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Alginate-Loaded Liposomes Can Protect Encapsulated Alkaline Phosphatase Functionality When Exposed to Gastric pH

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The present study has evaluated the potential of alginate-loaded liposomes as a vehicle for the oral delivery of bioactive proteins. The vesicles were prepared from the phospholipid dipalmatoyl phosphatidylcholine using a simple dry film hydration technique. Alkaline phosphatase (ALP) was used as a model bioactive protein, which was encapsulated in alginate-loaded liposomes and conventional liposomes. These vesicles were subject to physical characterization and stability analysis at varying pH values. The enzymatic activity of the ALP was evaluated following exposure to simulated gastric pH. The alginate-loaded liposomes were typically of the order of 10 μm; however, there was evidence of vesicle aggregation thought to be due to alginate present on the surface of the vesicles. The typical size of the aggregated vesicles was ~30 μm. The enzyme activity of APL following 2 h of exposure to simulated gastric pH was maintained at a significantly higher level when encapsulated in the alginate-loaded liposomes as compared to ALP loaded in conventional liposomes [80% as compared to 55% ($p < 0.05$), respectively].

KEYWORDS: Alginate; liposomes; alkaline phosphatase functionality; gastric pH; oral

INTRODUCTION

Developing foods that contain therapeutic macromolecules would benefit the food industry and consumer alike. However, delivering active proteins and peptides in full working order to the target site is frequently limited by processing and physiological barriers of the gastrointestinal tract (GIT). This is due to the functionality of such proteins often relying on structural conformation, which is easily denatured by organic solvents, high temperature, and extremes of pH (*1*, *2*). This fragile nature hinders the incorporation of functional bioactives into foods, as often proteins are denatured during production or on contact with the gastric fluid once ingested.

One way of overcoming these problems is by encapsulation, which can stabilize bioactives during processing and storage, provide protection from low pH and enzymatic degradation once ingested, and control the release of the entrapped bioactive at the desired target site. Liposomes (phospholipid vesicles) are one such platform used in encapsulation and controlled release and have been successfully utilized in pharmaceuticals (*3*, *4*), cosmetics (*5*), and food production (*6*, *7*). Recently, liposomes have been used to deliver flavors and nutrients within foods (*8*) and have also been investigated for their ability to incorporate food antimicrobials (*9*).

As with the delivery of proteins, stability problems in the GIT are the major drawback associated with the use of liposomes orally (*10*). This instability arises from large fluctuations in pH and the presence of various lipases and bile salts in gastric and intestinal fluid, all of which can destabilize liposomes (*11*). However, fully functional peptides and proteins, which are denatured by physiological conditions in the GIT, have been reported to be taken up by Peyer's patches in the intestine when encapsulated in liposomes, although only in very low concentrations (*12*).

There have been efforts to overcome the problems of instability in liposomes designed for oral consumption by coating the liposomes in mucoadhesive polymers (*13*−*16*) or by preparing liposomes with a gel core (*17*−*20*), whereby a gel-forming polymer is entrapped in the core of liposomes and subsequently cross-linked in situ. Indeed, Monshipouri and Rudolph (*19*) produced alginate beads within the core of large unilamellar vesicles (LUV) prepared from dipalmatoyl phosphatidylcholine (DPPC) and looked at the release profile of encapsulated cytochrome *c* and found that there was initially rapid release of cytochrome *c* for both preparations with slower subsequent release rates for the liposomes with encapsulated alginate beads (*21*). These methods of encapsulation are of interest when approaching the problem of formulation and delivery of bioactive proteins. The mild formulation procedure involves ambient temperatures, no organic solvents, or high shear, which may denature and inactivate the protein, often a problem when preparing gel microparticles. Therefore, using liposomes as controllable reaction vessels for creating gel beads could potentially overcome harsh processing conditions involved in conventional preparation. Moreover, control of particle size is entirely regulated by the size of liposomes prepared, which can range from the nanoscale up to ~100 μm,

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depending on the method of preparation. Incorporating a gel core into liposomes offers further tailorability of the delivery system and minimizes diffusion by providing a second layer of protection. It is also possible to select a polymer to be incorporated that has physiologically responsive properties that are triggered at a specific target site. While these studies have reported positive results, they have not investigated the protective nature of the particles on the structure-dependent functionality of bioactives.

Alginate is a well-characterized biopolymer that forms a gel when exposed to divalent cations and has been used previously as a matrix for immobilizing enzymes (*21*, *22*), for oral delivery of therapeutic proteins (*22*), and frequently in foods. Besides the mild cross-linking process, the acid insolubility of alginate gels makes it a good candidate for incorporation into liposomes to enhance stability in the stomach and to dissolve in the intestine, releasing the encapsulated contents. In this study, we have encapsulated an enzyme (alkaline phosphatase, ALP) within alginate gel core liposomes and evaluated the encapsulation efficiency, stability when stored at various pH values, and enzyme activity following exposure to simulated gastric pH.

MATERIALS AND METHODS

Materials. The neutral lipid DPPC was purchased from Avanti Polar Lipids, and the sodium alginate was from FMC Biopolymer (Norway) isolated from *Laminaria hyperborea* and was high in guluronate residues. All other reagents were purchased from Sigma-Aldrich Co. (Poole, United Kingdom).

Preparation of Liposomes. Multilamellar vesicles (MLVs) were prepared using the dry lipid film hydration method first developed by Bangham et al. (*23*). Briefly, DPPC was dissolved in a 9:1 solvent mixture of chloroform and methanol. The solvent was evaporated on a rotary evaporator to obtain a dry film, which was hydrated with a solution of ALP in 10 mM Tris HCl buffer, pH 7.4 (to give a final lipid concentration of 32 μmol/mL) in a round-bottomed flask at 45 °C with intermittent vortexing. Unentrapped ALP was removed from the liposome suspension by repeated centrifugation, decanting of the supernatant, and washing of the pellet with 10 mM Tris HCl until no ALP was detected in the supernatant.

Preparation of Gel-Loaded Liposomes. Liposomes loaded with sodium alginate and the enzyme ALP were prepared using the thin lipid evaporation method described previously and were hydrated with a mixture of 1% (w/v) sodium alginate containing 10 mg/mL ALP. Following removal of unentrapped alginate/ALP, the gel-loaded liposomes were suspended in 200 mM $CaCl_2$ for 30 min to induce gelation of the entrapped alginate. The diffusion of Ca^{2+} across the liposome bilayer was facilitated by increasing the temperature of the liposome suspension to the phase transition temperature (T_m) of DPPC (42 °C), which has the effect of increasing bilayer permeability (*17*). The $CaCl_2$ was then removed by centrifugation, and the liposomes were resuspended in 10 mM Tris HCl.

Sizing and ζ-Potential Measurements of Liposomal Dispersions. The volume mean diameter of the liposomes was measured using a Mastersizer (Malvern Instruments, Malvern, United Kingdom) at 25 °C by diluting 30 μL of the dispersion to the appropriate volume with double-distilled water. The ζ-potential, which is an indirect measurement of the liposome surface charge, was measured in 1 mM Tris HCl, pH 7.4, using a Zetamaster (Malvern Instruments).

Quantification of Entrapment. The concentration of entrapped ALP was determined using the bicinchoninic acid (BCA) assay (Sigma). Quantification of protein in aqueous solutions using this assay was determined by the extent of reduction of Cu^{2+} to Cu^{+}, which was measured colorimetricly using a UV/vis spectrophotometer at 562 nm.

Catalytic Activity of ALP. The catalytic activity of ALP was determined using an ALP activity kit (ABD Bioquest, CA). Briefly, *p*-nitrophenyl phosphate acted as the substrate, which was hydrolyzed by ALP releasing the chromomeric *p*-nitrophenol, which was measured using a UV/vis spectrophotometer at 450 nm. To quantify the protective effect of encapsulation, samples of ALP-loaded liposomes, gel core liposomes, and naked ALP were exposed to simulated gastric pH (pH 2) for up to 2 h. At selected time points, samples were taken, and the pH was raised to pH 8 by the addition of 1 M NaOH. The ALP was then released from the liposomes and gel core liposomes by treatment with 2% w/v sodium dodecyl sulfate and 2% w/v sodium citrate. The catalytic activity of the released ALP was then measured as a function of time exposed to pH 2. To confirm complete particle dissolution and hence total release of ALP, the samples were evaluated using a light microscope. The quantity of ALP released during incubation at pH 2 was also monitored. Samples were taken over a 2 h period and separated from the incubation media by centrifugation. The ALP content in the supernatant was then quantified using the BCA assay. The measured quantity of ALP in the supernatant was taken as the amount of ALP released at pH 2 as a function of time.

Table 1. Vesicle Size and ALP Loading in MLV Liposomes and Gel Core Liposomes

vesicle type	vesicle size (μm)	ALP entrapment (%)
MLV liposomes	13.4 ± 0.1	0.97 ± 0.10
gel core liposomes	30.8 ± 3.1	1.24 ± 0.51

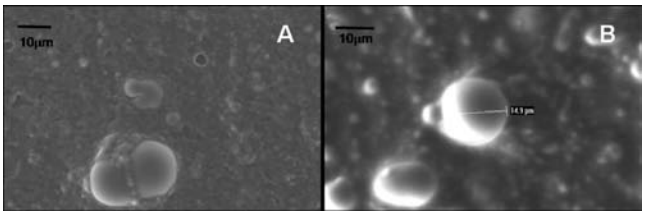

Figure 1. ESEM micrograph of (**A**) gel core liposomes and (**B**) conventional liposomes.

Stability Study. Samples of liposomes and gel core liposomes loaded with ALP were prepared and stored in 10 mM Tris HCl maintained at pH 3.8, 7.4, and 10 at 4 °C. The particle size of the samples was measured (using the method described above) during storage at selected time intervals up to 10 days.

Environmental Scanning Electron Microscopy (ESEM). The external morphologies of the liposomes and the gel core liposomes were visualized using ESEM. The internal morphologies of the liposomes were analyzed by Cryogenic ESEM (Cryo-ESEM). All ESEM analyses were performed with a Philips XL30 ESEM-FEG. The accelerating voltage was 3 kV, and the working distance was 5 mm. Sample preparation for the Cryo-ESEM was performed as follows: Liposome samples were frozen in liquid nitrogen slush (−196 °C) under vacuum. The samples were then fractured and coated with a thin layer of gold (approximately 10 nm) to make the sample conductive. Finally, each sample was placed on a stage cooled to around −140 °C to be imaged.

Statistical Analysis. Comparison of means was conducted using a one-way analysis of variance with a posthoc Tukey (HSD) test to identify significant differences ($p < 0.05$) between data sets.

RESULTS AND DISCUSSION

In an effort to protect the activity of ALP for the purpose of oral delivery, we dissolved the enzyme in a solution of alginate, encapsulated this mixture in liposomes, and then cross-linked the alginate by addition of $CaCl_2$ to the liposome suspension, creating liposomes with a cross-linked gel core. These particles were characterized and compared to liposomes prepared without alginate using particle size analysis, ESEM and Cryo-ESEM, entrapment efficiency, ζ-potential, and activity of the encapsulated enzyme following exposure to simulated gastric pH.

Particle size analysis of the ALP-loaded liposomes and gel core liposomes revealed a large difference in mean particle size. The gel core liposomes had over twice the average particle size diameter than that of the standard MLVs: 30.8 ± 3.1 and 13.4 ± 0.1 μm, respectively (**Table 1**). Entrapment, however, showed no significant difference ($p > 0.05$) between the vesicle types. When analyzed using ESEM, it was apparent that vesicle aggregation had occurred in the gel core liposome sample (**Figure 1A**) with the

individual particle size in the region of 10−15 μm, which is in the same order of size as the standard liposomes, in which aggregation did not appear (**Figure 1B**). This is likely to be due to alginate present on the surface of the liposomes entangling with adjacent liposomes prior to cross-linking; then, once cross-linking is initiated by the addition of calcium, gelation occurs, causing adhesion of the particles. The use of ESEM for studying changes in liposome morphology has been used previously, which confirmed the utility of this technique in providing a real-time alternative assay for liposome stability and formulation (*24*).

To determine whether any alginate and/or ALP were present on the surface of the liposomes, standard liposomes and gel core liposomes loaded with ALP were subjected to ζ-potential measurements, as indirect measurements of surface charge (*25*) were performed on liposomes loaded with ALP and gel core liposomes loaded with ALP before and after cross-linking. These measurements were also performed on samples without ALP. **Figure 2** shows that liposomes containing ALP and/or un-cross-linked alginate have a negative ζ-potential; however, when the alginate is cross-linked, the surface charge becomes positive. Interestingly, the cross-linked liposomes containing ALP gave a significantly greater ζ-potential ($p < 0.05$) than when there was no ALP encapsulated, 15.75 ± 1.28 and 12.38 ± 1.38 mV, respectively.

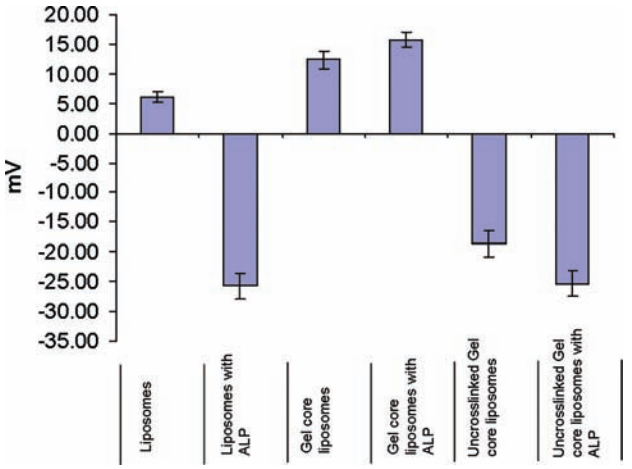

Figure 2. Measurements of ζ-potential for samples of liposomes and gel core liposomes (cross-linked and un-cross-linked) with and without ALP.

These measurements support the presence of alginate on the surface of the vesicles, as samples of gel-loaded liposomes gave a negative ζ-potential, indicating the presence of negatively charged sodium alginate. When the gel-loaded liposomes were cross-linked using calcium, the vesicles became positively charged due to the Ca^{2+} ions suppressing the negative charge of the alginate. The presence of ALP, which has an overall net negative charge (*26*), affected the ζ-potential on the standard liposomes, which indicated that there may be some protein incorporated in the external lipid bilayer. The binding of protein within the bilayers of liposomes is a well-known phenomenon and occurs due to favorable electrostatic interactions between the protein and the phospholipid (*27*). When ALP was incorporated with the cross-linked gel core liposomes, there was an increase in ζ-potential, which was surprising due to the overall net negative charge of the protein. ALP, however, is known to act as a calcium binding protein (*28*); therefore, the addition of Ca^{2+} as a cross-linker for the alginate may have also been bound to the ALP, shifting the overall charge of the ALP from negative to positive.

Further evidence of alginate incorporated onto the surface of the gel-loaded liposomes was revealed in the Cryo-ESEM micrograph given in **Figure 3A**, which shows the surface of a gel-loaded liposome having small solid sections upon the surface, thought to be cross-linked alginate. The presence of several thick layers is also evident when cross-sectioned (**Figure 3B**), indicating that gel is incorporated throughout the many lamellae of the liposome. Clusters of cross-sectioned gel-loaded liposomes are shown in **Figure 3C**, further indicating aggregation. Although it is clearly visible that the conventional liposomes are multilamellar as expected for MLVs (**Figure 3E**), the aggregation characteristics and the thick layered lamellae were not witnessed (**Figure 3D−F**); therefore, it can be argued that incorporation of alginate alters the structural characteristics of liposomes, potentially affecting the functional properties. The presence of calcium alginate on the

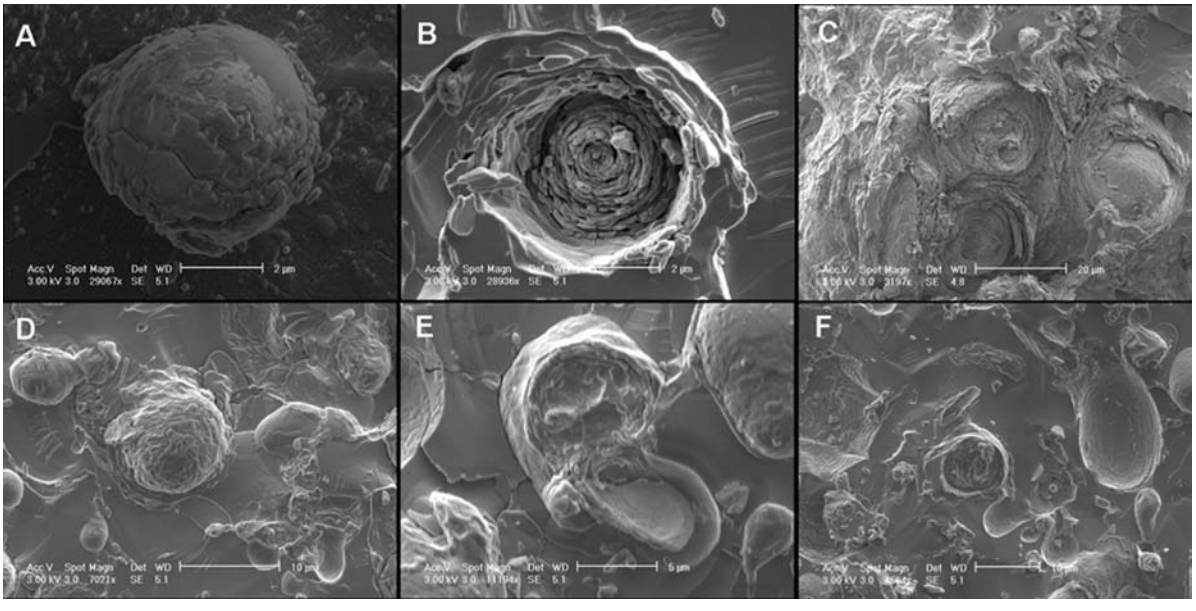

Figure 3. Cryo-ESEM micrographs of (**A**) gel core liposome before fracture, (**B**) cross-section of gel core liposome, (**C**) a cluster of cross-sectioned gel core liposomes showing aggregation, (**D**) ALP-loaded liposome before fracture, (**E**) cross-sectioned ALP-loaded liposome, and (**F**) several cross-sectioned liposomes with no evidence of aggregation.

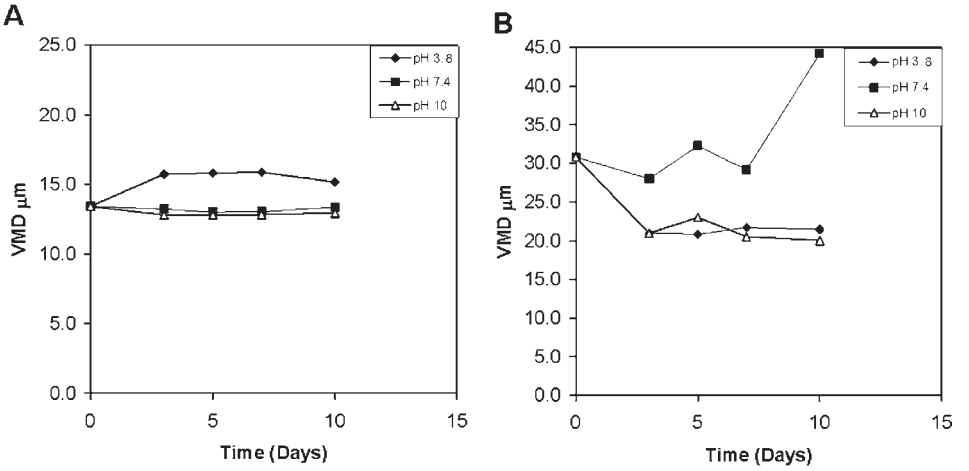

Figure 4. Effect of pH on the size of (**A**) MLVs loaded with ALP and (**B**) gel core liposomes over a period of 10 days.

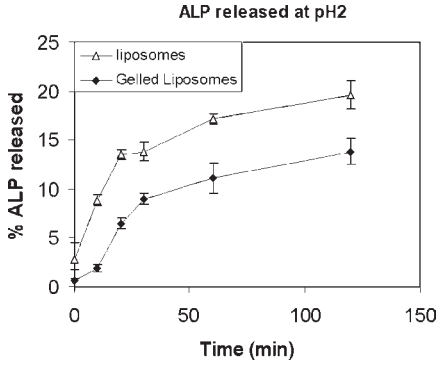

Figure 5. Release profiles of ALP form standard liposomes (open triangles) and gel core liposomes (filled rhombi). Points represent mean values with vertical error bars indicating ± standard deviation, $n = 3$.

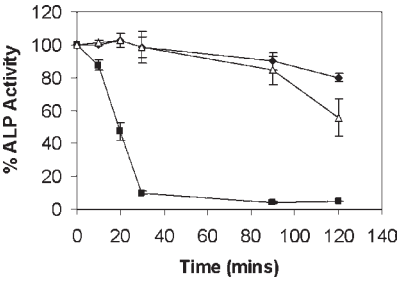

Figure 6. Percent ALP activity retained during 2 h of exposure to simulated gastric fluid, pH 2, for standard liposomes (open triangles), gel core liposomes (filled rhombi), and free ALP (filled squares). Points represent mean values with vertical error bars indicating ± standard deviation, $n = 3$.

surface of alginate-loaded liposomes has been hypothesized previously by Monshipouri and Rudolph (*19*) who used differential scanning calorimetry to indicate a marked reduction in the enthalpy of the main gel to liquid crystalline phase transition of the lipid in MLV and LUV dispersions when alginate was encapsulated in the core (*19*).

The effects of pH on the stability of both vesicle types were analyzed by monitoring vesicle size over a period of 10 days when incubated at pH 3.8, 7.4, and 10 (**Figure 4**). Standard MLV samples showed no dramatic change in mean diameter across the range of pH tested (**Figure 4A**). The gel core liposomes, however, appeared to decrease in size when stored at acidic pH, and at pH 7.4, there was a substantial rise in the mean diameter after 10 days of storage (**Figure 4B**). These results indicate that standard liposome samples remained stable over a range of pH (pH 3.8−10), perhaps showing slight instability at pH 3.8 highlighted by a slight increase in particle size (13−15 μm) (**Figure 4A**). The reduction in particle size of the gel core liposomes at pH 3.8 (below the isoelectric point of ALP but above the pK_a of the negatively charged carboxylate groups on the alginate) was possibly a result of the alginate gel contracting (*29*) as the pH approaches the pK_a of the carboxylate group. When stored at pH 7.4, the increased size of the alginate-containing liposomes following 10 days of incubation may be due to the alginate gel gradually swelling. In addition, as lipid bilayers are dynamic systems that are easily deformed in response to changes in the local environment, it is likely, therefore, that the lipids would have spread to cover the surface during the swelling. This 50% gain in size was similar to what was reported by Martins et al. for alginate beads (*30*).

To determine the protective effect that encapsulation in liposomes and gel core liposomes had on ALP, the loaded samples were exposed to simulated gastric pH (pH 2) for 2 h, and then, the enzyme activity of the encapsulated ALP was evaluated; in addition, the quantity of ALP released by the liposomes and gel core liposomes was also monitored. The release curves in **Figure 5** reveal that the gel core liposomes released 13% of its entrapped ALP as compared with 20% released from the standard liposomes over 2 h. MLVs are expected to be more stable than other liposome types due to the many bilayers surrounding the encapsulated material (*31*); however, the addition of alginate appeared to increase the stability in acid, where 7% less ALP was released from the gel core liposomes when incubated for 2 h at 37 °C at pH 2. This stability was highlighted further by evaluating release over 48 h at pH 2, which revealed that the alginate-containing liposomes retained 70% ALP as compared with 32% retained by the standard liposomes (results not shown), indicating the potential of these particles as sustained release delivery vehicles.

Although it has been shown that ALP was successfully encapsulated and that release in acidic pH was retarded by incorporation of alginate in the liposomes, this does not confirm whether the ALP was still active. In addressing this, an activity assay was performed on the encapsulated ALP during 2 h of exposure to pH 2. **Figure 6** compares the activity of APL in the gel core liposomes with standard MLV and free ALP. As expected, the activity of the free ALP was dramatically reduced on exposure to pH 2; however, both the MLV and the gel core liposomes protect the activity of ALP with no reduction in activity over the first 30 min of exposure. At 2 h, the exposure activity was significantly higher in the gel core liposomes (80% of original

activity) than in the standard liposomes (55% of original activity) ($p < 0.05$). The reduction in activity in the standard liposomes is a possible consequence of degradation of the liposomes or by acid ingress due to the pH gradient across the lipid bilayer. Moreover, evidence from the measurements of ζ-potential (**Figure 2**) of ALP on the surface of the liposomes could also explain the reduction in activity. In the gel core liposomes, the ALP activity was reduced over the 2 h period but at a slower rate than was seen in the standard MLV. This can be explained by the acid-insoluble properties of alginate gels. This acid insolubility, however, is thought not to prevent a reduction in activity over longer periods of time, as acid is gradually imbibed by the gel, forming an alginic acid gel. This reduction in pH within the gel would ultimately reduce the activity of the enzyme.

The tailorability of liposomes and biopolymers such as alginate provided scope for further investigation of these gel-loaded particles as oral delivery vehicles. Properties such as surface charge, particle size, and stability are easily manipulated further by selecting alternative lipids to form the liposomes (e.g., cationic phospholipids), adding secondary processing steps such as extrusion, incorporating a bilayer stabilizers such as cholesterol, and optimizing the preparation methods by evaluating the penetration of Ca^{2+} through the lipid bilayer under different conditions.

In conclusion, alginate was incorporated within the core of liposomes, which dramatically affected liposomal characteristics in terms of both morphology and stability. Incorporating alginate into liposomes appears to improve stability at acidic pH; however, at neutral pH, the alginate causes swelling and aggregation of the liposomes. This study also suggested that alginate was present on the surface as well as in the core of the liposomes. The alginate-loaded liposomes offer potential as a delivery platform for functional proteins, verified by maintaining 80% of the original activity of ALP following exposure to simulated gastric pH for a period of 2 h.

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