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The potential of pectin as a stabilizer for liposomal drug delivery systems

Gro Smistad[a,*], Silje Bøyum[a], Siv Jorunn Alund[a], Anne Berit C. Samuelsen[b], Marianne Hiorth[a]

[a] University of Oslo, School of Pharmacy, Department of Pharmacy, Oslo, Norway
[b] University of Oslo, School of Pharmacy, Department of Pharmaceutical Chemistry, Oslo, Norway

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ABSTRACT

The aim of the present study was to investigate the potential of different types of pectin as stabilizers for liposomal drug delivery systems. Positively charged liposomes were coated with commercially available and purified low-methoxylated (LM), high-methoxylated (HM) and amidated (AM) pectins. The samples were stored for up to 12 weeks at 4 °C, at room temperature and at 35 °C. The change in liposomal size and size distribution, zeta potential, pH, leakage of encapsulated carboxyfluorescein (CF), and lipid degradation were studied. All the types of pectin were found to protect the liposomes against aggregation during storage. The pectin coat did not affect the permeability of the liposome membrane. HM and LM pectin seemed to be the most promising types of pectin due to minimal changes in the zeta potentials during storage for these samples and no detectable lipid degradation. It is concluded that pectin may be used for stabilizing liposomal drug delivery systems.

1. Introduction

Liposomes are small vesicles of nano size, consisting of an aqueous core enclosed by one or more lipid bilayers. The bilayers are usually made of phospholipids. The application of liposomes as carriers of therapeutically active compounds in drug formulations is well-known (ElBayoumi & Torchilin, 2010). However, sufficient stabilization of this kind of colloidal formulations is a challenge. Liposomes may aggregate during storage. Fusion of the membranes and loss of encapsulated material may be a consequence. Also, loss of encapsulated material due to membrane permeability represents a problem. Another issue is the chemical stability of the lipids; both hydrolysis and oxidation may occur.

Long-term stability is essential for drug delivery systems. In several studies in the literature various polymers (e.g. chitosan, hyaluronic acid) have been coated on liposomes to improve the physical stability (Henriksen, Vagen, Sande, Smistad, & Karlsen, 1997; Laye, McClements, & Weiss, 2008; Rinaudo, Quemeneur, & Pepin-Donat, 2009). Also, polymers have been combined with liposomes to give the system mucoadhesive properties (Karn, Vanic, Pepic, & Skalko-Basnet, 2011; Rescia, Takata, de Araujo, & da Costa, 2011; Takeuchi, Matsui, Yamamoto, & Kawashima, 2003). Mucoadhesion of a pharmaceutical formulation may lead to improved delivery of the drug due to retention at the site of action.

Pectin is a polysaccharide shown to be mucoadhesive (Hagesaether, Bye, & Sande, 2008; Hagesaether & Sande, 2008; Liu, Fishman, Hicks, & Kende, 2005; Takahashi, Takeda, Seto, Kawano, & Machida, 2007; Thirawong, Thongborisute, Takeuchi, & Sriamornsak, 2008). Pectin is a complex anionic plant cell wall component that is composed of homogalacturonan regions with varying degrees of methylesterification and with different types of side chains such as galactans, arabinans or arabinogalactans linked to rhamnogalacturonan regions of the molecule. Furthermore, regions of single xylose residues and the well characterized side chains of rhamnogalacturonan II are also found linked to the galacturonan backbone (for review, see Voragen, Coenen, Verhoef, & Schols, 2009). However, in commercially available pectin most of the neutral side chains are lost upon isolation, leaving galacturonic acid as the major component. Thus, the exact structure varies with the source and the conditions applied during isolation. The acid groups of the galacturonic units can be methoxylated and/or amidated to varying degrees. The commercially available pectins are usually classified according to their degree of esterification (DE). High-methoxylated pectin has DE > 50% and low-methoxylated pectin has DE < 50% (Rolin, 1993). Pectin is well-known for its long and safe use in the food industry, e.g. as gelling and thickening agent and as a stabilizer. Also, pectin is a promising polymer for pharmaceutical use because of its bio-compatibility and non-toxic properties (Sriamornsak, 2011). In an earlier investigation we have studied in detail the coating procedure of liposomes with pectin, with the intention of developing a mucoadhesive liposomal

* Corresponding author at: School of Pharmacy, Department of Pharmacy, P.O. Box 1068, Blindern, N-0316 Oslo, Norway. Tel.: +47 22 85 65 92; fax: +47 22 85 44 02.
E-mail address: gro.smistad@farmasi.uio.no (G. Smistad).

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system for delivery of drugs to the oral cavity (Nguyen, Alund, Hiorth, Kjoniksen, & Smistad, 2011). The stability of the pectin coated systems, however, has not yet been investigated.

The aim of the present study was to investigate the influence of a pectin coat on the *in vitro* stability of the liposomal delivery system. Size stability, zeta potential stability, permeability of the liposome membrane and chemical stability during storage at different temperatures were investigated. Low-methoxylated, high-methoxylated and amidated pectins were included in the study. A stabilizing effect of pectin on the liposomal systems combined with its mucoadhesive properties would make pectin coated liposomes a promising candidate for site specific drug delivery, e.g. in the oral cavity and in ocular drug delivery.

2. Materials and methods

2.1. Materials

The main lipid dipalmitoyl phosphatidylcholine (DPPC) was obtained from Lipoid GmbH (Ludwigshafen, Germany). The cationic lipid dipalmitoyl trimethylammoniumpropane (DPTAP) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Lyso-phosphatidyl choline (lyso-PC) was from Sigma Chemical Co (USA). All types of pectin were obtained from CPKelco: Low-methoxylated (LM) pectin (Genu® pectin LM12 CG-Z) and high-methoxylated (HM) pectin (Genu® pectin 150 USA-SAG type B) from Grossenbrode, Germany; and amidated (AM) pectin (Genu® pectin LM-102 AS) from Lille Skensved, Denmark. The main raw material source of Genu® pectins is from citrus peel.

Sodium dihydrogen phosphate monohydrate and disodium hydrogen phosphate dihydrate used in the phosphate buffer, and chloroform used for liposome preparation were obtained from Merck (Darmstadt, Germany), and the fluorescent marker 5-carboxyfluorescein (CF) was from Sigma (USA). Ammoniumheptamolybdate-tetrahydrate, L(+)-ascorbic acid, and perchloric acid 70–72% used in the lipid quantification analysis, and copper sulfate pentahydrate and o-phosphoric acid (85%) used in the lipid degradation analysis, were all purchased from Merck (Germany). Triton X-100 used for liposome disintegration, was from Sigma Ultra (Germany). All chemicals were of analytical grade.

2.2. Purification of pectin

The commercially available pectins were purified before use. The pectins were dissolved in distilled water to a concentration of 1.5% (w/w) and stirred overnight at room temperature for complete dissolution. The pectin solutions were centrifuged for 60 min at 4400 rpm (Centra MP4, International Equipment Co., MA, USA), the solutions were decanted and the supernatants were centrifuged an additional 60 min at the same speed. The supernatants were dialysed against distilled water at 4 °C using Spectra/Por® 6 dialysis membrane (Spectrum Laboratories Inc., CA, USA) with a molecular weight cut-off of 8000 Da. The water was changed once a day for seven days. The remains after dialyzing were freeze dried (Christ Alpha 2–4 freeze drier, Christ, Osterode am Harz, Germany) and stored in the refrigerator.

2.3. Characterization of pectin

The molecular weights of the purified pectins were determined by asymmetric flow field-flow fractionation as described elsewhere (Nguyen et al., 2011). Carbohydrate composition analysis was carried out by methanolysis, TMS derivatisation and gas chromatography according to a method previously described (Chambers & Clamp, 1971) with modifications (Samuelsen et al., 1995) using 3 M HCl in anhydrous methanol (Supelco) at 80 °C for 20 h with mannitol as internal standard.

2.4. Preparation of liposomes

All liposomes contained 10 mol% of the positively charged lipid DPTAP. The liposomes were made according to the film method as follows: the phospholipids were dissolved in chloroform and the solutions were evaporated to dryness in a rotary evaporator. The films were further dried in vacuum in the Christ Alpha 2–4 freeze drier for about 20 h to remove organic residues. The thin films obtained were hydrated with 1.5 mM CF solution in phosphate buffer (pH 6.8) and swelled for 2 h, gently shaken intermittently, at a temperature above the gel to liquid-crystalline phase transition temperature (T_c), protected against light, and kept in the refrigerator overnight. Size reduction was performed at a temperature above T_c by extrusion with a Lipex extruder (Lipex Biomembranes Inc., Vancouver, Canada) using two-stacked 200 nm polycarbonate membranes (Nuclepore®, Costar Corp., Cambridge, USA). Non-encapsulated CF was removed by gel filtration through PD-10 desalting columns (Sephadex G-25) (GE Healthcare Biosciences AS, Sweden). Phosphate buffer 5 mM (pH 6.8) was used for eluation. The eluate was diluted to the final liposome concentration for use in further experiments which was 3 mM for most of the samples and 10 mM for some. The liposomes were coated with pectin immediately thereafter.

2.5. Coating of the liposomes with pectin

Purified pectin was dissolved in 5 mM phosphate buffer (pH 6.8) under stirring at room temperature overnight. To minimize the risk of dust and particles, the pectin solutions were filtered once through a 2 μm polycarbonate membrane at 45 °C with the Lipex extruder. Pectin concentrations used were 0.05 and 0.125% (w/w) for coating of 3 mM and 10 mM liposome solutions, respectively.

Pectin coated liposomes were prepared by adding one part of liposomal dispersion to four parts of pectin solution under continuous magnetic stirring. The liposomal dispersion was added in a drop wise manner (~3 ml/min) by means of a peristaltic pump (Watson-Marlow 520S IP3, Cornwall, UK). After the addition was completed the suspension was stirred for an additional five minutes. Three parallels were prepared for each formulation.

2.6. Stability testing

Each batch of the coated liposomes, and also uncoated liposome batches, were divided into three and stored in brown glass tubes. The tubes were stored protected from light in the refrigerator (4 °C), at room temperature (19–23 °C) and at 35 °C, respectively. In addition, pectin solutions (0.05% and 0.125% (w/w)) in phosphate buffer (pH 6.8) and seven dilutions of 1.5 mM CF solution in 5 mM phosphate buffer (pH 6.8) were stored in the refrigerator (4 °C) and at 35 °C to investigate the stability of the fluorescence marker during the study. Samples for analysis were withdrawn at various time points throughout the study. The samples were analysed for size and size distribution, zeta potential, pH, leakage of CF and lipid degradation.

2.6.1. Determination of size and size distribution

The intensity mean diameter of the liposomes, both uncoated and pectin coated, were determined by dynamic light scattering (DLS) using Coulter N4 Plus Submicron Particle Sizer (Coulter Corp., FL, USA) at 25 °C with 90° scattering angle. The refractive index and viscosity of pure water were used as calculation parameters and each sample was measured in triplicate using SDP analysis. All

samples were diluted with filtered (0.2 μm) 5 mM phosphate buffer (pH 6.8) to an appropriate counting rate prior to analysis.

2.6.2. Zeta potential determination

The zeta potential was measured by laser doppler micro-electrophoresis at 25 °C (Zetasizer 2000, Malvern Instruments Ltd., Worcestershire, United Kingdom) after dilution with 5 mM phosphate buffer (pH 6.8) to an appropriate counting rate. Five measurements were performed for each sample. The zeta potentials (ζ) were deduced from the mobility (U) by means of the Smoluchowski approximation, $U = \varepsilon\zeta/\eta$, where the viscosity ($\eta$) and the dielectric constant (ε) for pure water were used.

2.6.3. Determination of carboxyfluorescein leakage

Gel filtration columns PD-10 desalting columns (Sephadex G-25) were washed with phosphate buffer, and saturated with empty liposomes coated with the respective type of pectin. Columns for gel filtration of uncoated liposomes were saturated with uncoated liposomes. After saturation the columns were washed thoroughly with MilliQ water before use. At predetermined time point samples were withdrawn from the storage tubes and gel filtered using MilliQ water as eluent. The eluate was diluted with MilliQ water, and 50 μl were applied to the wells of a commercially available white flat-bottom polystyrene 96-wells micro titer plate (Nunclon) ($n = 8$ for each sample). Triton X-100 2% solution (50 μl) in 5 mM phosphate buffer (pH 6.8) was added to each well. After careful mixing the fluorescence was quantified using a Wallac Victor3 1420 multilabel counter (PerkinElmer, Boston, USA) (λ_{ex} 485 nm, λ_{em} 535 nm). Calibration solutions of carboxyfluorescein and blanks (MilliQ water plus Triton X-100 2%) were applied every time and to each plate.

The lipid content in the eluates were determined by phosphorus analysis using the Rouser method (Rouser, Fleische, & Yamamoto, 1970).

2.6.4. Determination of lipid degradation

Lipid degradation was estimated form the lyso-PC content in the samples and determined by high-performance thin layer chromatography (HPTLC). In short, aqueous samples and standard solutions of DPPC/DPTAP and lyso-PC in chloroform were applied to silica gel $60F_{254}$ HPTLC plates (E. Merck, Darmstadt, Germany) using Linomat IV sample applicator (CAMAG, Muttenz, Switzerland). The plates were developed in a horizontal developing chamber (CAMAG, Muttenz, Switzerland) using a mixture of chloroform:methanol:distilled water (32.5:12.5:2 v/v) as the mobile phase (Stensrud, Smistad, & Karlsen, 1996) and dried. The spots were visualized by immersing into a solution of 8% *ortho*-phosphoric acid and 10% copper sulphate pentahydrate (Touchstone et al., 1983) using a Camag chromatogram immersion device III (CAMAG, Muttenz, Switzerland). The plates were dried at 180 °C for 20 min in an incubator, then cooled to room temperature and scanned at 510 nm using a Camag TLC scanner III (Camag, Muttenz, Switzerland). Calibration graph was made from lyso-PC standard solutions, and peak area was used for quantification. All samples were analysed in duplicate.

2.7. Statistical analysis

For group comparisons of the results a one way analysis of variance (ANOVA) followed by Tukey's Post Hoc test were applied using $p < 0.05$ as level of significance (Minitab® 16 statistical software, Minitab Inc., USA).

3. Results

The characteristics of pectin are summarized in Table 1 showing that the main differences between the pectin tested are related to the DE and the degree of amidation. The monosaccharide composition was essentially the same for all three types of pectin. Galacturonic acid was the main monosaccharide present in all samples (85–87%). In addition, small amounts of rhamnose, arabinose, galactose and glucuronic acid were detected.

Table 1
Pectin characteristics of LM, HM, and AM: molecular weight (kDa), % degree of esterification (DE), % degree of amidation (DA) and monosaccharide composition (% of total carbohydrate content).

	LM	HM	AM
Mw (kDa)	76	110	96
DE	34.8[a]	70.2[a]	30.0[b]
DA	0	0	19.0[b]
Arabinose	<1	2.3	<1
Galactose	4.1	5.3	6.2
Rhamnose	4.4	2.9	3.3
Galacturonic acid	86.8	85.4	86.3
Glucuronic acid	4.7	4.1	4.2

[a] Information provided by the manufacturer.
[b] Hagesaether (2011).

In Fig. 1 the size stability at three different storage temperatures for all the liposomal formulations are shown. The size stability was acceptable for all the pectin coated liposomes during the time period investigated, and the polydispersity indices were relatively constant (in the range 0.1–0.4). The size increase was less than 6% for the LM- and HM-pectin coated liposomes and less than 14% for the AM-pectin coated liposomes. In contrast, the size stability of the uncoated liposomes was poor. After 5–6 weeks of storage the size started to increase at all storage temperatures, and the variation became very large (Fig. 1d). The size increases were in the range 100–200% for all temperatures.

In Fig. 2 the zeta potentials of the formulations during storage are shown. It can be seen from the figure that the zeta potentials of the uncoated liposomes remain stable during the period (Fig. 2d). There was a slight increase (<7%) of the zeta potentials to a less negative value for the LM-pectin coated liposomes stored at all temperatures and the HM-pectin coated liposomes stored at 4 °C. For the HM coated liposomes stored at 35 °C, however, there was a significant decrease of the zeta potential to a more negative value (Fig. 2c). The AM-pectin coated liposomes appeared as the least stable formulations. After about 40 days of storage the zeta potentials of the liposomes changed dramatically to less negative values, especially for storage at room temperature and at 35 °C (Fig. 2b). These findings are confirmed in Fig. 3 which shows that the LM- and HM-pectin coated liposomes still show only one peak for the coated liposomes after 12 weeks of storage at 4 °C. For the AM-coated liposomes, however, several peaks appear which may suggest some desorption of the polymer from the liposome surface and concequently liposomes with varying zeta potentials in the solution (Fig. 3c). The same trend, with even more peaks for the AM-pectin coated liposomes, was observed when liposomes were stored at room temperature and at 35 °C (plots not shown). During the whole storage period the pH values of the pectin solutions and the pectin coated liposomes were stable (or showed a slight decrease of about 0.2 pH units).

In Fig. 4 the leakage of CF from the different liposomal formulations during the storage period is shown. As can be seen from the figure, leakage from all the formulations were relatively low both at 4 °C and at room temperature. At 35 °C the leakage was higher for all the formulations, including the uncoated liposomes. No significant difference between the formulations could be detected, neither after 2 days nor 4 weeks of storage. The leakage during storage was calculated in per cent loss compared to the respective gel filtered sample at time point zero. The fluorescence stability of CF in solution was found to be good. No decrease in fluorescence was observed for the CF solutions

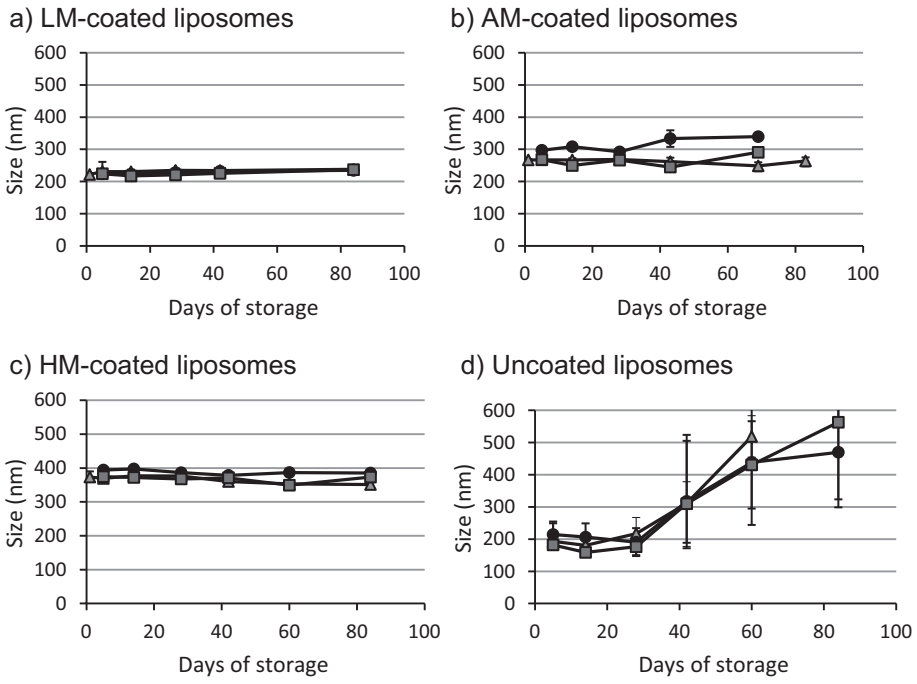

Fig. 1. Size stability of pectin coated (a–c) and uncoated (d) liposomes during storage at different temperatures. The samples were prepared from lipid concentration 3 mM and pectin concentration 0.05% (w/w). Symbols represent storage at 4 °C (▲), room temperature (●) and 35 °C (■). Bars represent max and min (n = 2–3).

stored at 35 °C during the storage period of 12 weeks (data not shown), and no difference was detected between the fluorescence in CF solutions stored at 4 °C and at 35 °C.

Lipids in solution are subjected to hydrolysis and the first step will be the formation of lyso-PC and free fatty acids (Grit, Desmidt, Struijke, & Crommelin, 1989; Grit, Underberg, & Crommelin, 1993). Free fatty acids are not detected with our method but lyso-PC will appear as a new peak in the HPTLC chromatograms. After 9 weeks of storage lyso-PC was detected only in the formulations stored at 35 °C. After 12 weeks of storage lyso-PC was detected in samples stored at 4 °C and room temperature, as well, but only for the AM-pectin coated liposomes. However, all levels of lyso-PC in the samples were low; below 3.5% for samples stored at 35 °C and even lower for storage at room temperature and at 4 °C (Fig. 5).

In Fig. 6 the size and zeta potential stability of liposomal preparations with higher lipid and pectin concentrations (prepared from lipid concentration 10 mM and pectin concentration 0.125%, w/w) are shown. The preparations were stored at 4 °C.

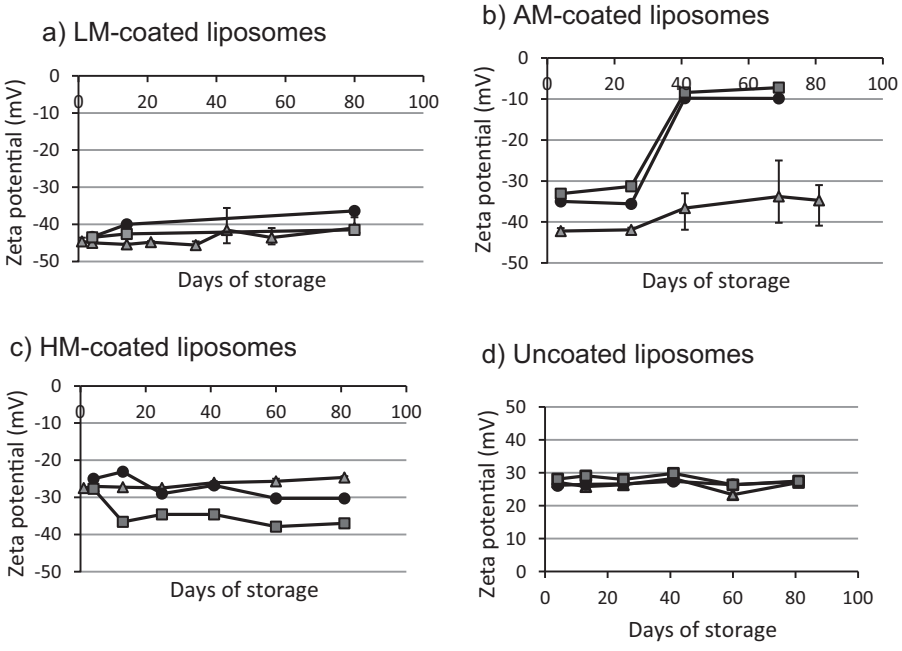

Fig. 2. Zeta potential stability of pectin coated (a–c) and uncoated (d) liposomes during storage at different temperatures. The samples were prepared from lipid concentration 3 mM and pectin concentration 0.05% (w/w). Symbols represent storage at 4 °C (▲), room temperature (●) and 35 °C (■). Bars represent max and min (n = 2–3).

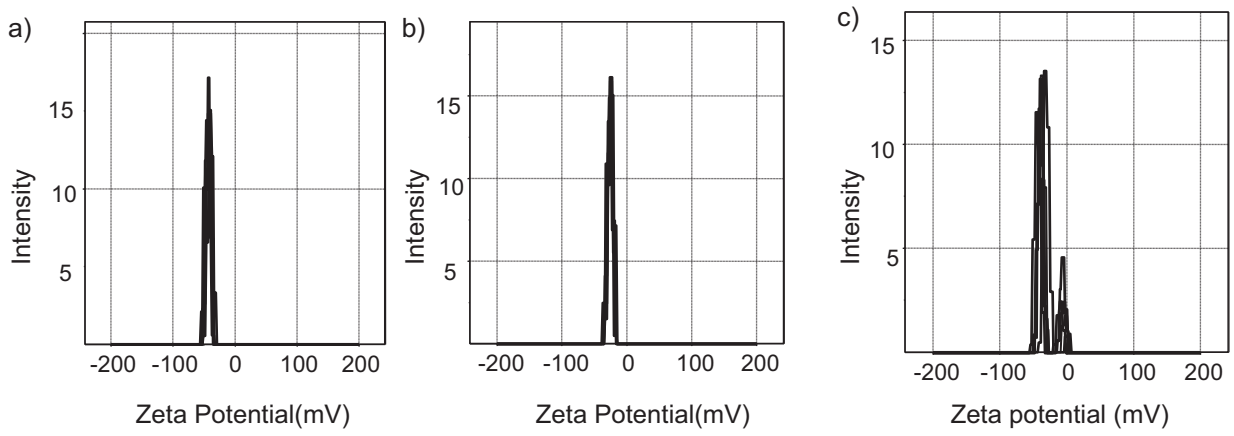

Fig. 3. Zeta potential intensity plots of (a) LM-pectin coated liposomes, (b) HM-pectin coated liposomes and (c) AM-pectin coated liposomes after 12 weeks of storage at 4 °C. The samples were prepared from lipid concentration 3 mM and pectin concentration 0.05% (w/w). The LM- and HM-pectin coated liposomes showed more or less one peak in contrast to the AM-pectin coated liposomes.

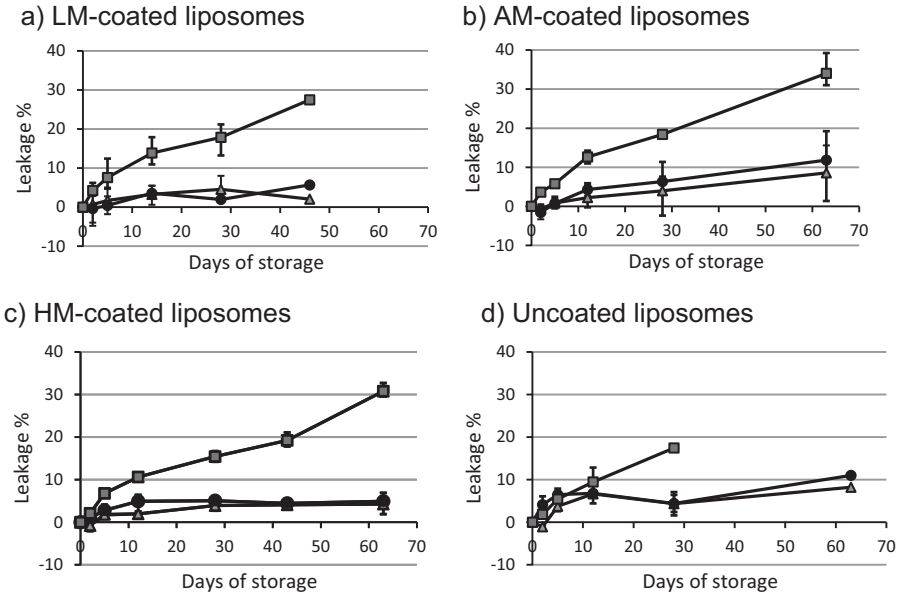

Fig. 4. CF leakage from pectin coated (a–c) and uncoated (d) liposomes during storage at different temperatures. The samples were prepared from lipid concentration 3 mM and pectin concentration 0.05%. Symbols represent storage at 4 °C (▲), room temperature (●) and 35 °C (■). Bars represent max and min ($n = 3$).

All formulations seemed to be relatively stable during the storage period, although there was a slight increase in the zeta potential to a less negative value for the AM-pectin coated liposomes. However, again the size and size distribution of the uncoated liposomes started to increase after 5–6 weeks of storage (data not shown).

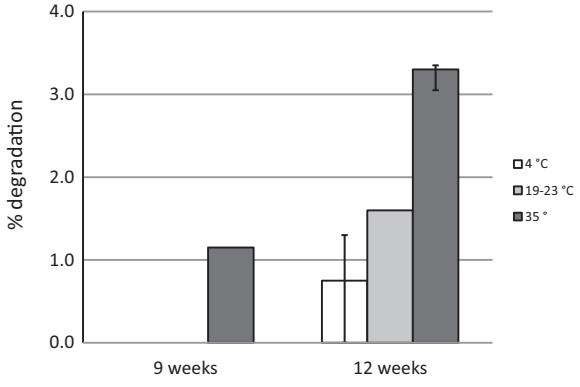

Fig. 5. Lipid degradation (calculated from the amount of lyso-PC detected in the samples) of AM-coated liposomes during storage.

4. Discussion

Stability of drug delivery systems is an important issue. The stability of liposomal systems may be divided into physical stability, i.e. aggregation, fusion and leakage, and chemical stability, i.e. oxidation and hydrolysis (Grit & Crommelin, 1993). In this study stability at three different temperatures was investigated, since the storage conditions may be expected to influence the stability of the systems. Refrigerator temperature (4 °C) and room temperature were chosen for storage. In addition 35 °C was chosen to accelerate degradation reactions. This temperature, and not a higher one, was chosen to keep the lipids in the gel state during the study, since the T_c of the main phospholipid is +41.5 °C (Cevc, 1993).

The size stability of uncoated liposomes has been investigated in several studies in the literature (Fang, Lin, Hsu, & Tsai, 1997; Grit & Crommelin, 1992; Nguyen et al., 2010; Yohannes, Pystynen, Riekkola, & Wiedmer, 2006; Zuidam, Gouw, Barenholz, & Crommelin, 1995). The size stability depends on the liposome

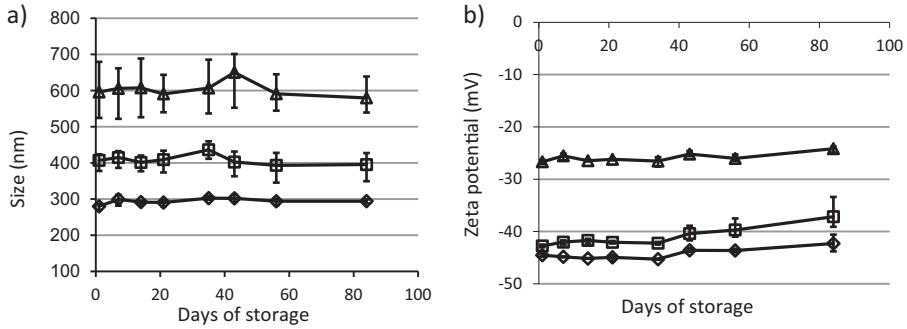

Fig. 6. Size (a) and zeta potential (b) stabilities during storage at 4 °C of liposomes 10 mM coated with 0.125% pectin. HM-pectin coated liposomes (△), AM-pectin coated liposomes (□), and LM-pectin coated liposomes (◊). Bars represent max and min ($n = 3$).

size and composition, and ionic strength of the medium. Incorporation of charged components in the liposome membrane is known to reduce the aggregation tendency due to electrostatic repulsion between the particles (Crommelin, 1984). Zeta potentials larger than 30 mV, optimally larger than 60 mV, have been reported to be sufficient for stabilizing nanoparticles from aggregation (Mosqueira, Legrand, Pinto-Alphandary, Puisieux, & Barratt, 2000; Ney, 1973; Schwarz, Mehnert, Lucks, & Muller, 1994). In our investigation the zeta potentials of the uncoated liposomes were in the range +26 to +31 mV. The zeta potentials remained at the same level throughout the whole storage period for all investigated temperatures (Fig. 2d). Nevertheless, the size of the uncoated liposomes increased significantly after storage for 5–6 weeks (Fig. 1d). This was assumed to be aggregation and not fusion since there was no corresponding increase in CF leakage (Fig. 4d). Aggregation of positively charged liposomes has been reported in the litterature (Crommelin, 1984; Nakamori et al., 1993). Aggregation was observed despite theoretically high enough surface potential for stabilization. Nakamori et al. (1993) suggested that above a certain level of positively charged component in the liposomes, the membrane may be partially solubilised during storage and aggregation will occur. The size of the pectin coated liposomes in our investigation remained quite stable for all types of pectin and at all storage temperatures (Fig. 1a–c). This shows that the pectin coat stabilizes the liposomes against aggregation during storage, which is expected due to both electrostatic repulsion and steric hinderance of aggregation. A stabilizing effect of polymer coating of liposomes has been described in the literature for other polymers, as well, such as polyvinyl pyrrolidone, chitosan, alginate, polyvinyl alchohol, and hyaluronic acid (Henriksen et al., 1997; Kawakami, Nishihara, & Hirano, 2001; Laye et al., 2008; Quemeneur, Rinaudo, Maret, & Pepin-Donat, 2010; Rinaudo et al., 2009; Sone et al., 1996).

Although stabilizing against aggregation, the pectin coat did not stabilize against leakage of CF. There was no difference between the leakage from the pectin coated and the uncoated liposomes during storage. The same trend has been reported in the literature for chitosan coated liposomes (Henriksen et al., 1997; Volodkin, Mohwald, Voegel, & Ball, 2007), while other polymers have been reported to increase the leakage of the liposomes (Berkovich, Orlov, & Melik-Nubarov, 2009; Thomas & Tirrell, 2000; Zhang, Peng, Cheng, & Zhuo, 2004). The leakage from the liposomes in our investigations was below about 10% for all the formulations stored at 4 °C and at room temperature. For formulations stored at 35 °C, however, the leakage was higher. Already after storage for about 10 days the leakage was higher than 10% for all formulations (both pectin coated and uncoated). This shows that the leakage was determined by the properties of the liposome membrane itself and not by the pectin coat. At 4 °C and at room temperature the lipid membrane is in the gel phase with low permeability. At 35 °C, however, the membrane is closer to T_c, which is +41.5 °C for DPPC (Cevc, 1993) and +44.5 °C for DPTAP (Filion & Phillips, 1997). At T_c the membrane changes from gel to fluid phase. The membrane is most leaky close to the T_c because of disorder in the membrane (New, 1990). No difference between the leakage from uncoated and pectin coated liposomes at 35 °C reveals that the polymer coat does not stabilize against leakage.

Pectin in solution is known to decompose by deesterification and depolymerisation (Rolin, 1993). The change to a more negative zeta potential for the HM-pectin coated liposomes stored at 35 °C may be a result of hydrolysis of methyl esters leading to free carboxylic groups and consequently more negative charge at pH 6.8. The same decrease in zeta potential was not observed for the LM-pectin coated liposomes. LM-pectin is known to be more stable than HM-pectin caused by the lower degree of esterification (Albersheim, Neukom, & Deuel, 1960). For the AM-pectin coated liposomes, however, there was a significant change in the zeta potential during the storage period (Fig. 2b). After about 5–6 weeks of storage, the zeta potentials became gradually less negative for all storage temperatures, and the intensity distributions showed more peaks (Fig. 3c). The large change in zeta potential and the appearance of more peaks with varying peak values may reflect desorption of pectin from the liposome surface. Desorption of the negatively charged polymer may lead to exposure of liposome surface areas with positive charge, and this will most probably affect the apparent zeta potential of the particle. However, the size determination did not reveal any strong indication of aggregation of the samples which could be expected when positively charged areas on the liposome surface is exposed to negatively charged polymer coated liposomes. This may suggest that the final desorption of the polymer takes place in the dilution step during the sample preparation procedure immediately prior to analysis. A corresponding change in zeta potentials were not seen for the LM and HM-pectin coated liposomes. This suggests stronger interactions between these types of pectin and the liposomes. LM-pectin is expected to bind strongly to positively charged liposomes due to the high negative charge of the pectin. HM-pectin has a less negative zeta potential. However, the HM-pectin is more hydrophobic and may possibly also bind to the liposomes by hydrophobic interactions. Although the AM-pectin is highly charged, the amide groups may interfere with the electrostatic interactions and lead to a more loosely bound pectin coat. Loosely bound pectin may be even more loosely bound during storage and finally desorb on dilution of the sample, as discussed above.

In liposomal formulations the amount of encapsulated drug will be affected by the lipid concentration. Formulations of 10 mM lipid concentration were therefore also included in the study. These were coated with pectin solutions of 0.125% (w/w) based on preliminary findings in our lab for promising combinations of lipid- and pectin concentrations. Both the size- and zeta potential stability of all these formulations seemed to be good during storage at 4 °C for 12

weeks (Fig. 6). However, we can see that higher pectin concentration resulted in larger liposome–pectin complexes for all the types of pectin (compare Figs. 1a–c and 6a). Also, we can see that for the HM-coated liposomes and for the AM-coated liposomes, as well, the variation in size was much larger (larger error bars) for the samples containing 10 mM lipid (Fig. 6a) compared to the samples containing 3 mM lipid (Fig. 1c and b, respectively), and the distributions were broader. This may suggest some aggregation tendencies of the coated liposomes. In the 10 mM lipid + 0.125% pectin samples the pectin-to-lipid ratio in fact is lower than in the 3 mM lipid + 0.05% pectin samples. This may result in insufficient coating of the liposomes which again may lead to some aggregation. Most probably we are at the border of complete coating and thus the aggregation is limited. The aggregation is expected to be more pronounced for the HM-pectin since this is the most hydrophobic type of pectin with the least negative zeta potential and the highest molecular weight. Again, a small change in zeta potential to a less negative value for the AM-pectin coated liposomes (10 mM lipid + 0.125% pectin) was observed as was observed for the 3 mM lipid + 0.05% AM-pectin coated samples (compare Figs. 2b (4 °C) and 6b (AM-liposomes)). No influence of increasing the lipid concentration from 3 mM to 10 mM for the uncoated liposomes was expected. The uncoated liposomes were still quite unstable and again showed aggregation tendencies after 5–6 weeks of storage at 4 °C.

Lipids in solution are subjected to hydrolysis. The first step will be formation of lyso-PC (Grit et al., 1989, 1993). Lipid hydrolysis is expected to destabilize the bilayer leading to increased permeability of the membrane due to structural changes. However, it has been shown that hydrolysis up to 10% degradation in fact reduces the leakage through the membrane (Grit & Crommelin, 1992). This was explained by a stabilizing effect of the combination of lyso-PC and fatty acids on the bilayer permeability, in that the fatty acids neutralised the destabilizing effect of lyso-PC. In our investigation lyso-PC was not detected in any of the samples in the first 7–8 weeks. After 9 weeks of storage lyso-PC was detected, but only in the samples stored at 35 °C. Lyso-PC may be further degraded to glycerophosphocholine (Grit & Crommelin, 1992), and by analysing only the lyso-PC level there is a possibility to underestimate the degradation. However, it has been shown that the rate constant of the first hydrolysis step (DPPC to lyso-PC) is larger than for the second step (lyso-PC to glycerophosphocholine) for saturated phospholipids (Grit et al., 1993). Since no lyso-PC was detected the first weeks it seems most probable that the hydrolysis rate in fact is very slow. The low degree of hydrolysis may be explained by the gel state of the lipid membrane and the positively charged surface, which both have been shown to be favourable for DPPC liposomes (Zuidam & Crommelin, 1995). Moreover, the pH of all the solutions were in the range 6.3–7.15, which is quite close to the pH 6.5 where minimum hydrolysis has been shown to occur both for unsaturated (Grit et al., 1989) and saturated (Grit et al., 1993) phospholipids.

The AM-pectin coated liposomes were the only formulation in which lyso-PC was detected in all the samples after 12 weeks of storage. The level of lyso-PC was highest in samples stored at 35 °C, which was expected since the rate of hydrolysis is affected by the temperature (Grit et al., 1989, 1993). This indicates that the AM-coated liposomes was the least stable combination. This was also confirmed by the large change and great variations in the zeta potentials of these samples (Fig. 2b). The degradation did not lead to increased leakage of CF from the liposomes, which may be explained by the low level of lipid degradation. There was no difference between the levels of lyso-PC in the LM- and HM-coated liposomes and the uncoated liposomes which means that LM and HM coatings neither protect the lipid bilayer from hydrolysis nor lead to increased hydrolysis.

5. Conclusions

This study shows that different types of pectin may be used for stabilizing liposomal drug delivery systems. Both LM-, HM- and AM-pectin protected the positively charged liposomes against aggregation during storage. The pectin coat did not affect the permeability of the liposome membrane, and the leakage was very low at 4 °C and at room temperature. LM- and HM-pectin appeared as the most favourable types of pectin for stabilizing due to minimal changes in the zeta potentials during storage for these samples and no detectable lipid degradation during storage at 4 °C and at room temperature.

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References

Albersheim, P., Neukom, H., & Deuel, H. (1960). Splitting of pectin chain molecules in neutral solutions. *Archives of Biochemistry and Biophysics*, *90*(1), 46–51.
Berkovich, A. K., Orlov, V. N., & Melik-Nubarov, N. S. (2009). Interaction of polyanions with electroneutral liposomes in a slightly acidic medium. *Polymer Science Series A*, *51*(6), 648–657.
Cevc, G. (1993). *Phospholipids handbook*. New York: Marcel Dekker.
Chambers, R. E., & Clamp, J. R. (1971). Assessment of methanolysis and other factors used in analysis of carbohydrate-containing materials. *Biochemical Journal*, *125*(4), 1009–1018.
Crommelin, D. J. A. (1984). Influence of lipid-composition and ionic strength on the physical stability of liposomes. *Journal of Pharmaceutical Sciences*, *73*(11), 1559–1563.
ElBayoumi, T. A., & Torchilin, V. P. (2010). Current trends in liposome research. In V. Weissig (Ed.), *Liposomes, methods in molecular biology*. Humana Press, pp. 1–27.
Fang, J. Y., Lin, H. H., Hsu, L. R., & Tsai, Y. H. (1997). Characterization and stability of various liposome-encapsulated enoxacin formulations. *Chemical & Pharmaceutical Bulletin*, *45*(9), 1504–1509.
Filion, M. C., & Phillips, N. C. (1997). Toxicity and immunomodulatory activity of liposomal vectors formulated with cationic lipids toward immune effector cells. *Biochimica Et Biophysica Acta-Biomembranes*, *1329*(2), 345–356.
Grit, M., & Crommelin, D. J. A. (1992). The effect of aging on the physical stability of liposome dispersions. *Chemistry and Physics of Lipids*, *62*(2), 113–122.
Grit, M., & Crommelin, J. A. (1993). Chemical stability of liposomes—Implications for their physical stability. *Chemistry and Physics of Lipids*, *64*(1–3), 3–18.
Grit, M., Desmidt, J. H., Struijke, A., & Crommelin, D. J. A. (1989). Hydrolysis of phosphatidylcholine in aqueous liposome dispersions. *International Journal of Pharmaceutics*, *50*(1), 1–6.
Grit, M., Underberg, W. J. M., & Crommelin, D. J. A. (1993). Hydrolysis of saturated soybean phosphatidylcholine in aqueous liposome dispersions. *Journal of Pharmaceutical Sciences*, *82*(4), 362–366.
Hagesaether, E. (2011). Permeation modulating properties of natural polymers—Effect of molecular weight and mucus. *International Journal of Pharmaceutics*, *409*, 150–155.
Hagesaether, E., & Sande, S. A. (2008). Effect of pectin type and plasticizer on in vitro mucoadhesion of free films. *Pharmaceutical Development and Technology*, *13*(2), 105–114.
Hagesaether, E., Bye, R., & Sande, S. A. (2008). Ex vivo mucoadhesion of different zinc-pectinate hydrogel beads. *International Journal of Pharmaceutics*, *347*(1–2), 9–15.
Henriksen, I., Vagen, S. R., Sande, S. A., Smistad, G., & Karlsen, J. (1997). Interactions between liposomes and chitosan: 2. Effect of selected parameters on aggregation and leakage. *International Journal of Pharmaceutics*, *146*(2), 193–203.
Karn, P. R., Vanic, Z., Pepic, I., & Skalko-Basnet, N. (2011). Mucoadhesive liposomal delivery systems: The choice of coating material. *Drug Development and Industrial Pharmacy*, *37*(4), 482–488.
Kawakami, K., Nishihara, Y., & Hirano, K. (2001). Effect of hydrophilic polymers on physical stability of liposome dispersions. *Journal of Physical Chemistry B*, *105*(12), 2374–2385.
Laye, C., McClements, D. J., & Weiss, J. (2008). Formation of biopolymer-coated liposomes by electrostatic deposition of chitosan. *Journal of Food Science*, *73*(5), N7–N15.
Liu, L. S., Fishman, M. L., Hicks, K. B., & Kende, M. (2005). Interaction of various pectin formulations with porcine colonic tissues. *Biomaterials*, *26*(29), 5907–5916.
Mosqueira, V. C. F., Legrand, P., Pinto-Alphandary, H., Puisieux, F., & Barratt, G. (2000). Poly(D,L-lactide) nanocapsules prepared by a solvent displacement process: Influence of the composition on physicochemical and structural properties. *Journal of Pharmaceutical Sciences*, *89*(5), 614–626.
Nakamori, K., Nakajima, T., Odawara, M., Koyama, I., Nemoto, M., Yoshida, T., et al. (1993). Stable positively charged liposome during long-term storage. *Chemical & Pharmaceutical Bulletin*, *41*(7), 1279–1283.

New, R. R. C. (1990). *Liposomes a practical approach*. Oxford, UK: IRL Press.
Ney, P. (1973). *Zetapotentiale und flotierbarkeit von mineralen*. New York: Springer.
Nguyen, S., Alund, S. J., Hiorth, M., Kjoniksen, A. L., & Smistad, G. (2011). Studies on pectin coating of liposomes for drug delivery. *Colloids and Surfaces B-Biointerfaces*, *88*(2), 664–673.
Nguyen, S., Solheim, L., Bye, R., Rykke, M., Hiorth, M., & Smistad, G. (2010). The influence of liposomal formulation factors on the interactions between liposomes and hydroxyapatite. *Colloids and Surfaces B-Biointerfaces*, *76*(1), 354–361.
Quemeneur, F., Rinaudo, M., Maret, G., & Pepin-Donat, B. (2010). Decoration of lipid vesicles by polyelectrolytes: Mechanism and structure. *Soft Matter*, *6*(18), 4471–4481.
Rescia, V. C., Takata, C. S., de Araujo, P. S., & da Costa, M. H. B. (2011). Dressing liposomal particles with chitosan and poly(vinylic alcohol) for oral vaccine delivery. *Journal of Liposome Research*, *21*(1), 38–45.
Rinaudo, M., Quemeneur, F., & Pepin-Donat, B. (2009). Stabilization of liposomes by polyelectrolytes: Mechanism of interaction and role of experimental conditions. *Macromolecular Symposia*, *278*, 67–79.
Rolin, C. (1993). Pectin. In R. Whistler, & J. N. BeMiller (Eds.), *Industrial gums. Polysaccharide and their derivatives*. California, USA: Academic press, Inc.
Rouser, G., Fleische, S., & Yamamoto, A. (1970). 2 dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorous analysis of spots. *Lipids*, *5*(5), 494–496.
Samuelsen, A. B., Paulsen, B. S., Wold, J. K., Otsuka, H., Yamada, H., & Espevik, T. (1995). Isolation and partial characterization of biologically active polysaccharides from Plantago major L. *Phytotherapy Research*, *9*(3), 211–218.
Schwarz, C., Mehnert, W., Lucks, J. S., & Muller, R. H. (1994). Solid lipid nanoparticles (SLN) for controlled drug-delivery: 1. Production, characterization and sterilization. *Journal of Controlled Release*, *30*(1), 83–96.
Sone, T., Hanamizu, T., Ichioka, M., Yokokura, T., Satoshi, Y. B., Yoshio, H. B., et al. (1996). Effect of poly(vinylpyrrolidone) on the stability of vesicles formed using monoglyceride. *Colloids and Surfaces A-Physicochemical and Engineering Aspects*, *108*(2–3), 273–276.
Sriamornsak, P. (2011). Application of pectin in oral drug delivery. *Expert Opinion on Drug Delivery*, *8*(8), 1009–1023.
Stensrud, G., Smistad, G., & Karlsen, J. (1996). Effect of gamma irradiation on the stability of liposome/phospholipids. I. *Drug Stability*, *1*, 152–160.
Takahashi, Y., Takeda, C., Seto, I., Kawano, G., & Machida, Y. (2007). Formulation and evaluation of lactoferrin bioadhesive tablets. *International Journal of Pharmaceutics*, *343*(1–2), 220–227.
Takeuchi, H., Matsui, Y., Yamamoto, H., & Kawashima, Y. (2003). Mucoadhesive properties of carbopol or chitosan-coated liposomes and their effectiveness in the oral administration of calcitonin to rats. *Journal of Controlled Release*, *86*(2–3), 235–242.
Thirawong, N., Thongborisute, J., Takeuchi, H., & Sriamornsak, P. (2008). Improved intestinal absorption of calcitonin by mucoadhesive delivery of novel pectin-liposome nanocomplexes. *Journal of Controlled Release*, *125*(3), 236–245.
Thomas, J. L., & Tirrell, D. A. (2000). Polymer-induced leakage of cations from dioleoyl phosphatidylcholine and phosphatidylglycerol liposomes. *Journal of Controlled Release*, *67*(2–3), 203–209.
Touchstone, J. C., Levin, S. S., Dobbins, M. F., Matthews, L., Beers, P. C., & Gabbe, S. G. (1983). (3-sn-phosphatidyl)cholines (lecithins) in amniotic fluid. *Clinical Chemistry*, *29*(11), 1951–1954.
Volodkin, D., Mohwald, H., Voegel, J. C., & Ball, V. (2007). Coating of negatively charged liposomes by polylysine: Drug release study. *Journal of Controlled Release*, *117*(1), 111–120.
Voragen, A. G. J., Coenen, G. J., Verhoef, R. P., & Schols, H. A. (2009). Pectin, a versatile polysaccharide present in plant cell walls. *Structural Chemistry*, *20*(2), 263–275.
Yohannes, G., Pystynen, K. H., Riekkola, M. L., & Wiedmer, S. K. (2006). Stability of phospholipid vesicles studied by asymmetrical flow field-flow fractionation and capillary electrophoresis. *Analytica Chimica Acta*, *560*(1–2), 50–56.
Zhang, L., Peng, T., Cheng, S. X., & Zhuo, R. X. (2004). Destabilization of liposomes by uncharged hydrophilic and amphiphilic polymers. *Journal of Physical Chemistry B*, *108*(23), 7763–7770.
Zuidam, N. J., & Crommelin, D. J. A. (1995). Chemical hydrolysis of phospholipids. *Journal of Pharmaceutical Sciences*, *84*(9), 1113–1119.
Zuidam, N. J., Gouw, H., Barenholz, Y., & Crommelin, D. J. A. (1995). Physical (in)stability of liposomes upon chemical hydrolysis—The role of lysophospholipids and fatty-acids. *Biochimica Et Biophysica Acta-Biomembranes*, *1240*(1), 101–110.