CHAPTER 3

CM-CHITIN-COATED LIPOSOMES

CHAPTER 3

Part A

QUANTITATIVE DETERMINATION OF CM-CHITIN IN POLYMER-COATED LIPOSOMES[2]

3A.1. Introduction

Carboxymethyl chitin (CM-chitin), a derivative of a poly-(1-4)-linked N-acetyl-D-glucosamine, has been used to stabilize egg yolk phosphatidylcholine vesicles (*Izawa et al., 1986*), especially in liposome-type artificial red blood cells (*Kato et al., 1984*). Used in this manner, CM-chitin is electrostatically bound to the surface of the liposomes thereby strengthening the liposomes and providing increased stability to the vesicles (*Kato et al., 1983 & 1987*). No quantitative direct determination of CM-chitin in the presence of liposomes has been reported. *Clark et al. (1977)* employed a method of polysaccharide determination involving the analysis of the mixture of monosaccharides that is released from the polymer by hydrolytic or enzymatic digestion. However, this method is inconvenient and does not particularly lend itself to quantitative determination of CM-chitin, especially in liposome systems.

The aim of this investigation was to develop a convenient, quantitative determination of CM-chitin in coated liposome systems using a turbidimetric method.

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[2]A version of this part has been published in *J. Microencapsulation*, 8, 153-160, 1991.

3A.2. Materials and Methods

3A.2.1. Materials

CM-chitin was obtained from Nanyo Kasei Co., Ltd.,Tokyo, Japan. Dimyristoylphosphatidylcholine (99%, DMPC), dipalmitoylphosphatidylcholine (99%, DPPC), distearoylphosphatidylcholine (99%, DSPC), and dicetyl phosphate (DCP) (*Sigma Chemical Co.*) were used as received. Egg phosphatidylcholine (EPC) was extracted from eggs according to the method of *Singleton et al. (1958)* then purified by column chromatography over alumina. Fractions, which yielded a single spot by TLC, were collected and stored at 5°C prior to use. All other chemicals were reagent grade and water was twice-distilled.

3A.2.2. Selection of Solvent System

Several solvent systems, including methanol, ethanol, isopropanol, acetone and their aqueous solutions, were tested for their solvency of phospholipids but their nonsolvent action for CM-chitin by visual inspection and by spectrophotometric means at 400 nm (*Beckman Spectrophotometer, Model 25*).

3A.2.3. Preparation of Standard Solutions

Standard solutions of CM-chitin were prepared by diluting a 1% stock solution in water (20-200 μl) to 5 ml with sodium acetate buffer solution at pH 2.5, 4.0, or 5.6, then further dilution to 10 ml with isopropanol.

3A.2.4. Preparation of Samples

Samples were prepared containing phospholipid and CM-chitin in the same manner as for the standard solutions, except the isopropanol for dilution contained the required amounts of phospholipid.

3A.2.5. Coating Efficiency of DPPC Liposomes by CM-chitin

Liposomes were prepared by the reverse-phase evaporation method (*Szoka et al., 1978*). Briefly, phosphate buffer solution (pH 7.0) was emulsified in dichloromethane containing DPPC, then sonicated (*Model W-375 sonicator, Heat Systems-Ultrasonics, Inc., cup-horn, model 431*) at 10 Khz for 4 min. Subsequently, CM-chitin solution was added to the w/o emulsion and vortex-mixed to form a w/o/w emulsion. The organic solvent was slowly removed under reduced pressure by rotary evaporation resulting in polymer-coated liposomes. The CM-chitin-coated liposomes were then separated from free CM-chitin by gel permeation chromatography (*Sephadex G-25, 1.6 × 70 cm*) using pH 5.6 acetate buffer solution as the eluting solvent. Fractions of the coated liposomes eluted from the column were collected and pooled, allowed to sediment ($\sim$ 12 hr), the supernatant was removed by Pasteur pipette, then the sediment was diluted to 10 ml with buffer solution. Following this treatment, 5 ml were removed and mixed with 5 ml of isopropanol and the turbidty was measured spectrophotometrically at 400 nm. In some experiments pre-formed liposomes prepared by this method were diluted with CM-chitin solution, equilibrated, and treated as before. The coating efficiency of liposomes by CM-chitin was

calculated as the ratio of the weight of CM-chitin (mg) determined from the polymer-coated liposomes to the weight of DPPC per ml of liposome suspension (25 mg).

3A.3. Results and Discussion

3A.3.1. Selection of Solvent

Aqueous solutions of methanol, acetone, ethanol, or isopropanol were poor solvents for CM-chitin but had varying solvent effects on liposomal phospholipids. However, among these only aqueous isopropanol solutions were able to dissolve DMPC, DPPC, or EPC and, thus, were chosen as the solvent systems at 1:1 v/v aqueous buffer:isopropanol. The sensitivity of the measurement (*i.e.* degree of turbidity) was a function of the wavelength as depicted in Fig. 3A.1. Thus, the sensitivity of CM-chitin determination decreased as the wavelength of detection increased as predicted according to *Raleigh's law* of light scattering (*Wieding et al., 1989*). The wavelength of 400 nm was considered optimum for sensitivity in the visible light range and has also been previously chosen by others for turbidity measurements (*Regen et al., 1980*).

3A.3.2. CM-chitin Turbidity at Different pH

Fig. 3A.2 describes the effect of pH on the sensitivity of measurement at 100 μg/ml CM-chitin and an ionic strength of 0.2. The results gave a maximum turbidity at pH 2.0~2.5 and 5.6 but a mimimum at pH 3.5 and negligible turbidity at pH 1.0 or above pH 7.0. The disappearance of turbidity at low and high pH values can be

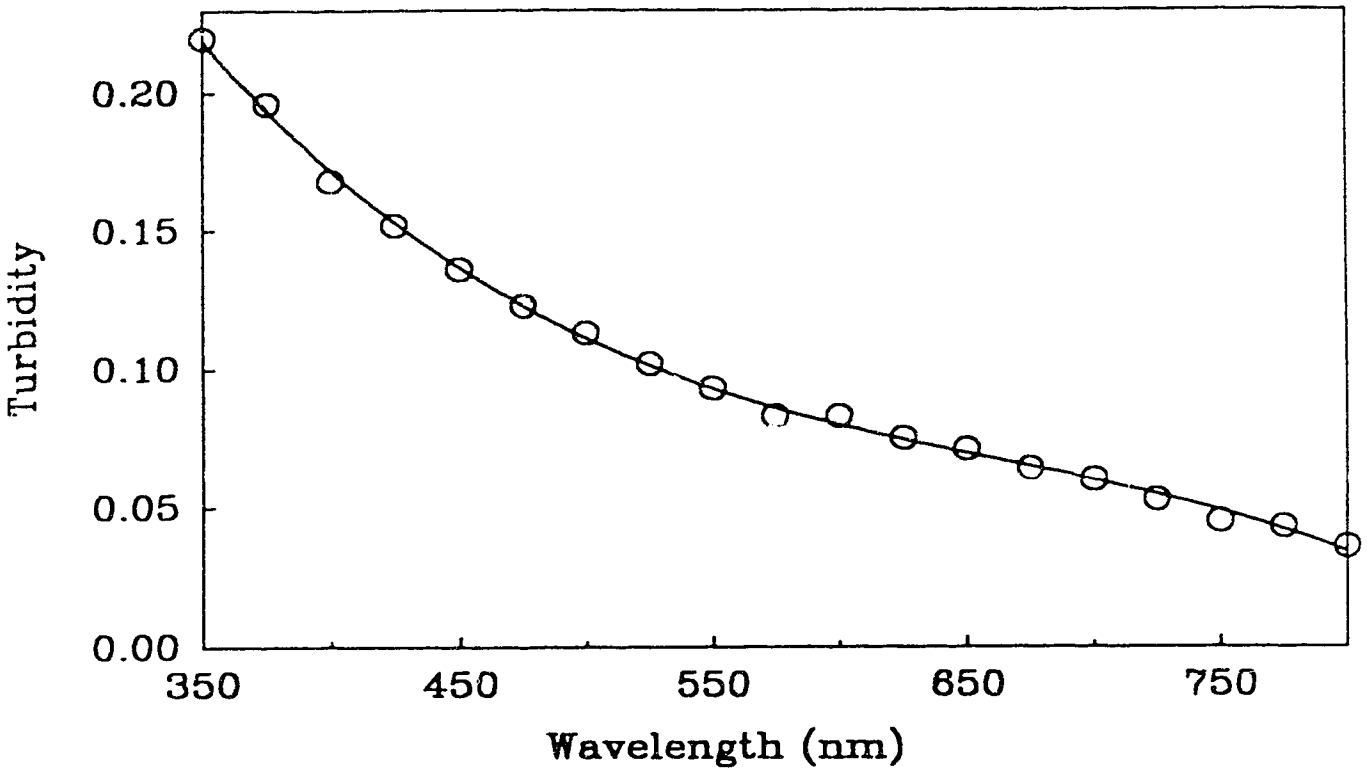

Fig. 3A.1. CM-chitin turbidity as a function of the wavelength of light. The solvent system was 1:1 v/v isopropanol:acetate buffer solution (pH 5.6); the initial CM-chitin concentration was 100 μg/ml; the ionic strength was 0.2.

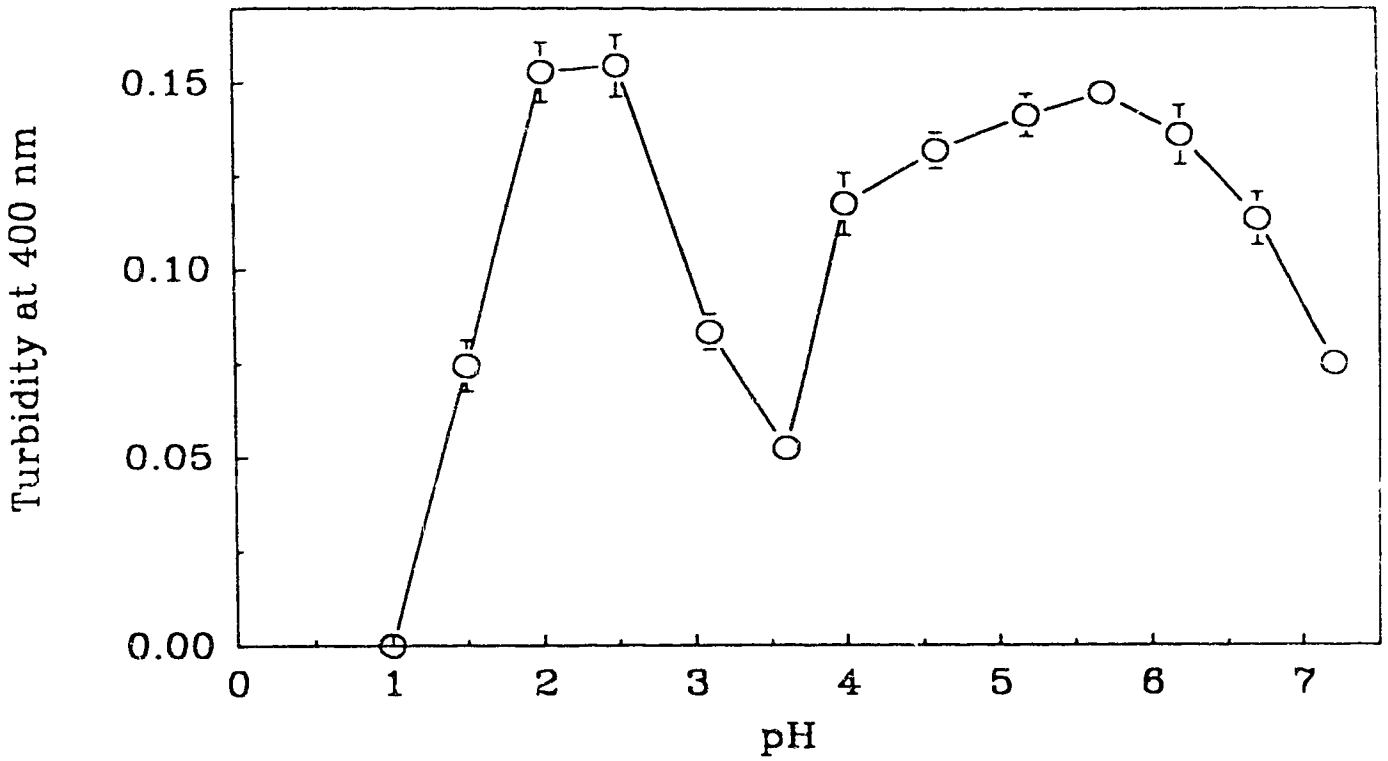

Fig. 3A.2. Dependence of the CM-chitin turbidity on the pH in 1:1 v/v isopropanol:acetate buffer solution. The CM-chitin concentration was 100 μg/ml; the ionic strength was 0.2.

explained by the high degree of ionization of the glucosamine residues or the carboxyl groups of CM-chitin, respectively, giving rise to increased solubility in the aqueous solvent system. On the other hand, it is possible that only partial ionization of the basic and acidic groups at pH 2.0 ~ 2.5 and 5.6 resulted in a minimum in solubility of CM-chitin in the solvent system and the corresponding maxima in turbidity, respectively. The reason for decreased turbidity at pH 3.5 is less clear. Assuming that this is approximately the pH of total charge neutralization, the possible formation of molecular association complexes may have resulted in a solubilization phenomenon and, consequently, decreased turbidity.

3A.3.3. The Stability of CM-chitin Turbidity

The stability of the measured turbidities due to CM-chitin was shown to be a function of both the CM-chitin concentration and the pH of the medium (Fig. 3A.3). The turbidities were most stable (for at least 20 min) when the CM-chitin concentration was low, *i.e.* up to 100 μg/ml, regardless of the pH. However, at the higher concentration of 500 μg/ml turbidities were stable only at pH 4.0 suggesting that the solubility of any association complexes that may have formed has been exceeded at this concentration. The instabilities in turbidity may be the result of a slow solubilization phenomenon due to a gradual association of the CM-chitin molecules at the higher concentration. Thus, standard calibration curves could be constructed at pH 2.5, 4.0 and 5.6 for CM-chitin concentrations up to 300 μg/ml (Fig. 3A.4). The correlation coefficients, r, determined from regression analysis at

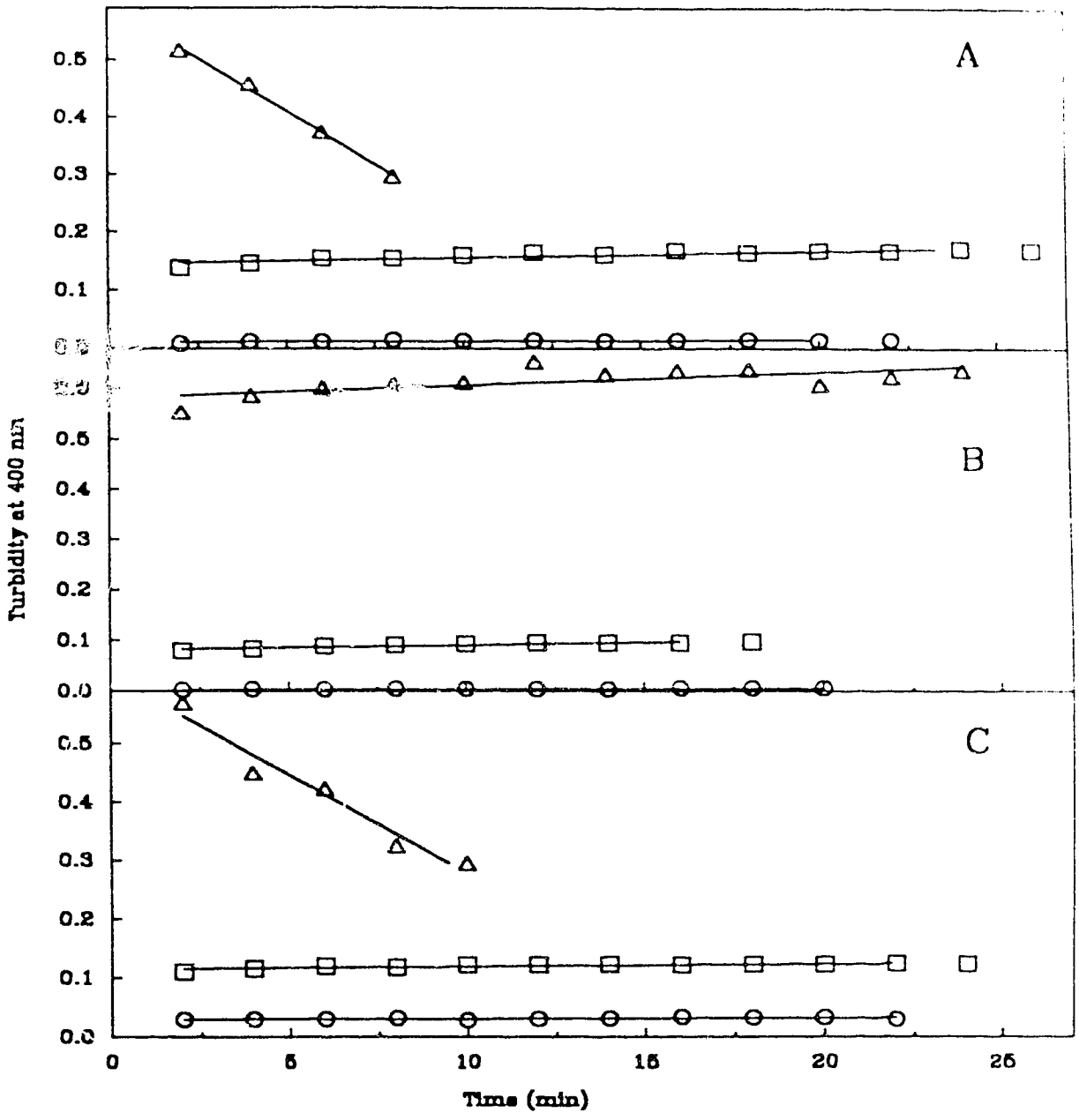

Fig. 3A.3. Stability of the CM-chitin turbidity as a function of the CM-chitin concentration at pH 5.6, 4.0, and 2.5 in 1:1 v/v isopropanol:acetate buffer solution. A. pH 5.6; B. pH 4.0; C. pH 2.5. ○ , 20 μg/ml; □ , 100 μg/ml; △ , 500 μg/ml. The ionic strength was 0.2.

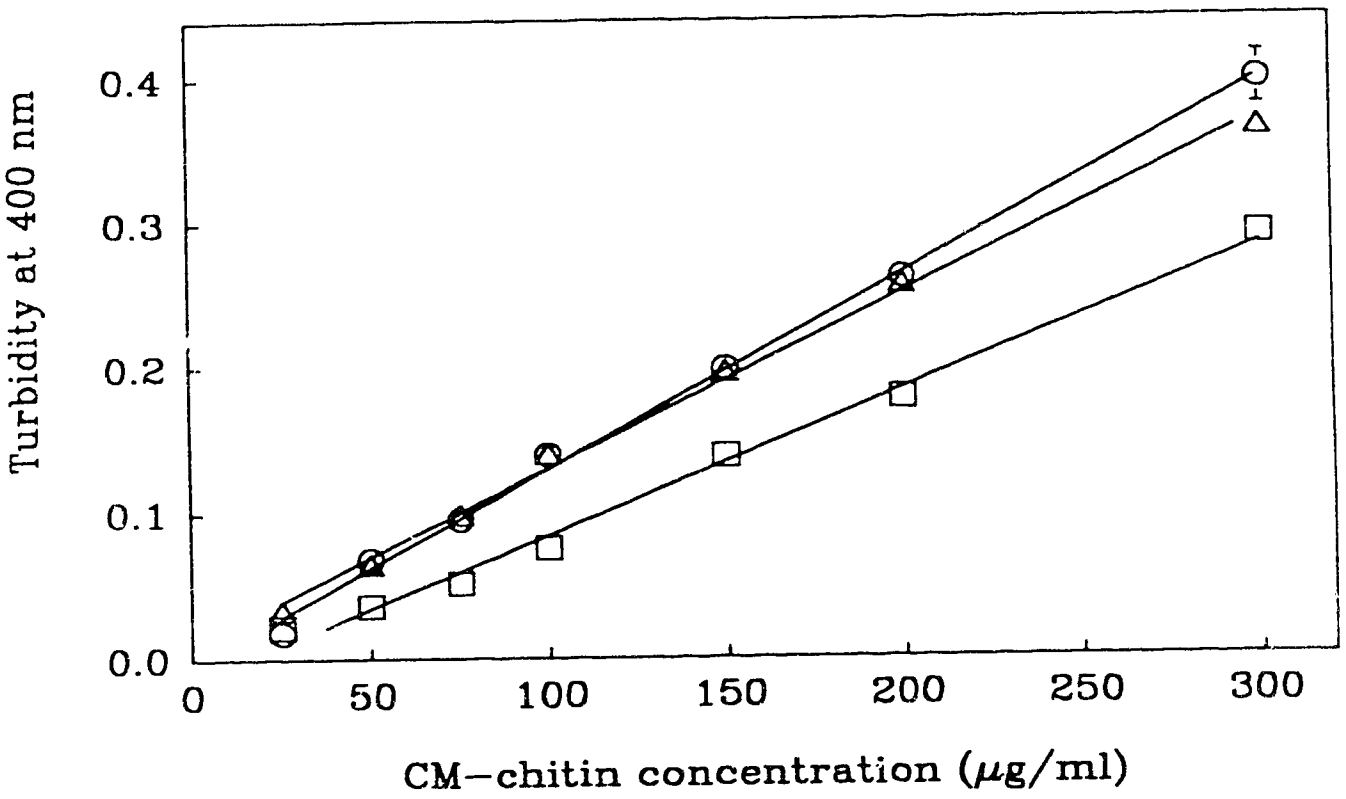

Fig. 3A.4. Standard calibration curve for the turbidimetric determination of CM-chitin in 1:1 v/v isopropanol:acetate buffer solution. ○ , pH 5.6, r = 0.999; □ , pH 4.0, r = 0.996; △ , pH 2.5, r = 0.998. Means ± SEM (n = 3) are shown.

the above pHs were 0.998, 0.996, and 0.999, respectively. The lower limit of detection was approximately 25 μg/ml of CM-chitin. If the sample concentration was below this limit, nephrometric method was suggested. Thus, when CM-chitin concentrations in liposome systems are greater than 300 μg/ml dilution of the sample should be made prior to mixing with the aqueous isopropanol solution to avoid aggregation into large particles.

3A.3.4. CM-chitin Turbidity at Different Ionic Strength

The effect of the ionic strength of the aqueous medium in the sample on the turbidity measurements can be seen in Fig. 3A.5 as a function of pH. Generally, there is a sharp increase in turbidity from 0.05 to about 0.15 ionic strength at pH 4.0 and 5.6 followed by relative insensitivity to ionic strength up to 0.5. In contrast, at pH 2.5 the sensitivity to ionic strength is only slight, up to 0.2, but becomes increasingly sensitive at higher ionic strengths. In all cases it would appear that increases in the ionic strength suppress the solubility of CM-chitin in the medium in a manner somewhat dependent on the pH, leading to increased turbidities. Thus, it would be important to control the ionic strength in these determinations, but if maintained at 0.15 to 0.3 the turbidities would be the least sensitive to ionic strength at each pH.

3A.3.5. Recovery of CM-chitin in the Presence of Lipids

Recoveries of CM-chitin and reproducibilities of the analyses using this

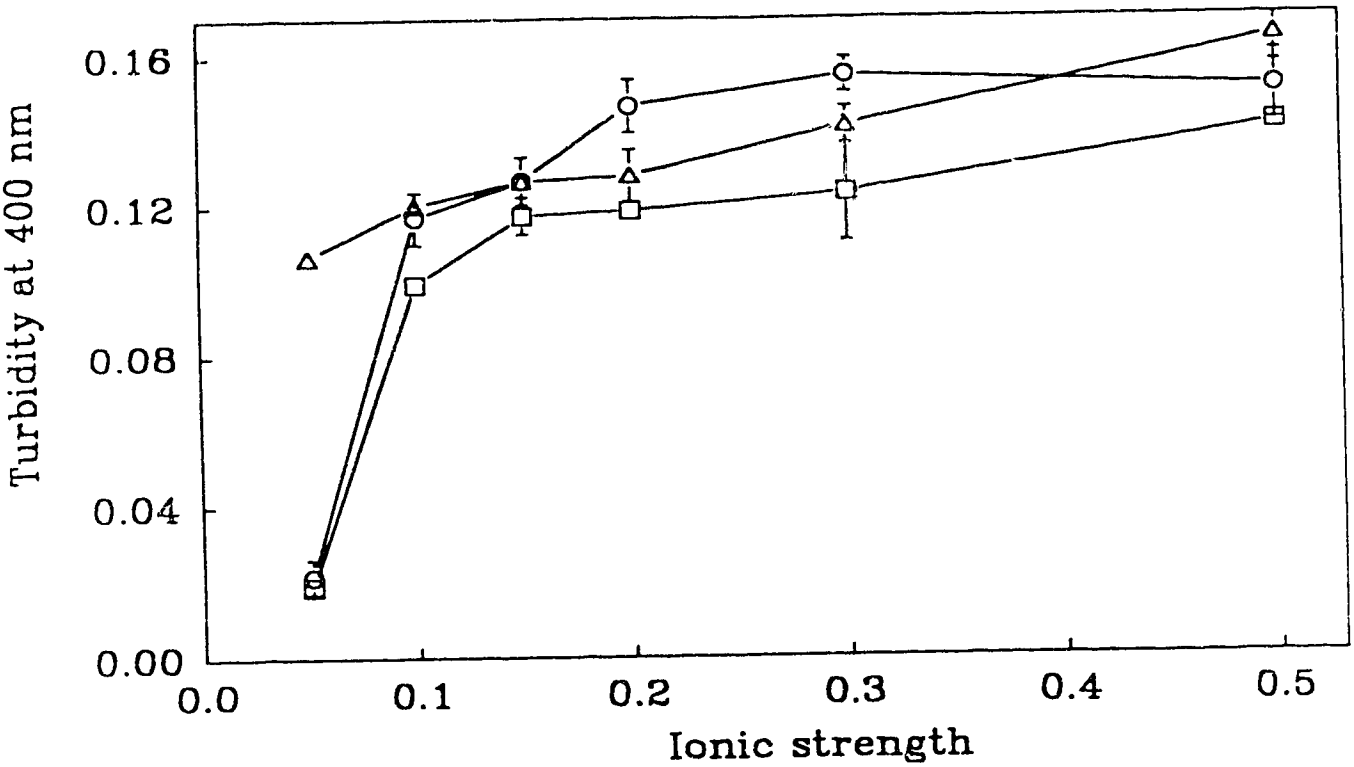

Fig. 3A.5. Dependence of CM-chitin turbidity on the ionic strength at pH 5.6, 4.0, and 2.5 in 1:1 v/v isopropanol:acetate buffer solution. ○ , pH 5.6; □ , pH 4.0; △ , pH 2.5. The concentration of CM-chitin was 100 μg/ml. Means ± SEM (n = 3) are shown.

technique are compared in Tables 3A.1 and 3A.2. Generally, the recoveries of CM-chitin from the analyses of CM-chitin-coated DPPC liposomes were excellent at each concentration examined, with the possible exception at pH 4.0 for an initial concentration of 200 μg/ml (F-test, p=0.05). It can be seen from analyses at the various pHs (R.T. ~ 22°C), that the recovery of CM-chitin from DMPC liposomes at pH 4.0 was significantly less than theoretical whereas recoveries from EPC liposomes at pH 5.6 and pH 2.5 were significantly greater than theoretical (F-test, p=0.05). Both DMPC and EPC liposomes are in the liquid crystalline state at this temperature therefore, the reduction in turbidity at pH 4.0 in DMPC systems and the increase in turbidity at pH 2.5 or 5.6 in EPC systems reflect the differences in the interactions between the phospholipid and CM-chitin molecules at the respective pHs.

3A.4. Summary

Since the determination of a polymer such as CM-chitin in a complex system of CM-chitin-coated liposomes in the presence or absence of drug would be difficult without interferences or requiring separations, the turbidimetric method as described here could offer a simple, rapid spectrophotometric means of determination. The method was applicable to the phospholipids DMPC, DPPC, and EPC using a 1:1 v/v isopropanol:acetate buffer solution but could be extended to other compositions providing a solvent system is found which dissolves the lipids but not CM-chitin. Also, in principle, the method could be applied to other polymer-coated liposome sytems as well.

Table 3A.1. Percent recovery of CM-chitin in the prescence DPPC as a function of pH and CM-chitin concentration.

pH	CM-chitin concentration (μg/ml)		
	50	100	200
5.6	99.9 (4.9)	103.7 (3.7)	98.5 (1.7)
4.0	92.1 (4.3)	98.6 (7.2)	114.1 (6.6)*
2.5	100.5 (6.3)	104.1 (3.6)	103.0 (2.4)

[a] Number in the brackets is the standard deviation of 6 measurements in acetate buffer:isopropanol (1:1 v/v) solution;

[b]F-test, comparsion with theratical value, p=0.05;

*Significant.

Table 3A.2. Percent recovery of CM-chitin at 100 μg/ml as a function of pH and phospnolipid.

pH	DPPC	DMPC	EPC
5.6	103.7 (3.7)	101.0 (5.4)	112.2 (3.0)*
4.0	98.6 (7.2)	86.2 (9.3)*	97.5 (5.9)
2.5	104.1 (3.6)	102.7 (6.4)	115.3 (4.8)*

[a] Number in the brackets is the standard deviation of 6 measurements in acetate buffer:isopropanol (1:1 v/v) solution;

[b]F-test, comparsion with theratical value, p=0.05;

*Significant.

3A.5. References

Clark, J. M. Jr., and Switzer, R. L. (1977). Microanalysis of carbohydrate mixtures by isotopic, enzymic, and colorimetric methods, in Experimental Biochemistry, Chapter 14, 2nd ed., W. H. Freeman and Co., San Francisco, pp. 153-158.

Izawa, H., Arakawa, M., and Kondo, T. (1986). Distribution by surfactants of egg yolk phosphatidylcholine vesicles, Biochim. Biophys. Acta, 855, 243-249.

Kato, A., Arakawa, M., and Kondo, T. (1983). Flow properties of hemolysate-loaded liposome suspensions, Biorheology, 20, 593-601.

Kato, A., Arakawa, M., and Kondo, T. (1984). Preparation and stability of liposome-type artificial red blood cells stabilized with carboxymethyl chitin, J. Microencapsulation, 1, 105-112.

Kato, A., and Kondo, T. (1987). A study of liposome-type artificial red blood cells stabilized with carboxymethyl chitin, Polym. Sci. Tech., 35, 299-310.

Regen, S. L., Czeceh, B., and Singh, A. (1980). Polymerized vesicles, J. Am. Chem. Soc., 102, 6638-6639.

Singleton, W. S., Gray, M. S., Brown, M. L., and White, J. L. (1958). Chromtographically homogeneous lecithin from egg phospholipids, J. Am. Oil Chemists Soc., 44, 53-56.

Szoka, F., and Papahadjopoulos, D. (1978). Procedure for preparation of liposomes with large internal aqueous space and hign capture by reverse-phase evaporation, Proc. Natl. Acad. Sci. USA, 75, 4194-4198.

Weiding, J. U., Eisinger, G., and Kostering, H. (1989). Determination of soluble fibrin by turbidimetry of its protamine suphate-induced paracoagulation, J. Clin. Chem. Biochem., 27, 57-63.

CHAPTER 3

Part B

CM-CHITIN-COATED LIPOSOMES: PREPARATION, STABILITY AND RELEASE OF ACETYLSALICYLIC ACID[3]

3B.1. Introduction

The majority of liposome studies has involved the administration of liposomes intravenously, or intraperitoneally as drug carriers for the delivery of therapeutic or diagnostic agents to specific target tissues. However, there has been increasing interest in the potential use of liposomes for drug delivery by the oral route (*Woodley, 1985*). The main problems associated with orally-administered liposomes pertain to their relatively poor stabilities in the milieu of the GIT and, consequently, leakage of entrapped agents.

Many attempts have been made to enhance the stability of liposomes. Two major approaches, polymerized liposomes (*Regen et al., 1981 & 1985; Tirrell et al., 1985; Tackigawa and Tirrell, 1985; Ohno et al., 1987; Rinsdorf and Schlarb, 1988; Hub et al., 1990*) and polymer-coated liposomes (*Takada et al., 1984; Sunamoto et al., 1985, 1987 & 1988; Sato et al., 1986*), have been proposed thus far. For instance, *Hupfer and Ringsdorf (1981)* have successfully produced polymerized

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[3]A version of this part has been accepted by *J. Control. Rel.* (in press).

vesicles that withstand 50% ethanol dilution and dehydration procedures during sample preparation for scanning electron microscopy. *Tirrell et al. (1984)* have reported that coatings of poly(acrylic acid), poly(methacrylic acid) and poly(α-ethylacrylic acid) can be used to modify the properties and behavior of liposomes. *Kondo et al. (1983, 1984, 1985 & 1987)* and *Izawa et al., (1986)* have used carboxymethyl chitin to produce stable artificial red blood cells (ARBC) and *Sunamoto et al. (1983)* have used other specialized polysaccharides to increase the stability of liposomes and decrease their leakage of entrapped solute after intravenous administration. Subsequently, these same workers tested *O*-palmitoylpullulan- and *O*-palmitoylamylopectin- coated egg phosphatidycholine liposomes, where the polymers were added to preformed small unilamellar vesicles, for 6-carboxyfluorescein release in the presence of bile salts at pH 8.6 and found increased release of the marker (*O'Connor et al., 1985*). This unexpected result was, perhaps, due to an unfortunate choice of composition and conditions as will be demonstrated from the results of the present study.

The aim of this work was to determine the optimum conditions for coating liposomes with the polysaccharide, carboxymethylchitin (CM-chitin), which bestows increased stability of the liposomes in sodium cholate solutions and decreased release of the model water-soluble drug molecule, acetylsalicylic acid (ASA). In this regard, the coating efficiency of a fully-saturated, synthetic phospholipid liposome by CM-chitin, comparison of methods of preparation, the encapsulation efficiency of ASA, and the kinetics of release of ASA from the liposomes have been determined.

Particular attention was given to selecting liposomes in the gel phase and at a pH at which CM-chitin has been found to be stable.

3B.2. Materials and Methods

3B.2.1. Materials

Dipalmitoylphosphatidycholine (DPPC) was purchased from Sigma Chemical Co. Acetylsalicylic acid and sodium cholate from Aldrich Chemical Co. were used as received. Carboxymethyl chitin (pk_a = 3.5; viscosity of 1% solution = 14 cps) was a gift from Nanyo Kasei Co., Japan. All other chemicals were reagent grade, and water was twice-distilled.

3B.2.2. Preparation Methods

Two methods were used to prepare CM-chitin-coated liposomes. One method involved adding CM-chitin during liposome preparation as follows: Twenty-five mg of DPPC in 3 ml of dichloromethane were combined with 1 ml of pH 7.0 (0.085 M) phosphate buffer, then sonicated (*Model W-375 Sonicator, Heat System-Ultrasonics, Inc., Cup-horn, Model 431*) at 10 Khz for 4 min to form a w/o emulsion. The emulsion was diluted with 1 ml of CM-chitin aqueous solution then vortex-mixed to form a w/o/w emulsion. Subsequently, the organic solvent was slowly removed under reduced pressure by rotary evaporation resulting in CM-chitin-coated liposomes. When ASA (30 mM) was included it was incorporated together with the phospholipid in the organic solvent. The other method involved preparing liposomes

as above, except in the absence of CM-chitin, then diluting the liposome preparation (2 ml) with CM-chitin solution (1 ml) followed by incubation at room temperature for about one hour.

3B.2.3. Microscopic Observation

A drop of liposomes or polymer-coated liposomes was placed on a slide with coverslip and observed at 1000× magnification using a *Zeiss Standard 14* microscope fitted with a 35 mm camera. Individual frames were exposed for 4 sec using *Kodak Pan X film (100 ASA).*

3B.2.4. Particle Size Determination

Liposomes were prepared as described, then treated by gel filtration chromatography (*Sephadex G-25, 1.6 × 70 cm*) to remove free CM-chitin using pH 5.6 acetate buffer as the eluting solvent. Liposome fractions were pooled and diluted with the same buffer (20 μl to 3 ml of buffer) and sized by laser light scattering (*Brookhaven Particle Sizer, Model BI-90*) as follows: 2500 cycles/run, 4 runs for each of triplicate samples at a count rate of about 27 kcps.

3B.2.5. ASA Encapsulation Efficiency

The encapsulated ASA was separated from free ASA by centrifugation (*Beckman Model L8-55 ultracentrifuge*) at 135,000 × g for 20 min. The pellet containing entrapped ASA was dissolved in chloroform:isopropanol (1:4v/v) to 10 ml

then analyzed for ASA by HPLC as follows: solvent delivery system (*Waters, Model 501*), UV detector at 280 nm (*Waters, Lambda-Max, Model 481*), Rheodyne injector (*Spectra Physics, Model 7125*), 20 μl injection loop, C_{18} reverse phase chromatography column (*Waters, 15-cm Novapak*, 5 μm particle size), integrator-plotter (*Waters, Model 740*). The mobile phase was 20% acetonitrile aqueous solution containing 0.1% phosphoric acid at pH 2.7 delivered at a flow rate of 1.5 ml/min.

3B.2.6. CM-chitin Coating Efficiency

The CM-chitin coating efficiency was determined as described in 3A. Briefly, CM-chitin-coated liposomes were separated from free CM-chitin by gel filtration (*Sephadex G-25, 1.6 × 70 cm*) using pH 5.6 acetate buffer as the eluting solvent; eluted fractions of the coated liposomes were collected and pooled. After allowing sedimentation of the polymer-coated liposomes for 12 hr at 8°C, the supernatant was removed by Pasteur pipette then the sediment was diluted to 10 ml with buffer solution. Subsequently, 5 ml were removed, mixed with 5 ml of isopropanol and the turbidity was measured at 400 nm. The coating efficiency was calculated as the ratio of the weight of associated CM-chitin (mg) per 100 mg of DPPC.

3B.2.7. Stability in Sodium Cholate Solutions

One-ml samples of either uncoated or CM-chitin-coated liposomes were mixed with different volumes of 20 mM sodium cholate solution, then diluted to 10 ml with

pH 5.6 acetate buffer at room temperature (22°C). The turbidity of the mixture was measured spectrophotometrically at 400 nm. The relative stabilities of the liposomes were assessed by the equilibrium turbidity changes as a function of the added sodium cholate concentrations.

3B.2.8. ASA Release Studies

CM-chitin-coated liposomes containing ASA were prepared as described, then treated by gel permeation chromatography (*Sephadex G-25, 1.6 × 70 cm*) to remove free ASA using pH 5.6 acetate buffer as the eluting solvent. Liposome fractions were pooled then incubated at 37°C (*Dubnoff Metabolic Shaker*) following which aliquots (1 ml) of the thoroughly-mixed preparation were removed at 20 min intervals, centrifuged, and the supernatants were analyzed by HPLC. The following was used to calculate the release of ASA:

$$\text{ASA release (\%)} = (C\text{-}C_o)/(C_t\text{-}C_o) \times 100$$

where C_o is the initial ASA concentration in the supernatant before incubation, C_t the total ASA and SA concentrations in the liposome suspension, and C the ASA and SA concentrations in the sample supernatant after time t.

3B.3. Results and Discussion

3B.3.1. Morphology of CM-chitin-Coated Liposomes

The particle size of the liposome preparations was estimated to be in the range of 1.3 to 8.9 μm (p=0.1) by laser light scattering. CM-chitin-coated liposomes,

coated after liposome formation, resulted in increased average diameters of liposomes ranging from 4.16 μm (control liposomes) to 4.56 μm while liposomes coated with CM-chitin during liposome formation had smaller diameters than the control liposomes (Table 3B.1), suggesting different mechanisms of CM-chitin coating. CM-chitin coatings formed after liposome formation were based on hydrophilic adsorption at the liposome surfaces, thus, the liposome core and the CM-chitin coating determined the size of the particle. In contrast, CM-chitin coating formed during liposome formation not only covered the surfaces of the liposomes but also participated in the formation of liposomes. In the other words, CM-chitin mixed with and became anchored to the bilayers causing a condensation of the phospholipid molecules in the liposomes resulting in a smaller overall size. It can be observed from the data in Table 3B.2 that this was a concentration-dependent effect.

Optical microscopic inspection of individual particles clearly revealed the multilamellar structure of the control (uncoated) liposomes (Fig. 3B.1A) whereas this was obscure for the CM-chitin-coated liposomes (Fig. 3B.1B). In the latter case the particles were aggregated and appeared to be enclosed by a polymer shell.

The results of the gel permeation technique to remove free ASA from liposomal ASA are illustrated in Fig. 3B.2. Under these conditions the liposomes were eluted in the void volume as fractions 8-15 and the free ASA was eluted in fractions 15-26, approximately. Liposome fractions were turbid while ASA had no light absorption at 400 nm. Likewise, liposomal components in isopropanol solution were shown not to interfere with the UV absorption of ASA at 276 nm. This elution

Table 3B.1. Particle size range of CM-chitin-coated liposomes as a function of liposome preparation methods.

CM-chitin added	Average diameter μm, (STD)	Diameter range (μm, p=0.1)
0	4.16 (0.25)	1.35 ~ 7.96
1% CM-chitin added during liposome formation	3.92 (0.19)	1.29 ~ 7.47
1% CM-chitin added to preformed liposomes	4.59 (0.69)	1.42 ~ 8.87

Table 3B.2. Particle size range of CM-chitin-coated liposomes as a function of CM-chitin concentration.

CM-chitin concentration (%)	Average diameter μm (STD)	Diameter range (μm, p=0.1)
0	4.16 (0.25)	1.35 ~ 7.96
0.5	4.58 (0.38)	1.55 ~ 8.62
1.0	3.92 (0.19)	1.29 ~ 7.47
1.5	3.80 (0.22)	1.92 ~ 6.13
2.0	3.32 (0.43)	1.34 ~ 5.91

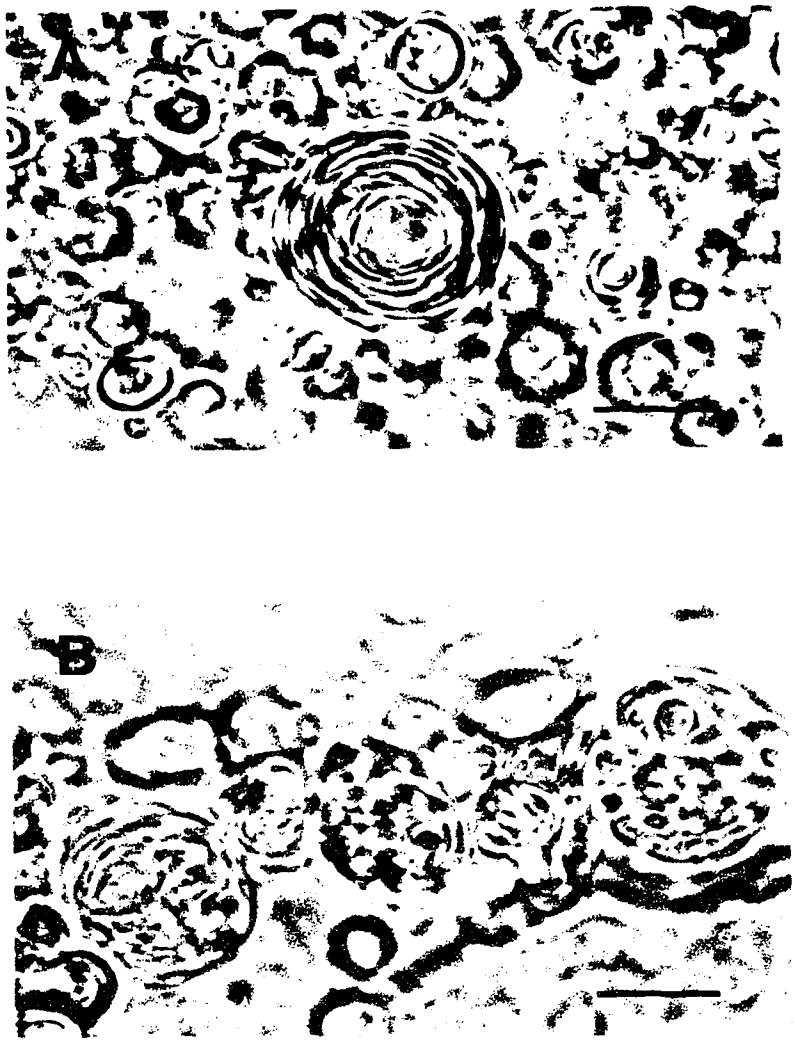

Fig. 3B.1. Micrographs from optical microscope showing the microstructure of uncoated and CM-chitin-coated DPPC liposomes. A) Uncoated liposomes, B) 1.0% CM-chitin-coated liposomes coated during liposome preparation. Bar length = 10 μm.

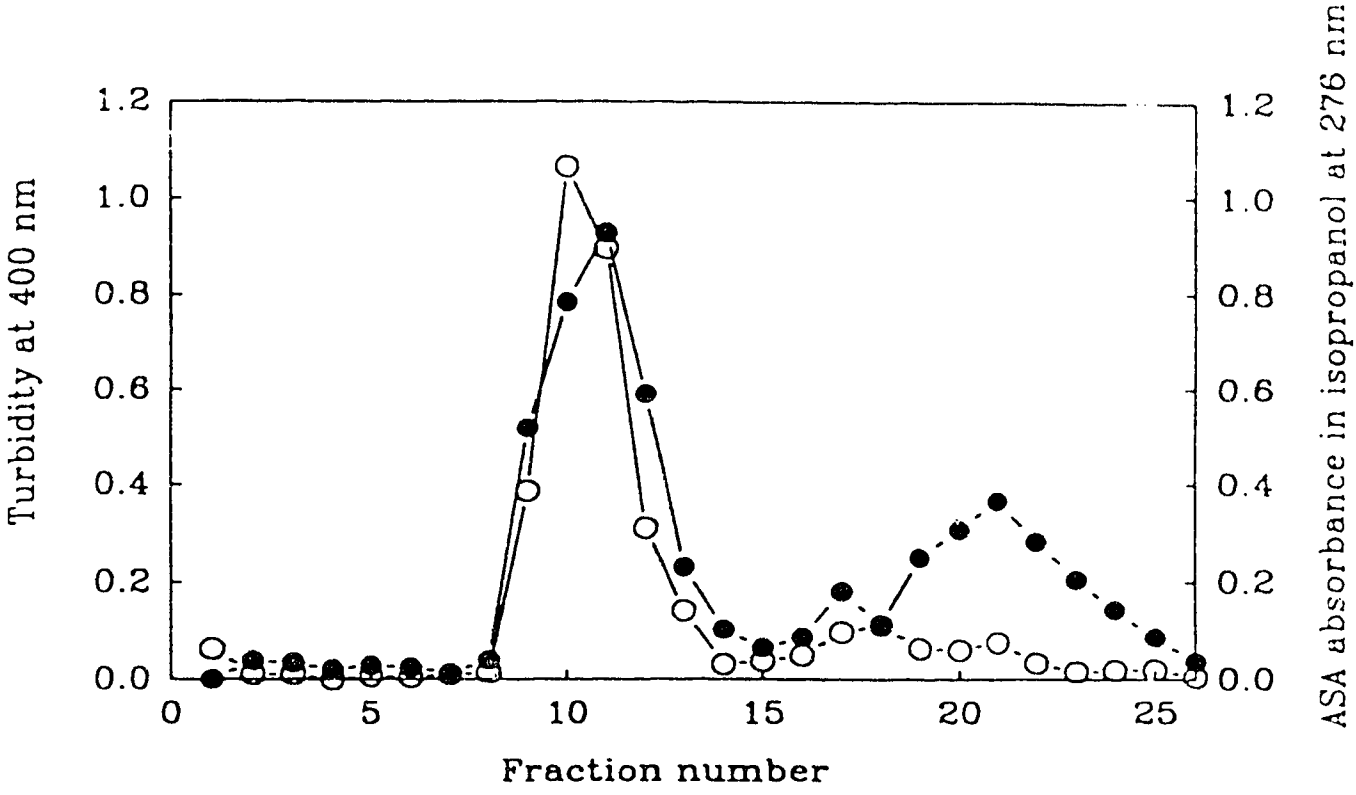

Fig. 3B.2. Profile of the separation of entrapped ASA in DPPC liposomes from unentrapped ASA by gel permeation chromatography, Sephadex G-25, 1.6 X 70 cm column, pH 5.6 sodium acetate buffer. ○ , turbidity at 400 nm; ●, ASA absorbance in isopropanol at 276 nm.

pattern was reproducible for either uncoated or CM-chitin-coated liposomes demonstrating application of gel permeation chromatography to polymer-coated liposomes.

3B.3.2. ASA Encapsulation Efficiency

The encapsulation efficiency of ASA in uncoated and CM-chitin-coated DPPC liposomes is described in Table 3B.3 as a function of the initial ASA concentration. The encapsulation efficiency gradually increased with ASA concentration up to 60 mM, but then increased dramatically at higher concentrations, being approximately 20-fold greater following an 8-fold increase in the initial ASA concentration. It is also apparent that the CM-chitin coating of liposomes did not significantly influence the ASA encapsulation efficiency.

3B.3.3. CM-chitin Coating Efficiency

The coating efficiency of liposomes by CM-chitin was dependent on the initial CM-chitin concentration and the coating procedure. The results, illustrated in Fig. 3B.3, indicate the differences in coating efficiency at various initial CM-chitin concentrations up to 2 percent. When the CM-chitin was added during liposome formation more of the polymer became associated with liposomes at initial CM-chitin concentrations up to 1.5 percent compared to when the polymer was added to preformed liposomes. This is not surprising since the CM-chitin molecules were intimately in contact with all of the bilayers comprising the multilamellar liposome

Table 3B.3. Comparison of ASA encapsulation efficiency in uncoated and 1.0% CM-chitin-coated DPPC liposomes. Number in brackets is the standard deviation of triplicate measurements with a DPPC concentration of 74 mM.

Initial ASA Concentration (mM)	Encapsulation efficiency (ASA:DPPC mole ratio %)	
	Uncoated liposomes	Coated liposomes
15	1.5 (0.2)	1.5 (0.1)
30	2.2 (0.1)	2.3 (0.1)
60	4.8 (0.1)	5.1 (0.7)
90	18.6 (0.6)	18.1 (2.2)
120	27.2 (1.7)	24.6 (8.2)

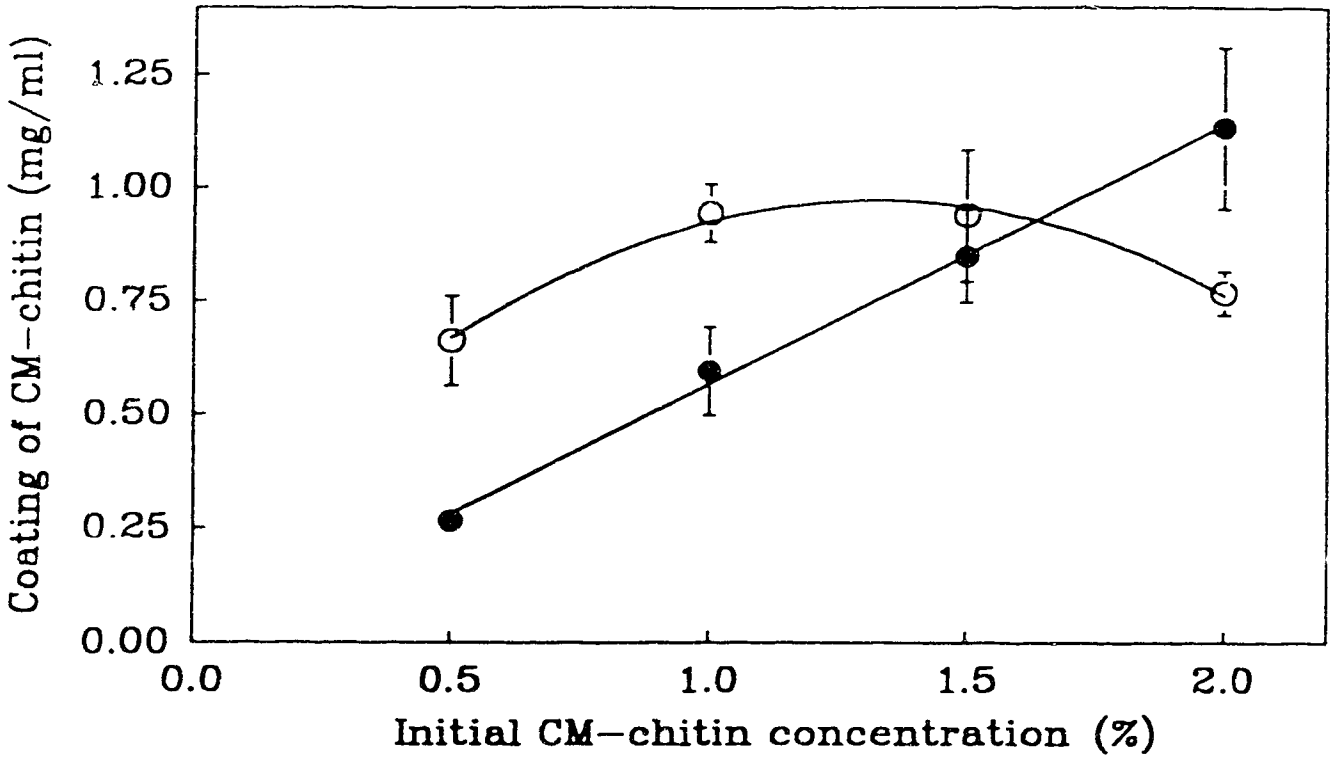

Fig. 3B.3. CM-chitin coating efficiency as a function of initial CM-chitin concentration and coating method. Experiments were conducted at room temperature (~22°C) at a DPPC concentration of 74 mM. ○ , liposomes coated during liposome formation; ● , coated preformed liposomes. ± SD (n=3).

structure when added during liposome formation whereas only the outer bilayers of the liposomes were available for interaction with the CM-chitin molecules when added to preformed liposomes. In the former case, the coating efficiency rose only slightly with additional CM-chitin, reached a maximum, then actually decreased when 2 percent CM-chitin was added. This suggests a capacity-limited interaction process which would occur if the CM-chitin molecules interdigitated the ordered DPPC molecules in the bilayers. On the other hand, in the latter case, the coating of preformed liposomes by CM-chitin is proportional to the CM-chitin added which indicates surface binding. Since the coating efficiency increased linearly up to 2 percent of added CM-chitin, the surface film formed is probably multimolecular. The chemical structure of CM-chitin (Fig. 3B.4) would suggest that it would undergo both hydrophobic interaction in the hydrocarbon region of the bilayers as well as polar group interactions which could have the effect of altering the permeability to entrapped solutes.

3B.3.4. Liposome Stability Studies

The degree of physical stability of CM-chitin-coated liposomes in sodium cholate solution was also a function of the liposome coating procedure. Fig. 3B.5 shows uncoated liposomes to be unstable in 2 mM sodium cholate solution as expected. However, each of the CM-chitin-coated liposome preparations was stable in sodium cholate solutions as high as 3-4 mM. In comparison, only about 65% of the original turbidity of the uncoated liposomes remained after addition of 2 mM of

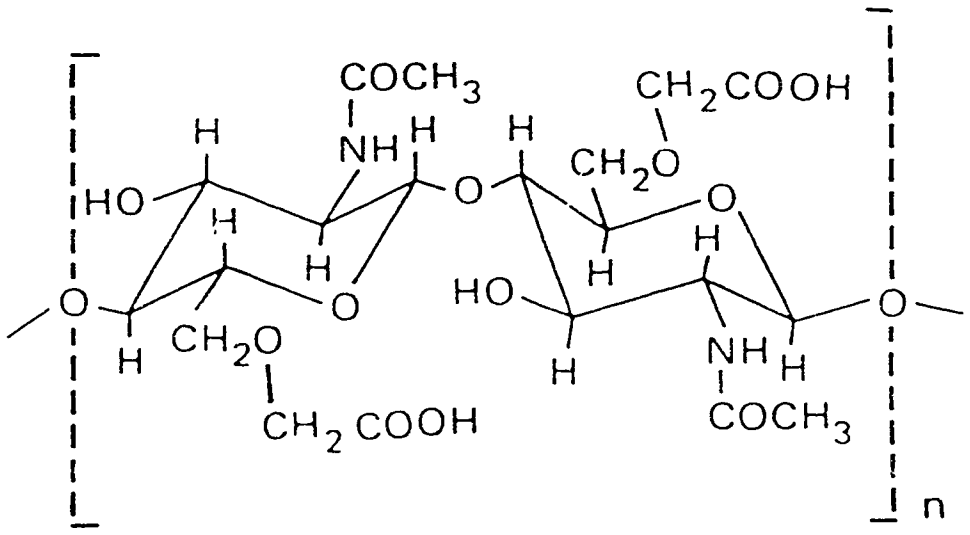

Fig. 3B.4. Chemical structure of carboxymethyl chitin.

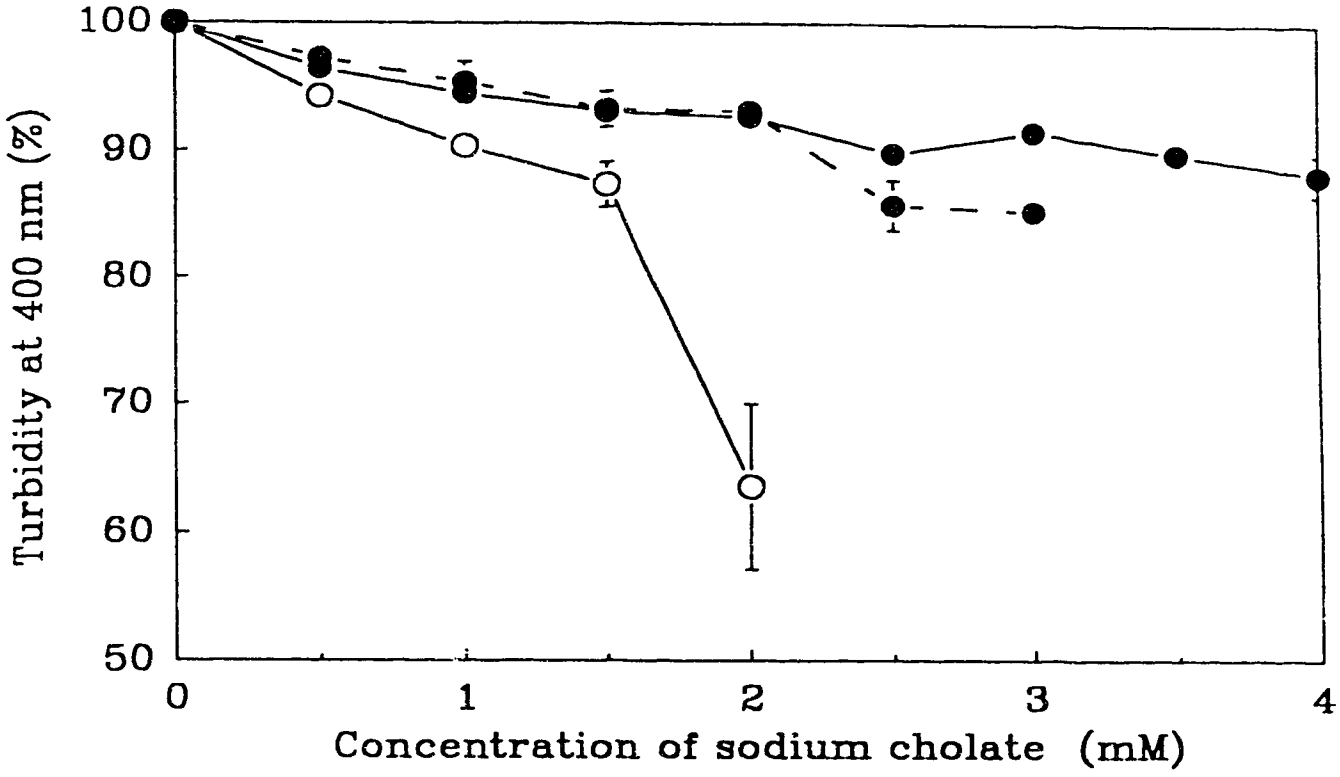

Fig. 3B.5. Turbidity of CM-chitin-coated and uncoated DPPC liposomes after dilution in sodium cholate solution as a function of coating procedure, in pH 5.6 acetate buffer at 22°C. ○ ——— ○ , uncoated liposomes; ● --- ● , 1.0% CM-chitin added to preformed liposomes; ● ——— ● , 1.0% CM-chitin added during liposome formation. ± SD (n=3).

sodium cholate solution whereas more than 90% of the original turbidity of 1% CM-chitin-coated liposomes, coated during liposome formation, remained after dilution with 3 mM sodium cholate solution and about 85% for the preformed liposomes. Statistically, the stability between CM-chitin-coated and uncoated liposomes was significant especially at 2 mM sodium cholate concentration (F-test, $p=0.05$), however, there was no significant difference between two procedures.

Fig. 3B.6 illustrates that the stability of liposomes, coated during liposome formation, was also a function of the initial CM-chitin concentration used to coat the liposomes. It is apparent that after the addition of 0.5% CM-chitin, the coated liposomes became stable at 2 mM but not at 2.5 mM sodium cholate concentration. Increasing the initial CM-chitin concentration to 1.0% produced even greater stability of the liposomes and were stable even in the presence of 3 mM sodium cholate solution (F-test, $p=0.05$). Furthermore, increasing the initial CM-chitin concentration to 2.0% appeared to yield similar, although slightly lower liposome stability at sodium cholate concentrations greater than 2mM. The stability results show a reasonable correlation with the measured CM-chitin coating efficiencies since liposomes coated during liposome formation from a 1.0% initial CM-chitin concentration yielded the greatest coating efficiency (Fig. 3B.3). The results at 2% CM-chitin concentration point towards reduced coating efficiency and stability of the liposomes, suggesting a possible increased perturbation of the lipid bilayers at the higher CM-chitin concentration.

The turbidimetric method, also used by others (*Regen et al., 1980*) to study

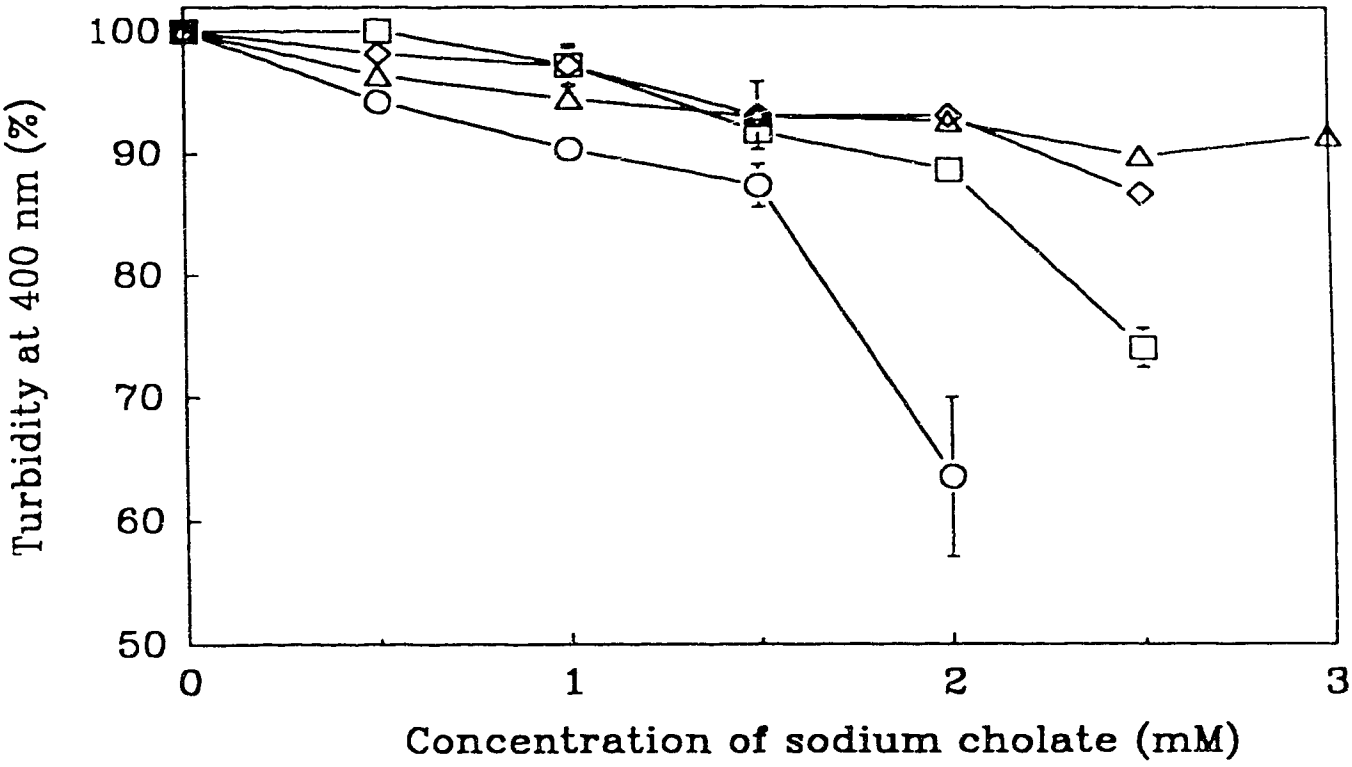

Fig. 3B.6. Turbidity of CM-chitin-coated and uncoated DPPC liposomes as a function of initial CM-chitin concentration after dilution in sodium cholate solution . ○ , uncoated liposomes; □ , 0.5% CM-chitin-coated liposomes; ▵ ,1.0% CM-chitin-coated liposomes; ◇ , 2.0% CM-chitin-coated liposomes. ± SD (n=3).

the stability of polymerized liposomes in ethanol solutions, supports the use of a 400 nm wavelength. In this study, the same principle was used without interference by sodium cholate and the other solutes in the system. Likewise, CM-chitin-coated egg phosphatidycholine (EPC) liposomes were demonstrated to be more stable against the detergent effect of sodium 1-dodecyl sulfate using a turbidimetric method (*Kato and Kondo, 1987*). Since sodium cholate is prevalent as an endogenous detergent in the GI tract, solutions of it represent a more appropriate vehicle for the demonstration of liposome stability following oral administration. Bile salt damage of liposomes, like other detergents, requires a critical detergent:lipid ratio before mixed micelles begin to form and solubilization of the liposomes occurs (*Lichtenberg, 1985; Rogers et al., 1990*). Attainment of this critical ratio is a function of the detergent concentration and the intermolecular energies of the lipid molecules in the liposomal bilayers. The latter is determined by the fluidity of the hydrocarbon chains and the integrity of the outer hydrated polar group region. The inclusion of additives which increase hydrocarbon chain separation and decrease the surface energy barrier will result in a reduction of the detergent:lipid ratio for mixed micelle formation. Thus, the coating of egg phosphatidylcholine liposomes in the liquid crystalline state by *O*-palmitoylpullulan or *O*-palmitoylamylopectin at pH 8.6 as reported by *O'Connor et al. (1985)* would be expected to decrease interactions between the lipid molecules in the bilayers, thereby increasing the ease with which detergents can participate with the lipid molecules in mixed micelle formation, leading towards increased permeability of the 6-carboxyfluorescein marker. In contrast, it is concluded that the interaction of

CM-chitin with gel phase DPPC liposomes at pH 5.6 contributes to the coherent structure of the bilayer membranes but only over a narrow concentration range (approx. 0.5%-1%) giving a polymer-coated liposome structure which is more stable against sodium cholate than uncoated liposomes. This behavior of CM-chitin occurs whether the polymer is included during liposome formation or added to preformed liposomes, but the magnitude of the effect differs slightly as shown in Fig. 3B.5, particularly in sodium cholate solutions greater than 2 mM.

3B.3.5. ASA Release Studies

The release of encapsulated ASA from uncoated and CM-chitin-coated DPPC liposomes in pH 5.6 buffer at 37°C is described in Fig. 3B.7. ASA was rapidly released from uncoated DPPC liposomes, about 85% being released within 20 min and the remaining being gradually released over a period of an hour. In contrast, CM-chitin-coated liposomes yielded decreased release of ASA, to the extent of about 25% within 20 min from liposomes coated by CM-chitin during liposome formation, or 70% in 20 min from preformed liposomes coated with CM-chitin, the release of the remaining ASA was so slow that there was not significant amount released for two hours, however, the extent of ASA release was significantly different among these three formulations (F-test, $p=0.05$). These different results are interpreted to mean that the manner and extent to which CM-chitin is associated with the liposomes plays an important role in the degree of cohesiveness and continuity of the bilayers in the liposome structure and in the polymer film as a physical barrier towards diffusing

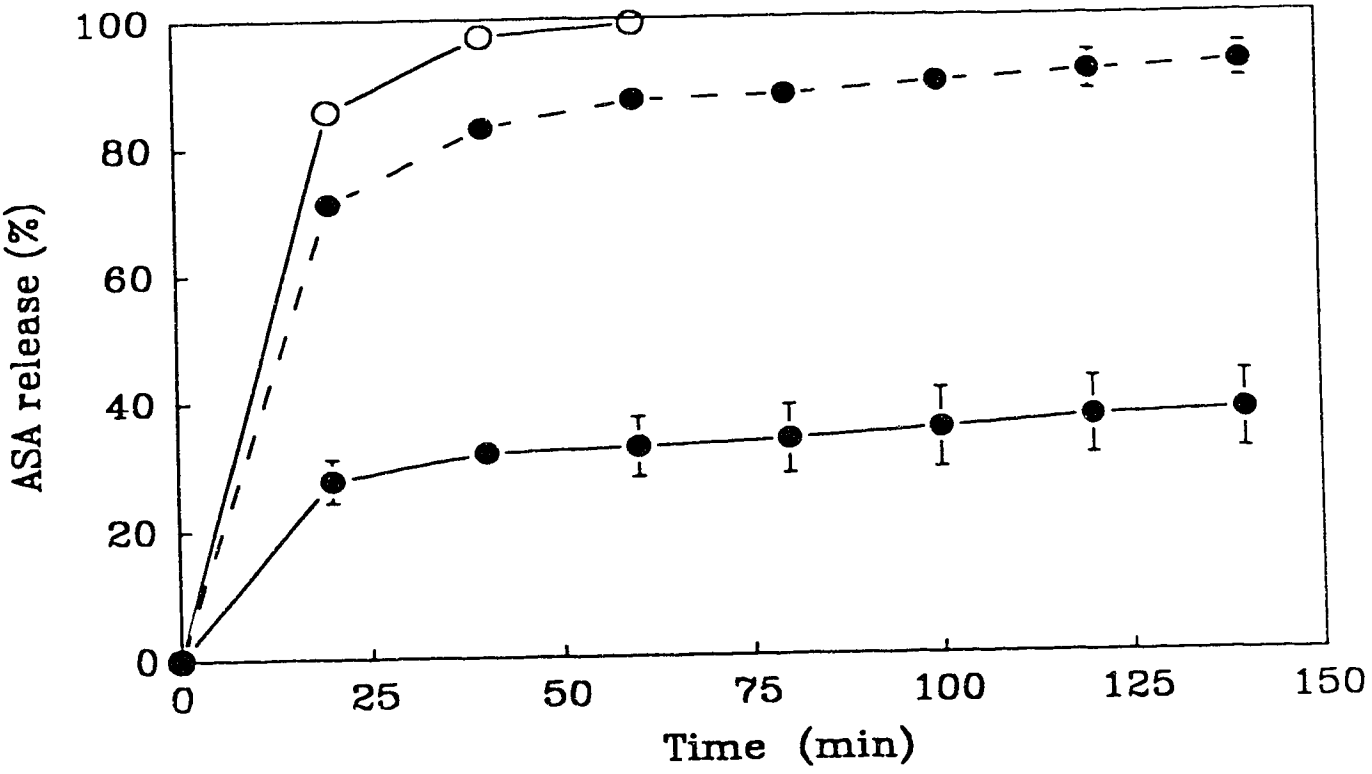

Fig. 3B.7. The release of ASA from ASA-encapsulated CM-chitin-coated and uncoated DPPC liposomes as a function of the coating procedure in pH 5.6 acetate buffer at 37°C. ○ ——— ○ , uncoated liposomes; ● --- ● , 1.0% CM-chitin added to preformed liposomes; ● ——— ● , 1.0% CM-chitin added during liposome formation.

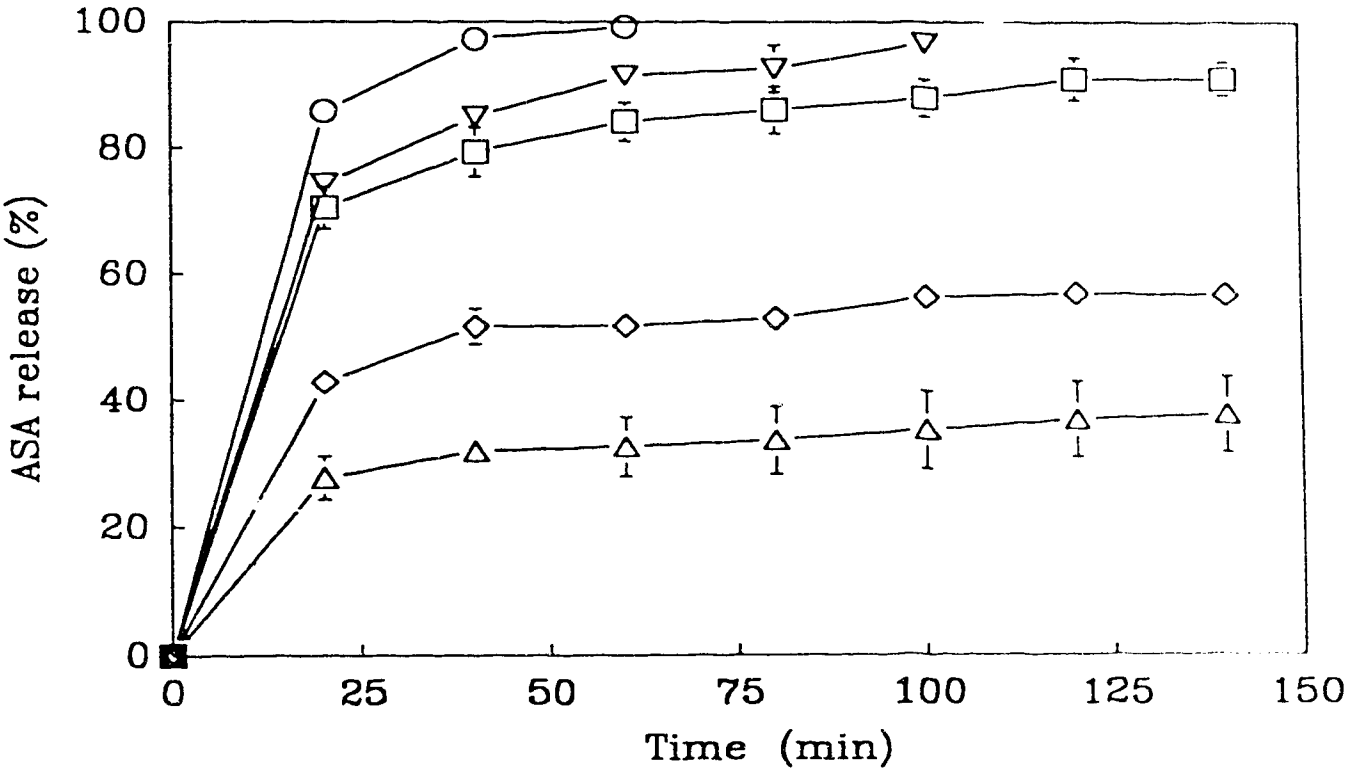

Fig. 3B.8. The release of ASA from ASA-encapsulated CM-chitin-coated (during liposome formation) and uncoated DPPC liposomes as a function of CM-chitin concentration in pH 5.6 acetate buffer at 37°C. ○ , uncoated liposomes; □ , 0.5% CM-chitin-coated liposomes; △ , 1.0% CM-chitin-coated liposomes; ◇ , 1.5% CM-chitin-coated liposomes; ▽ , 2.0% CM-chitin-coated liposomes. ± SD (n=3).

solute. Since the release was carried out close to the DPPC phase transition temperature (42°C), CM-chitin added during liposome formation was at least partially located in the bilayers and could have increased the ordered structure of the bilayers (with decreased fluidity). This would result in a decreased permeability and leakage of ASA.

The release of ASA from CM-chitin-coated DPPC liposomes, coated during liposome formation, was also influenced by the initial CM-chitin concentration as shown in Fig. 3B.8. At a CM-chitin concentration of 0.5%, the release of ASA after 50 min was reduced by about 20%. At 1% levels, the release of ASA was reduced approximately 3-fold. This compares with the decreased release of carboxyfluorescein (CF) from EPC liposomes using derivatized pullulan (*Sunamoto et al., 1987*). However, at higher CM-chitin concentrations a surprising reversal took place. Thus, at 1.5% the release of ASA was reduced approximately 2-fold only whereas at 2% CM-chitin concentration the release of ASA was hardly 10% less than that from uncoated liposomes. Thus, it would appear that the main effect of polymer coating on drug release is the influence which the polymer exerts on the bilayer structure of the liposomes rather than the polymer film acting as a barrier to ASA release. Over a narrow range of concentration of polymer added during liposome formation, the barrier to the transport of solute increases whereas larger amounts of added polymer lead to a reduction of the barrier function of the bilayers to transport.

3B.4. Summary

The results of this study demonstrate a clear effect of the coating of DPPC liposomes by CM-chitin on liposome stability and the release of ASA from CM-chitin-coated liposomes. The effects produced were somewhat dependent on the coating procedure. Adding CM-chitin during liposome formation enabled the polymer to become associated with the surfaces of each of the bilayers in the multilamellar structure whereas adding the polymer to preformed liposomes only coated the outer bilayers and had a smaller influence on the release of encapsulated drug. The main significance of these studies is the possibility of developing a liposome system which possesses considerably greater stability in sodium cholate solutions then uncoated liposomes and which the leakage of entrapped drug, such as ASA, was at a much lower level by appropriate selection of the polymer concentration and the method used to form polymer-coated liposomes. This could have important implications in the design of a liposome drug delivery system for oral administration.

3B.5. References

Hub, H. H., Hupfer, B., Koch, H., and Ringsdorf, H. (1980). Polymerizable phospholipid analogues-new stable biomembrane and cell models, Angew. Chem., Int. Ed. Engl., 19, 938-940.

Hupfer, B., and Ringsdorf, H. (1981). Polymeric monolayers and liposomes as models for biomembranes and cells, ACS Symp. Ser., 15, 209-232.

Izawa, H., Arakawa, M., and Kondo, T. (1986). Disintegration by surfactants of egg yolk phosphatidycholine vesicles stabilized with carboxymethylchitin, Biochim. Biophys. Acta, 855, 243-249.

Kato, A., Arakawa, M., and Kondo, T. (1983). Flow properties of hemolysate-loaded liposomes suspensions, Biorheology, 20, 592-601.

Kato, A., Arakawa, M., and Kondo, T. (1984). Preparation and stability of liposome-type artificial red blood cells stabilized with carboxymethyl chitin, J. Microencapsulation, 1, 105-112.

Kato, A., Arakawa, M., and Kondo, T. (1985). A study of liposome-type artificial red blood cells stabilized with carboxymethyl chitin, Polym. Mater. Sci. Eng., 53, 654-658.

Kato, A., and Kondo, T. (1987). A study of liposome type artificial red blood cells stabilized with carboxymethyl chitin, Polym. Sci. Tech., 35, 299-310.

Lichtenberg, D. (1985). Characterization of the solubilization of lipid bilayers by surfactants, Biochim. Biophys. Acta, 821, 470-478.

O'Connor, C. J., Wallace, R. G., Iwamoto, K., Taguchi, T., and Sunamoto, J. (1985). Bile salt damage of egg phosphatidylcholine liposomes, Biochim. Biophys. Acta, 817, 95-102.

Ohno, H., Takeoka, S., Iwai, H., and Tsuchida, E. (1987). Polymerization of liposomes composed of diene-containing lipids by radical initiators. II. Polymerization of monodiene-type lipids as liposomes, J. Polym. Sci., Part A, Polym. Chem., 25 2737-2746.

Regen, S. L., Czech, B., and Singh, A. (1980). Polymerized vesicles, J. Am. Chem. Soc., 102, 6638-6640.

Regen, S. L., Singh, A., Oebeme, G., and Singh, M. (1981). Polymerized phosphatidyl choline vesicle . Stabilized and controllable time-release carriers, Biochem. Biophys. Res. Commun., 101, 131-136.

Regen, S. L., Samuel, N. K. P., and Khurana, J. M. (1985). Polymerization of macrocyclic phospholipid- and surfactant-based vesicles, J. Am. Chem. Soc., 107, 5804-5805.

Ringsdorf, H., and Schlarb, B. (1988). Preparation and characterization of unsymmetrical "liposomes in a net", Makromol. Chem., 189, 299-315.

Rogers, J. A., Betageri, G. V., and Choi, Y. W. (1990). Solubilization of liposomes by weak electrolyte drugs. I. Propranolol, Pharm. Res., 7, 957-961.

Sato, T., Koyima, K., Ihda, T., and Sunamoto, J. (1986). Macrophage activation by poly(maleic acid-alt-2-cyclohexyl-1,3-dioxap-5-ene) encapsulated in polysaccharide-coated liposomes, J. Bioact. Comp. Polym., 1, 448-460.

Seki, K., and Tirrell, D. A. (1984). pH-dependent complexation of poly(acrylic acid) derivatives with phospholipid vesicle membranes, Macromolecules, 17, 1692-8.

Sunamoto, J., Iwamoto, K., Takada, M., Yuzuriha, T., and Katayama, K. (1983). Improved drug delivery to target specific organs using liposomes as coated with polysaccharides, Polym. Sci. Tech., 23, 157-168.

Sunamoto, J., and Iwamoto, K. (1985). Protein-coated and polysaccharide-coated liposomes as drug carriers, CRC Crit. Rev. Ther. Drug Carrier Syst., 2, 117-136.

Sunamoto, J., Sato, T., Hirota, M., Tukushima, K., Hirarani, K., and Hara, K. (1987). A newly developed immunoliposome-an egg phosphatidycholine liposome coated with pullulan bearing both a choiesterol moiety and an IgMs fragment, Biochim. Biophys. Acta, 898, 323-330.

Sunamoto, J., Sakai, K., Sato, T., and Kondo, H. (1988). Molecular recognition of polysaccharide-coated liposomes. Importance of sialic acid moiety on liposomal surface, Chem. Lett., 1781-1784.

Takada, M., Yuzuriha, T., Katayama, K., Iwamoto, K., and Sunamoto, J. (1984). Increased lung uptake of liposomes coated with polysaccharides, Biochim. Biophys. Acta, 802, 237-244.

Takigawa, D. Y., and Tirrell, D. A. (1985). Disruption of phospholipid packing by branched poly(ethylenimine) derivatives, Macromolecules, 18, 338-342.

Tirrell, D. A., Turek, A. B., Wilkinson, D. A., and McIntosh, T. J. (1985). Observation of an interdigitated gel phase in dipalmitoylphosphatidyglycerol bilayers treated with ionene-6,6, Macromolecules, 18, [illegible]-1513.

Woodley, J. F. (1985). Liposomes for oral administration of drugs, CRC Crit. Rev. Ther. Drug Carrier Syst., 2, 1-18.

CHAPTER 4

ACACIA-GELATIN-MICROENCAPSULATED LIPOSOMES: PREPARATION, STABILITY AND RELEASE OF ACETYLSALICYLICACID[4]

4.1. Introduction

The development of liposomes as drug delivery systems is often halted because of poor stability in the biological environment in which they are intended to be administered. The oral route in particular has not been successful due to hydrolytic influences of enzymes and pH and the solubilizing action of the bile salts. Attempts to improve liposome stability have used polymerized liposomes (*Regen, 1987*), polymer-encased liposomes (*Fukuda et al., 1986*), polymer-coated liposomes (*Ringsdorf and Schlarb, 1988; Sunamoto et al., 1983; Tirrell et al., 1985*), or microencapsulated liposomes (*Kibat et al., 1990; Nixon and Yeung, 1989; Yeung and Nixon, 1988*). In addition, controlled release of protein (*Ozden and Hasirci, 1990*) and sustained release of an ionized drug molecule from these modified liposomes have been demonstrated.

Microencapsulation of liposomes by polymers may have the advantage of stabilizing liposomes against hydrolytic influences in the gastrointestinal tract more efficiently than polymer-coated liposomes because of thicker shells which can be produced. Although synthetic polymers, such as the polyamides (*Nixon and*

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[4]A version of this chapter has been submitted for publication to *Pharm. Res.*

Yeung, 1989; Yeung and Nixon, 1988) may be suitable in some circumstances, nontoxic naturally-occurring polysaccharides are more appropriate for oral liposomes with which it is also possible to microencapsulate liposomes without the use of organic solvents.

This chapter describes the preparation of microencapsulated liposomes and the effect of this process on the encapsulation of an ionic solute and its release behavior. In addition, the degree of stability of the microencapsulated liposomes compared to the liposomes themselves in bile salt solutions has been measured. These properties of microencapsulated liposomes have been compared to similar properties previously observed with other polymer-coated liposomes.

4.2. Materials and Methods

4.2.1. Materials

L-α-Dipalmitoylphosphatidylcholine (DPPC) and gelatin (300 bloom, type A) were obtained from Sigma Chemical Co., St. Louis, MO. Acacia, formaldehyde (Fisher Scientific Co., Fairlawn, NJ), acetylsalicylic acid (ASA) and sodium cholate (Aldrich Chemical Co., Inc., Milwaukee, WI) were used as received. All other chemicals were at least reagent grade and deionized distilled water was used throughout.

4.2.2. Microencapsulated Liposomes by Complex Coacervation

Multilamellar liposomes (MLVs) were prepared by a simple hydration

method. Typically, DPPC (34 μmol) and ASA (30 μmol) dissolved in 5 ml of chloroform were dried as a film in a round-bottom flask by rotary evaporation at 50°C then flushed with N_2 and left overnight in a vacuum oven at 30°C. Subsequently, the film was hydrated with 1 ml of pH 5.6 buffer solution (15 mM acetic acid + 157 mM sodium acetate) at 50°C and dispersed by vortex-mixing. MLVs (1 ml) were mixed with equal volumes (0.5 ml, 1.5 ~ 5% w/v in each case) of acacia and gelatin solutions then the preparation was adjusted to a pH of 4.0 using a pH 2.5 acetate buffer. The mixture was cooled to 8°C for 10 min to form microencapsulated liposomes. The microcapsule shell was hardened by the addition of 37% formaldehyde (1 ml) and the excess formaldehyde was removed by centrifugation and washing with pH 5.6 buffer solution.

4.2.3. Electron Microscopic Studies

a) *Negative staining*. The procedure for negative staining of a sample liposome preparation was as follows: a drop of liposome preparation was applied to a formvar film-coated copper grid and the excess was removed by filter paper. Phosphotungstic acid solution (1%, pH 7.0) was dropped onto the grid and after 10 sec it was removed with filter paper. The stained samples were examined in a Hitachi H-7000 electron microscope at 75 KV.

b) *Ultrathin section technique*. Liposomes were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer solution at pH 7.5 for 2 h. After three times rinsing with the buffer solution samples were further fixed with

1% osmium tetroxide in the same buffer solution for 2 h. After rinsing with water to remove excess osmium tetroxide 1% uranyl acetate was added and the sample left overnight. Subsequently, after rinsing with distilled water the samples were dehydrated with successive 10 min washings of acetone solutions (50%, 70%, 95%, and 2×100%). This was followed by successive 30 min washings of acetone:Spurrs' medium[5] (1:1 v/v, 1:3 v/v) then Spurrs' medium (twice for 1 h). The specimens were placed in flat embedding moulds containing Spurrs' medium and put in an oven at 70°C to polymerize for 8 h. Thin sections of approximately 60 ~ 90 nm were cut from the sample with a glass knife on an ultramicrotome and collected on 400 mesh copper grids. Post-staining was carried out with 2% uranyl acetate then the specimens were examined under the electron microscope.

4.2.4. Particle Sizing and Wall Thickness Determinations

Liposome preparations (20 μl) were diluted with pH 4.0 acetate buffer solution (3 ml) and sized by dynamic light scattering (*Brookhaven Instruments Particle Sizer, Model BI-90*) using a protocol of 2500 cycles/run, 5 runs for each of triplicate samples at a count rate of ca. 18 kcps. Wall thicknesses of microcapsules were estimated from the differences in mean size between microencapsulated and control liposomes.

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[5]Spurrs' medium consists of 23.6% v/v 4-vinylcyclohexene dioxide (ERL 4206), 14.2% v/v diglycidyl ether of polypropylene glycol (DER 736), 61.3% v/v nonenyl succinic anhydride (NSA) and 0.9% v/v dimethylaminoethanol (DMAE).

4.2.5. Analysis of Encapsulated ASA

Either liposome-encapsulated ASA or microencapsulated liposomal ASA was determined by centrifugation (135,000 x g for 10 min, *Beckman Model L8-55 Ultracentrifuge*) and removal of the supernatant. The pellet was then dissolved in chloroform:isopropanol solution (1:4 v/v) and analyzed by reverse phase HPLC (*Waters*) with spectrophotometric detection at 280 nm. The mobile phase was 20% acetonitrile at pH 2.7 (0.1% phosphoric acid) at a flow rate of 1.5 ml/min.

4.2.6. Liposome Stability Studies

A 1-ml aliquot of either control or microencapsulated liposomes was mixed with different volumes of 20 mM sodium cholate solution buffered to pH 5.6 then diluted to 10 ml with buffer at room temperature. Turbidities were monitored spectrophotometrically at 400 nm (*Regen et al., 1980*) (*Beckman spectrophotometer, Model 25*). Decreased turbidities were considered indicative of decreased stability (*Nagata et al., 1990; Roda et al., 1990*). Some studies were also conducted in pH 7.0 buffer solution (50 mM NaH_2PO_4, 35 mM NaOH).

4.2.7. Drug Release Studies

Microencapsulated liposomes containing ASA (0.24mmol) at pH 5.6 were incubated (*Dubnoff Metabolic Shaker*) with gentle agitation either at room temperature (23°C) or 37°C. At 20 min intervals 1 ml aliquots were withdrawn and centrifuged. The amount of ASA and SA in the supernatant was analyzed as

before. The fraction of ASA released was calculated from,

$$\% \text{ ASA released} = (C-C_o)/(C_t-C_o) \times 100$$

where C_o is the initial ASA concentration in the aqueous medium before incubation, C_t is the total ASA and SA concentrations in the liposome suspension, and C is the ASA and SA concentrations in the sample supernatant after time, t.

4.3. Results and Discussion

4.3.1. Preparation of Microencapsulated Liposomes

Microcapsules prepared by coacervation usually employ an organic disperse phase of an oil-in-water emulsion to contain the material to be encapsulated. In the present study MLVs constituted the disperse phase on the surfaces of which the coacervate of acacia and gelatin were deposited. Evidence of this is seen in Figures 4.1 and 4.2. In Fig. 4.1 electron micrographs of a negatively-stained DPPC liposome is shown before (A) and after (B) being microencapsulated by a shell of acacia and gelatin coacervate. It indicates that the material on the surface was not liposomal but presumably the acacia-gelatin polymer. Fig. 4.2 provides additional evidence from the ultrathin section technique that the multilamellar structure of the liposomes was retained after microencapsulation. Particle size data also suggest that microencapsulation has significantly increased the size of the liposomes at 3.0% and 5.0% acacia-gelatin concentration (F-test, $p=0.05$) and that this was due to the formation of the polymeric shell surrounding the liposomes (Table 4.1). At 5 percent acacia-

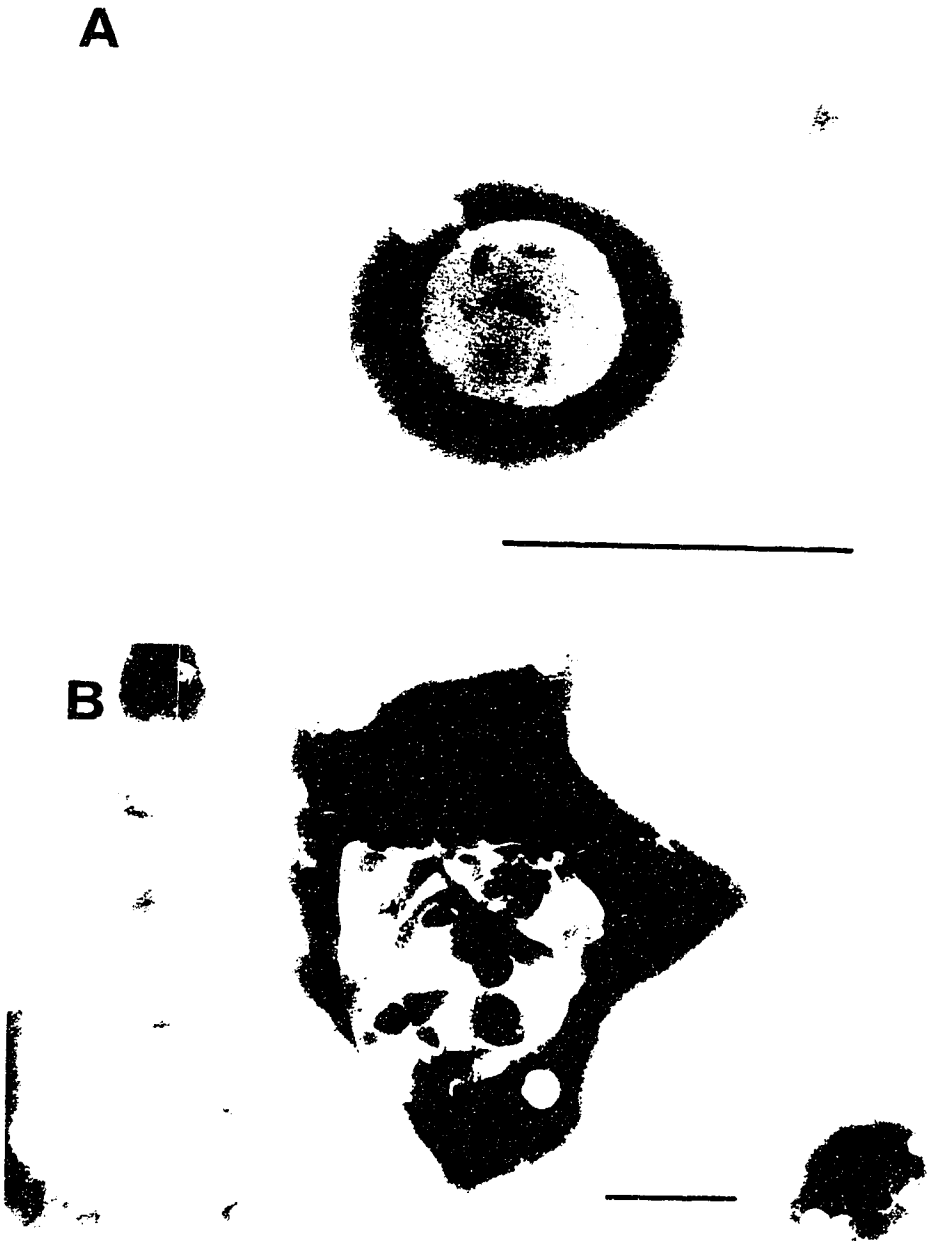


Fig. 4.1. Negative stain electron micrographs showing the microstructures of A) control liposomes; and B) 3% acacia-gelatin-microencapsulated DPPC liposomes. Bar length = 1 μm.

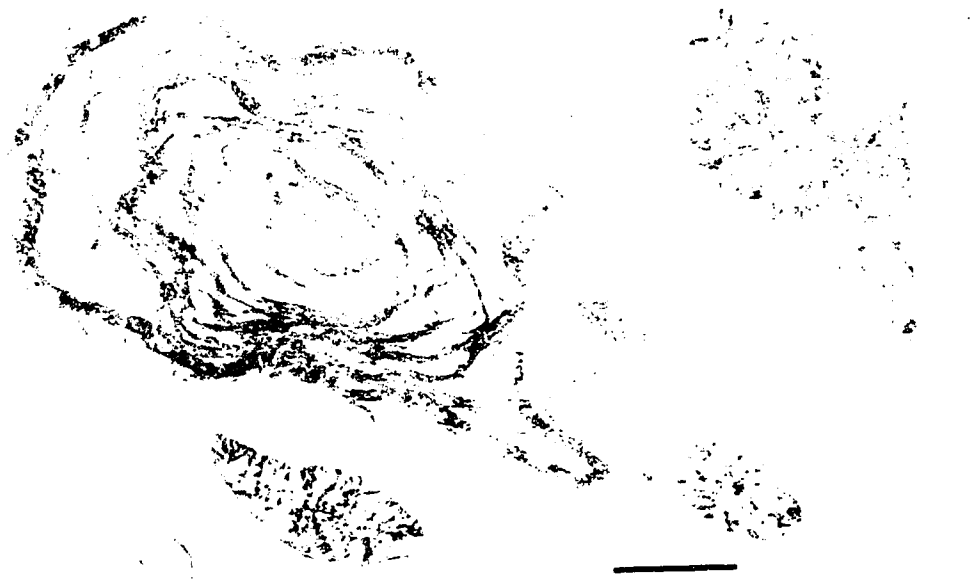


Fig.4.2. Electron micrograph of acacia-gelatin-microencapsulated DPPC liposomes by fixation and thin section technique. Bar length = 1 μm.

Table 4.1. Mean diameter, and size range of acacia-gelatin-microencapsulated liposomes as a function of acacia-gelatin concentration with a hardening time of 1 hr.

Acacia-gelatin Conc. (%)	Average diameter μm (SD)	Diameter range (μm, p=0.1)
0	2.76 (0.16)	1.25 ~ 4.69
1.5	2.96 (0.23)	0.92 ~ 5.74
3.0	3.53 (0.13)	1.08 ~ 6.86
5.0	3.50 (0.21)	0.97 ~ 6.98

gelatin, excess coacervate was removed by rinsing or it penetrated the liposome. Also, the particle sizes were not influenced by formaldehyde hardening times of 1 to 4 h.

4.3.2. Evaluation of Liposome Stability

As shown previously, and confirmed here, turbidity changes occurring in the liposome preparations are proportional to the number of liposomes, and in this case microencapsulated liposomes, after irradiation at 400 nm (*Regen et al., 1980*). The sodium cholate reduces the number of liposomes by solubilization without itself interfering with the turbidity measurement. Zero turbidity resulted after complete disruption of liposomes. The relative stabilities of microencapsulated and control liposomes at pH 5.6 and 7.0 are depicted in Fig. 4.3. The liposomes are shown to be fairly stable in sodium cholate solutions at pH 7.0, hence no difference could be seen between microencapsulated and control liposomes. In contrast, control liposomes rapidly disintegrated upon addition of cholate solutions at pH 5.6 whereas microencapsulated liposomes were significantly resistant (F-test, $p=0.05$), only about 30 percent reduction in the turbidity occurring with concentrations as high as 8 mM sodium cholate. At pH 7.0 negligible amounts of cholic acid exist ($pK_a = 5$) (*Roda et al., 1990*) compared to pH 5.6 (20 percent unionized) suggesting that the incidence of mixed micelle formation of phospholipid and cholate over this concentration range is attributed primarily to cholic acid levels in the system (*Nagata et al., 1990*). The detergent

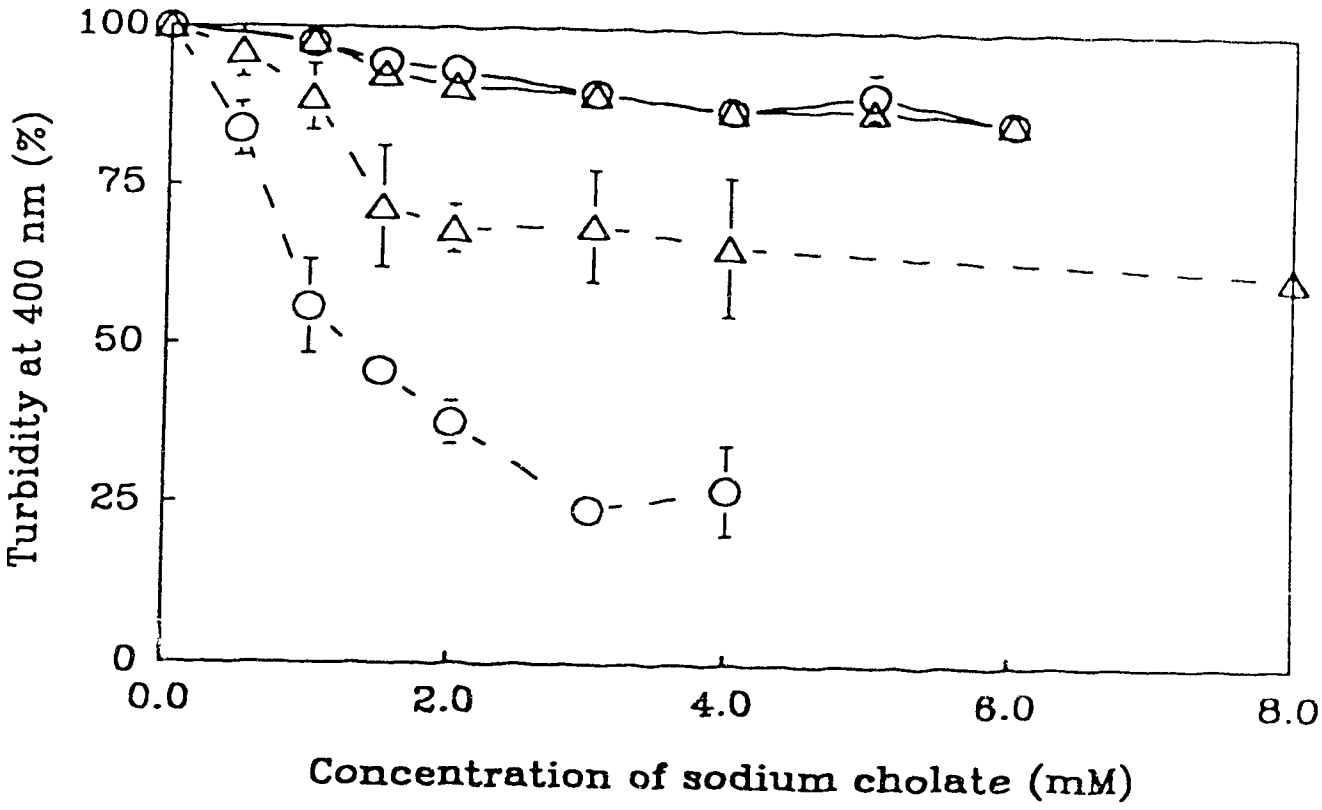


Fig. 4.3. Physical stability of liposomes at 23°C as a function of pH. ○, control liposomes; Δ, 3.0% acacia-gelatin-microencapsulated DPPC liposomes at pH 7.0 (—), and pH 5.6 (---).

activity of sodium cholate has been reported to be greater at pH 5.6 due to protonation, which can be near the pH in the duodenum where bile salts are excreted into the GIT (*Notari, 1987*), than at pH 7.0 (*Nagata et al., 1988 & 1990*). Hence, microencapsulated liposomes could have a distinct advantage in this situation.

When the concentration of acacia-gelatin is increased the coating thickness and, hence, the stability in sodium cholate solution might be expected to increase accordingly. Fig. 4.4 shows these results at pH 5.6 and 23°C. Not unexpectedly, there exists a fairly narrow range of concentration over which microencapsulation is able to occur and the wall thickness reaches a maximum. Thus, at 1.5 percent acacia-gelatin no increase in stability of the liposomes was found, indicating the presence of insufficient polymer coacervate to result in the formation of a uniform layer around the liposomes. At the other extreme, 5 percent acacia-gelatin at a hardening time of 1 h yielded microcapsule wall properties that were more susceptible to cholate than at 3 percent which yielded the greatest stability. Since the particle size also did not increase at 5 percent it appears that the excess polymer penetrated the liposomal bilayers causing a reduction in the overall integrity of the liposomes leading to a lower energy requirement of the cholate to mix with the phospholipid molecules and cause partial solubilization, *i.e.* reduction in turbidity compared to liposomes microencapsulated with 3 percent acacia-gelatin. A similar observation has been made previously with other polysaccharide-coated liposomes (*O'Connor et al., 1985*). Another factor affecting

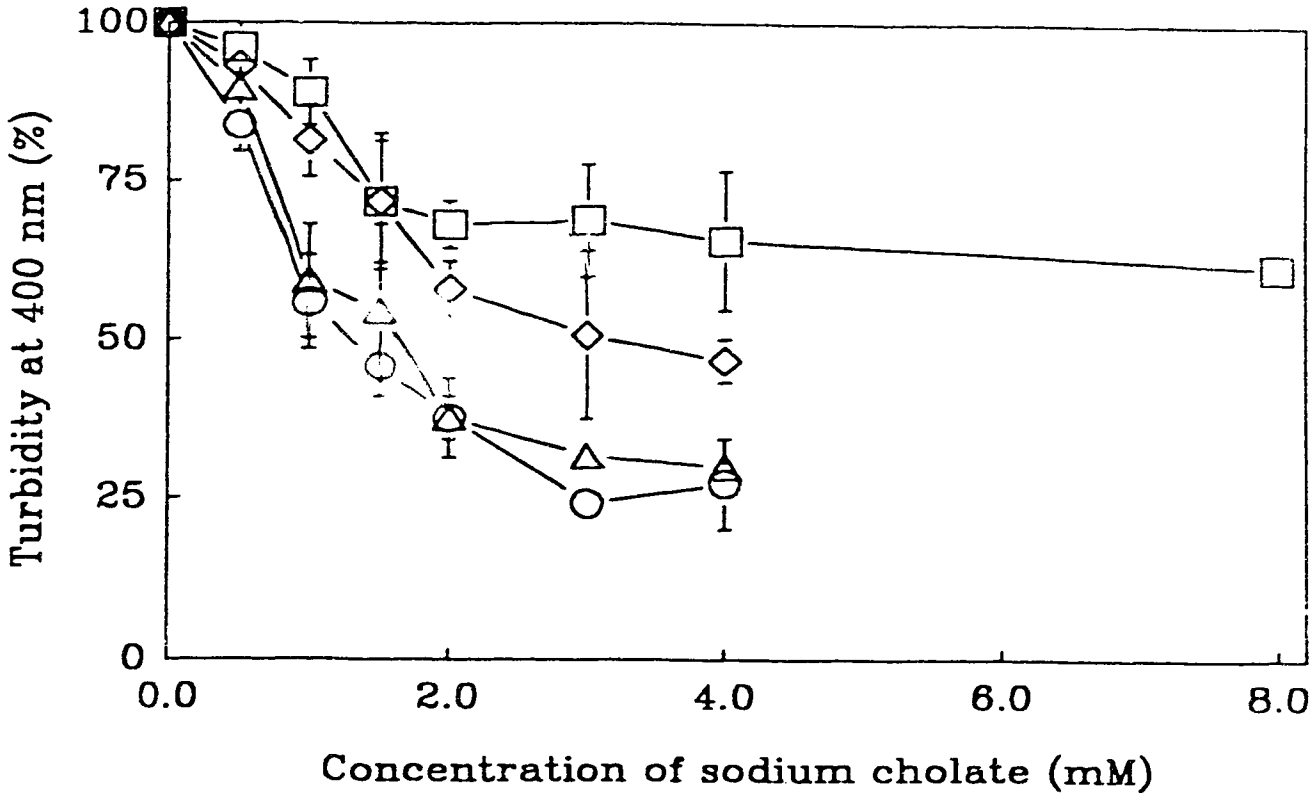


Fig. 4.4. The effect of acacia-gelatin concentration on the physical stability of microencapsulated DPPC liposomes in pH 5.6 acetate buffer at 23°C. ○ , control liposomes; microencapsulated liposomes with an acacia-gelatin concentration of, Δ, 1.5%; □ , 3.0%; and ◇ , 5.0%.

the formation and coherent structure of the microcapsule wall is the formaldehyde hardening time. While it might be expected that a minimum hardening time would be required it is apparent in Fig. 4.5 that 1 h was sufficient before harvesting the microcapsules and 2 h made no significant difference. On the other hand, there appeared to be a deterioration in the microcapsule wall if it was left to harden for 4 h since the turbidity decreased significantly at 3 mM cholate compared to 1 or 2 h hardening times. Although the reason for this is unclear the prolonged presence of the formaldehyde may have influenced the integrity of the microcapsule wall through denaturation and cracking (*Deasy, 1984*).

4.3.3. Effect of ASA Release

The encapsulation efficiency of ASA in liposomes was determined to be $8.3 \pm 1.3\%$ ($n = 3$) and was unchanged after the microencapsulation process. The influence of acacia-gelatin concentration and temperature on the release of ASA from microencapsulated liposomes is shown in Figures 4.6 and 4.7. There is a dramatic difference in the amount of ASA released within the first 20 min at room temperature and 37°C, less than 5 percent and approximately 90 percent, respectively. Nevertheless, concentrations of acacia-gelatin reduced this happening. Even at 1.5 percent acacia-gelatin some reduction in the release of ASA was observed, although at this concentration it had been shown that no increase in particle size or decrease in stability of the liposomes occurred. However, at 3.0 or 5.0 percent the initial release of ASA was diminished the most

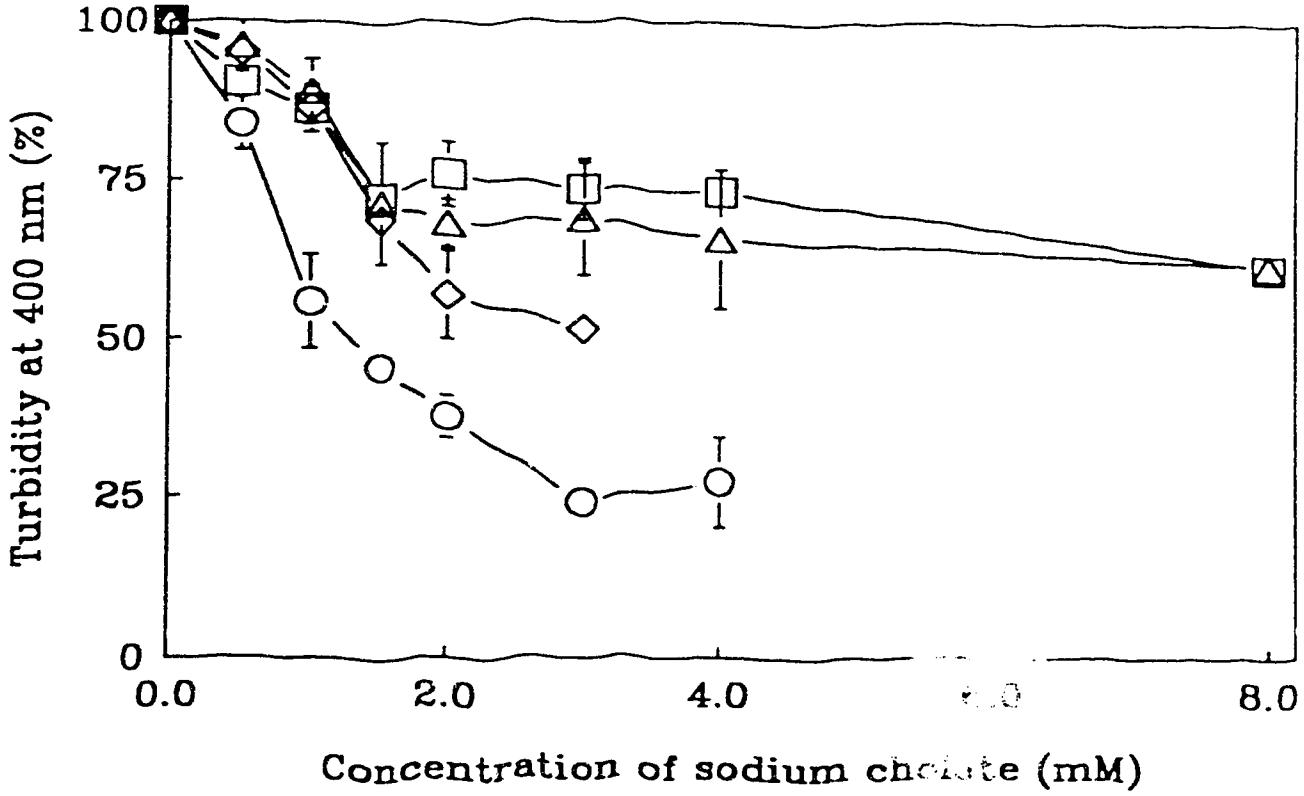


Fig. 4.5. The effect of formaldehyde hardening time on the physical stability of acacia-gelatin-microencapsulated DPPC liposomes in pH 5.6 acetate buffer at 23°C. ○, control liposomes; 3.0% acacia-gelatin-microencapsulated liposomes with a hardening time of, Δ, 1 hr; □, 2 hr; and ◇, 4 hr.

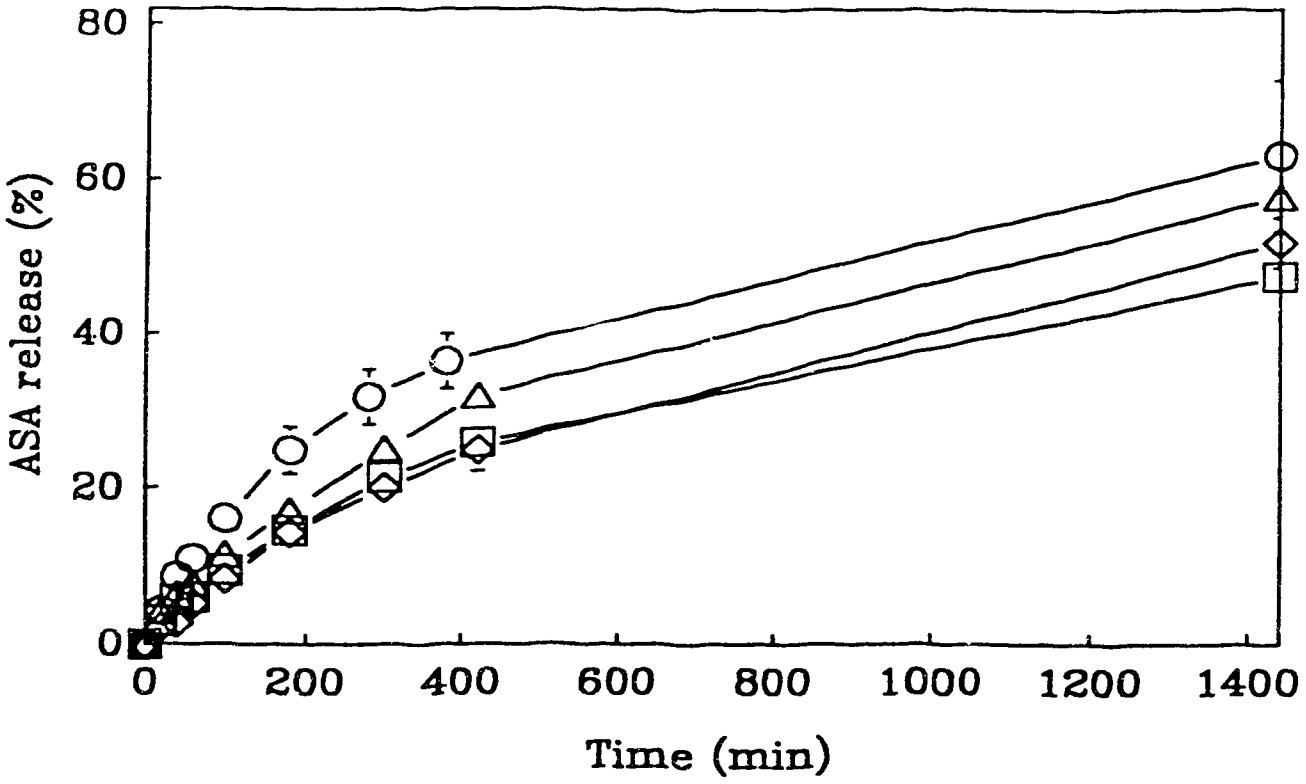


Fig.4.6. The effect of acacia-gelatin concentration on the release of ASA from microencapsulated DPPC liposomes in pH 5.6 acetate buffer at 23°C. ○, control liposomes; microencapsulated liposomes with an acacia-gelatin concentration of, Δ, 1.5%; □ , 3.0%; and ◇ , 5.0%.

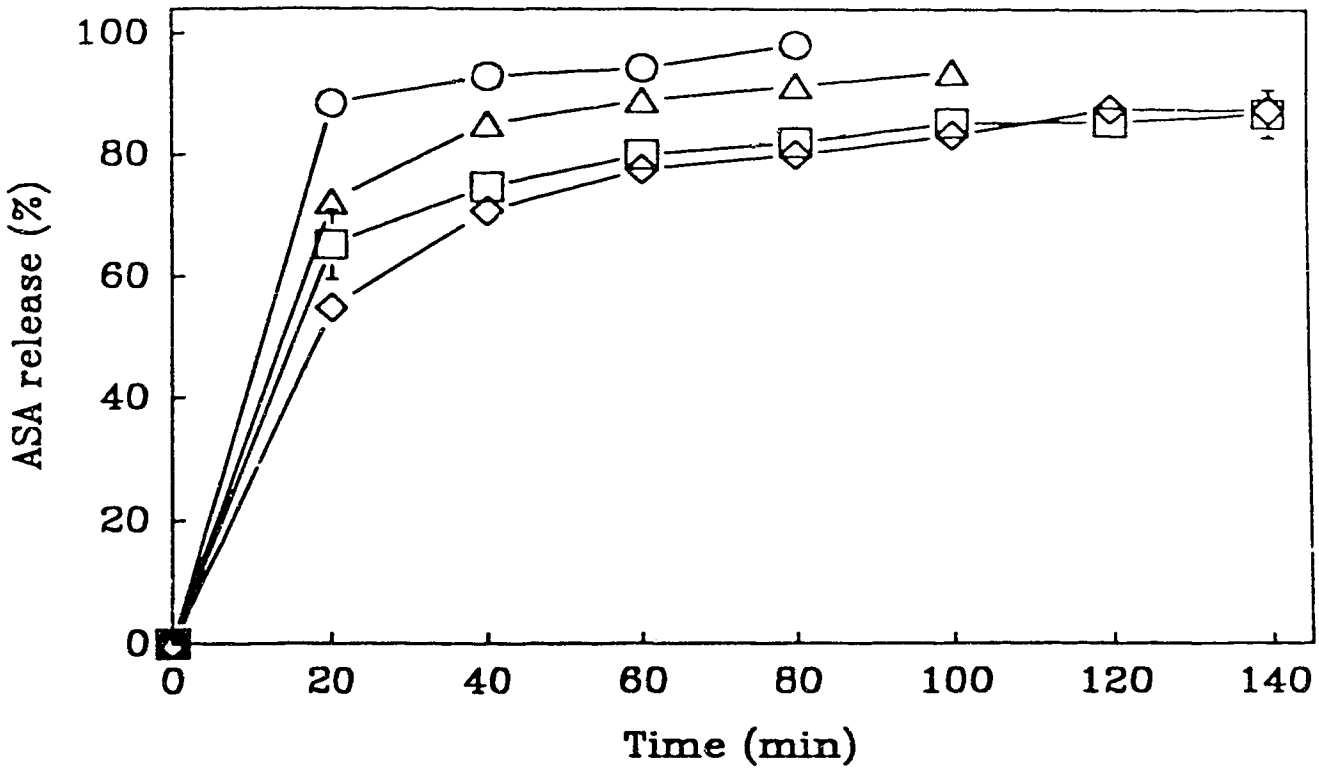


Fig. 4.7. The effect of acacia-gelatin concentration on the release of ASA from microencapsulated DPPC liposomes in pH 5.6 acetate buffer at 37°C. ○ , control liposomes; microencapsulated liposomes with an acacia-gelatin concentration of, Δ, 1.5%; □ , 3.0%; and ◇ , 5.0%.

and was sustained with kinetics which were approximately equal for all formulations, including the control liposomes (F-test, p=0.05). These results suggest a matrix-type release at 23°C and Fig. 4.8 confirms adherence to the equation (*Higuchi, 1961*),

$$Q = K\, t^{1/2}$$

where the amount of ASA released, Q, is a function of $t^{1/2}$ and the linear relationship of Q versus $t^{1/2}$ has a correlation coefficient, r, of 0.987. Although similar kinetics are followed at 37°C, the release is dominated by a rapid phase in the first 20 min, probably due to slight osmotic effects due to the encapsulated ASA but mainly due to the change in temperature from R.T. to 37°C. Compared to control liposomes, the slower release of ASA from microencapsulated liposomes is due to the necessity of diffusion through two barriers, the liposomal bilayers then the microcapsule wall. This mechanism has even been suggested as a means of delivering a drug by pulsed release (*Kibat et al., 1990*). A hardening time in excess of 2 h was also found to be detrimental for sustained release of ASA (Fig. 4.9) consistent with the decreased stabilities found for microcapsules subjected to extended hardening times.

4.4. Summary

The microencapsulation of DPPC liposomes using acacia-gelatin and a coacervation technique yielded particles ranging in size from about 1 μm to 7 μm in diameter. The stability of the microcapsules in sodium cholate solutions was

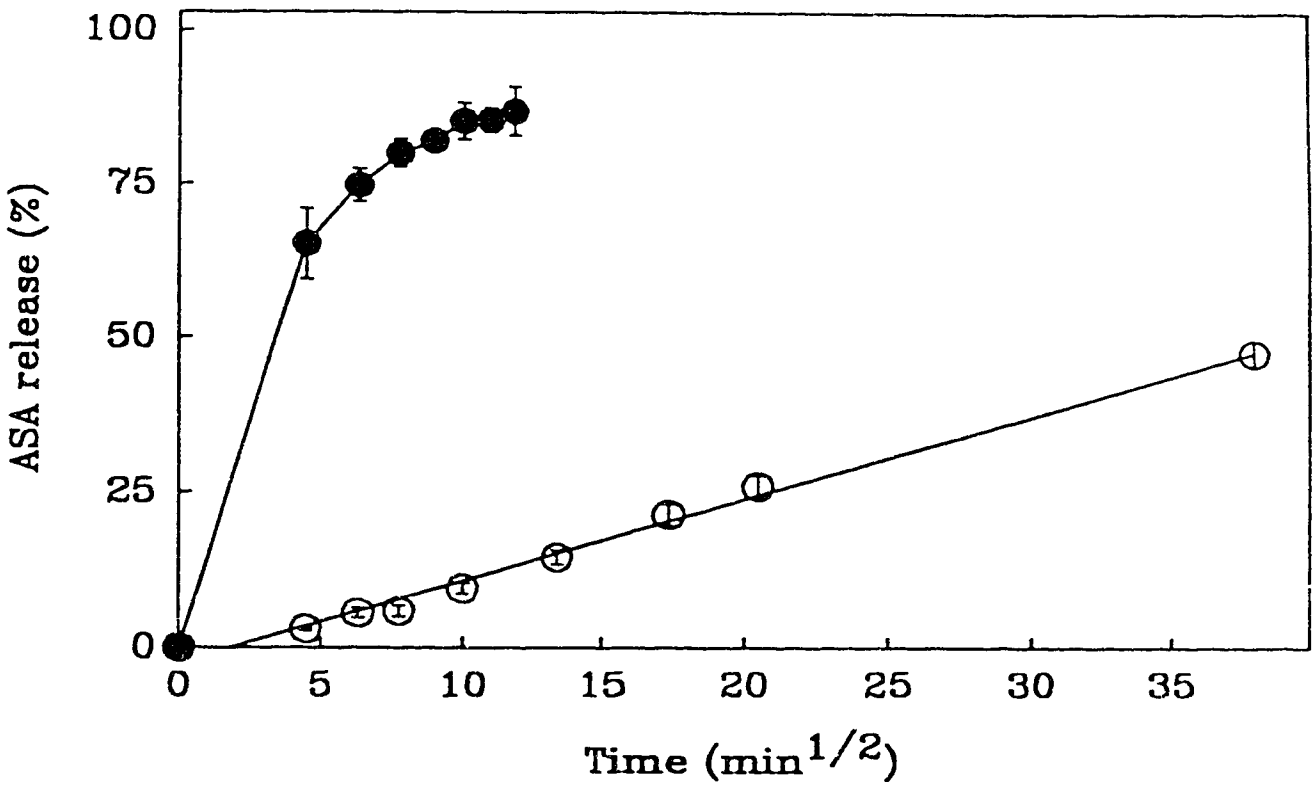


Fig. 4.8. Release of ASA as a function of $t^{1/2}$ from 3% acacia-gelatin-microencapsulated DPPC liposomes at 23°C, ○ , and 37°C, ● at pH 5.6.

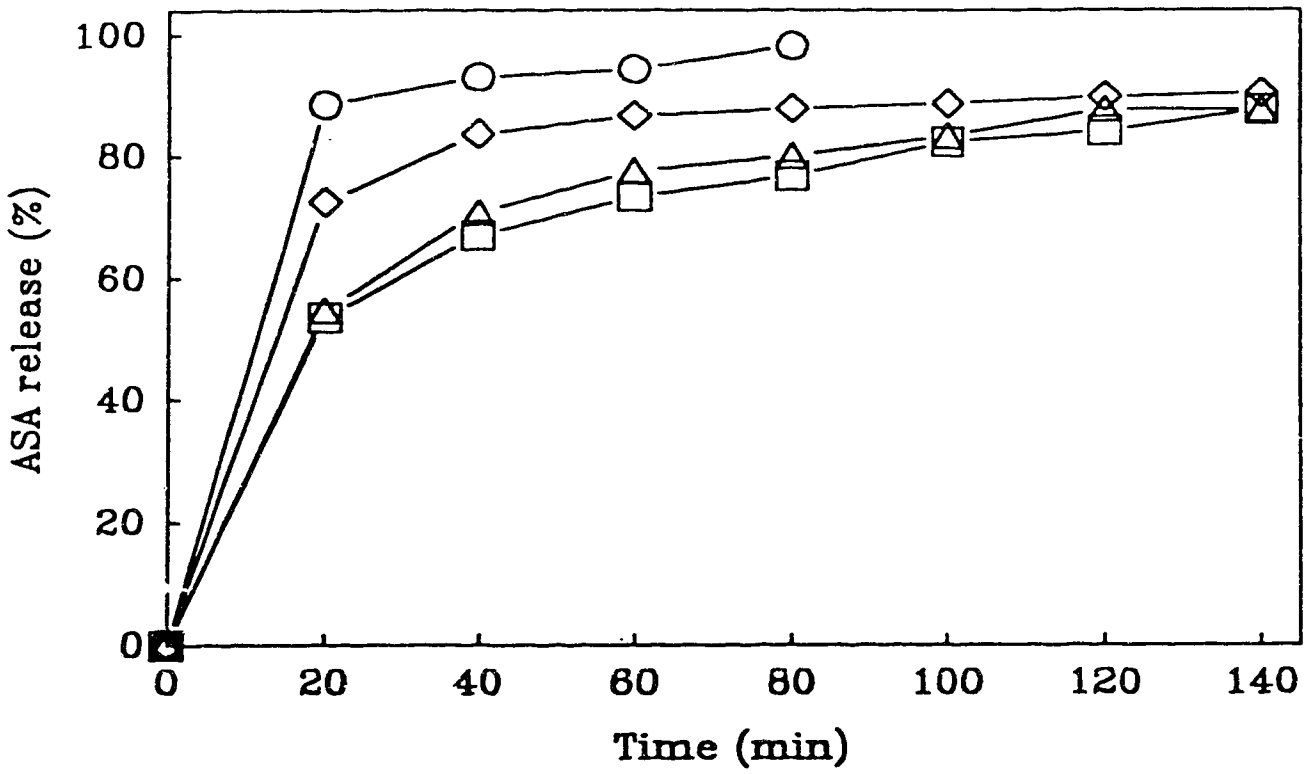


Fig. 4.9. The effect of formaldehyde hardening time on the release of ASA from acacia-gelatin-microencapsulated DPPC liposomes in pH 5.6 acetate buffer at 37°C. ○ , control liposomes; 3.0% acacia-gelatin-microencapsulated liposomes with a hardening time of, Δ , 1 hr; □, 2 hr; and ◇ , 4 hr.

much greater than the corresponding liposomes. The encapsulation efficiency of ASA was unaltered by the microencapsulation process. Optimum composition and conditions for stability as well as release of ASA were 3.0 percent acacia-gelatin and 1 h of hardening time with formaldehyde. The kinetics of ASA release conformed to a model of transport across two diffusion barriers, namely, the lipid bilayers and the microcapsule wall of which the rate-determining step after the initial release phase was diffusion from the liposomes. A marked increase in the amount released at 37°C (cf. 23°C) but without change in the release pattern substantiates this conclusion. These properties of microencapsulated liposomes suggest their possible use as oral drug delivery systems.

4.5. References

Deasy, P. B. (1984). Coacervation-phase separation procedures using aqueous vehicles, in Microencapsulation and Related Drug Processes, Marcel Dekker, Inc., New York, pp. 61-95.

Fukuda, H., Diem, T., Stefely, J., Kezdy, F. J., and Regen, S. L. (1986). Polymer-encased vesicles derived from dioctadecyldimethylammonium methacrylate, J. Am. Chem. Soc., 108, 2321-2327.

Higuchi, T. (1961). Rate of release of medicaments from ointment bases containing drugs in suspension, J. Pharm. Sci., 50, 874-875.

Kibat, P. G., Igari, Y., Wheatley, M. A., Eisen, H. N., and Langer, R. (1990). Enzymatically activated microencapsualted liposomes can provide pulsatile drug release, FASEB J., 4, 2533-2539.

Nagata, M., Yotsuyanagi, T., and Ikeda, K. (1988). A two-step model of disintegration kinetics of liposomes in bile salts, Chem. Pharm. Bull., 36, 1508-1513.

Nagata, M., Yotsuyanagi, T., and Ikeda, K. (1990). Bile salt-induced disintegration of egg phosphatidylcholine liposomes: A kinetic study based on turbidity changes, Chem. Pharm. Bull., 38, 1341-1344.

Nixon, J. R.; Yeung, V. W. (1989). Preparation of microencapsulated liposomes, II. Systems containing nylon-gelatin-acacia walling material, J. Microencapsulation, 6, 43-52.

Notari, R. E. (1987). Biopharmaceutics and Clinical Pharmacokinetics, Chapter 5, Marcel Dekker, Inc., New York, pp. 130-220.

O'Connor, C. J., Wallace, R. G., Iwamoto, K., Taguchi, T., and Sunamoto, J. (1985). Bile salt damage of egg phosphatidylcholine liposomes, Biochim. Biophys. Acta, 817, 95-102.

Özden, M. Y., and Hasirci, V. N. (1990). Enzyme immobilization in polymer coated liposomes, Brit. Polym. J., 23, 229-234.

Regen, S. L., Czech, B., Singh, A. (1980). Polymerized vesicles, J. Am. Chem. Soc., 102, 6638-6640.

Regen, S. L. (1987). Polymerized liposomes. in M. J. Ostro (ed.), Liposomes: From Biophysics to Therapeutics, Marcel Dekker, Inc., New York, pp.73-108.

Ringsdorf, H., and Schlarb, B. (1988). Preparation and characterization of unsymmetrical "liposomes in a net", Markromol. Chem., 189, 299-315.

Roda, A., Minutello, A., Angellotti, M. A., and Fini, A. (1990). Bile acid structure-activity relationship: Evaluation of bile acid lipophilicity using 1-octanol/water partition coefficient and reverse phase HPLC, J. Lipid Res., 31, 1433-1443.

Sunamoto, J., Iwamoto, K., Takada, M., Yuzuriha, T., and Katayama, K. (1983). Improved drug delivery to target specific organ using liposomes as coated with polysaccharides, Polym. Sci. Tech., 23, 157-168.

Tirrell, D. A., Takigawa, D. Y., and Seki, K. (1985). pH sensitization of phospholipid vesicles via complexation with synthetic poly(carboxylic acid)s, Ann. N. Y. Acad. Sci., 446, 237-247.

Yeung, V. W., and Nixon, J. R. (1988). Preparation of microencapsulated liposomes, J. Microencapsulation, 5, 331-337.

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

5.1. General Discussion

Liposomes have been extensively studied as drug delivery systems by different routes of administration, such as intravenous (*Alving et al., 1978; New et al., 1978; Gabizon et al., 1989*), intrapulmonary (*Mihalko et al., 1988*), intraperitoneal (*Markman et al., 1986*), intramuscular (*Eppstein and Felgner, 1988*), subcutaneous (*Kim and Howell, 1987*), ocular (*Guo et al., 1988*), topical (*Mezei, 1988*), and oral (*Patel et al., 1982*). The oral route is the most convenient, versatile, and patient acceptable. However, this route has not been successful because of liposome instability in the GIT, especially due to the detergent action of bile salts. Many attempts have been made to overcome these problems, but little progress has been made so far.

In the early stages of development, improvements in liposome stability concentrated on alterations of liposome composition. Liposomes of DSPC:CHOL (2:1) which were believed to be the most stable were unable to survive in the GIT (*Chiang, 1986*). An alternative method was to use modified liposomes such as polymerized liposomes (*Regen, 1987*) or polymer-coated liposomes (*Sunamoto et al., 1987*). Both approaches have been shown to stabilize liposomes. In comparison, polymerized liposomes significantly changed the bilayer characteristics of liposomes, whereas polymer-coated liposomes appeared to retain their bilayer properties similar

to uncoated liposomes, but confirmation of this has not been made. The objectives of this study were to develop polymer-coated liposome systems, test their stabilities against the detergent effect of bile salt solutions, and quantitate the kinetics of release of entrapped solute in order to increase the possibilities of developing effective liposomes for oral administration.

In principle, liposomes can be coated with polymers via hydrophilic adsorption (*Seki and Tirrell, 1984*), ionic forces (salt formation) (*Aliev et al., 1984*), or due to penetration of the bilayers by hydrophobic anchors attached to the polymer (*Sunamoto et al., 1987*). In practice, one or more mechanisms can be operative for a particular polymer. The application of poly(4-VP) (*Aliev et al., 1984*) and CM-chitin (*Izawa et al., 1986*) to coat liposomes and a coacervate of nylon-gelatin-acacia (*Nixon and Yeung, 1989*) to microencapsulate liposomes have been reported. However, none of these have been developed as oral drug delivery systems.

Perhaps degradation of ASA was important, and the stability of ASA was not the objectives of this thesis, because this has been dealt by others (*Habib, 1987*).

In the first instance, the ability to prepare poly(4-VP)-coated liposomes of DMPC and containing varying amounts of the negatively-charged amphiphile, DCP, was investigated. This was in contrast to the use of only DCP liposomes used by Aliev et al. (1984) to demonstrate the principle. Furthermore, the effects of polymerization on the encapsulation of ASA as a water-soluble solute, and on the resultant ASA release kinetics were explored. The outcome of these studies were very encouraging. Not only could poly(4-VP)-coated liposomes be prepared with an optimum DMPC:DCP, 1:1 mole ratio, but it was shown by TEM that the lamellar

structure of the liposomes was retained and that the coating process had no influence on the encapsulation efficiency of ASA. The release kinetics further indicated that poly(4-VP)-coated liposomes released a smaller fraction of ASA during the initial rapid phase than the corresponding uncoated liposomes. The appearent slow release during the second phase was similar for the coated and uncoated liposomes which meant that the period over which ASA could be released was extended when using the poly(4-VP)-coated liposomes. Although the potential toxicity of poly(4-VP) (even after total removal of residual 4-VP monomer) is unknown and has not been tested, the principle of formulating a stable, sustained release phospholipid liposome by coating with a polymer was demonstrated. Most importantly, the stability of poly(4-VP)-coated liposomes was found to be considerably greater in sodium cholate solutions than the uncoated liposomes.

Another approach was to determine the extent to which a polysaccharide could stabilize liposomes and alter the release kinetics of ASA. CM-chitin chosen for this purpose was incorporated in two ways - addition to preformed liposomes or addition via the aqueous phase during liposome preparation, *i.e.* during hydration of the lipid film. The coating efficiency of DPPC liposomes varied with CM-chitin concentration but was optimum at 1 percent. Regardless of which procedure was used in preparing the polymer-coated liposomes they were spherical and appeared as uniform spheres by optical microscopic inspection indicating that polymer resided at the surfaces of the liposomes. Again, it was demonstrated that the polymer coating process did not influence the encapsulation efficiency of ASA. The release kinetics further indicated that CM-chitin-coated liposomes reduced the release rate of entrapped ASA compared

to uncoated liposomes, the magnitude of which was a function of the amount of polymer added. In comparison to poly(4-VP)-coated liposomes, the decreased release was more pronounced. Similar to poly(4-VP)-coated liposomes, the physical stability of CM-chitin-coated DPPC liposomes was greater than uncoated liposomes by several orders of magnitude at the optimum 1% CM-chitin concentration added during liposome formation. At a lower CM-chitin concentration (0.5%), the stability against solubilization by detergent was less, presumbly because of a thinner polymer coating at the surfaces, although this could not be determined directly. At a 2 percent CM-chitin concentration, the stability was not any better than at 1 percent indicating a limit in the properties of the polymer film resistant to the action of the bile salt. Furthermore, *O'Connor et al. (1985)* have shown that linear polysaccharide-coated liposomes, which includes CM-chitin, were more stable in serum than branched polysacchride-coated liposomes. It was shown that turbidity measurements, as an indication of the degree of liposome solubilization, could not only be used to provide stability information but also be applied in the quantitation of the CM-chitin associated with the liposomes.

A slightly different approach in preparing a polymer-coated liposome was to employ a microencapsulation method which did not involve organic solvent as is usually the case using this technique. This was also in contrast to the microencapsulated liposomes prepared by Nixon (*Nixon and Yeung, 1989; Yeung and Nixon, 1988*) from an emulsion of cyclohexane/chloroform in aqueous liposome suspension. *Kabat et al. (1990)* prepared microencapsulated liposomes in which each microcapsule contained multiple liposomes whereas close examination of the acacia-

gelatin microcapsules by TEM in this study revealed individually microencapsulated liposomes of which the lamellar bilayer structure was preserved. As with poly(4-VP)- or CM-chitin-coated liposomes, the microencapsulation process did not alter the encapsulation efficiency and the microencapsulated liposomes released ASA at a slower rate than uncoated liposomes. These systems possessed stability and release rate characteristics intermediate between poly(4-VP)- and CM-chitin-coated liposomes although more similar to the latter systems.

Thus, three polymers have been successfully used to coat/microencapsulate on the surface of the liposomes without any apparent alteration of the bilayer integrity at the optimum concentrations or changing the ASA encapsulation efficiency. Furthermore, all polymer-coated or microencapsulated liposomes decreased ASA release by different magnitudes and the decrease was in the order of CM-chitin-coated > acacia-gelatin-microencapsulated > poly(4-VP)-coated liposomes. This indicates that different release rates can be achieved by selection of different polymer coatings and changes in polymer concentrations. It can also be predicted that different hydrophobicity of the drug as well as its molecular weight will also significantly influence drug release. One might eventually envision formulations which control drug release at pre-determined desired rates. Most importantly, the stability of polymer-coated/microencapsulated liposomes were demonstrated to be considerably more stable in sodium cholate solutions than control liposomes. It is anticipated that these stabilized liposomes can protect liposomes from destruction in the GIT when given orally. The appropriate level of stability can be achieved again by selection of suitable polymer systems. This characteristic plus the controlled release of drug are

the basic requirements to be achieved by polymer coating or microencapsualtion of liposomes intended for oral administration.

Potential applications could include macromolecules such as insulin or other proteins, hormones, polypeptides in which oral administration of polymer-coated/microencapsulated liposomes would result in an increased bioavailability by protecting the drug from the destructive elements of the GI fluid, controlling the drug release rates and possibly facilitating drug absorption. Approaches to the latter might involve increasing the residence times of formulations by virtue of a mucoadhesive or bioadhesive characteristic of the surfaces of polymer-coated liposomes. This may also entail attachment of a site specific determinant to the liposome surfaces such as tomato lectin, which has been proposed as a binding ligand to intestinal mucosa (*Woodley and Naisbett, 1988; Naisbett and Woodley, 1989*).

5.2. Conclusions

1. Liposomes can be coated with poly(4-VP) or CM-chitin and microencapsulated with acacia-gelatin. The coating procedures neither alter the integrity of liposomes nor influence the ASA encapsulation. The optimum concentrations for polymer coatings were 8.5 mM 4-VP, 1% CM-chitin, and 3% acacia-gelatin.
2. ASA release from poly(4-VP)-coated liposomes was significantly reduced compared with control liposomes. However, the reduction in extent of release of ASA was most pronounced from CM-chitin-coated liposomes followed by acacia-gelatin-microencapsulated liposomes.

3. The physical stabilities of polymer-coated liposomes in sodium cholate solutions were greatly increased compared to control liposomes. Again, CM-chitin-coated liposomes were found to be the most stable formulations followed by acacia-gelatin-microencapsulated liposomes then poly(4-VP)-coated liposomes.

4. Two of the most important properties of an oral liposome drug delivery system has been shown to exist by employing selective polymers to coat or encapsulate the liposomes. These are increased stability in a typical bile salt solution and slower release of entrapped ASA, representing a small water-soluble permeant, compared to the corresponding uncoated liposomes. Further testing both *in vitro* and *in vivo* is therefore warranted.

5.3. Future Work

Future work could involve improvements in the poly(4-VP) type coated liposomes. For example, other vinylpyridinium analogues such as 1,2-dimethyl-5-vinylpyridinium methyl sulfate could be used (*Kabanov et al., 1968*). Alternately, other positively-charged monomers, such as choline methacrylate, bis(2-methacryloyloxyethyl)dimethylammonium bromide (*Ringsdorf and Schlarb, 1988*) could be used instead of a vinylpyridium monomer. More recently, it has been suggested that polysaccharides such as pullulan, mannan, dextran or amylopectin derivatized with cholesterol to anchor the polysaccharide to the liposomal surfaces might yield liposomes of improved stability and functionality. These have the advantages of low toxicity, biodegradability and low cost. However, ultimately, a

better understanding of the mechanisms involved and requirements for particulate delivery of drug across the GIT is required in order to achieve the successes warranted by these novel approaches to improved drug therapies by the oral route.

5.4. References

Aliev, K. V., Ringsdorf, H. and Schlarb, B. (1984). Liposomes in a net: Spontaneous polymerization of 4-vinylpyridine on acidic liposomal surfaces, Makromol. Chem., Rapid Commun., 5, 345-352.

Alving, C. R., Steck, E. A., Chapman, Jr., W. L., Waits, W. B., Hendrick, L. D., Swartz, Jr., G. M., and Hanson, W. L. (1978). Therapy of leishmaniasis: Superior efficiencies of liposome-encapsulated drugs, Proc. Natl. Acad. Sci. USA, 75, 2959-2963.

Chiang, C. (1986). Liposomes as a potential oral drug delivery system, Ph.D. thesis in University of Michigan, Ann Arbor, MI, U.S.A.

Eppstein, D. A., and Felgner, P. L. (1988). Applications of liposome formulations for antimicrobial/antiviral therapy, in Liposomes as Drug Carriers: Recent Trends and Progress, (G. Gregoriadis, ed.), John Wiley and Sons, Chichester, pp. 311-323.

Gabizon, A., Sulkes, A., Peretz, T., Druckmann, S., Goren, D., Amselem, S., and Barenholz, Y. (1989). Liposome-associated doxorubicin: Preclinical pharmacology and exploratory clinical phase, in Liposomes in the Therapy of Infectious Diseases and Cancer, (G. Lopez-Berestein and I. J. Fidler, ed.), Alan R. Liss, Inc., New York, pp. 391-402.

Guo, L. S. S., Sarris, A. M., and Levy, M. D. (1988). A safe bioadhesive liposomal formulation for ophthalmic applications, Invest. Ophthalmol. Visual. Sci. (Suppl.), 29, 439.

Habib, M. J. (1987). The stabilization of drugs in liposomes: theory and practice, Ph.D. in University of Alberta, Edmonton, Alberta, Canada.

Izawa, H., Arakawa, M., and Kondo, T. (1986). Disintegration by surfactants of egg yolk phosphatidylcholine vesicles stabilized with carboxymethylchitin, Biochim. Biophys. Acta, 855, 243-249.

Kabanov, V. A., Patrikeeva, T. I., Kargina, O. V., and Kargin, V. A. (1968). Organized polymerization of vinyl pyridinium salts, J. Polym. Sci. Part C, 23, 357-363.

Kibat, P., Igari, Y., Wheatley, M. A., Eisen, H. N., and Langer, R. (1990). Enzymatically activated microencapsulated liposomes can provide pulsatile drug release, FASEB J., 4, 2533-2539.

Kim, S., Kim, D. J., and Howell, S. B. (1987). Multivesicular liposomes containing cytarabine for slow release s.c. administration, Cancer Treatm. Rep., 71, 447-450.

Markman, M., Cleary, S., Lucas, W., Weiss, R., and Howell, S. B. (1986). Ip chemotherapy employing a regimen of cisplatin, cytarabine, and bleomycin, Cancer Treatm. Rep., 70, 755-760.

Mezei, M (1988). Liposomes in the topical application of drugs: A review, in Liposomes as Drug Carriers: Recent Trends and Progress, (G. Gregoriadis, ed.), John Wiley and Sons, Chichester, New York, pp. 663-677.

Mihalko, P. J., Schreier, H., and Abra, R. M. (1988). Liposomes: A pulmonary perspective, in Liposomes as Drug Carriers: Recent Trends and Progress, (G. Gregoriadis, ed.), John Wiley and Sons, New York, pp. 679-694.

Naisbett, B., and Woodley, J. (1989). Uptake of tomato lectin by the adult rat small intestine *in vitro*, Biochem. Soc. Trans., 17, 883.

New, R. R. C., Chance, M. L., Thomas, S. C., and Peters, W. (1978). Antileishmanial activity and antimonials entrapped in liposomes, Nature, 272, 55-56.

Nixon, J. R., and Yeung, V. W. (1989). Preparation of microencapsulated liposomes, II. Systems containing nylon-gelatin and nylon-gelatin-acacia walling material, J. Microencapsulation, 6, 43-52.

O'Connor, C. J., Wallace, R. G., Iwamoto, K., Taguchi, T., and Sunamoto, J. (1985). Bile salt damage of egg phosphatidylcholine liposomes, Biochim. Biophys. Acta, 817, 95-102.

Patel, H. M., Stevensen, R. W., and Parsons, J. A. (1982). Use of liposomes to aid intestinal absorption of entrapped insulin in normal and diabetic dogs, Biochim. Biophys. Acta, 716, 188-193.

Regen, S. L. (1987). Polymerized liposomes, in Liposomes from Biophysics to Therapeutics, (M. J. Ostro ed.), Marcel Dekker, Inc., New York, pp. 73-108.

Rinsdorf, H., and Schlarb, B. (1988). Preparation and characterization of unsymmetrical "liposomes in a net", Makromol. Chem., 189, 299-315.

Seki, K., and Tirrell, D. A. (1984). pH-dependent complexation of poly(acrylic acid) derivatives with phospholipid vesicle membranes. Macromolecules, 17, 1692-1698.

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

Woodley, J. F., and Naisbett, B. (1988). The potential of lectins for delaying the intestinal transit of drugs, Proceed. Intern. Symp. Control. Rel. Bioact. Mater., 15, 125-126.

Yeung, V. W., and Nixon, J. R. (1988). Preparation of microencapsulated liposomes, J. Microencapsulation, 5, 331-337.

APPENDIX

CALCULATION OF IONIZATIONS OF 4-VP AND DCP

The pK_a of an organic base can be quantitatively calculated by the Hammet equation (*Perrin, 1980*):

$$\log(k/k^o) = (\Sigma\sigma)\,\rho \qquad (1)$$

Here k and k^o are the ionization constants for the substituted and the unsubstituted compounds, respectively. σ is a constant for the particular substituent and ρ is constant for a particular equilibrium reaction. From (1),

$$pK_a = pK_a^\circ - (\Sigma\sigma)\,\rho \qquad (2)$$

The pK_a° of pyridine is 5.25, ρ is 5.9, and σ is -0.08 (*Clark and Perrin, 1964; Perrin, 1980*). Hence, for 4-VP

$$pK_a = 5.25 - (-0.08) \times 5.9 = 5.72$$

The pK_a of DCP has been reported to be 4.5 (*Maitani et al., 1990*), which was obtained by a ζ-potential method.

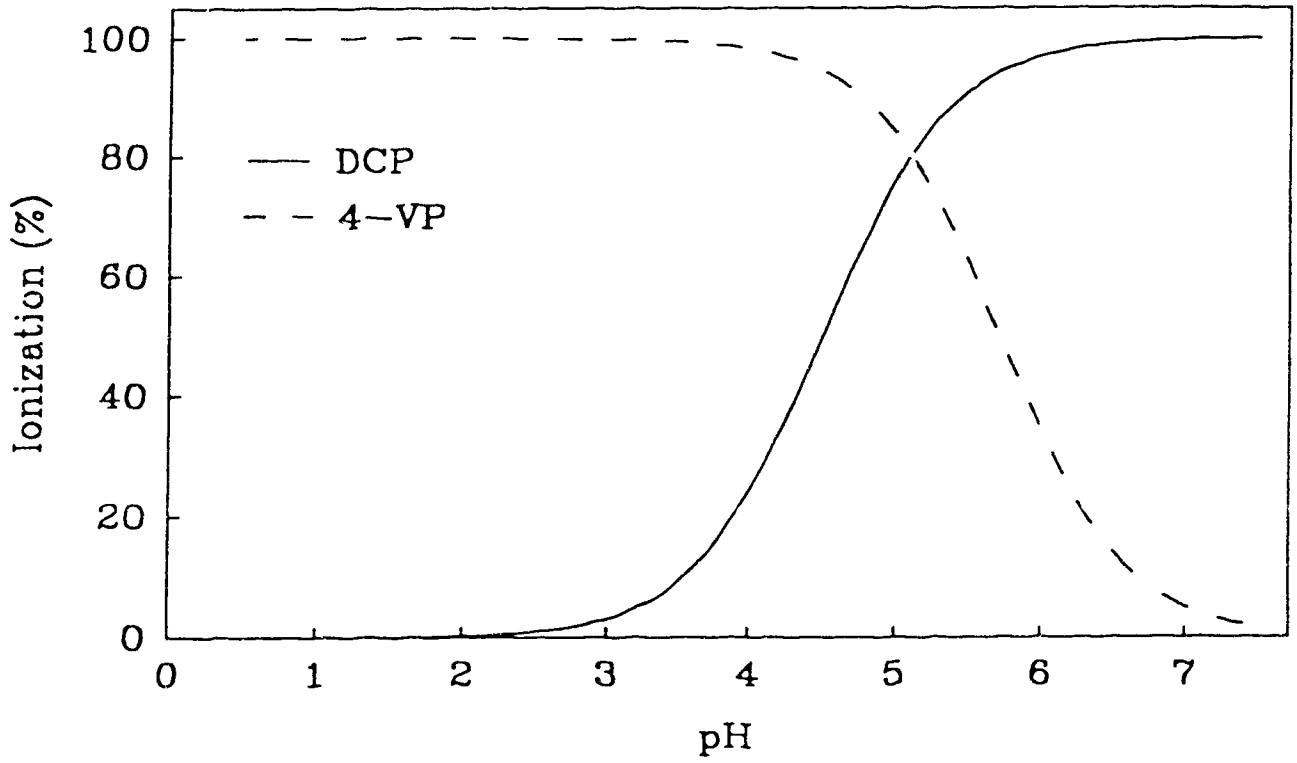
Calculations of percentage ionization at different pHs are based on the following equations (*Martin et al., 1983*):

$$\text{Ionization \% of DCP} = 100 \times 10^{(pH-4.5)}/(1 + 10^{(pH-4.5)}) \qquad (3)$$

$$\text{Ionization \% of 4-VP} = 100 \times 10^{(5.72-pH)}/(1 + 10^{(5.72-pH)}) \qquad (4)$$

Since the pK_a is also a function of the solvent used, the predicted pK_a can only be an approximate value. However, this is sufficiently accurate in making predictions of degree of ionization of 4-VP and DCP.

pH versus percent ionization of both DCP and 4-VP is illustrated as follows.



Percentage ionizations of DCP and 4-VP as a function of pH.

The percent ionizations at selected pHs calculated from equations 3 and 4 are illustrated as follows:

pH	Percentage ionization	
	DCP	4-VP
1.0	0	100
2.5	1	100
4.0	24	98
7.0	100	5

References

Clark, J. and Perrin, D. D. (1964). Prediction of the strengths of organic bases, Quarterly Rev., 18, 295-320.

Maitani, Y., Nakagaki, M. and Nagai, T. (1990). Surface potential of liposomes with entrapped insulin, Int. J. Pharm., 64, 89-98.

Martin, A., Swarbrick, J., and Cammarata, A. (1983). Physical Pharmacy, Chapter 9, 3rd ed., Lea & Febiger, Philadelphia, PA, pp. 187-221.

Perrin, D. D. (1980). Prediction of pK_a values, In S. H. Yalkowsky, A. A. Sinkula, S. C. Valvani (eds.), Physical Chemical Properties of Drugs, Chapter 1, Marcel Dekker, Inc., New York, pp. 1-48.