

Ca²⁺ Efflux from Temperature Sensitive Liposomes and In Situ Formation of Metal Cholate Liposome Gels: Basic Studies and Potentials for Sustained Site-Specific Drug Delivery

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Abstract

Liposomes or vesicles are biomimetic close containers for the delivery of drugs at the local site for an extended period of time. On the other hand hydrogels which are three-dimensional hydrophilic matrices are another class of popular materials for sustained release. Such hybrids combine the features of liposomes and polymer to ensure a sustained local drug delivery. In the present work a novel liposome/hydrogel soft assembly is explored which may be potentially useful for drug delivery applications. We report thermally triggered release of Ca²⁺ from temperature sensitive liposomal compartments constituted as 90 mol% DPPC and 10 mol% DMPC to induce rapid gelation of a solution of calcium cholate and AgNO₃ (extravesicular precursor fluid). Calcium chloride loaded liposomes were prepared using the lipid film rehydration method. The formation of unilamellar bilayer were supported by the fluorometric studies using a compatible and labeled fluoropore (NBD-PS). Hydrogels were obtained by mixing Ca²⁺ loaded liposome with extravesicular precursor fluid and incubating the content at 37°C. The concentration of the cholate during gelation was sublytic in order to avoid vesicle solubilization. The integrity of liposomes within the hydrogels were preserved during gelation as confirmed by transmission electron microscopy (TEM). The presence of low concentration of cholate (for example 0.05 mM) also permitted spectrophotometric monitoring of Ca²⁺ efflux for Ca-vesicles employing calcium sensitive dye, Arsenazo III. We expect that this simple experiment may also be useful for developing implantable as well as rapidly gelling injectable biomaterials for site-specific drug delivery. The present work is also significant as the antimicrobial properties of hydrogels containing silver has been widely recognized for its therapeutic profile.

Keywords: Liposome, triggered release, metal cholate liposome gel, site-specific delivery.

1. Introduction

Phospholipids are amphiphatic biomolecules which self-assemble into spherical vesicles called 'liposomes' constituted with single or multiple concentric lipid bilayers (Al-Jamal *et al.* 2011). Liposomes loaded with therapeutic payloads have been extensively employed as vectors in targeted delivery (Torchilin 2005). On the other hand polymer hydrogels which are three-dimensional hydrophilic matrices constitutes another class of materials for sustained release of drugs (Peppas *et al.* 2000). Particularly attractive are the liposome/hydrogel soft assembly that blends the features of both liposomes and polymer hydrogels in attaining local control of payload release to ensure a sustained local drug delivery and in applications that require implantable biomaterials (Lee *et al.* 2012; Maria-Teodora *et al.* 2011). In this context in-situ forming hydrogels triggered by environmental stimuli have emerged as a promising injectable strategy for various biomedical applications and are also attractive for site-specific drug delivery (Chiu *et al.* 2009; Ganta *et al.* 2008; Thompson *et al.* 1996). These in situ forming hydrogels are of particular interest because they can be administered through a minimally invasive procedure to achieve a sustained therapeutic outcome, offering a significant advantage over conventional implantable devices (Jin *et al.* 2009). In recent years, considerable effort has been focused on designing liposomes which can induce to release entrapped compounds from liposomal compartments in response to an applied stimulus to induce rapid gelation and a few molecules endowed with such features such as have been investigated (Lee *et al.* 2012; Westhaus *et al.* 2001).

The present work describes temperature as external stimuli to trigger the release of liposomal Ca²⁺ and initiate in situ rapid gelation of a solution of calcium cholate and AgNO₃ (extravesicular precursor fluid). Calcium chloride loaded liposomes were prepared using the lipid film rehydration method (Karim *et al.* 2010). The formation of unilamellar bilayer were supported by the fluorometric studies using a compatible and labeled fluoropore (NBD-PS). Hydrogels were obtained by incubating the Ca²⁺ loaded liposome at 37°C in a solution of sodium cholate and AgNO₃ as the precursor fluid. Sodium cholate is a mild biological detergent often used in protein membrane

reconstitution experiments (Rigaud *et al.* 1995). and earlier studies have shown that the exovesicular addition of cholate increases the permeability of phospholipid bilayers to entrapped inorganic ions (Hunt & Jawaharlal 1980). The concentration of the cholate during gelation was sublytic and was 6mM in order to avoid vesicle solubilization (Hunt & Jawaharlal 1980; Treyer & Oberholzer 2002). Hydrogels was confirmed by visual observations and the integrity of liposomes within the hydrogels were preserved during gelation as confirmed by transmission electron microscopy (TEM). Ca^{+2} efflux from liposomes were also monitored at low concentrations of cholate by employing calcium sensitive dye, Arsenazo III which undergoes a sizeable spectral shifts in the visible region upon complexation with extravesicular Ca^{+2} (Sokolove & Kester 1989). We expect that this simple experiment potentially be useful for developing implantable as well as rapidly gelling injectable biomaterials which are attractive for site-specific drug delivery. Further, the present work is also significant as silver based hydrogels are known to exhibit a broad range of antimicrobial activity (Wu *et al.* 2009).

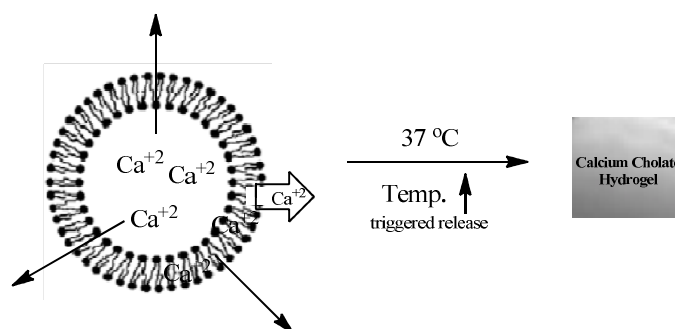


Figure (1): Gelation of Silver ion Doped Calcium Cholate Hydrogels Triggered By In Situ Release of Ca^{+2} from Temperature Sensitive Liposomes.

2. Experimental

2.1 Materials and Methods

1,2-bis(palmitoyl)-sn-glycero-3-phosphocholine (DPPC, >99%), 1,2-bis(myristoyl)-sn-glycero-3-phosphocholine (DMPC, >99%) and 1, 2-dioleoyl-sn-glycero-3-phospho-L-serine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-PS) were obtained from Avanti Polar Lipids and used as received. The purity of the lipids was confirmed by thin-layer chromatography, yielding a single spot on silica gel using a mobile phase of CHCl_3 : CH_3OH : H_2O (65:25:4). TLC were performed on Merck aluminium sheets precoated with silica gel. Reagent grade $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and PBS was obtained from Fisher Scientific and used as received. HEPES, EGTA, EDTA, arsenazo III (AIII), and all other reagents were obtained from Sigma Chemical Company. Silver nitrate and sodium cholate were purchase from Merck India Ltd. All materials were of analytical grade and were used without purification.

2.2 Preparation of Calcium Chloride Loaded Liposomes

Calcium chloride loaded liposomes were prepared using the lipid film rehydration method. Briefly, phospholipid 32 μmol (90 mol% DPPC and 10 mol% DMPC) were dissolved in 5 ml of chloroform and dried under an nitrogen stream to form a lipid film. The lipid was rehydrated overnight with 5 mL of CaCl_2 (0.29M) and hepes (5 mM), pH 7.4. and vortexed periodically over 1 h. The solutions were extruded through a 100 nm polycarbonate membrane using a syringe extruder yielding unilamellar vesicles. Subsequently, 0.75 ml were applied to small (1x18 cm) "pre-washed Sepharose 4B and collected as 1.0 ml fractions, employing 0.145M NaCl-KCl equimolar and hepes (5 mM), pH 7.4 as eluant to get final liposomal lipid concentration of 10 mM lipid after elution (Weissman *et al.* 1976).

2.3 Liposome Unilamellarity Assay (Pedersen *et al.* 2010; Plaunt *et al.* 2012)

The formation of a unilamellar bilayer were further supported by the fluorometric studies of reformulated liposome using a compatible and labeled fluoropore (NBD-PS). Fluorescent liposomes (90 mol% DPPC, 9.85 mol% DMPC and 0.15 mol% NBD-PS) were prepared by the thin film hydration method as described using an appropriate buffer (5 mM hepes, 0.145 M NaCl-KCl (equimolar) pH 7.4. The fluorescence of the NBD group (ex: 470 nm, em: 540 nm) was monitored over time before and after the addition of dithionite ($\text{S}_2\text{O}_4^{-2}$), 1.0 M $\text{Na}_2\text{S}_2\text{O}_4$ (30 μL) in 1.0 M Tris buffer (pH 10) at 60 s which functions as a quenching agent. At the end of the

experiment, the detergent Triton X-100 20 % (v/v) was added to solubilize the lipid vesicles after 180 s and causing a 100% quenching. Only the NBD-PS fluorophore in the outer leaflet of the outer vesicle membrane is chemically quenched by the initial dose of $\text{Na}_2\text{S}_2\text{O}_4$ and all of the NBD-PS is quenched after treatment with Triton. The relative NBD fluorescence at 540 nm was calculated from equation.

$$\text{Relative NBD fluorescence} = \frac{f_t - f_f}{f_i - f_f} \times 100$$

where f_i and f_f are the initial and final fluorescent intensities respectively and f_t is the fluorescent intensity at time t . Figure 2 shows that 70% of the NBD-PS is exposed on the outer surface which is indicative of liposomes that are primarily unilamellar.

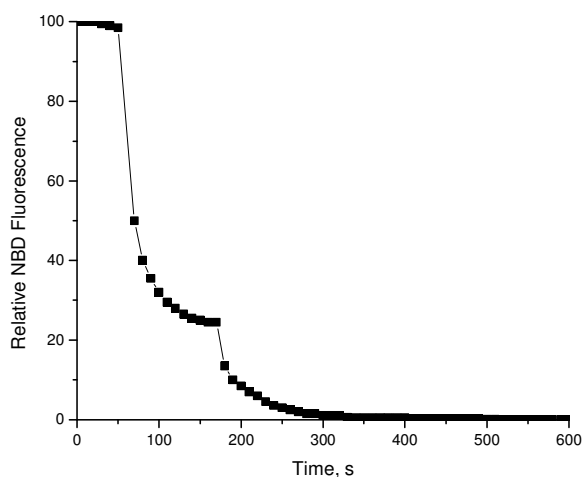


Figure (2): Reduction of NBD-PS labeled liposomes (90 mol% DPPC, 9.85 mol% DMPC and 0.15 mol% NBD-PS) upon exposure to 1 M $\text{Na}_2\text{S}_2\text{O}_4$ (30 μl) in 1.0 M Tris buffer (pH 10) at 70 s and 20% (v/v) Triton X-100 (20 μl) at 180 s.

2.4 Addition of Chololate containing Silver nitrate to preformed DPPC:DMPC Ca-vesicles

To 900 μl of Ca^{+2} loaded vesicles, which were first prepared at a concentration of 10 mM in 0.145 M NaCl-KCl (equimolar) containing hepes (5 mM) pH 7.4, were added 100 μl of precursor solution (50 μl of 0.2M sodium chololate solution and 50 μl of 1.6 mM silver nitrate solution) resulting in a mixture containing 6 mM sodium chololate and 0.08 mM silver nitrate at pH 7.4.

2.5 Formation of Calcium Chololate Hydrogel

The AgNO_3 doped calcium chololate hydrogel was prepared in the following fashion. 200 μL solution containing ion loaded vesicles in a solution of sodium chololate and AgNO_3 as prepared above was incubated at room temperature to 37°C for up to 24 h which resulted in the formation of a calcium chololate hydrogel.

2.5 Thermally Triggered Release of Ca^{+2}

Thermally triggered release of liposome entrapped Ca^{+2} were also monitored by UV spectroscopic studies at low concentration of chololate. For this to a 250 μl of the vesicles of 10 mM concentration in 0.145M NaCl-KCl (equimolar) containing hepes (5mM) pH 7.4, were added 10 μl of 0.2M sodium chololate solution and then 10 μl of 1.6 mM silver nitrate solution resulting in a mixture containing 0.042 mM sodium chololate and 0.0032 mM silver nitrate at pH 7.4. To this 10 μl of AIII solution (0.2 mM AIII in 10 mM hepes, 0.3 M NaCl, pH 7.4) was added. The contents were incubated for 2 days. The amount of encapsulated Ca^{+2} released from the Ca-vesicles was determined using Asenazo III (AIII, $\lambda_{\text{max}} = 560 \text{ nm}$), which undergoes a colour change upon complexation with Ca^{+2} (Ca^{+2} -AIII, $\lambda_{\text{max}} = 655 \text{ nm}$). A 5 μL aliquot of Ca-vesicles suspension was taken and UV absorption at 655 nm was measured. Absorption intensity of the 655 nm band allowed calculations for the % release of Ca^{+2} from the liposomes. Subsequent addition of 10% Triton-X-100 (3 μL) to this mixture leads to vesicle disruption and complete release of Ca^{+2} . Control experiments showed that this assay was linear up over 5-100 μM calcium

concentration range. The amount of Ca released (% Ca_{rel}), was expressed as a % of total encapsulated, was calculated by:

$$\% \text{Ca}_{\text{rel}} = (A/A_{\text{total}} - A_i) \times 100,$$

Where A_i is the initial absorption at 655 nm. A_{total} was determined by adding a lipid surfactant (20 μL of a 20% solution of Triton X-100) to the suspension.

2.6 Swelling Studies

Fully dried hydrogels were weighted and equilibrated in distilled water at 37°C for three days. The equilibrium swelling capacity or swelling ratio (Q) of the hydrogel was calculated employing the following equation (Mohan *et al.* 2006).

$$Q = W_e/W_d$$

Where, W_e is the swollen hydrogel weight and W_d is the dry hydrogels.

2. Results and Discussion

Calcium chloride loaded liposomes were prepared using the lipid film rehydration method as described. The binary mixture of 90% DPPC and 10% DMPC was selected because it has T_m near body temperature, 37°C (Weinstein *et al.* 1979). The formation of a unilamellar bilayer were confirmed by the fluorometric studies using NBD-PS labeled liposomes. The fluorescence of the NBD group was monitored over time before and after the addition of dithionite ($\text{S}_2\text{O}_4^{2-}$) which functions as a quenching agent. An abrupt decrease in fluorescence intensity was observed after 70 s which corresponded to quenching of the dye in the outer leaflet. Thereafter a gradual and much slower decay of fluorescence was observed. The changes in fluorescence intensity of around 15%, are significant and changes of similar magnitude has been employed to test unilamellarity (McIntyre & Sleight 1991; Huster *et al.* 2001). In contrast, fluid vesicles undergoes quenching of both leaflets simultaneously and effectively in single exponential-like decay in the NBD fluorescence is observed (Langner & Hui 1993).

Liposomes loaded with Ca^{+2} were used to trigger hydrogelation of extravesicular precursor solution containing sodium cholate and silver nitrate. Of particular relevance here is the knowledge that sodium cholate solution forms hydrogels in the presence of metal ion Ag^{+1} (Chakrabarty *et al.* 2012). The concentration of the lipid was determined by the Stewart assay and a corresponding calibration (Stewart 1980). The concentration of the cholate was kept sublytic and was 6 mM in order to avoid vesicle solubilization. It has been previously shown that the addition of cholate, a micelle-forming detergent to phospholipid vesicles leads to an increase in the permeability of vesicle bilayer if the concentration of the added cholate is below the concentration at which vesicle solubilization occurs (Treyer *et al.* 2002).

Thermal release of Ca^{+2} and the formation of hydrogel was achieved by incubating preformed liposomes as described in experimental at 37°C in an iso-osmotic buffer containing precursor solution for 24 hrs at pH 7.4. The gel formed could immobilize complete volume of water after cooling at room temperature. A semi transparent hydrogel formation was confirmed by turning the vial upside down (Fig.3). The solvent molecules retained with the solid like mass under the influence of gravity. The hydrogels were immersed in distilled water for four days and the water was changed every 12 hrs in order to remove residual monomers. The swollen gels were dried under vacuum oven at 40 °C to attain a constant weight and was stable for at least more than four weeks at room temperature. The gel formed showed the ability to trap water molecules upon gelation and the gel concentration was below 8 wt% at room temperature.



Figure (3): Thermally triggered release of liposome entrapped Ca^{2+} and formation of calcium cholate hydrogel doped with silver stable to tube inversion.

Control experiments with liposomes containing only DPPC: DMPC vesicles showed no propensity for hydrogel formation when exposed to a solution of sodium cholate stock solution containing silver nitrate as upon incubation. Under the identical mixing assay Ca-vesicles stored at room temperature for 72 hrs resulted in a viscous semi transparent fluid. Slow diffusion of ions from vesicle interior as a result of permeability enhancement by sodium cholate was confirmed by the addition of calcium sensitive dye AIII to the suspension which showed a gradual and visible colour change due to the formation of Ca^{2+} -AIII complex. As expected, the formation of hydrogel was arrested in the presence of the Ca^{2+} chelator EDTA (Zhang & Smith 1998). Increasing the temperature also increased the gel formation rate. It is proposed that the increased hydrophobicity of cholate ions with increasing temperature leads to stronger hydrogen bonding between cholate ions, which accounts for the unique heating-enhanced gelation behavior (Cai *et al.* 2012). The integrity of liposomes within the hydrogels were preserved during gelation as confirmed by transmission electron microscopy (TEM) as shown in figure 4.

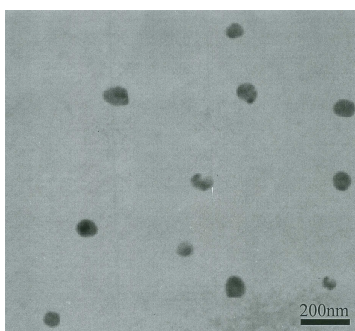


Figure (4): TEM image of Liposome in metal cholate hydrogel pH 7.4.

The presence of low concentration of cholate (for example 0.05 mM) allowed for the spectrophotometric monitoring of Ca^{2+} efflux for Ca-vesicles. For this, to the ion loaded vesicles of 10 mM concentration were added 0.2 M sodium cholate solution and 1.6 mM silver nitrate solution resulting in a mixture containing 0.042 mM sodium cholate and 0.0032 mM silver nitrate at pH 7.4. Subsequently, AIII solution was added to the mixing content and incubated at 37°C for 48 hrs. This resulted in 80% leakage of aqueous contents. However, Ca^{2+} release began after 5-6 h as observed by the study of UV spectrum which showed a characteristic spectral shifts [Fig 5]. The peak absorbance shifted from 560 nm to two peak at longer wavelengths: 605 and 655 nm. Samples were assayed for calcium by determining increase in absorbance of AIII at 655 nm caused by formation of the AIII-Ca complex. Upon 72 hrs of incubation, the formation of hydrogel were extensive. The addition of Triton-X after incubation was followed by the addition of EDTA. This resulted in a sizeable loss of peak at 655 nm which represented a reversal of the dye-Ca complex and chelation of Ca with EDTA..

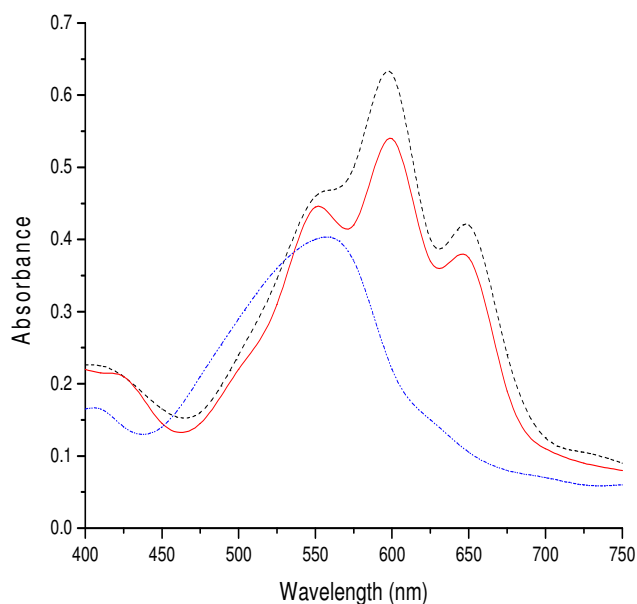


Figure (5): (a) — Absorbance spectra of Ca^{+2} liposomes containing arsenazo III after 48 hrs of incubation in the presence of .042 mM cholate concentration. (b) --- Absorbance spectra of Ca^{+2} liposomes containing arsenazo III after 24 hrs incubation and subsequent addition of Triton X. (c) ... Reversal of UV absorption (blue \rightarrow red shift) spectrum on addition of Calcium chelator EGTA.

3. Conclusions

In conclusions we have demonstrated that Ca^{+2} escape from temperature sensitive liposomes can be used to trigger gelation of calcium cholate hydrogels doped with Ag^+ ions. The liposomes under investigation releases greater than 80% of entrapped calcium upon incubation. Spectrophotometric monitoring of Ca^{+2} efflux from vesicles interior was done at low concentrations of cholate. Critical is the concentration of cholate which was kept sublytic in order to avoid vesicle solubilization. Visual observations confirmed the hydrogel formation and the integrity of liposomes within the hydrogels were preserved during gelation as studied by optical microscopy. This property might enable these hydrogels to be useful for sustained drug release applications and with promising applications in wound dressings, reconstructive surgery, tissue repair, scaffolds for cell delivery and tissue engineering. This approach may also be useful for developing gelling injectable materials that can be stored at room temperature and injected in a minimally invasive manner into a body tissue or cavity. Furthermore, silver based hydrogels are also attractive for various therapeutic applications.

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