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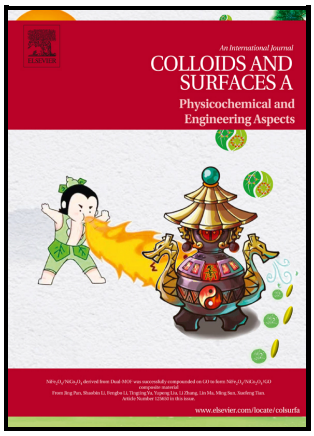

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Preparation of stable polymer-liposome complexes by a novel approach employing a one-pot method

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ABSTRACT

The purpose of this study was to prepare stable polymer-liposome complexes using a new, improved one-pot method. Complexes prepared by the conventional method were used as reference. The one-pot method combines the vesicle formation and complexation with the polymer in a single step allowing for fewer steps compared to the conventional thin-film method followed by coating. This has the possibility of complexing the polymer on the surface and within the aqueous core of the liposomes. Soya phosphatidylcholine (SoyPC) in combination with different amounts of either dioleoyl trimethylammoniumpropane (DOTAP, positively charged) or egg phosphatidylglycerol (Egg PG, negatively charged) were complexed with alginate or chitosan, respectively. All the alginate-liposome complexes were larger compared to the naked liposomes and the zeta potential changed from positive to negative after complexation with the polymer. Polydispersity index (PDI) analysis showed narrow size distributions implying homogenous populations. The best stability was obtained by employing 20 mol% DOTAP in the membrane. This formulation was stable during the whole four months period when the stability test was performed. The chitosan-liposome complexes were generally difficult to extrude and were larger than the alginate-liposome complexes due to the need for a larger pore size in the membrane during extrusion. Based on these results, the one-pot method can be used for preparing stable alginate-liposome complexes. For the chitosan-liposome complexes alternative methods for size reduction should be investigated.

KEY WORDS: one-pot method, polymer-liposome complexes, liposomes, alginate, chitosan, coating.

ABBREVIATIONS: Alg-Lip$_{pos}$, alginate-liposome complexes from positively charged lipids; Alg-Lip$_{neu}$, alginate-liposome complexes from neutral lipids; Chi-Lip$_{neg}$, chitosan-liposome complexes from negatively charged lipids; Lip$_{pos}$, positively charged naked liposomes; Lip$_{neg}$, negatively charged naked liposomes.

1. INTRODUCTION

Liposomes are spherical structures composed of one or more phospholipid bilayers surrounding an aqueous core. The structures have been studied extensively for use as drug delivery systems by encapsulating both hydrophilic and hydrophobic substances [1]. Liposomes can be prone to physical instability such as aggregation, fusion, and leakage of encapsulated ingredients during storage, and chemical instability such as oxidation and hydrolysis of the phospholipids [2-5]. Long-term stability of liposomes is important if their use as drug delivery systems is to be successful. Liposomal suspensions as colloidal systems may be stabilized by covering the surface of the liposomes with charged polymers. The repulsive forces between the particles help to stabilize the suspension by preventing aggregation and fusion as previously shown for alginate and chitosan [6, 7]. Stabilization could also be due to steric hindrance when the polymers prevent the liposomal particles from aggregating. However, the amount of polymer should be high enough to cover the surface of the liposomes[8], otherwise bridging flocculation will take place. On the other hand, excessive polymer amounts may lead to depletion flocculation [9]. It has also been shown that liposomes combined with polymers could increase the in vivo stability by avoiding rapid clearance from the circulation [10, 11]. Also, liposomes combined with polymers could confer the system mucoadhesive properties, which could be essential to prolong the residence time at the site of action [12-15].

Electrostatic deposition has been widely reported as one of the complexation methods for liposomes and polymers. For instance, alginate and chitosan have been complexed with liposomes in several studies [15-17]. This is achieved by exploiting the charge of the

polymers and the oppositely charged liposomes. It has also been shown that liposomes and polymers with the same charge can complex with each other, but the interaction is weaker compared to those of opposite charge [18]. Another widely used method for complexing liposomes and polymers involves the use of polymers with long alkyl side chains which can interact with the liposomes by inserting the lipophilic chains into the liposomal bilayer [19]. Polymers with the desired properties but lack of lipophilic side chains have been hydrophobically modified and successfully complexed with liposomes in some studies [20, 21]. Another complexation method reported by Andersen et al. [22] also termed the one-pot method, used a modified solvent injection method to prepare pectosomes (pectin-liposome complexes) and chitosomes (chitosan-liposome complexes). In this method the formation of the liposomes and the complexation with the polymers was performed in one step.

It is apparent that the choice of method for the polymer-liposome complexation will depend on factors such as type of lipids, polymers, and chemical properties of the active pharmaceutical ingredient (API). Also, the easiness and the possibility of scaling up the production for commercial use are important parameters. The ideal method should be simple with few steps, it should be mild with limited use of organic solvents and able to complex different types of polymers. The method should also prepare stable homogenous polymer-liposome complexes with narrow size distribution. In this study, a new and improved one-pot method for polymer-liposome complexation with the possibility of satisfying the criteria above is presented. In addition, this new method gives the opportunity of complexing the polymers not only on the surface of the liposomes but also in the core. This could be particularly useful in the delivery of rehydration substances to dry mucosa, for example to the mouth, the eye, or the vagina. Dry mouth is a condition that has a huge burden on the quality of life of the sufferers. There are lots of different products on the market for relieving the symptoms of dry mouth such as tablets, sprays, gels, and mouth washes[23-26]. However, these remedies are ineffective or inefficient in severely affected individuals and the need for better products is apparent[27]. One effect of dry mouth is the development of dental carries that has been shown to influence the quality of life of affected individuals negatively and continues to be a problem even at old age [25, 27, 28].

The aim of this study was to scrutinize the new one-pot method for preparing stable alginate-liposome and chitosan-liposome complexes with the potential of rehydrating dry mouth symptoms. The study design was divided into 2 parts. The aim of part 1 was to investigate if it was possible to prepare stable polymer-liposome complexes using the one-pot method. This

was done by employing the same amounts of lipids and polymers that have been shown to be stable with the conventional coating method. In part 2, the aim was to investigate if the amount of charged lipid could affect the stability of the polymer-liposome complexes prepared by the one-pot method. As a reference, liposomes prepared by the conventional thin-film method and subsequently coated with alginate or chitosan were included.

2. MATERIALS AND METHODS

2.1 Materials

Phosphatidylcholine from soyabean (SoyPC, Lipoid S PC, Approximate Mw = 787 Da, >98% phosphatidylcholine) was a kind gift from Lipoid GmbH (Ludwigshafen, Germany). Dioleoyl trimethylammoniumpropane (DOTAP) a cationic lipid and phosphatidylglycerol (Egg PG) an anionic lipid, were purchased from Avanti Polar Lipids, Inc. (Alabaster, USA). Chitosan hydrochloride (Protasan UPCL 213, Novamatrix DD = 83%, Mw = 3.1 x 10^5 Da) was purchased and sodium alginate (Protanal LF 10/60, Mw = 1.47 x 10^5 Da) was a gift, from FMC Biopolymer AS (Sandvika, Norway). Sodium dihydrogen phosphate monohydrate, disodium hydrogen phosphate dihydrate and chloroform of analytical grade were purchased from Merck (Darmstadt, Germany).

2.2 Preparation of polymer solutions

The chitosan hydrochloride was of ultrapure quality and was used without further purification.

The commercially available polymer sodium alginate, however, was purified by dissolution, dialyzing and freeze-drying as described elsewhere[29]. In short, the polymer was dissolved in distilled water to a concentration of 1.5% (w/w). The solution was dialyzed against distilled water for 8 days using a dialysis membrane with a molecular weight cutoff of 8000 Dalton and changing the dialysis water intermittently. The dialyzed solution was then freeze-dried to obtain the purified alginate.

Polymer solutions for the experiments were prepared by dissolving the polymer in 5 mM phosphate buffer (PB) pH 6.8 under magnetic stirring overnight. The resulting solutions were filtered through a sterile 5µm syringe filter (Versapor® Membrane, Pall Corporation, NY, USA) to minimize the risk of contamination from dust and other particles.

2.3 Preparation of polymer coated liposomes by the thin-film method

Preparation of liposomes

The liposomes were prepared according to the thin-film method [30]. The lipids were dissolved in chloroform and evaporated to dryness in a round bottom flask using a rotary evaporator (Heidolph W 2001 rotavapor, Heidolph Instruments GmbH & Co. KG, Kelheim, Germany). The resulting lipid film was vacuum dried overnight in a Christ Alpha 2 – 4 freeze drier (Christ, Osterode am Harz, Germany) to remove any remaining organic solvent. The film was hydrated with PB (5 mM, pH 6.8) and stirred on a rotavapor for 10 min. The flask was then kept in a dark place at room temperature (20 °C) for two hours and the suspension was gently hand-shaken intermittently. The multi-lamellar vesicles were further hydrated in a refrigerator (4 °C) overnight. The liposomes were extruded (room temperature) 10 times through a two-stacked polycarbonate membrane with a pore size of 200 nm (Nucleopore®, Costar Corp., Cambridge, USA) using a Lipex extruder (Lipex Biomembranes Inc., Vancouver, Canada). The extrusion pressure was < 10 bar ensuring minimal loss of lipids. The final lipid concentration was 3 mM. After extrusion, the liposomal suspension was transferred into glass vials, flushed with nitrogen gas to replace headspace air, and stored in the refrigerator.

Coating of liposomes with polymer

The liposomes were added to the polymer solution in a 1:4 ratio (v/v) under magnetic stirring. The resulting total volume was 5 mL. Using a peristaltic pump (Watson-Marlow 520S, Watson-Marlow Pumps Group, Falmouth, UK), the liposomes were added dropwise at a flow rate of 1.6 mL/min and further stirred for 5 min after all the liposomes were added. The final liposome- and polymer concentrations were 0.6 mM and 0.08% (w/v), respectively. The prepared liposomes were stored in the refrigerator overnight before further analysis.

2.4 Preparation of polymer-liposome complexes by the one-pot method

The preparation of liposomes by the one-pot method was similar to the thin-film method except that the dry lipid film was hydrated with a solution of the chosen polymer (0.08% w/v) dissolved in PB (5 mM, pH 6.8). The extrusion was done similarly, but the chitosan-liposome complexes were extruded through 800 nm polycarbonate membranes, as it was impossible to extrude through a smaller pore size. The extrusion was done 5 times.

2.5 Stability during storage

Samples were taken out at predetermined intervals for measuring the size and the zeta potential. The formulations were stored in the refrigerator, but prior to analysis, the glass vials were kept at room temperature for about 1 hour before the samples were taken out. The samples were mixed carefully and inspected for possible precipitation before measuring.

2.6 Characterization methods

The hydrodynamic diameter (size) and the electrophoretic mobility (zeta potential) were determined by dynamic light scattering using a Zetasizer Nano (Malvern Instruments Ltd, Worcestershire, UK) at 25 °C with a backscatter detection at a scattering angle (θ) of 173°. Polystyrene latex particles were chosen under the material settings of the instrument and the refractive index, and the viscosity of water were chosen as calculation parameters. Each sample was diluted with 5 mM PB (pH 6.8) in the ratio 1:9 (v/v) before measuring. For the determination of the size, disposable cuvettes (Sarstedt AG & co. kg, Nümbrecht, Germany) were used, the measurements were done in triplicates and the average value was calculated. For the zeta potential, the instrument uses a combination of laser Doppler velocimetry and phase analysis light scattering (PALS) technology to measure the electrophoretic mobility of the particles and calculate the zeta potential. The Smoluchowski approximation was used. The cells used for the analysis were disposable folded capillary cells (Malvern Instruments Ltd, Worcestershire, UK). The average value of five measurements of each sample was used.

2.7 Experimental design

2.7.1 **Part 1: The one-pot vs the thin-film method**

SoyPC was used as the main lipid for the experiments. SoyPC is generally regarded as a neutral lipid and the value of the zeta potential is normally between -2 and -1.3 mV [15, 17, 31]. The liposomes prepared from only SoyPC will therefore be referred to as neutral liposomes. DOTAP was giving the liposomes a positive charge for further complexation with alginate, and Egg PG was giving the liposomes a negative charge for complexation with chitosan.

The compositions of all the liposomal formulations are shown in table 1.

In Part 1, the aim was to investigate the possibility of preparing stable polymer-liposome complexes using the one-pot method. Polymer-liposome complexes were also prepared by the

thin-film method for comparison. SoyPC mixed with 10 mol% DOTAP was complexed with alginate, and SoyPC mixed with 10 mol% Egg PG was complexed with chitosan. SoyPC alone (0 mol% DOTAP) was prepared and complexed with alginate. The prepared polymer-liposome complexes were characterized and tested for stability during storage.

2.7.2 **Part 2: Influence of the amount of charged lipid on the stability of the polymer-liposome complexes prepared by the one-pot method.**

In part 2, the aim was to investigate if the concentration of the charged lipid affected the stability of the polymer-liposome complexes prepared by the one-pot method. SoyPC mixed with either 5 mol% or 20 mol% DOTAP was complexed with alginate (see table 1). For complexation with chitosan, SoyPC was mixed with either 5 mol% or 20 mol% Egg PG. The samples were characterized and tested for stability during storage.

2.8 Visualization of the stable polymer-liposome complexes

Transmission electron microscopy (TEM) and atomic force microscopy (AFM) pictures were taken of selected samples.

Sample preparation for TEM: a drop of approximately 25 microliters of sample was deposited on parafilm and a glow discharged 300 mesh copper grid with formvar film was placed on top for 5 minutes. Subsequently, the grids were washed 6 times 1 minute on drops of milli-Q® water, then a drop of 2% Uranyl Acetate (UA) was deposited on parafilm and the grid with adhered sample was placed on top (sample facing the drop of UA) for 1 minute and air-dried. Images were taken in a JEOL 1400plus TEM (Jeol USA Inc., Peabody, MA, USA) equipped with a Ruby camera at 120 kV.

Sample preparation for the AFM: The sample (0.6 mM lipid+ 0.08% alginate) was diluted 1:10 with 5 mM PB pH 6.8. Ten microliters of the diluted sample were applied onto freshly cleaved mica. After 10 seconds adsorption time, the excess liquid was removed with a filter paper and the sample was allowed to air-dry at room temperature to the next day. AFM imaging was performed using the NanoWizard AFM (JPK Instruments AG, Berlin, Germany). The stage was mounted onto an inverted optical microscope (Nikon Eclipse TE2000-S) placed on an antivibration table (Halcyonics MOD-1M, Accurion GmbH, Germany). For imaging, HQ-NSC35/AlBS Ultrasharp Silicon Cantilevers (MicroMasch, Spain) were used applying tapping mode in air. Z-range was set to 3 micrometers. The cantilever with resonance frequency 150 kHz and force constant 5.4 N/m (given by the manufacturer) was chosen for imaging.

3. RESULTS

3.1 Part 1: The one-pot vs the thin-film method

3.1.1 Size and zeta potential of Alginate-liposome complexes

The results presented in figure 1a show the size and the polydispersity index (PDI) of the naked and the alginate-liposome complexes. The size of the naked SoyPC liposomes was 174.6 ± 2 nm. The sizes of the alginate-liposome complexes prepared from SoyPC (without charged lipids included) by both methods were relatively similar and there was no clear increase in size compared to the naked liposomes. The sizes of the alginate-liposome complexes prepared from the charged lipids (SoyPC-DOTAP 10 mol%), however, were larger compared to the naked liposomes. The alginate-liposome complexes prepared by the thin-film method and subsequent coating were smaller (200 nm) than the polymer-liposome complexes prepared by the one-pot method (240 nm). The size increase after interaction with alginate was much larger for the charged liposomes compared to the neutral.

The zeta potential values can be seen in figure 1b. The surface charge of the neutral lipids before complexation with the polymer was -2 mV. The surface charge became more negative (-25 mV) after complexation with the negatively charged alginate. The SoyPC-DOTAP liposomes had a positive zeta potential before complexation, and the charge reversed to negative after the complexation.

The intensity distribution plots for both the size and zeta potential for the SoyPC liposomes (both naked and complexed with alginate) show a unimodal distribution (supplementary material to figure 1).

3.1.2 Size and zeta potential of Chitosan-liposome complexes

Figure 2 shows the results in terms of size and zeta potential of the chitosan-liposome complexes. The liposomes were prepared from SoyPC and 10 mol% Egg PG. It can be seen from the figure that the sizes increase when the liposomes are complexed with chitosan.

The chitosan-liposome complexes prepared by the thin-film method had a mean size of 293 ± 2.5 nm. Furthermore, the mean size of the chitosan-liposome complexes prepared by the one-pot method was much larger (605 ± 15 nm). It is important to note that the chitosan-liposome complexes prepared by the one-pot method were extruded through an 800 nm membrane instead of the regularly used 200 nm membrane. Experiments showed that extrusion through the 200 nm membrane, a 400 nm membrane and a 600 nm membrane was impossible since the pores became clogged. The extrusion through the 800 nm membrane was done only 5

times as this membrane also began to clog, and some material was observed on the membrane after 5 passages. The labels on the bars in figure 2a denote the PDI of the size measurements (PDI < 0.3) and show that the size distributions were relatively narrow for complexes prepared by both methods.

Regarding the zeta potential values in figure 2b, it can be observed that the charge of the negative liposomes was reversed after complexing with the positively charged chitosan. There was no significant difference between the zeta potentials of the chitosan-liposome complexes prepared by the two methods.

3.1.3 **Stability of the polymer-liposome complexes during storage**

The storage stability of the alginate-liposome complexes and the chitosan-liposome complexes was monitored over several weeks by measuring the size, the PDI and the zeta potential. It is generally accepted that lipid-based drug delivery systems, such as liposomes, are considered homogenous when the PDI value is 0.3 or less [32]. This value was therefore chosen as a cutoff in the present study. The results from the stability study are shown in figure 3.

Alginate-liposome complexes

From figure 3a, it can be seen that the sizes of all the formulations remained unchanged until week 6. At week 7, the formulation composed of charged liposomes complexed with alginate by using the one-pot method (**Alg-Lip$_{pos}$**, filled symbols), became very large and the PDI (not shown) at this point was higher than 0.3. The size distribution intensity plot at week 7 showed two peaks indicating possible aggregation of the vesicles. The same trend was observed for the zeta potential for this formulation (figure 3b). At week 9, it was concluded that the liposomes prepared from neutral lipids and complexed with alginate by the one-pot method (**Alg-Lip$_{neu}$**, filled symbols) were unstable. The size of the vesicles was reduced from an initial mean value of 171 nm to 113 nm and the zeta potential changed from -26 mV to -17 mV. The alginate-liposome complexes prepared by the thin-film method were stable with regards to the size and the zeta potential at least beyond week 12 when the measurements were discontinued.

Chitosan-liposome complexes

The chitosan-liposome complexes prepared by the one-pot method were not included in the stability study. They were deemed unsuccessful since it was generally difficult to extrude them. The chitosan-liposome complexes prepared by the thin-film method (**Chi-Lip$_{neg}$**),

however, were stable beyond week 16 as shown by the size and zeta potential analysis, figure 3.

Naked liposomes

The cationic naked liposomes (**Lip_{pos}**) prepared using SoyPC and 10 mol% DOTAP were stable beyond week 11, whereas the anionic naked liposomes (**Lip_{neg}**) prepared from SoyPC and 10 mol% Egg PG were less stable (figure 3). Measurements performed at week 14 showed a reduction in the mean size from 136 nm to 84 nm. The PDI was 0.2 and the zeta potential was unchanged at -53 mV.

3.2 Part 2: Influence of the amount of charged lipid on the stability of the polymer-liposome complexes prepared by the one-pot method

To investigate the influence of the amount of charged lipid (DOTAP and Egg PG) on the stability, formulations with 5 and 20 mol % charged lipids were included in the study. The results of the initial size, PDI and zeta potential measurements of all the experiments are shown in table 2. The results from these experiments showed that formulations with 5 mol% DOTAP together with SoyPC and complexed with alginate were very large. After extruding the alginate-liposome complexes containing 5 mol% DOTAP through the polycarbonate membrane with pore size 200 nm 10 times, the size was still larger than 1000 nm and the PDI was large and around 0.4. On the contrary, the alginate-liposome complexes with 20 mol% DOTAP had an acceptable size. Looking at the results shown in table 2 this formulation had an initial mean size of 193 nm after interaction with the polymer as against 135 nm for the naked liposomes. The zeta potential of the alginate-liposome complexes was – 52 mV against + 41 mV of the naked liposomes.

The chitosan-liposome complexes containing 5 mol% Egg PG were impossible to extrude, and the membrane pores were immediately clogged. Extrusion was tried both at room temperature (20 °C) and at 40 °C without success. The chitosan-liposome complexes containing 20 mol% Egg PG were also difficult to extrude, even through a membrane pore size of 800 nm. The membrane was clogged after 5 extrusions and there was material retained on the membrane. The analyses of these complexes were discontinued after the initial measurements. On the contrary, naked liposomes using SoyPC and 20 mol% Egg PG were easy to extrude through the 200 nm polycarbonate membrane. The initial mean size of these liposomes was 138 nm with a PDI of 0.1. The zeta potential was – 58 mV.

The results from the stability study of the alginate-liposome complexes and the naked liposomes containing 20 mol% DOTAP are shown in figure 4. Both the polymer-liposome complexes and the naked liposomes were still stable after 16 weeks of storage in the refrigerator when the measurements were discontinued. The results for both the size and zeta potential showed good stability as there were no major changes in their values over time. The size distribution plots (not shown) were unimodal during the test period.

3.3 Visualization of the stable polymer-liposome complexes

Transmission electron microscopy (TEM) and atomic force microscopy (AFM) pictures were taken to visualize the polymer-liposome complexes prepared by both the one-pot and the thin-film method. These results are shown in Figure 5. From the pictures, it can be clearly seen that liposomes were successfully prepared by both methods. Looking at the TEM pictures, the structures have sizes around 200 nm for the alginate-liposome complexes and less than 200 nm for the naked liposomes, though caution must be taken in trying to deduce size from the pictures since sample preparation can affect the sizes of the complexes. Regarding the AFM pictures, the vesicles have “clouds” surrounding them and these clouds may be the polymers covering the surface of the liposomes (see supplementary material to figure 5). No apparent difference between the polymer-liposome complexes prepared by the two methods could be observed.

4. DISCUSSION

The preparation of stable polymer-liposome complexes using the thin-film method followed by coating of the liposomes with appropriate polymers has been reported in several articles [8, 33, 34]. The purpose of this study was to evaluate the possibility of preparing stable polymer-liposome complexes using a new and simpler method, in this paper referred to as the one-pot method. The newly developed method has the advantage of combining the vesicle formation and the complexation of the polymer in a single step. The method allows for the incorporation of the polymers onto the surface and probably also within the core of the vesicles.

Evaluation of using the one-pot method to prepare stable polymer-liposome complexes

In this part of the study, the one-pot method was developed and evaluated by comparing the resulting polymer-liposome complexes with the product produced by the thin-film method, with respect to size, PDI, zeta potential and stability during storage.

One technique of producing polymer-liposome complexes is electrostatic deposition of polymers on the surface of the liposomes of opposite charge. The ratio between the amount of polymer and the liposomes is critical for obtaining complete coverage and successful coating. For finding the optimal concentration of the polymer, different polymer concentrations are normally investigated. The optimal polymer concentrations for alginate and chitosan have been determined in previous studies for the thin-film method [15, 17]. The same concentrations were used in this study.

The zeta potential is important for the stability of the formulations at the time of preparation and during storage. It was observed that the surface charge of the liposomes was changed after complexation with the polymers. This was observed for both the neutral and the charged liposomes and can be interpreted as successful "coating".

For most of the formulations, the size of the liposomes increased after complexation with the polymers, both for the one-pot and the thin-film method. This is in agreement with previously reported studies where complexation with polysaccharides led to size increase [16, 31, 35], although no size change has also been observed [36]. This size increase is due to the polymers covering the surface of the liposomes thereby increasing the hydrodynamic volume.

The size increase of the polymer-liposome complexes prepared from the neutral lipids was less than from the charged lipids. This can be explained by the fact that there are more attractive forces between the charged polymers and the charged lipids thereby leading to improved ionic interactions. In fact, there was no size difference between the neutral liposomes before coating with polymer and those of the alginate-liposome complexes prepared by the one-pot method. Probably this is an indication that only small amounts of polymer are covering the surface of these neutral liposomes. However, the zeta potential of the neutral liposomes before complexation with alginate was ≈-2 mV and this changed to ≈-25 mV after complexation with the polymer. Although no size increase, the change in zeta potential indicates some type of interaction between the neutral lipids and alginate. It has previously been reported that adsorption of polyelectrolytes on vesicles is possible even if the polyelectrolytes and the vesicles have the same charge sign, but the amount adsorbed would be less [18]. The size distribution by intensity and the zeta potential distribution plots (supplementary material to figure 1) of the polymer-liposome complexes from the neutral lipids showed unimodal distributions, which implies homogenous species.

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The liposome complexes prepared from the charged lipids (SoyPC-DOTAP 10 mol%) markedly increased in size after complexation with the polymers, and the size increase was more pronounced than observed for the neutral lipids. Also, the size increase for the alginate-liposome complexes from the one-pot method was larger than from the thin-film method. The reason could be that the interaction of polymers with lipids results in different structures when prepared by the two different methods. In the one-pot method the polymer is present during the hydration step of the lipid film, whereas in the conventional method the polymer interacts with preformed liposomes. The zeta potentials on the other hand were relatively similar (≈ -45 mV for one-pot and ≈ -53 mV for thin-film).

The liposomes complexed with chitosan showed a much larger size increase after complexation compared to the alginate-liposome complexes, with a large size increase for the one-pot complexes. The large size of these complexes was expected as they were extruded through an 800 nm pore membrane instead of 200 nm. Also, the larger size of the thin-film chitosan complexes compared to the thin-film alginate complexes was expected due to the different structure and charge properties of these polymers. Alginate in solution is expected to have an extended conformation being able to adsorb flat on the surface of the liposomes (so called “trains”) whereas chitosan is more bulky and do not pack densely on the surface of the liposomes (“loops” and “tails”) hence the bigger sizes [16, 37]. The zeta potential of the chitosan-liposome complexes changed from -54 mV to +16 mV after complexation with the polymer which can be interpreted as enough for covering the surface of the liposomes.

Stability of the polymer-liposomes complexes.

It has been shown in previous studies that liposomes prepared and coated subsequently with different polymers including alginate and chitosan by the thin-film method using 0.6 mM lipid with 10 mol% charged lipids, 0.08 % (w/v) polymer in 5 mM PB, pH 6.8 will produce stable liposomes [15, 17]. This combination was also used in this study and the findings confirmed the previously reported results for the thin-film method. Looking at the results presented in figure 3a at week seven, the alginate-liposome complexes with 10 mol% charged lipids prepared by the one-pot method, started to aggregate as shown by size distribution analysis. This is an indication that the amount of charged lipids used was not optimal and therefore the preparation of stable liposomes through the one-pot method was unsuccessful. It can be considered that since it took weeks for aggregation to occur, the process is probably not bridging flocculation [38]. The alginate-liposome complexes prepared by the one-pot method using neutral lipids had their mean size markedly reduced at week nine, probably due

to either disintegration of the vesicles and/or dislodging of the polymer from the liposomal surface.

On the other hand, the alginate-liposome complexes prepared from the neutral and the cationic liposomes by the thin-film method were stable during the investigation period since there were no major changes in size, PDI and zeta potential. The naked cationic liposomes (SoyPC-DOTAP 10 mol%) were also stable throughout the period of investigation.

The chitosan-liposome complexes prepared using SoyPC and 10 mol% Egg PG by the thin-film method were stable during the period of the investigation. However, the naked anionic liposomes (SoyPC-Egg PG 10mol%) seemed to be less stable since the size decreased. This may indicate that chitosan tends to prevent the liposomes from degrading and increases the long-term stability.

Influence of the amount of charged lipid on the stability of polymer-liposome complexes by the one-pot method

To further investigate the influence of the amount of charged lipid on the stability of formulations prepared by the one-pot method, new polymer-liposome complexes were prepared with 5 mol% and 20 mol% charged lipids in combination with neutral SoyPC. The new polymer-liposomes complexes showed a marked difference in storage stability when compared to the alginate-liposome complexes with 10 mol% DOTAP. While formulations with 10 mol% DOTAP were stable for between 6 and 8 weeks (figure 3), the formulations with 20 mol% DOTAP were stable during the four months period when the stability study was performed (figure 4). The naked liposomes with 20 mol% DOTAP were stable for the whole period as well. The formulations with 5 mol% DOTAP, however, were unstable and investigations were discontinued. This clearly shows that the amount of charged lipid is critical, also for the one-pot method and that the two preparation methods have different optimal amounts of charged lipids. The main purpose of adding charged lipids to SoyPC is to confer a particular charge to the liposome surface to promote interaction with an oppositely charged polymer. A higher amount of charged lipid is expected to provide more contact points for interaction on the liposomal surface and improve the complexation. Considering that 10 mol% DOTAP was sufficient for preparing stable polymer-liposome complexes by the thin-film method while 20 mol% was required for the one-pot method indicates that the one-pot method craves higher concentration of charged lipid to produce stable complexes compared to the thin-film method. By looking at the two methods and how the formation of liposomes and

complexation with the polymer take place, it is obvious that for the thin-film method all charged lipids are part of the liposomal double layer structure since the liposomes are formed first and then complexed with the polymer. By the use of 10 mol% charged lipids, the amount of charged lipid on the outside of the liposomes will be enough to prepare a stable polymer-liposome complex. When it comes to the one-pot method, the lipids and the polymer are mixed before the formation of the liposomes. It might be assumed that some of the charged lipids try to neutralize some of the alginate chains thereby reducing the amount of charged lipids being part of the liposomal bilayer. More charged lipids are, then, needed to prepare a stable polymer-liposome complex, since some of the charged lipids are tied into the interaction with alginate. A similar mechanism has been seen for preparation of mRNA and lipids being encapsulated into liposomes[39]. The charged lipids neutralize the charge on the mRNA. Even though the alginate chain is longer and has a more complex structure than mRNA it is reasonable to assume that this can also happen in the preparation of polymer-liposome complexes. These alginate-lipid complexes will then be encapsulated in the core of the liposomes. This hypothesis is supported by the fact that both the size (~200 nm) and the zeta potential (~ -50 mV) of the complexes prepared from both methods are similar implying that the structure of the complexes are similar even though prepared by different methods, which was also supported by the TEM and AFM pictures.

For the chitosan-liposome complexes, formulations prepared using either 10 mol% or 20 mol% Egg PG by the one-pot method, were difficult to extrude and considered unsuccessful and therefore storage stability could not be investigated. Chitosan is a very large molecule compared to alginate and can be considered a “bulky” molecule. This implies that chitosan, when complexed with charged lipids, can physically clog the pores of the polycarbonate membranes used for the extrusion. Therefore, the results presented for the chitosan-liposome complexes using the one-pot method can be considered as a feasibility study, with the conclusion that extrusion seems not to work well for reducing the sizes of the multilamellar vesicles.

The chitosan-liposome complexes prepared by the thin-film method using SoyPC with 10 mol% Egg PG were stable during the whole period of investigation.

5. CONCLUSIONS

The study has shown that alginate-liposome complexes were successfully prepared using the newly developed and improved one-pot method. The alginate-liposome complexes containing

20 mol% DOTAP were the most stable and were stable for more than four months. The production of chitosan-liposome complexes using both 10 mol% and 20 mol% Egg PG was unsuccessful. The alginate-liposome complexes prepared from SoyPC with 10 mol% DOTAP were stable for a short duration i.e.,6-8 weeks. The use of 5 mol% charge lipids was found to be insufficient for preparing stable polymer-liposome complexes for both alginate and chitosan. In comparison, the alginate-liposome and the chitosan-liposome complexes prepared by the thin-film method using 10 mol% charged lipids (DOTAP or Egg PG) mixed with SoyPC were stable for at least 12 weeks. This study has shown that the one-pot method is simple and gentle, has few steps, and can be used for complexing alginate with liposomes. Such polymer-liposome complexes have the potential to be used in formulation of drugs intended for local delivery to the mouth, eye, skin, or vagina especially in the delivery of rehydration substances. Regarding the use of chitosan with intermediate molecular weight i.e., 310 kDa in preparing the chitosan-liposome complexes, however, other methods for size reduction should be investigated.

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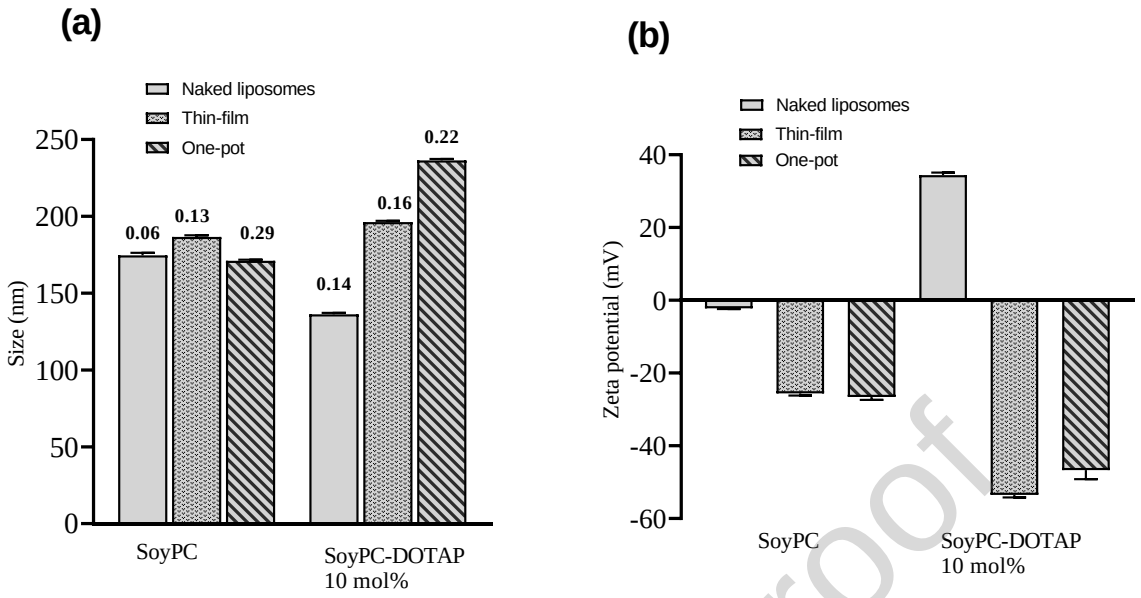

Figure 1. Naked liposomes and alginate-liposome complexes prepared by the one-pot and the thin-film method. (a) Size and (b) zeta potential measured 1 day after preparation. The error bars denote standard deviations of the size measurements (n = 3), the zeta potential measurements (n=5) and the number labelling above the bars in (a) denote the PDI of the size measurements.

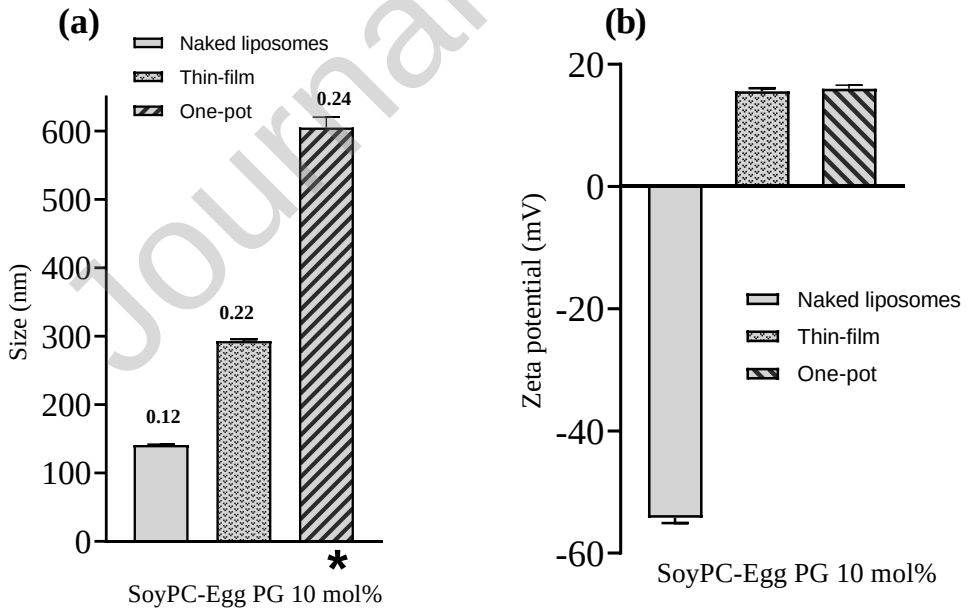

Figure 2. Naked liposomes and chitosan-liposome complexes prepared by the one-pot and the thin-film method. (a) Size and (b) zeta potential measured 1 day after the preparation. The error bars denote standard deviations of the size measurements (n = 3), zeta potential measurements (n=5) and the number labelling above the bars in (a) denote the PDI of the size measurements.

* Extruded through 800 nm polycarbonate membranes.

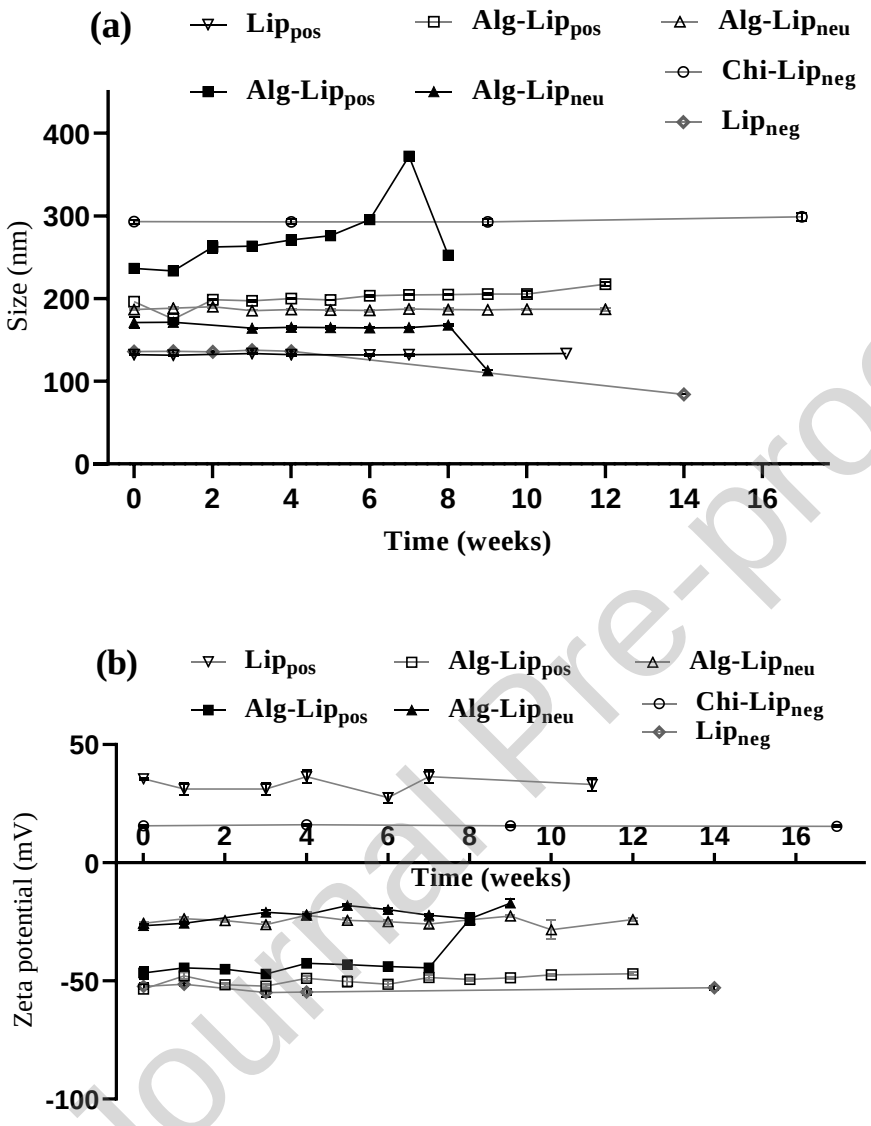

Figure 3. Stability of alginate-liposome complexes prepared by the one-pot (filled symbol) and thin-film (open symbol) method. (a) Size and (b) zeta potential of alginate-liposome and chitosan-liposome complexes measured over several weeks during storage in refrigerator. The error bars denote standard deviations (n=3 for size and n=5 for zeta potential) and the points without error bars have values that are equal to or less than the markers. Alg-Lip$_{pos}$ = alginate-liposome complexes prepared from SoyPC with 10 mol% DOTAP, Alg-Lip$_{neu}$ = alginate-liposome complexes prepared from neutral SoyPC, Lip$_{pos}$ = naked liposomes prepared from SoyPC with 10 mol% DOTAP, Lip$_{neg}$ = naked liposomes prepared from SoyPC mixed with 10 mol% Egg PG. Chi-Lip$_{neg}$ = chitosan-liposome complexes prepared from SoyPC mixed with 10 mol% Egg PG.

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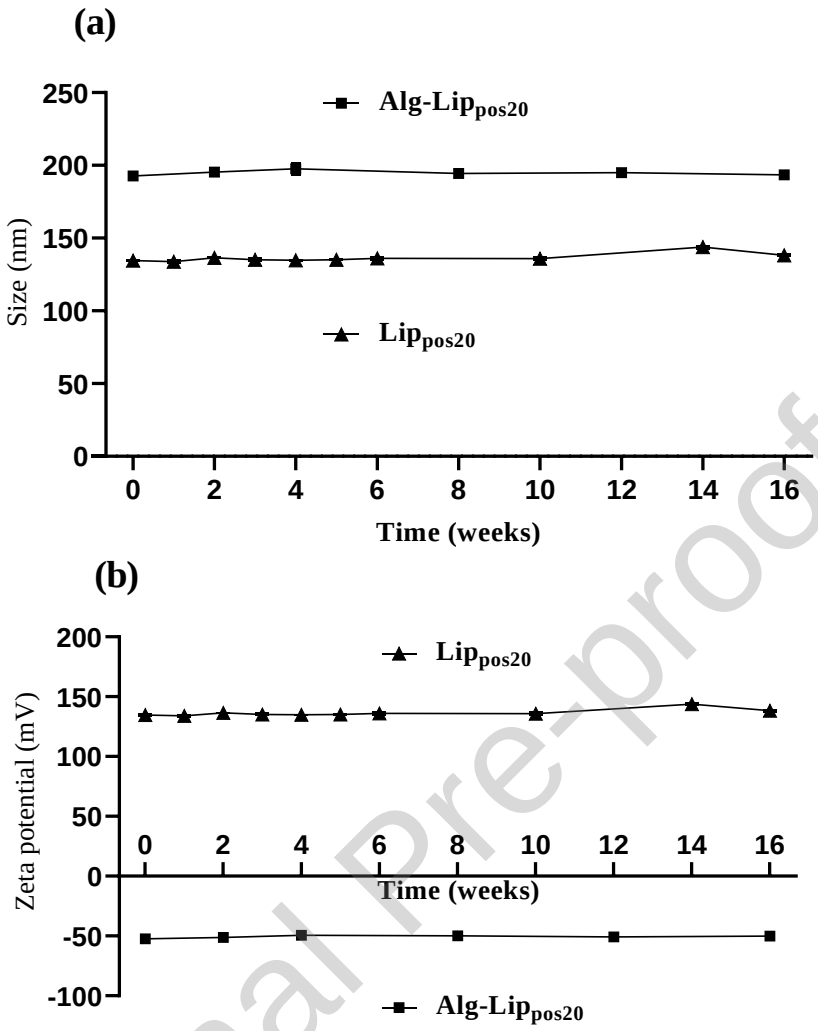

Figure 4. Stability of liposomes prepared by the one-pot method using SoyPC with 20 mol% DOTAP as charged lipid. (a) Size and (b) zeta potential of naked liposomes and alginate-liposome complexes. The error bars denote standard deviation (n=3 for size and n=5 for zeta potential) and the points without bars have values that are equal to or less than the markers. Lip_{pos20} = naked liposomes prepared from SoyPC with 20 mol% DOTAP. Alg-Lip_{pos20} = alginate-liposome complexes with SoyPC mixed with 20 mol% DOTAP.

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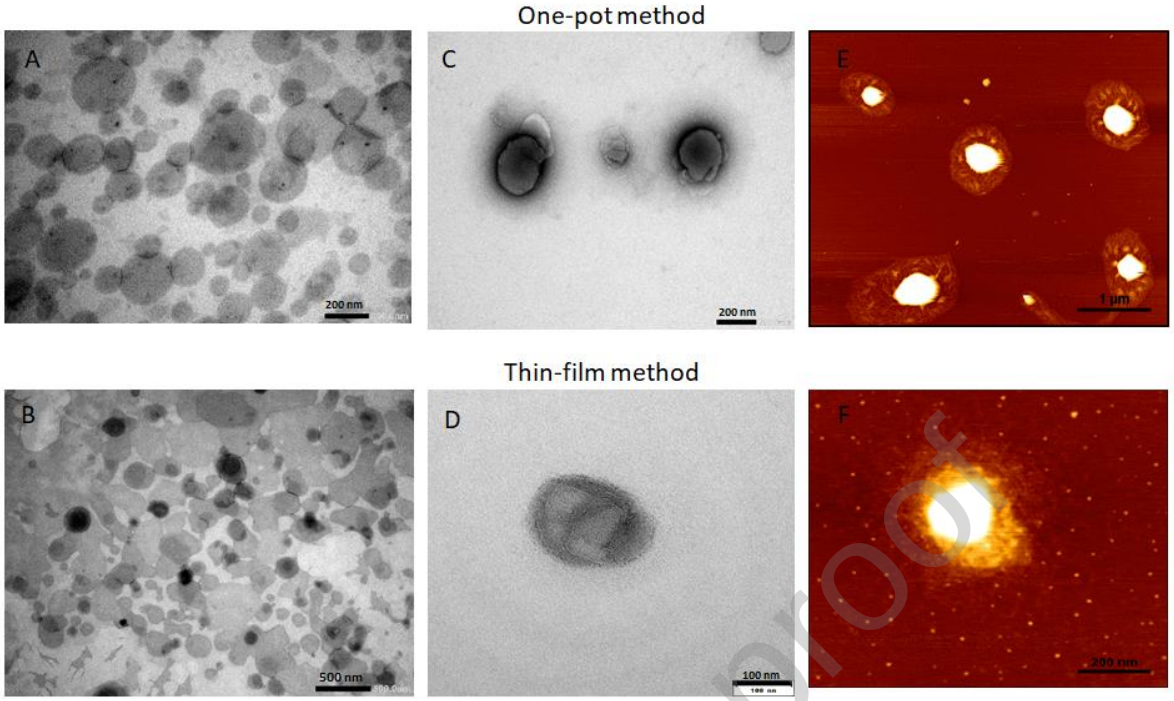

Figure 5. TEM (A-D) and AFM (E-F) pictures of naked liposomes and alginate-liposome complexes. One-pot method: Naked liposomes (SoyPC+DOTAP 20 mol%) (A), complexed with alginate (C, E). Thin-film method: Naked liposomes (SoyPC + DOTAP 10 mol%)(B), complexed with alginate (D, F)

Graphical abstract

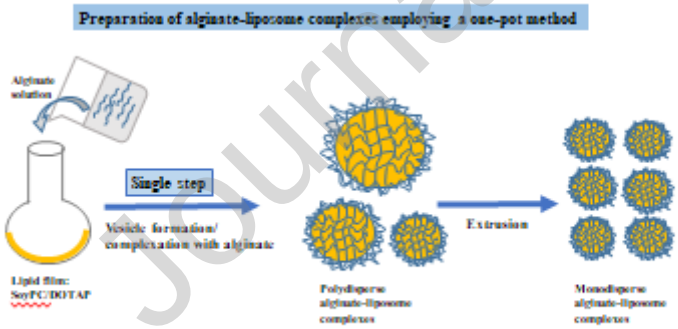

Table 1. The compositions of the liposomal samples.

Sample composition	Method	Charge	Amount of charged lipid (mol%)
Part 1			
SoyPC liposomes (naked)	Thin-film	Neutral	0 %
Alginate coated SoyPC liposomes	Thin-film	Negative	0 %
Alginate-SoyPC-liposome complexes	One-pot	Negative	0 %

SoyPC-DOTAP liposomes (naked)	Thin-film	Positive	10 %
Alginate coated SoyPC-DOTAP liposomes	Thin-film	Negative	10 %
Alginate-SoyPC-DOTAP liposome complexes	One-pot	Negative	10 %
SoyPC-Egg PG liposomes (naked)	Thin-film	Negative	10 %
Chitosan coated SoyPC-Egg PG liposomes	Thin-film	Positive	10 %
Chitosan-SoyPC-Egg PG liposome complexes	One-pot	Positive	10 %
Part 2			
Alginate-SoyPC-DOTAP liposome complexes	One-pot	Negative	5 %
Chitosan-SoyPC-Egg PG liposome complexes	One-pot	Positive	5 %
Alginate-SoyPC-DOTAP liposome complexes	One-pot	Negative	20 %
Chitosan-SoyPC-Egg PG liposome complexes	One-pot	Positive	20 %

Table 2. Initial size (n=3), PDI and zeta potential values (n=5) of samples in part 2.

Sample composition	**Amount of charged lipid (mol%)**	**Size ± SD (nm)**	**PDI**	**Zeta potential ± SD (mV)**
Part 2				
SoyPC-DOTAP	20	135 ± 1	0.1	+41 ± 1
SoyPC-Egg PG	20	138 ± 0.3	0.1	-58 ± 1
Alginate-SoyPC-DOTAP liposome complexes	5	1007 ± 99	0.4	-39 ± 1
Chitosan-SoyPC-Egg PG liposome complexes	5	-	-	-
Alginate-SoyPC-DOTAP liposome complexes	20	193 ± 1	0.2	-52 ± 1
Chitosan-SoyPC-Egg PG liposome complexes*	20	704 ± 12	0.2	+16 ± 1

*Extruded through 800 nm pore membranes 5 times instead of 10 times.

Contributions:

Joseph Azumah: Methodology, Investigation, Formal analysis, Validation, Writing - Original Draft, Writing - Review & Editing, Visualization. Gro Smistad: Conceptualization, Methodology, Visualization, Supervision, Writing - Review & Editing. Marianne Hiorth: Conceptualization, Methodology, Supervision, Writing - Review & Editing, Project administration.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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