.00)	219	0.48	0.50	45.0
MS pure error	4.2	2×10^{-5}		
(B) Formula	Size (nm)	PD	PDest	Residual
14 (pH 4.91, ratio 2.23)	396	0.53	0.54	−0.01
15 (pH 4.91, ratio 5.75)	452	0.59	0.65	−0.06
16 (pH 5.20, ratio 0.50)	366	0.46	0.51	−0.05
17 (pH 5.20, ratio 2.23)	472	0.50	0.57	−0.07
18 (pH 5.20, ratio 5.75)	741	0.64	0.71	−0.07
19 (pH 5.67, ratio 4.00)	1478	0.80	0.74	0.06

(a) The original two-variable central composite design and the measured responses with each of the formulas. (b) Additional formulas, with the measured size and polydisperity (PD), estimated PD according to the model (PDest) and the residual.

particle size at two levels in a 2^3 factorial design (Tables 1 and 2) (Montgomery, 1991). The second part considered in more detail the effect of ionic strength on particle size. Here, three different chitosan qualities were studied at four NaCl concentrations (a 3×4 design, Davies, 1954, Table 1). The third study was a two-variable central composite design where the effects of pH and lipid/chitosan ratio on size and polydispersity were screened (Montgomery, 1991). The original design (Table 3A) consisted of 13 experiments, including five replicates of the center point to allow an estimate of the experimental error. This design was extended with six more formulae (Table 3b). The fitted model was evaluated using MODDE® software made by UMETRI, Umeå, Sweden.

2.3. Production of liposomes and chitosomes

Liposomes were produced using egg PC and egg PG 10:1. Chloroform solutions of the phospholipids were mixed and evaporated to dryness in a 250 ml round bottom flask. The lipid film was hydrated with the appropriate buffer, depending on the desired pH and ionic strength (see below). The hand-shaken liposomes were allowed to swell for 2 h before ten extrusions through a two-stacked 0.1 μm polycarbonate membrane, as recommended by the manufacturer (Nuclepore).

In the 2^3 factorial design (Table 1), 5.5 mg·ml^{-1} lipid was hydrated in 10 mM acetic buffer pH = 5.0 ± 0.1 (+ 154 mM NaCl to achieve the high level of factor C, see Table 1). The same buffer was used in the second experiment (Fig. 1) (3×4 design, Davies, 1954) and again NaCl was

added to obtain the desired ionic strength. In the third study HCl, acetic acid and sodium acetate were used to adjust the pH. NaCl was used to obtain similar ionic strengths in the different pH buffers.

In the leakage study, 50 $mg \cdot ml^{-1}$ lipid was hydrated with 50mM Quinine sulfate in 0.1 M HNO_3. The liposomes were freezed and thawed five times in liquid nitrogen in order to increase encapsulated volume, and 0.2 μm membranes were used for extrusion.

Chitosan chloride 0.2% solutions were prepared in the same buffer as the liposomes, (except for the leakage experiment, see below), and the pH adjusted. The coating procedure was as follows: 1.0 ml of liposomes was added to 4.0 ml of chitosan solution at a constant speed using a tube pump. When the pH was varied in the experiment, the viscosity (η) of the chitosan solutions was kept constant by dilution. Therefore, the volume of chitosan solutions would vary between 4–8.6 ml. During this addition the suspension was mixed with a magnetic stirrer at a selected speed. The stirring was continued for 5 min after mixing. The resulting Chitosomes were stored under N_2 atmosphere in a refrigerator overnight.

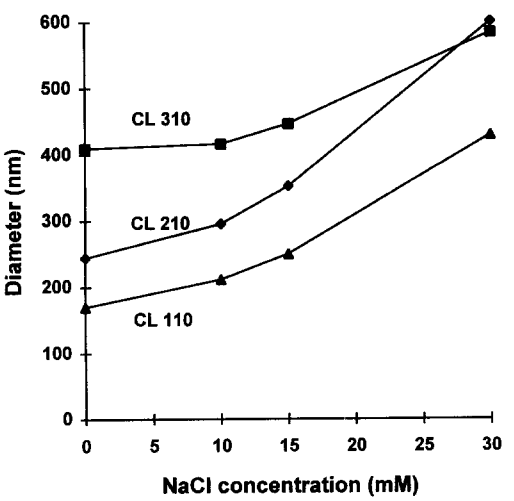

Fig. 1. Graphical presentation of the results of the 3×4 factorial design.

2.4. Particle size measurements

Determination of particle size was carried out via photon correlation spectroscopy (PCS) using a Coulter N4 MD at a 90° angle. Since the chitosan-liposome aggregates generally have a broad particle size distribution, the unimodal analysis was chosen as the response (Henriksen et al., 1994). The measurements were done at least in triplicate.

2.5. Zeta potential measurements

The zeta potential of the particles was measured using a Coulter DELSA 440® (Doppler-Electrophoretic Light Scattering Analyzer). The zeta potential (ζ) is deduced from the mobility (U) based on the equation: $\zeta = 4\pi\eta U/\epsilon$, where η is the viscosity of the medium and ϵ denotes the dielectric constant. The viscosities of test solutions to be compared were measured and the conductivity was kept constant. Measurements were performed at four different angles and in both stationary layers.

2.6. Stability of chitosomes during storage

Two Chitosome productions (three parallels of each) were stored under N_2 in refrigerator for up to 30 weeks, and their size measured at intervals. Both were produced according to the 2^3 factorial design (Table 1) in 10 mM Acetic buffer and with the pump speed 1.09 ml/min, but with the stirring set at 100 (Batch 1) and 300 (Batch 2) rpm respectively.

2.7. Cryo-electron microscopy

Chitosomes were prepared with chitosan chloride CL110 in 10 mM acetic buffer, pH = 5.0. The size of liposomes and Chitosomes was measured using Coulter N4. The zeta potential of Chitosomes after centrifugation and resuspension of the pellet was also measured to confirm that the liposomes were coated.

The liposome and Chitosome suspensions were vitrified in thin films on 200 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.

2.8. Quinine leakage from liposomes and chitosomes

Non-encapsulated quinine was removed by passing the liposomes through PD10 Pharmacia G-25 columns. Liposomes (2.5 ml) were applied to the pre-equilibrated column and eluted with 3.5 ml 0.1 M $NaNO_3$/10 mM Acetic buffer (pH = 5.0). The eluate was immediately diluted to either 250 ml with the same buffer, or to 150 ml with the buffer and then subsequently added to 100 ml of 0.2% chitosan (CL210b) in $NaNO_3$/Acetic buffer (the pH adjusted if necessary) during stirring. The batches of liposomes and Chitosomes were prepared in triplicate, and the leakage of quinine was analysed using the previously described fractional dialysis technique (Henriksen et al., 1995). The dialysis was performed at 37°C. The liposome and Chitosome samples were kept in dark bottles in a refrigerator (4°C) overnight. H_2SO_4 (2 mM) was added to the dialysate before measuring the fluorescence at exitation wavelength 354 nm and emission wavelength 448 nm. The percentage leaked out from the liposomes was calculated from the appropriate dialysis standard curve (with or without chitosan present).

The degree of encapsulation was calculated from the fluorescence measured after rupturing 0.1 ml of the eluted liposomes with Triton X-100 and appropriate dilution.

The interaction between liposomes and quinine was studied by means of zeta potential measurements. Neutral PC-liposomes or negative PC/PG-liposomes were diluted in buffer or in 0.02 mM, 0.2 mM, 0.4 mM, 0.8 mM or 2 mM quinine sulfate solutions.

3. Results

3.1. Chitosome size and charge

The diameter of extruded liposomes was 107 nm (no NaCl added to buffer) and 115 nm (0.154 M NaCl added).

The levels of the factors in the 2^3 factorial design are given in Table 1 and the responses of each treatment combination in Table 2. As Table 2 shows, the relative S.D.'s for the high ionic strength treatments (treatment c, ac, bc and abc) were much larger than for the low ones (1, a, b and ab). A closer analysis of this variation showed that this was partly due to the method of measurement (higher relative S.D.'s ($n = 9$)) and consequently even larger absolute S.D.'s on the same sample when the size distribution was large), but also because of large interbatch variations when the extent of aggregation was high (the interbatch deviations were several magnitudes higher than the intrabatch deviations). Since the ANOVA method assumes equal variance, it was decided to split the factorial design and treat it as two 2^2 designs. ANOVA of 1, a, b and ab showed that only factor B had a significant effect (in average −13 nm) at the low ionic strength (significance level: $\alpha < 0.01$). Analysis of c, ac, bc and abc showed that only factor A had a significant effect (−192 nm) at the high ionic strength ($\alpha < 0.05$). A t-test comparing the high and the low ionic strength treatments (C) showed a highly significant effect of this factor ($\alpha < 0.01$).

The effect of ionic strength of the polymer solution on aggregation was further investigated in the 3×4 design, where three chitosan grades were compared at four different NaCl concentrations. Fig. 1 depicts the responses. The ANOVA showed that also the chitosan grade had a significant effect on particle size, and there was a significant interaction between the factors ($\alpha < 0.01$). Furthermore, a Tukeys Studentized Range test with the ionic strength as a class variable showed that the effect of this factor was significant above 10 mM. Fig. 2 shows a representative micrograph of Chitosomes produced with chitosan chloride CL 110 and no NaCl added (see section 2.6). The size as measured on N4 was 183 nm and the zeta

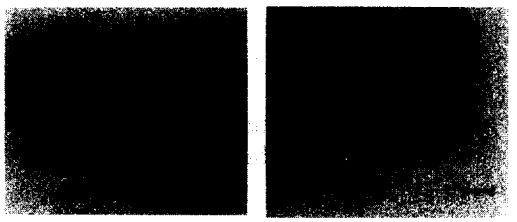

Fig. 2. Cryo-electron micrograph of Chitosomes produced with chitosan chloride CL 110. Bar represents 100 nm.

potential after washing was +30 mV. Before coating the liposomes had a zeta potential of −60 mV and diameter 118 nm.

The measured responses from the central composite design are shown in Table 3A. The polydispersity index (PD) is reported by the instrument, and is used to calculate the S.D. When PD is below 0.1, it indicates a narrow monomodal sample, whereas a PD of 0.1–1.0 indicates a broad monomodal or possibly a multimodal sample. It was attempted to fit a response surface to the effects of pH and lipid/chitosan ratio (R)(weight/weight). However, no model was found that gave an adequate fit with the measured size or polydispersity data (see discussion). Therefore, more data were collected (Table 3B) and a response surface could be developed using the polydispersity index (PD)(Fig. 3). The resulting model was:

$$PD = 1.075 - 0.316 \times pH - 0.075 \times R + 0.039 \times pH^2 + 0.022 \times pH \times R$$

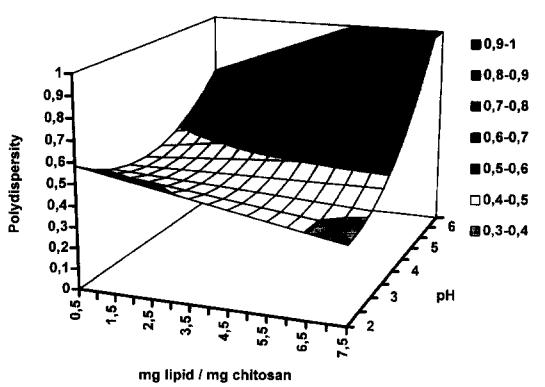

Fig. 3. Response surface showing the polydispersity index as a function of pH and lipid/chitosan weight ratio.

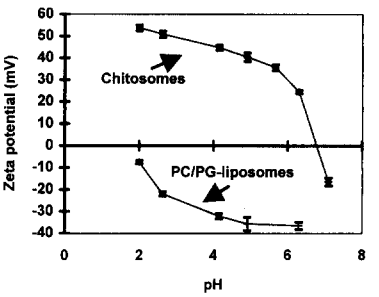

Fig. 4. Zeta potential of PC/PG liposomes and Chitosomes as a function of pH. $N = 8$ or more, error bars denote S.D.

The regression was significant ($\alpha < 0.001$) and all the parameters (pH, R, pH^2 and pH × R) in the model were significant ($\alpha < 0.05$). The estimated PD's and the residuals are reported in Table 3B and were found to be acceptable. The significant lack of fit observed was due to the extremely low pure error calculated from the five replicates in the centerpoint (formulae 9–13 in Table 3A.

The lipid/chitosan ratio did not have any significant effect on the zeta potential of the formulae. The pH profile of the zeta potential for the Chitosomes and the liposomes is shown in Fig. 4. The Chitosomes were produced and measured at the pH values as shown, except for the data point at pH 7.1. This zeta potential was obtained by centrifugation of Chitosomes produced at pH 4.15 and subsequent dilution of the pellet in borate buffer (same conductivity as the other measurements). The supernatant was removed to avoid interference from precipitated chitosan particles.

3.2. Stability of chitosomes during storage

Batch 1 was 244 nm at day 1, 212 nm after 4 weeks and 245 nm after 30 weeks. The PD index decreased from 0.51 to 0.42 after 30 weeks. Batch 2 was 226 nm at day 1, decreased to 194 nm after 6 weeks and 183 nm after 30 weeks. The PD index decreased from 0.5 to 0.38 during the same period (all: $n = 3$).

There was no visible sedimentation or particles in any sample during this period.

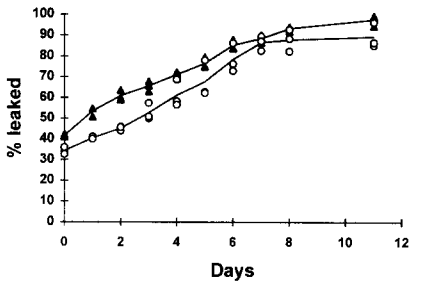

Fig. 5. Leakage of quinine sulphate from liposomes (○) and Chitosomes (▲) in 0.1 M $NaNO_3$/10 mM Acetic, pH = 5.0 buffer. Mean curves and datapoints of the three parallels shown.

3.3. Chitosome leakage

Fig. 5 compares the leakage profiles from non-coated and coated liposomes. The liposomes had leaked 34.3 ± 1.7% at the first measurement and 89.4 ± 6.0% at the last. The Chitosomes had leaked 41.7 ± 0.6% at the first measurement and 97.7 ± 2.6% at the last (all: $n = 3$). The coefficient of variation was 0.6–9.0% for liposomes and 0.6–2.6% for Chitosomes.

The degree of encapsulation in the liposomes was 13.6%. The liposome size was 137 nm at the start of experiment and 150 nm after 11 days. The polydispersity index (PD) increased from 0.09 to 0.19. The Chitosomes size was 323 nm (PD 0.35) and no change was found after 11 days. The liposomes had a zeta potential of about − 12 mV and the Chitosomes + 55 mV.

The zeta potential of liposomes diluted in different quinine sulphate solutions is shown in Fig. 6. The results show that the liposome zeta potential is increasingly positive when the quinine sulfate concentration is increased and that the effect is reversed immediately upon dilution.

4. Discussion

4.1. Chitosome size and charge

The interaction between a charged particle and a strong polyelectrolyte of the opposite charge has in most cases been interpreted in terms of a

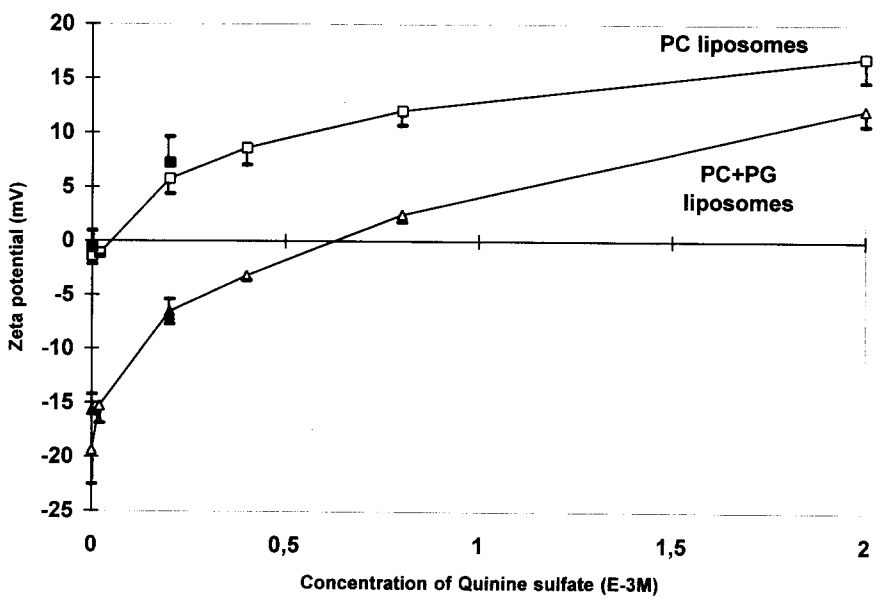

Fig. 6. Zeta potential of PC and PC + 10% PG liposomes in 0.1 M NaNO3/10 mM acetic buffer, pH = 5.0 as a function of quinine sulphate concentration. $N = 24$ measurements, error bars denote S.D. Open symbols: diluted and measured at the quinine sulphate concentration given. Filled symbols: diluted with quinine sulphate, then ten fold diluted with buffer to the given concentration and measured.

'patch' or 'charge mosaic' model (Gregory, 1973; Mabire et al., 1984). The effect is sometimes also called an adsorption coagulation. This mechanism of aggregation is described as an initial adsorption of polymer to the particle surface and then an alignment of these positive surface patches with negative patches on approaching particles. The phenomenon is used in particle flocculation with small quantities of polymer, or in particle stabilization with an excess of polymer (e.g. Demarger-Andre and Domard, 1993, 1994; Henriksen et al., 1994). In the latter case, restabilization occurs due to a charge reversal of the polymer-coated particle. However, a polymer-colloid system is complex, and in many cases other mechanisms of flocculation will be present (e.g. polymer bridging and double-layer coagulation) depending on experimental conditions. For simplicity, the following two main reactions may be used to describe the charge mosaic interaction between chitosan (C) and the liposomes (L): (1) $C + L \Rightarrow CL$; (2) $CL + L \Leftrightarrow LCL$, where (1) represents the interaction between chitosan and liposome (irreversible coating). If chitosan is present in excess, this reaction will proceed until the particle is restabilized at a positive surface charge. In the present work, the polymer (chitosan) is present in a large excess of that required for restabilization to occur. (2) represents the interaction between a partly coated liposome with a non-coated (part of) liposome which leads to an increase in measured particle size. For the purpose of the discussion, these two reactions may be seen as competing: when (1) is rate limiting, aggregation will be pronounced whereas when (2) is rate limiting, aggregation should be limited (i.e. restabilization occurs first).

All steps in the production of polymer-coated particles need to be carefully standardized to achieve a reproducible result. The 2^3 factorial design and the central composite design were used as a screening to identify important factors. The 2^3 design can only show whether there is a significant effect of increasing a factor from a low to a high level.

The factors to be investigated were chosen on the background of previous experimental results and data reported in the literature. Mabire et al. (1984) studied the flocculation of 185 nm silica particles with strong cationic polyelectrolytes. The effect of mechanical agitation was found to be important for the resulting floc size. Gregory (1973) emphasized that attention must be paid to the method of mixing the polymer solution and the suspension.

Due to this it was decided to study both the rate of addition and the stirring rate in the 2^3 design. The small effect of increasing the stirring rate that can be seen at the low ionic strength level (-13 nm) is negative, as opposed to the increased size seen in the work of Mabire et al. (1984). The difference may be due to the excess of polymer as well as to the small sizes: According to the theories of Smoluchowski (1917), particle aggregation is mainly governed by brownian motion when cluster size is less than 0.5 μm and by mechanical agitation for larger sizes. Increasing the rate of addition (factor A) causes a significant reduction in the measured size when the ionic strength is high. This can be explained by the charge-mosaic model described above: With a too slow rate of addition, the liposomes that are added first will be partly coated with chitosan and may easily interact with liposomes added later, resulting in aggregation (reaction 2).

There is also a significant effect of factor C (ionic strength). The importance of ionic strength has been stated by several authors as being due to a reduced Stern-layer with a resulting increase in flocculation rate (faster reaction 2) (Gregory, 1973, Demarger-Andre and Domard, 1993). A positive AC interaction was found in the full 2^3 ANOVA which means that the effect of increasing the rate of addition is larger at the high ionic strength than at the low one. This seems reasonable in light of the above discussion.

The ionic strength seemed to have the most pronounced effect, therefore this factor was studied more closely at four levels and with three grades of chitosan chloride (3×4 design, Fig. 1). The NaCl concentrations were below 50 mM, because it was supposed that the small particles would be the most stable and therefore most interesting to use. The size increase is significant above 10 mM NaCl with all three grades. Fig. 2 shows a representative micrograph of the Chito-

somes CL 110 (lowest point on Fig. 1). This illustrates that even with this small measured size (183 nm, less than two liposomes per aggregate) the realities are more complex. Both large aggregates and single liposomes are present as restabilized 'Chitosomes'.

The effect of molecular weight in the 3×4 design is also according to the charge-mosaic theory of interaction. At least at low ionic strengths, the particle size decreases when a lower molecular weight chitosan is used. A lower molecular weight polymer is expected to cause a more uniform adsorption and thereby charge, on the particle surface. This will in turn reduce the probability of interaction with other particles. The mechanism probably has a lower molecular weight limit, where the polymer chains are too short to produce a charge reversal of particles (Henriksen et al., 1994) and a higher molecular weight limit, where bridging effects predominates (Demarger-Andre and Domard, 1994). Theoretically, particle size should increase at both limits. The significant interaction between chitosan molecular weight and ionic strength means that the magnitude of the effect of increasing the latter, will depend on the molecular weight of the chitosan.

The difficulties encountered in fitting the measured particle sizes to a response surface (central composite design) were due to several causes. Firstly, the effect of pH was very pronounced between pH 4.15 and 5.67 in the original design (Table 3A). To achieve an adequate model, more data were collected in this area (Table 3B). However, the fit was still not satisfactory, probably because an abrupt shift in aggregation mechanism takes place between pH 5.2 and 5.67 (vide infra). Secondly, the largest sizes resulted in problems with the increased relative S.D.'s of these measurements, as explained in section 3.1. The lack of fit to any model could therefore also be related to both a reduced accuracy in the (intrabatch) measurements ($n = 6$) and to an expected larger interbatch variation ($n = 1$). The polydispersity index was found to be a more robust response, and therefore it was chosen to develop a response surface based on this parameter, to obtain a predictive picture of the area of interest.

Fig. 4 is useful for interpreting the effect of pH on size and polydispersity. Generally, the smallest Chitosomes are produced at a low pH where the liposomes have the lowest negative charge. This is consistent with the charge-mosaic model of aggregation, since a low particle charge will reduce the driving force for reaction (2). Above pH = 5, the liposomal zeta potential is constant, whereas the Chitosome potential decreases rapidly above pH 5.67 and is reversed around pH 6.5, the approximate pKa value of chitosan. Above this pH the chitosan chains are rapidly deprotonated and the Chitosome charge will eventually approach the liposomal charge. According to Claesson and Ninham (1992), chitosan adsorbs in a flat manner when the pH is low, but above pH 6.2 this will change so that increasing fractions of the polymer will be in loops and tails as the polyelectrolyte becomes less strongly charged. This increases the probability of a bridging mechanism of aggregation. In conclusion, the sharp increase in particle size seen when the pH is increased towards 6.0 is probably due to a shift in aggregation mechanism to a combination of adsorption coagulation (liposomal charge is neutralized but not reversed) and bridging between liposomes.

The effect of lipid/chitosan ratio above pH 3.0 is as expected: when more liposomes are present, more collisions and therefore more extensive aggregation is expected. This is also seen in the work of Demarger-Andre and Domard (1993) with chitosan and undecylenic dispersions in pH 5.8/0.15 M acetate buffer. More surprisingly, at the lower pH the effect is opposite: the size and PD decrease as the ratio increases. It cannot be ruled out that the chitosan concentration is a confounded facor in the central composite design, since it was chosen to keep the viscosity constant over the range of pH's, rather than the concentration. The response surface has the same shape when sizes are plotted, but the size effects are much larger: when the polydispersity increases from 0.6 to 0.8, the sizes increase from about 450 to 1500 nm. With this in mind, the surface gives information as to what pH and lipid/chitosan ratio should be chosen to achieve a certain particle size/size distribution. It may also serve as an indication as to what result to expect if a certain pH and lipid/chitosan ratio is desired in a specific experiment.

4.2. Chitosome leakage

The $NaNO_3$/Acetic buffer was chosen because it was compatible with both chitosan (solubility) and quinine (no quenching of fluorescence). Quinine was chosen as marker because it is a drug substance which is easily detected. Since the purpose was to study the effect of chitosan on leakage from liposomes, the fractional dialysis technique was used to avoid any influence of, e.g. separation on the detected leakage (Henriksen et al., 1995). The liposomes were produced with a pH gradient to increase the encapsulation of quinine. This was found necessary because quinine interacts with the liposomal membrane to such an extent that a drug gradient over the membrane was difficult to obtain (Girke et al., 1989). Fig. 6 illustrates the problem: Quinine adsorption to the membrane increases at least up to 2 mM quinine sulphate, but this adsorption is reversed as the liposomes are diluted with buffer.

Fig. 5 shows that liposomes leak at the same rate with and without chitosan present, but in the first case the starting point is somewhat higher. The high starting values may be partly due to the release of adsorbed quinine when the eluted liposomes are diluted, as described above. It is also possible that chitosan displaces quinine on the membrane surface and therefore produces a high initial release.

Fig. 5 also shows increasing deviations between the three liposome parallels with time, whereas the three Chitosome parallels are very consistent. A similar leakage experiment was performed using egg PC liposomes (results not shown). Interestingly, the same difference in scatter between liposomes and Chitosomes was seen here. An increase in liposome size during storage has been seen in stability studies and is due to increasing liposomal fusion. One explanation may be that chitosan stabilizes the liposomes against fusion, and thereby gives a more predictable release rate, although the average release profile may be the same.

5. Conclusion

Factorial designs were useful in the screening of the Chitosome production. If a small extent of aggregation is desired, a high mixing rate should be used in combination with a low ionic strength, a low molecular weight chitosan and a low pH. If a higher pH is desired during production, the lipid/chitosan weight ratio should be low. Chitosomes of size 200–250 nm are physically stable and can be stored in refrigerator for at least 30 weeks. The leakage rates of quinine from PC/PG liposomes and Chitosomes were similar, but leakage from Chitosomes was more consistent.

The interaction between chitosan and negative PC/PG liposomes can in most cases be explained by the charge mosaic model.

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