Asian Journal of Chemistry Vol. 22, No. 1 (2010), 66-78

Fabrication and Stability Study of Multilayer Liposome

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Multilayer nanocapsules were obtained with layer-by-layer (LBL) technique. Liposomes were employed as templates and obtained through extrusion method. The polymers as shells were biocompatible and biodegradable materials. The obtained multilayer nanocapsules with alginate-chitosan complex were fabricated successfully. The z-potential result confirmed that the different polyelectrolyte layers were absorbed on the liposomes, alternatively. The obtained multilayer liposomes were treated with ethanol [ethanol:water are 1:10 and 1:1 (v/v)] and SDS solution, respectively. The results showed that the obtained capsules were stable to low concentration of ethanol and SDS solution but not stable to high concentration of ethanol solution. Such capsules have potential application in drug delivery system.

Key Words: Adsorption, Aggregation, Separation, Nanocapsules, Mixing, Stability.

INTRODUCTION

Surface modification of materials is one of primary importance for biomedical application[1]. Among the different techniques used to modify surfaces, the deposition of polyelectrolyte multilayers (PEM) has emerged as easy handling and versatile tool[2,3]. The layer-by-layer adsorption of oppositely charged species is an approach which was initially employed to construct multilayer films of polymers, proteins, dyes *etc*. on flat surfaces[2] and recently was used to submicrometer, especially to micrometer-sized charged colloidal particles as the adsorbing surfaces for a wide range of substrates to produce colloid-supported PEM then the core templates were removed and multilayer polyelectrolyte microcapsules are obtained[4,5]. Melamine formaldehyde (MF) particles are mostly used to construct multilayer polyelectrolyte capsules. However, there are several disadvantages *e.g.*, non-biocompatibility, formation of oligomers which remain partly within the shells during the dissolution with time and high cost. Considerable scientific efforts have focused on the fabrication of composite micro- and nanoparticles which consist of either organic or inorganic cores coated with shells. The shells have different chemical composition[4,6-8]. The polymers widely used to construct multilayer polyelectrolyte capsules are sodium poly (styrene sulfonate, sodium salt (PSS) and poly (allylamine hydrochloride)

(PAH). They are synthetic polymers and are not biocompatible or biodegradable, so the application of such multilayer polyelectrolyte capsules in biological system will have limitations[6,7]. To continue the research in this field, many groups are searching for completely biocompatible and sacrificial templates which would yield capsules with definite properties. We joined in this searching and tried to construct multilayer biocompatible hollow nanocapsules. Our interest is in nanocapsule because its size is small, which allows them to be administered intravenously without any risk of embolization and can be considered as a 'reservoir' system. Their cores may be aqueous or composed of lipophilic solvent. Because oil-based nanocapsules are unable to encapsulate water-soluble compounds and nanocapsules with an aqueous core in an oily phase are not suitable for intravenous administration, efforts have been made to prepare nanocapsules with an aqueous core suspending in an aqueous medium. This technology was developed for the efficient encapsulation of water-soluble compounds, which are generally very difficult to include within carrier systems[8,9]. According to this requirement, liposomes were selected as templates because they have various advantages *e.g.*, biodegradable, low toxicity and they can encapsulate a hydrophilic substance within an aqueous environment and lipophilic material within the lipid phase[10].

The charged polymers used in the experiment are chitosan and alginate, respectively. Chitosan is natural cationic polysaccharide with biodegradable, biocompatible and bioactive characteristics that has been investigated repeatedly for its potential use in the pharmaceutical and biomedical field[11]. Chitosan and chitosan-polyanion microparticles and nanoparticles have been continued to be investigated as delivery systems for drugs, proteins, DNA and other oligonucleotides. Among the chitosan-polyanion complexes investigated, the combination of chitosan and alginate is most interesting as a drug delivery systems[12-15]. In this manuscript, we have employed cationic mixed 1,2-dipalmitoyl-sn-glycerol-phosphatidylcholine (DPPC) liposomes as templates to construct a new type of multilayer biocompatible nanocapsules and avoided using bio-incompatible polyelectrolytes as shell components completely. Charged polyelectrolytes which have opposite charges with the liposomes can be adsorbed on the liposomes based on the electrostatic interactions and then inverts the surface charge enabling a subsequent layer to be adsorbed[16]. In each cycle, the excess polymer was removed through filtration. The aim of the work presented in this paper is to show that more than one polyelectrolyte layers can be adsorbed on the liposomes and study the obtained multilayer liposomes` stability to detergent and ethanol. One possible application for such device will be the incorporation of transforming growth factors in the polyelectrolytes for bone engineering. Polymers that are widely used in bone engineering such as chitosan and alginate have been chosen for the investigation. They are biodegradable, protein release can be obtained through natural polymer degradation. As surface modified liposomes and also as nanocapsules, it can not only carry oily drugs but also hydrophilic drugs. It may also be used in drug delivery systems.

EXPERIMENTAL

1,2-Dipalmitoyl-sn-glycerol-phosphatidylcholine (DPPC), alginic acid (sodium salt, low viscosity), N-dioctadecyldimethylammonium bromide (DDAB) and cholesterol were obtained from Sigma Chemical Company. Sodium dodecyl sulfate (SDS), ethanol, chitosan (low viscosity, Mw 10 kDa) and glacial acetic acid were purchased from saiji company, P.R. China. Polycarbonate membranes were purchased from Avanti Polar lipids, USA (0.2 μm, product code 800281) and from Osmonics Inc., USA (0.1 μm, product code 1215056). All solutions were prepared in milliQ water (18.1 mΩ). All other solvents were analytical grade.

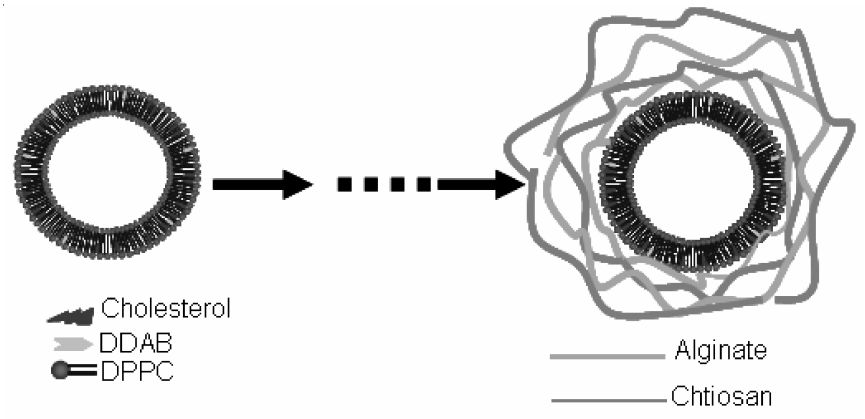
Preparation of the liposomes: Unilamellar liposome vesicles were prepared using the extrusion technique[17,18]. All liposomes were made of DPPC, DDAB and cholesterol (38 mg total mass) and were made more or less positive by incorporating various amounts of cationic surfactant DDAB (from 4 mol % DDAB to 19 mol % DDAB). The lipid mixtures were dissolved in methanol/chloroform (v/v 1:4). The solvent was rotoevaporated at 60 ºC then left dry lipid film on the glass surface. The lipid film was hydrated with 3 mL of preheated ultrapure water (18.1 mΩ) and the solution was vortexed to form white cloudy solution of multi-lamellar vesicles (MLV). The MLV solution was kept in water bath at 60 ºC and 250 μL aliquots were sucked in a glass syringe that was introduced in the extruder barrel. The vesicles were extruded through 100 or 200 nm polycarbonate filters using a mini-extruder (Avanti Polar Lipids, USA). The MLVs were passed through filters at least 20 times. The resulting vesicles (VETs) were stored at room temperature. The sizes of the liposomes were analyzed with dynamic light scattering using Malvern HPPS instrument (Malvern Instrument Inc., Malvern, UK). The net surface charge of the liposomes was measured by zeta potential (Zetaplus, Brookhaven Instruments, USA). The obtained liposomes are positively charged, so they can be employed as templates to adsorb negatively charged polymers on the surface in the next step.

Polyelectrolyte adsorption on cationic liposomes: Alginate solutions (1.0 mg/mL) were made up in ultrapure water. Chitosan solutions were prepared in 1 % acetic acid aqueous solutions. The pH value of the final chitosan solution was adjusted to 5 with 1 M NaOH. All solutions were filtered and degased before use. Aliquots of VETs (total lipid concentration 12.66 mg/mL) ranging from 25 μL to 200 μL were diluted in 2 mL of alginate solution (1.5 mg/mL) in 8 mL ultracentifugation tubes and placed on a rotating wheel overnight or at least 1 h.

Separation of adsorbed and non-adsorbed polyelectrolytes

Ultracentrifugation method: Samples were ultracentifuged at 55,000 rpm for 2 h. The supernatant was collected and the zeta potential was measured. The pellet was washed with 2 mL ultrapure water, sonicated and the tubes placed on the rotating wheel for 0.5 h. The pellet was resuspended in ultrapure water, the dispersion is homogenized and the zeta potential is measured. After measurements, tubes were ultracentrifuged again and the supernatant discarded. The pellet was resuspended

in 2 mL chitosan (1.5 mg/mL) and incubated overnight on a rotating wheel. The washing process was done as previously described and further layers were adsorbed as shown in **Scheme-I**.



Scheme-I: Multilayered polyelectrolyte nanocapsules constructed by layer-by-layer technique

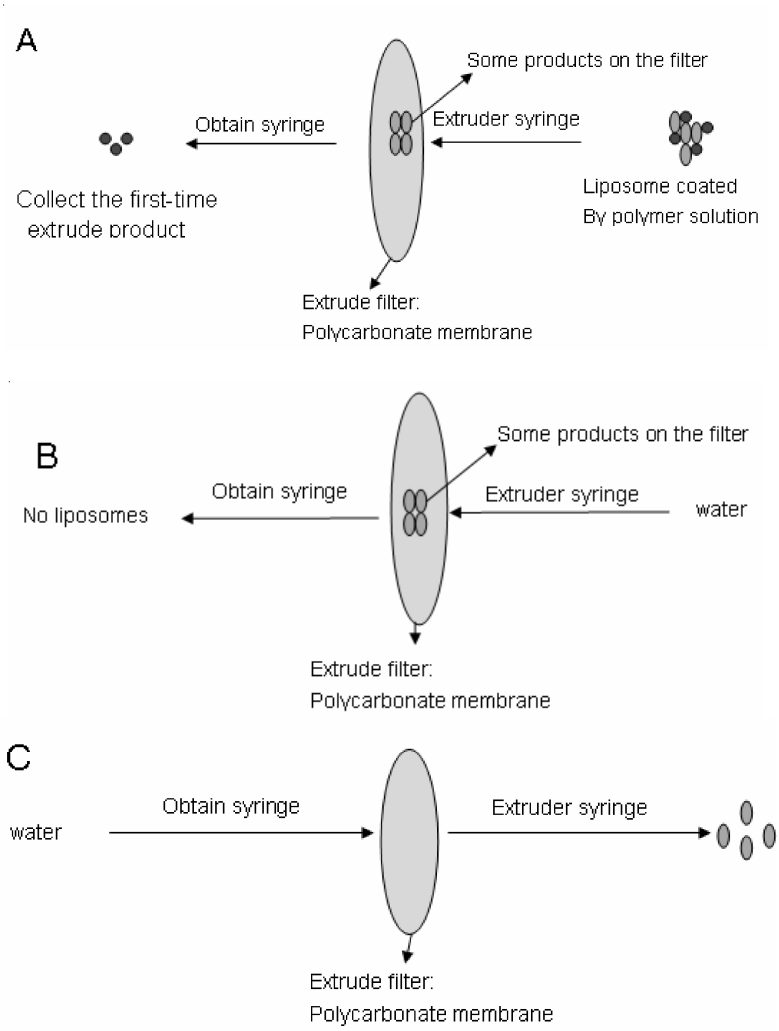
Filtration method: Separation of adsorbed and non-adsorbed polyelectrolytes was carried out by filtration using a mini-extruder. The filtration membrane pore size was 80 nm to avoid leakage of the liposomes. Liposome solution is not a good monodisperse system, there are many liposome aggregates in the solution[19].

Non-adsorbed polymer and part of liposomes which adsorbed polymers can go through the membrane and would be collected in the obtained syringe, part of liposome adsorbed polymers can pass through the membrance because they are flexible hollow balls and they can deform during extrusion[20]. The content of the syringe was removed. Ultra-pure water was placed in the syringe and washed the products on the filter for three times then the products were collected in the empty syringe by pushing back to the water in another direction and for the next adsorption. The process was shown in **Scheme-II**.

Preparation of the sample for the measurements of transition electron microscopy (TEM): A drop of sample solution was placed on the copper grid and the excess materials were removed with filter paper. 2 % Uranyl acetate solution was dropped onto the grid. An excess of staining solution was removed with filter paper in 45 s. The grid with liposomes was examined under a transition electron microscope (Philips, JEM- 100CX) at 80 kV.

Measurement of dynamic light scattering (DLS): In order to study the liposome's diameter, DLS (ELS 8000) was employed in the experiment.

Stability study of the obtained multilayer liposomes: In order to study the obtained multilayer liposome's stability, the ethanol (1:10, 1:1) and sodium dodecyl sulfate (SDS, 0.01 mg/mL) were added in the liposome solution then observed by TEM.



Scheme-II: Extrude method in the experiment: A Step I liposome coated by polymer solution in the extruder will be filtered and the product which can pass through the filter will be collected in the obtain syringe; B Step II using water to wash the product on the filter; C Step III reversing wash, the product on the filter will be collected in extruder syringe

RESULTS AND DISCUSSION

Separation of adsorbed and free polymer on liposomes: Most studies have concentrated on the adsorption of polyelectrolytes on micron sized particles or solid particles because such particles are easily separated from the free polymer solution after each deposition cycle. So, the major problem associated with the preparation of nanosized coated particles lies in separating the coated particles

from the unadsorbed polymer solution. According to prepared multilayer polyelectrolyte microcapsule, ultracentrifugation, filtration or dialysis was the only method that could be applied to separate present systems after each polyelectrolyte adsorption cycle. Results obtained after dialysis were not satisfactory and the duration of the experiment was not practical (results not shown). The ultracentrifugation technique was then investigated. TEM image of the liposomes was shown in Fig. 1. One can find that there are still many single liposomes in the solution. At the same time, there are some liposome aggregations in the solution, which is not good to employ liposome as templates with the layer-by-layer technique. We are interested in the single liposome. During centrifugation, the close particles encounter together and induce unfavourable interactions of the polyelectrolyte films leading to the destruction of the film upon subsequent particle dispersion.

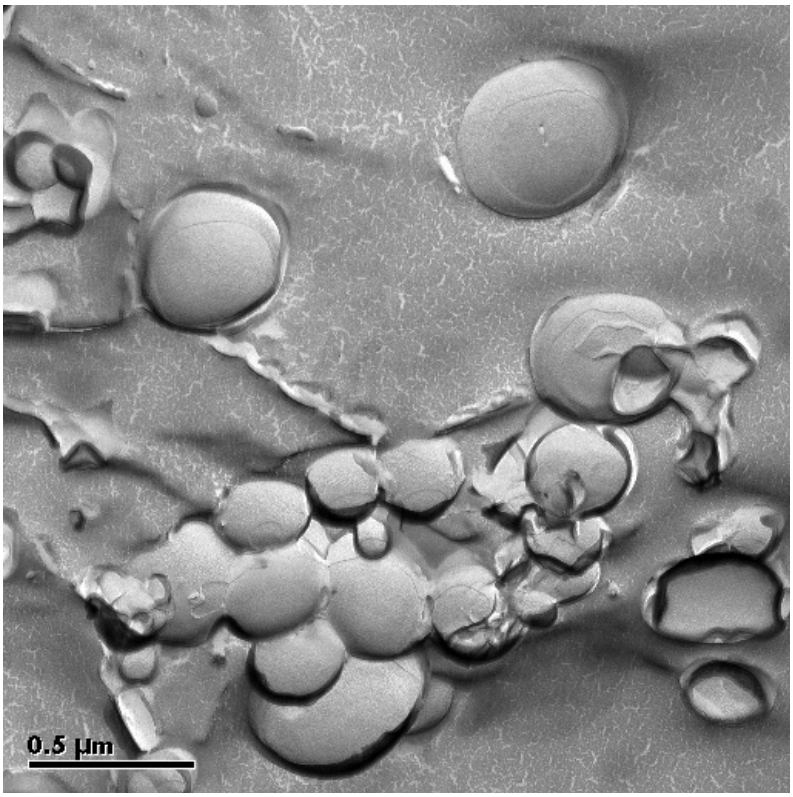


Fig. 1. Free-fracture TEM image for liposomes obtained by extrusion method

Purification methods were then developed using the filtration properties of the Avanti mini-extruder and mild centrifugation. The reliability of the method was proved by measuring the zeta potential values, size of the initial solutions, the resulting particle solutions on one hand and the washing solutions on the other

hand. Results are shown in Fig. 2. After incubation of the polymer solutions with the liposomes, the sizes of the obtained liposomes in the resulting solution were measured without being purified. The peak at 300 nm indicates the presence of the coated nanoparticles and the peak at 424 nm corresponds to the particle aggregates. After filtration through 400 nm filters, the first peak moved to 225 nm and the second one disappeared. To eliminate any potential small particle aggregates, a mild centrifugation was done. The peak was then displaced to 200 nm. That the small peak shifts to left, the possible reason may be because the surface charge of the liposomes, created by charged DDAB is insufficient to overcome the ionic and hydrophobic polyanion/liposome interactions, resulting in the polymer desorption from the highly curved liposome surface[21].

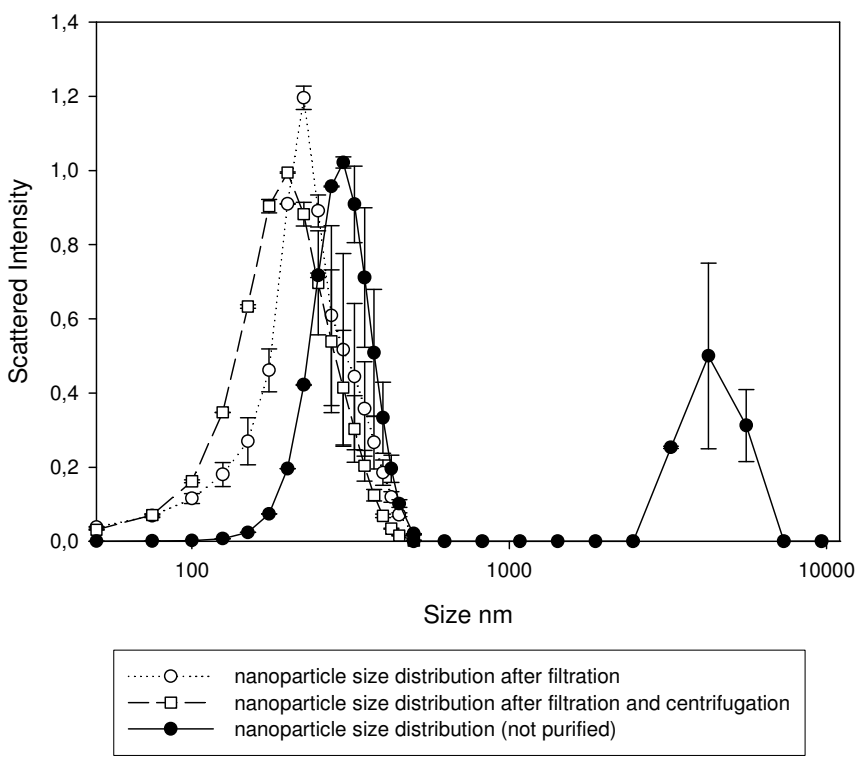


Fig. 2. DLS measurements for particle size distribution (one layer polymer coating, n = 1; Error bar mean SD)

Optimum conditions for adsorption

Liposome composition: Experiments combining the use of different concentrations of sodium alginate with the variation of the liposome external charge were carried out and the results were shown in Fig. 3. After incubation three types of particle solutions were obtained: clear nanoparticle solution, cloudy solutions and

clear aggregates. Fig. 3 shows that nanoparticles are obtained when low polymer concentrations are used and the ratios [lipid]/[alginate] are small. From the result shown in Fig. 3, external charge of the liposomes does not seem to influence the particle formation. Cloudy solutions result of a mixture of nanoparticles and other more opaque complexes corresponding to small aggregates. These small aggregates could be easily removed using present purification method. Aggregates were only obtained when high lipid concentrations were used or liposome solutions were mixed with polymers in PBS, water and NaCl. (these results were not plotted on Fig. 3 for the understanding). In the following experiments, 100 and 150 μL of liposome solutions were incubated with 1 to 1.5 mL in 1.0 mg/mL sodium alginate solutions.

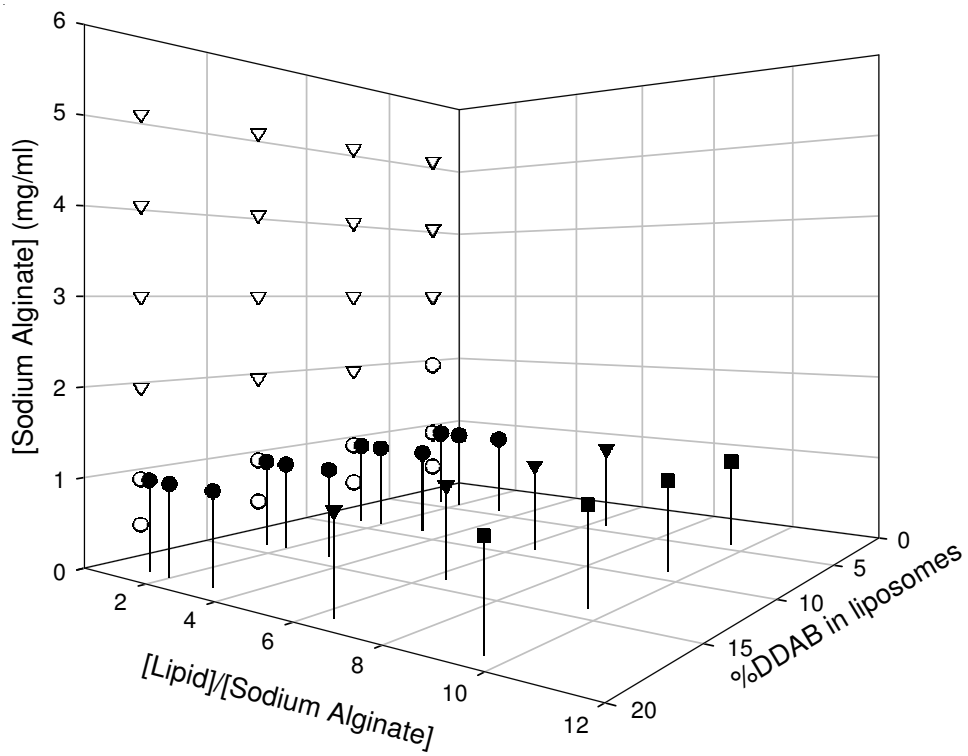


Fig. 3. 3D representation of the optimum parameters for nanoparticle formation ▽ ▼ □ Cloudy solutions that can be filtered and give nanoparticles. □ ■ Aggregates: can not be used result in a thick pellet. ○ ● nanoparticle suspension

Adsorption of alginate and chitosan on cationic vesicles: size and zeta characterization on the effect of [lipid]/[alginate] ratios and liposome charge on the adsorption of the first layer: 4, 9, 14, 19 mol % DDAB/chol/DPPC liposomes were incubated with 1.0 mg/mL of sodium alginate solutions. Results are shown in Fig. 4. The error bars were obtained by grouping data from at least three different

experiments. The large deviation can be explained by the difference in the liposome initial sizes. From these figures, the first alginate modified liposomes' diameters were measured as a function of the liposome charge density and the increasing lipid concentration. The alginate concentration was chosen at 1.0 mg/mL and remained fix. Overall, in Fig. 4a-4c, the diameter increases with the increase of charge density. It is logical to think that as the charge increases, the polymer chains are more attracted to the liposome surfaces. However, some distinctions can be made among these four graphs. In Fig. 4a, the diameter increases until the composition is 14 mol % DDAB/chol/DPPC. An explanation for this could be due to the increase in charge density, the polymer covers more uniformly on the liposome surfaces. However, saturation has not been reached as the layer thickness increases with the charge density independently of the liposome concentration. In Fig. 4b, compared to 4 mol % DDAB/chol/DPPC liposomes, the layer thickness increases of about 20 nm when 9 mol % DDAB/chol/DPPC were used. The layer does not increase significantly after this ratio. Therefore, the charge effect on adsorption becomes small after 9 mol % composition. In Fig. 4c, the layer thickness increases about 20 nm from 9 mol % to 14 mol %. A more important layer covers 19 mol % liposomes. Adsorption depends on charge density. In Fig. 4d, there is a constant increase until 14 mol % and then no significant increase. The size of the error bars generally is more important with results obtained with 19 mol % DDAB. Additionally, results shown in Fig. 3 indicate that some particle aggregation happened with [lipid]/[alginate] = 6.35. It is possible that the layer thickness increases correspond to some aggregation or doublet formation[10]. From this four figures, one can see that for 4 and 9 % DDAB liposomes as template, the first layer thickness changes gently while for the liposomes composed of 14 and 19 % DDAB, it changes dramatically. The reason may be because for 4 and 9 % DDAB, the surface charge densities are small, the polymer can be adsorbed on the surface to form a homogenous alginate layer on the liposome surface[10,21]. For the 14 and 19 % DDAB liposomes, the surface charge densities are much higher than those of 4 and 9 % DDAB liposomes[22]. Such high charges concentrated on a small surface curve, it is easily to cause particle aggregations, not only liposome themselves but also after negatively charged alginate solution was added into the systems, which will result in a dramatically increase in the diameter measurements.

Zeta potential measurements: According to the above analysis, 4 % DDAB liposomes were selected for further study in order to avoid particle aggregation seriously. Zeta potential measurements were taken after adsorption of four polymer layers on 4 mol % DDAB liposomes (Fig. 5). From Fig. 5, it is observed that four alginate/chitosan layers were adsorbed on the different composition liposome surfaces successfully. After first alginate layer was adsorbed on the liposome surface, the surface charge were transferred to negative, which will be induced next positively charged chitosan layer to be adsorbed on the surface to make the surface charge reverse, which proves that different layer was on the templates' surface.

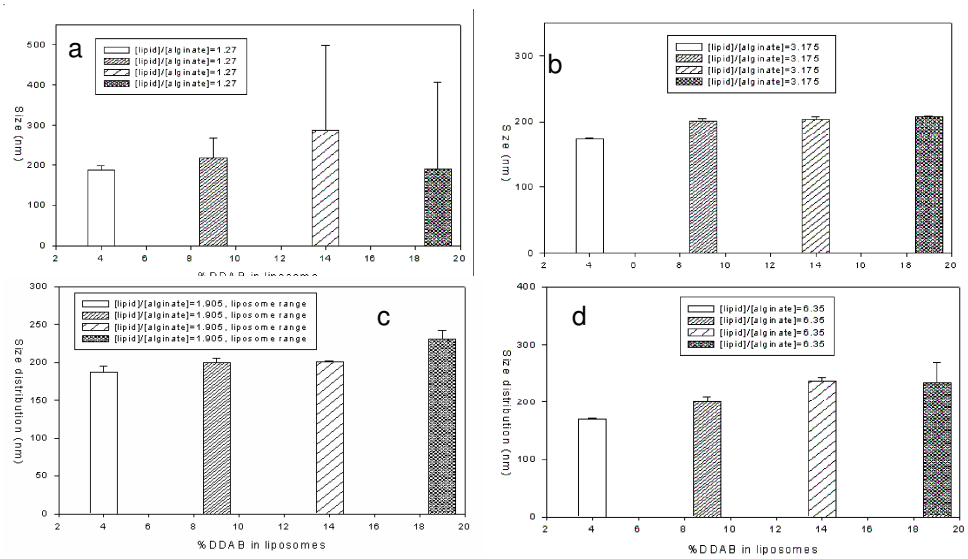


Fig. 4. (a) the first alginate layer thickness at different composition of liposomes [lipid]/ [alginate]= a) 1.27; b) 3.175; c) 1.9; d) 6.35, respectively. (one layer polymer coating, n = 1; Error bar mean SD)

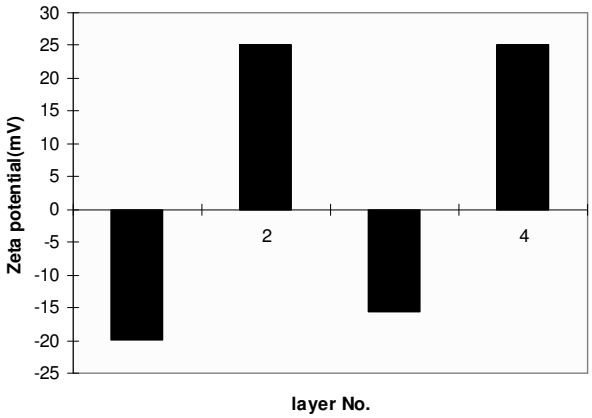


Fig. 5. Zeta potential measurement for after adsorption of four polymer layers on 4 mol % DDAB liposomes

Diameter measurement by dynamic light scattering (DLS): In order to avoid aggregations, 4 % DDAB liposomes were selected as templates and were measured average diameters with DLS. Fig. 6 shows the peak location of every sample. In Fig. 6, 0 means the average size of liposomes, 1 means the average diameter liposome/alginate, 2 means the average diameter liposome/alginate/chitosan, 3 means the average diameter of liposome/alginate/chitosan/ alginate and 4 means the average diameter of liposome/alginate/chitosan/ alginate/ chitosan. One can find easily with

the layer number increases that the average diameter increases too, which shows the layer construction is successful. From the DLS results, it can be find that after alginate or chitosan was adsorbed on the surface, the layer thickness increases obviously, which is much more obvious than PSS/PAH layers on the surface[5], but this result is consistent with Elbert's and Picart's results, who described a new type of polyelectrolyte multilayer films constructed by polysaccharides and polypeptides, which are characterized by an exponential growth of both the mass and the thickness of the film with the number of deposition steps[23,24].

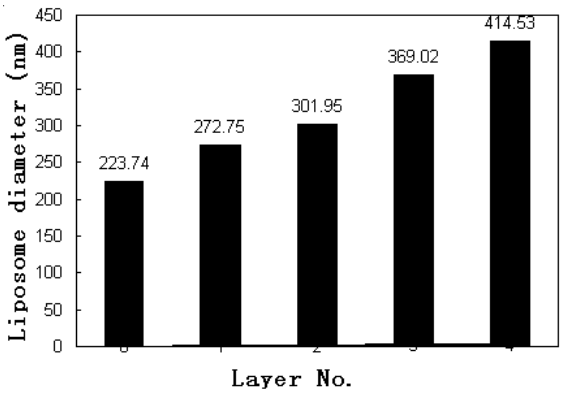


Fig. 6. DLS data for the thickness increase with the layer number

Stability study of the obtained multilayer liposomes: According to our previous publication, L-a-dimyristoylphosphatidic acid (DMPA) liposomes covered by one layer polyelectrolyte can have limited stability to detergent, such as SDS[22]. We also have performed another experiment on polymer stabilized phospholipid vesicles with an inner support of polyelectrolyte multilayer capsules. Experimentally it was found that phospholipid vesicles on polyelectrolyte multilayer shells could be stabilized against ethanol by coating a single cationic polyelectrolyte. Confocal laser scanning microscopy (CLSM) proved that the lipids were stabilized by cationic polyelectrolytes and the permeability for small hydrophilic dyes was decreased[7]. In this experiment, we fabricate four layer polyelectrolytes to coat on the liposomes then test its stability to 1:10 ethanol (Fig. 7a), 1:1 ethanol (Fig. 7b) and SDS (Fig. 7c). Fig. 7a shows that after treated with 1:10 ethanol, the multilayer liposomes can keep its shape, *i.e.*, they are stable to 1:10 ethanol. This result is consistent with the behaviour of lipid coating multilayer polyelectrolyte capsules[7]. If we increased the ratio of ethanol to water to 1:1 then treated the multilayer liposomes (Fig. 7b), one can find that the multilayer liposomes disappeared and the micelles appeared. This is because high concentration ethanol will damage the intact bilayer structures of the inner liposomes[7]. Fig. 7c shows that the obtained multilayer liposomes are stable to SDS, which is consistent with the behaviour of polymer protected small liposomes[22].

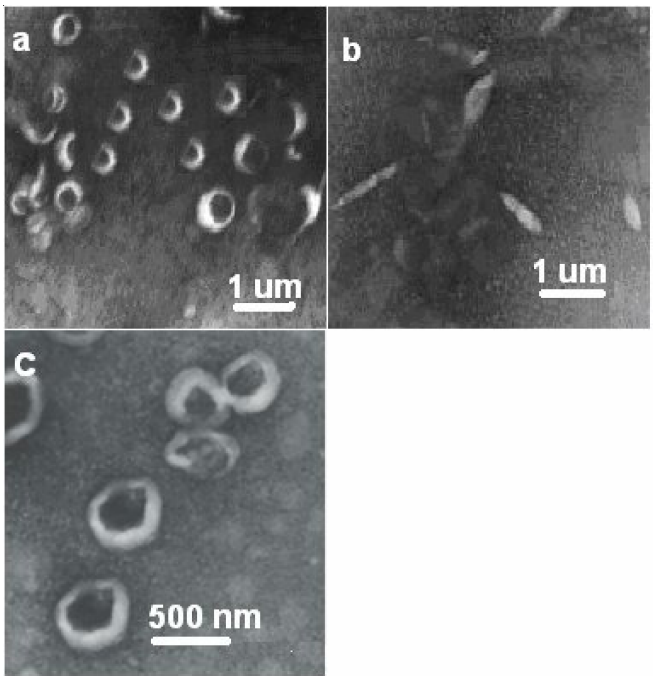


Fig. 7. TEM images of the obtained multilayer capsules were treated with a) 1:10 ethanol (v/v) b) 1:1 ethanol (v/v) c) SDS (0.01 mg/mL)

Conclusion

In this paper, we probed to use liposome as templates to construct a new type of multilayer capsules by layer-by-layer technique and studied its stability to detergent and ethanol. The liposomes are obtained with high extrusion method and the morphologies of the liposomes are studied with TEM. Zeta-potential measurements and DLS measurements were used to record the multilayer formation. Present results show that the obtained multilayer liposomes are stable to low concentration ethanol and SDS but they are not stable to high concentration ethanol. Comparing scarify templates to construct multilayered polyelectrolyte capsules, there are many advantages for this new type of multilayered polyelectrolyte capsules. The obtained capsules have great application not only in practical but also in academic fields. These multilayered liposomes are also surface modified liposomes and may be widely used in drug delivery systems.

ACKNOWLEDGEMENTS

One of the authors, Liqin Ge acknowledges the financial support from the excellent young teacher's program for teaching and researching from Southeast University, P.R. China. Thanks are also due to Prof. Dr. Maryam Tabrizian, McGill University, Canada for his support and direction.

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(*Received*: 22 October 2008; *Accepted*: 3 September 2009) AJC-7806