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Original Research Article

Preparation and Purification of Calcein Loaded Temperature-Sensitive Liposomes

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ABSTRACT

Temperature-sensitive liposomes are drug carriers that can encapsulate drug molecules and carry them safely to their target sites for release. They ensure that the encapsulated drug is released for it to become cellularly internalized at the site of action. They also have a wide range of medical applications, however the most popular is chemotherapy.
In this study, the objective was to prepare and purify calcein loaded temperature-sensitive liposomes. Calcien loaded liposomes were prepared using the thin-film hydration method and calcein was loaded passively. To separate non-encapsulated calcein from the loaded liposomes three purification methods were utilized: ion exchange beads, centrifugation, and size exclusion methods. The formulated liposomes were characterized for zeta potential, average particle size, phase transition temperature, and release of loaded calcein molecules at 21, 37, and 45°C. Average size of temperature-sensitive liposomes after preparation and purification was < 200 nm and the poly-disparity index < 0.2. The stability and release efficiency of the temperature-sensitive liposome formulation at 37°C in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer solution and 5% Foetal bovine serum was optimal with a percentage leakage of less than 5% in both media. The release ratio, which was calculated as the ratio of fluorescence intensity of calcein in heated temperature-sensitive liposomes (45°C) to the fluorescence intensity of non-heated temperature-sensitive liposomes at room temperature ($R= I_{21°C}/I_{45°C}$) was 1.489, the target-release ratio to be achieved was 1.5. The application of temperature-sensitive liposome formulations in the delivery of anticancer drugs will be beneficial as they exhibited low drug leakage and efficient release.

***Keywords*:** Temperature-sensitive, Liposomes, Calcein, Release, Kinetics.

Introduction

Temperature-sensitive liposomes are drug carriers that can encapsulate drug molecules and carry them safely to their target sites for release.[1] Release of encapsulated drugs is facilitated by heating liposomes to their transition temperature.[2] Temperature-sensitive liposomes do not only encapsulate water-soluble chemotherapeutic agents but also encapsulate lipophilic chemotherapeutics as well. If the liposome surface is decorated with polyethylene glycol it can protect itself and the encapsulated drug from mononuclear phagocytic uptake and biochemical recognition by the body's opsonin.[3] One conventional challenge with drug delivery to tumour tissue is the intracellular internalization of chemotherapeutics. Temperature-sensitive liposomes ensure that the encapsulated drug is released for it to become cellularly internalized and therapeutically active at the site of action. Thermal elevation on accumulation at tumour tissue allows for a phase transition of the lipid bilayer of the liposomes from a gel-to-sol state. The bilayer develops transient pores, and the encapsulated chemotherapeutic agent can sip through its pores into the surrounding media.[3] Temperature-sensitive liposomes can be made from biocompatible and biodegradable phospholipids. Phospholipids such as phosphatidylcholine, phosphatidylethanolamine, and phosphatidylglycerol are frequently used.

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The choice and blend of lipids determine the transition temperature of the resulting liposomes.[4] Two major phospholipids used in the preparation of temperature-sensitive liposomes are dipalmitoylphosphatidylcholine (DPPC) and di-stearoyl phosphatidylcholine (DSPC). These phospholipids contribute majorly to the heat sensitivity of liposomes. A combination of dipalmitoylphosphatidylcholine and di-stearoyl phosphatidylcholine gives a transition temperature between 41-54°C. Lysolipids are also incorporated to ensure phase transition and the release of liposome content.[3] The heating of temperature-sensitive liposomes through localized hyperthermia can be mediated by focused ultrasound. When temperature-sensitive liposomes accumulate in tumour tissue, heat is released through focused ultrasound as a release trigger mechanism. The drug is rapidly released within seconds. Circulation and accumulation of liposomes may be monitored through magnetic resonance image guidance.[5] Temperature-sensitive liposomes have a wide range of medical applications, however its most popular application is in chemotherapy. Cancer remains the third leading cause of death in the world. So far, more than 8.2 million lives have been lost globally because of the disease. Chemotherapy remains the most clinically applicable and cheapest form of cancer therapy. One major setback with chemotherapy is that anti-cancer drugs have narrow therapeutic cytotoxicity and have poor pharmacokinetic profiles. This results in the experience of gruesome side effects such as nausea, vomiting, alopecia, and kidney failure.[6] The major goals of cancer therapy are to improve the patient's quality of life, increase their time of survival, and ultimately provide a complete cure for the diseased state. It is a clear fact that to achieve these stated goals, innovation and research that will effectively translate to positive clinical outcomes specifically in liposomal drug delivery for cancer agents is necessary.[7] A lot of investigations have been carried out in this field. In a study by Cheung et al, temperature-sensitive liposomes were developed using the microfluidic technique, results from the experiment showed that the

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liposomes were stable, however encapsulation efficiency was slightly low.[8] In another study by Jose et al, temperature sensitive liposomes were formulated using phospholipids and surface-active agents. Tamoxifen and Imatinib were co-encapsulated in the liposomes unfortunately drug release and encapsulation efficiency turned out to be both sub-optimal.[9] In an investigation by Du et al, F7 (a novel drug molecule highly resistant to cyclin-dependent kinase) and topotecan were co-loaded into temperature-sensitive liposomes. The size, stability, encapsulation efficiency, and release were investigated. The average size of liposomes was 103 nm and the encapsulation efficiency was above 95% but liposomes were highly unstable at body temperature as leakage of encapsulated content was observed. The slight lapses observed in recent studies show that the quest for stable, efficient, and functional liposomal formulation with optimal release kinetics for the systemic delivery of anticancer agents to tumour tissues is yet to be conclusively achieved.[10] An ideal method or technique that is guaranteed to yield stable and efficient liposomes is yet to be developed.[11] This scientific study intends to fill this lapse in literature, as it deploys the preparation of liposomes and purification as independent stages allowing liposome size regulation using the extrusion method and purification using three different methods.[12] Purification methods considered included size exclusion chromatography, separation by ion exchange beads, and ultra-filtration by centrifugation. The size, stability, and release kinetics of the liposomes purified using these three methods were also assessed and compared to identify the best method and technique combinations for preparing stable and effective liposomes.[13]

Materials and Methods

Materials

1,2-dipalmitoyl-3-phosphocholine (DPPC), 1,2distearoyl-3-phosphocholine (DSPC),1-myristoyl-2-stearoyl-3-phosphocholine (MSPC),1,2-distearoyl-3-phosphoethanolamine-N-[methoxy(polyethylene-glycol)2000] (ammonium salt), (DSPE-Me-O-PEG_{2000}) and doxylstearic acid (DSA) Avanti (Polar lipids, U.S.A), Methanol and chloroform (analytical grade), were purchased from Fisher scientific reagents (U.K). HEPES (4-(2 hydroxyethyl) piperazine-1-ethanesulfonic acid), sodium chloride glucose, weakly basic anion exchange beads, and Calcein disodium (Sigma Aldrich, St. Louis, USA), Liquid nitrogen and foetal bovine serum were purchased from (Sigma Aldrich, St. Louis, USA).

Preparation and characterization of blank temperature-sensitive liposomes

Temperature-sensitive liposomes composed of the phospholipid formula: (1,2-dipalmitoyl-3-phosphocholine (DPPC), 1,2distearoyl-3-phosphocholine (DSPC),1-myristoyl-2-stearoyl-3-phosphocholine (MSPC),1,2-distearoyl-3-phosphoethanolamine-N-[methoxy(polyethylene-glycol)2000] (ammonium salt), (DSPE-Me-O-PEG_{2000}) and doxylstearic acid (DSA) = 53: 5: 5: 7: 30 (molar percentage) was prepared by thin-film method.[6] The phospholipids were dissolved in organic solvents; DPPC, DSPC, and DSA were dissolved in chloroform (10 mg/ml). MSPC was dissolved in a 50:50 mixture of methanol/chloroform (5 mg/ml), while DSA was dissolved in methanol (10 mg/ml). A mixture of phospholipids was made based on their molecular percentage. The mixture of organic solvents was evaporated, using a rotary evaporator (Buchi, Switzerland), to yield a dry thin lipid film. The film was then rehydrated, with 1 ml of (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) HEPES buffer (pH 7.4). The hydrated film was subjected to five freeze-thaw cycles (freezing in liquid nitrogen and then melting in sonicator water bath at 60°C). The liposome solution was sonicated for 30 min to give small unilamellar vesicles. Size control of liposomal sample was carried out by extruding liposomes at 60°C through a 200 nm polycarbonate membrane ten times using a hand extruder Avanti (Polar Lipids, U.S.A). The average size, liposome count, and the poly-disparity index of liposome samples were measured by dynamic light scattering Nano series-ZEN1600, (Malvern, U. K) liposomes were stored in the fridge at 4°C. [14]

Preparation and characterization of calcein-loaded temperature-sensitive liposomes

Temperature-sensitive liposomes composed of the phospholipid formula: (1,2-dipalmitoyl-3-phosphocholine (DPPC), 1,2distearoyl-3-phosphocholine (DSPC),1-myristoyl-2-stearoyl-3-phosphocholine (MSPC), 1,2-distearoyl-3-phosphoethanolamine-N-[methoxy(polyethylene-glycol)2000] (ammonium salt), (DSPE-Me-O-PEG_{2000}) and doxylstearic acid (DSA) = 53: 5: 5: 7: 30 (molar percentage) was prepared by thin-film method and the calcein was passively loaded [12]. The phospholipids were dissolved in organic solvents; DPPC, DSPC, and DSA were dissolved in chloroform (10 mg/ml). MSPC was dissolved in a 50:50 mixture of methanol/chloroform (5 mg/ml), while DSA was dissolved in methanol (10 mg/ml). A mixture of the phospholipids was made based on their molecular percentage. The mixture of organic solvents was evaporated, using a rotary evaporator (Buchi, Switzerland), to yield a dry thin lipid film. The film was then rehydrated, with 67 mg/ml of calcein disodium dissolved in 1 ml of HEPES buffer (pH 7.4). The hydrated film was subjected to five freeze-thaw cycles (freezing in liquid nitrogen and then melting in sonicator water-bath at 60°C). The liposome solution was sonicated for 30 min to give small unilamellar vesicles. Size control of liposomal sample was carried out by extruding liposomes at 60°C through a 200 nm polycarbonate membrane ten times using a hand extruder Avanti (Polar Lipids, U.S.A). Three sets of liposomes were made and labelled IL (Ion exchange bead purified liposomes), SL (Size exclusion purified liposomes**)**, and CL (Liposomes purified by centrifugation). The average size, liposome count, and the poly-disparity index of liposome samples were measured by dynamic light scattering Nano series-ZEN1600, (Malvern, U. K). Liposomes were stored in the fridge at 4°C.[14]

Purification of Calcein-loaded liposomes using three methods

Size exclusion chromatography (gel filtration method)

Sephadex G-25 column was washed successively with aqueous 2-[4-(2-hydroxyethyl) piperazin-1-yl] ethane sulfonic acid (HEPES buffer, pH 7.4), and then the column was equilibrated with the HEPES buffer. The already prepared liposomes labelled SL were filtered through the column and fractions of 1.5 ml of liposomes were collected over time as the liposomes eluted the column. Each fraction collected was analysed for average liposome size (nm), size distribution (PDI –poly disparity index), and liposome count/ml, using dynamic light scattering, Malvern Instruments (U.K).[15, 16]

Ion exchange beads

Exactly 300 mg of weakly basic anion exchange resin beads was weighed out and rinsed successively with deionized water to get rid of the bad odour due to the presence of ammonium ion (NH_3^+) in the polymer beads. The polymer beads were then rinsed once with 2-[4-(2-hydroxyethyl) piperazin-1-yl] ethane sulfonic acid (HEPES buffer, pH7.4). One millilitre of already prepared calcein loaded liposomes labelled IL was transferred into a glass tube containing 100 mg of the clean beads. The tube was sealed and kept on a roller mixer for 10 min to allow interaction and binding of the free calcein to the beads. The clear liposome solution was separated from the coloured 'calcein-bead complex' by filtering. The liposomal suspension was labelled IL. The purified liposome solution was evaluated for physical characteristics, average size, size distribution, poly disparity index, and liposome count using dynamic light scattering, Malvern Instruments (U.K).[17]

Ultrafiltration by centrifugation method

A Millipore centrifugal tube (Billerica, USA) with filter membrane (molecular weight cut off-1Kda) was used for ultrafiltration of liposomes by centrifugation, to completely remove the un-encapsulated calcein present in the buffer from loaded liposomes. Exactly 1 ml of liposome solution labelled CL was pipetted into the Millipore tube. It was then diluted with 3 ml of HEPES buffer, pH 7.4 to give a 0.25% dilution. The diluted liposome solution was then centrifuged at 4,000 rpm for 15 min (4°C). After centrifugation, the filtrate composed of (un-entrapped free calcein and buffer) was discarded and the concentrated liposomal solution was again diluted with 3 mL buffer.

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The centrifugation step was repeated five times the filtrate was discarded each time and the liposome solution was re-diluted. The resulting liposomal suspension was labelled CL, collected, and analysed for physical characteristics by dynamic light scattering.[18]

Short-term stability studies

A stock solution of 0.1 mg/mL of calcein disodium in deionized water was prepared and 1:2 serial dilutions were made (8 dilutions). The fluorescence intensity of calcein dilutions was measured in a 96-well micro-plate by a monochromatic fluorescence plate reader (Tecan, Switzerland). The excitation was set at 450 nm and emission at 530 nm. 200 µL of HEPES buffer was pipetted into six wells in the micro-plate and 10 µL of liposomes were pipetted into three wells. Fluorescence intensity readings of the formulations were taken at 0, 15, 30, 45, 60, 75, and 90 min. A calibration curve of fluorescence intensity against the concentration of calcein was plotted. The percentage release was also calculated using equation 1.

$$R\% = I_t - I_0 / I_{100} - I_0 \times 100 \quad \text{Equation}\ldots\ldots\ldots\ldots\ldots\ldots\ 1$$

where I_t is the fluorescence intensity at time t, I_0 is the fluorescence intensity at 0 min (I_0) and 100 min (I_{100}) is the fluorescence intensity on the complete release of calcein (above 45°C).[19]

In-vitro release of temperature-sensitive liposome-encapsulated Calcein

Temperature-sensitive liposomes were heated to 45°C which is above the T_m (Transition temperature) and the fluorescence intensity of calcein was measured in a 96 well plate reader. The fluorescence intensity of calcein in the liposomes at room temperature before heating was also measured. The release ratio was calculated using equation 2.

$$R = I_{45°C} / I_{21°C} \quad \text{Equation}\ldots\ldots\ldots\ldots\ldots\ 2$$

where R is the release ratio, $I_{45°C}$ is the fluorescence intensity of liposomes heated to 45 °C and $I_{21°C}$ is the fluorescence intensity of liposomes at room temperature (21°C).[20]

Statistical analysis

One-way ANOVA (Analysis of variance) was carried out for multiple data groups (SL, IL, and CL), and Student's T-test for the calcein release in buffer and serum. All results were represented as the mean of three values with a standard deviation. The standard level of confidence is 95% (i.e., a statistical significance level of 0.05).[20]

Results and Discussion

Purification of Liposomes and its effect on liposome size and count

Purification using Ion Exchange Beads

Figure 1 shows a clear reduction in the liposomes count after purification by ion exchange bead method. Liposomes were allowed to interact with ion-exchange beads to allow the separation of free calcein from liposome formulation. Ion-exchange beads are resins or polymers that function as a platform for ionic exchange. They are non-soluble structural matrixes that appear as small spherical micro-beads which may be white or yellow in colour. The beads are usually porous, with enough surface area around and within them for binding to ions and subsequent release of other ions, hence the name ion exchange bead. A clear reduction in liposome count was observed after the separation of non-encapsulated, free calcein using ion-exchange beads. These beads are insoluble polymers with basic functional groups that bind to oppositely charged molecules in aqueous suspension. Interaction of the ammonium NH_3^+ functional groups of the polymer beads with the COO^- functional groups of free calcein molecules in solution results in covalent bonding. Therefore, separation of the "free calcein-ion exchange bead complex "from loaded liposomes can easily be achieved by decanting or filtration [21]. The NH_3^+ functional group of the basic ion exchange polymer beads also reacts with the phosphorus oxide groups on the polar head of the phospholipids that make up the bilipid layer of the liposome. The binding of the polymer beads to the phosphorus oxide groups of the phospholipids leads to an increase in the cross-sectional area (a_o) of the phospholipid polar head. The difference in the area occupied by the polar head to the hydrocarbon tail may cause a change in molecular shape (from cylindrical to cone) this also affects the critical packing parameter and leads to a transition in the aggregate structure of the lipid bilayers from lamellar to micellar phase, this transition causes rapid leakage of liposome content, aggregation, fusion of liposomes and then finally, liposomal destruction (hence a reduction in liposome population).[22]

Purification of liposomes using Centrifugation method

Figure 1 shows no change in the liposomes count after purification by centrifugation. Centrifugation is a technique which separates materials such as liposomes and free calcein suspended in a liquid medium in this case HEPES buffer. It acts by applying centrifugal force using the principle of sedimentation. Materials will separate based on their varying density, shape, size, and viscosity of the medium. A minimal change was observed in liposome count when purified by centrifugation. Calcein loaded liposomes were separated from free calcein by ultrafiltration through a filter membrane. The force driving the solution through the membrane was provided by the centrifugal field created during spinning at 4000 rotations per minute. Liposomes purified by centrifuging reflected the minimal change in liposome count per ml, after removal of free calcein indicating that the spinning did not have a negative effect on the stability of the liposomes. There was a slight change in the polydispersity index from 0.219 to 0.197. Polydispersity refers to the level of heterogeneity of the liposomal sample in terms of size. A reduction in the poly-disparity index means that there was a reduction in the variation of liposome size and enhanced uniformity of liposome size.[23] During centrifugation the liposomes moved rapidly away from the centrifugal axis towards the cellulose filter membrane of the Millipore filter (membrane-1000 g/mol cut off). There was therefore a tendency for liposomes to clog the membranes during centrifugation. Care was taken when washing the membrane with buffer after each centrifugation round to prevent the loss of liposomes [24]. There was a reduction in liposomes size after centrifugation and minimal change in the liposomes population this is due to the high centrifugal acceleration which acts as a source of mechanical energy that facilitates lipid dispersion and liposome formation.[25]

Purification using Size Exclusion Chromatography

Figure 1 shows some level of reduction in the liposomes count after purification by size exclusion. Size-exclusion chromatography may also be termed as molecular sieve chromatography. It is a chromatographic separation technique in which molecules in solution are separated by their size or molecular weight.

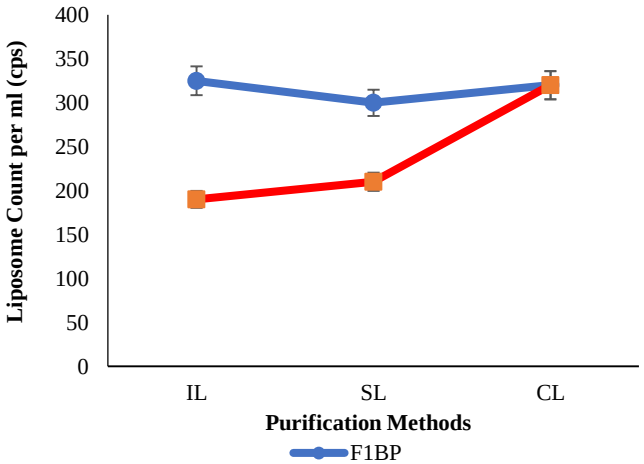

Figure 1: Change in liposome count per ml of liposome suspension, after removal of free calcein by different purification methods IL (Ion exchange purification), SL (Size Exclusion Chromatography), and CL (Purification by Centrifuging). F1BP represents liposome count before purification and F1AP represents liposome count after purification. (n = 3 ± S.D).

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It is frequently employed in the purification of liposome due to its simplicity and cost efficacy. Purification by size exclusion chromatography reflected a significant reduction in liposome count, due to dilution of the sample by the mobile phase. The stationary phase of the size exclusion chromatography column is made of a cross-linked matrix of porous spherical beads and is equilibrated with mobile phase (HEPES buffer, pH 7.4), this occurred to ensure the flow of the sample. Liposome fractions were eluted from the column along with the mobile phase. The resultant liposome solution was diluted by the mobile phase hence a reduced liposomal count per millilitre.[23]

Average Hydrodynamic Diameter

No significant change in liposome size was observed in Figure 2 by liposomes purified through size exclusion chromatography; however, there was a slight increase in liposome size after purification by ion-exchange chromatography due to the chemical interaction between the COO^- functional group on the polar head of the phospholipid and the anion charge of the ion exchange beads. A decrease in the liposome size was observed from 200nm to 150nm after centrifugation due to the spinning effect over time, enhancing the stability of the liposomes. [24] Once the liposomal suspension is sealed in the centrifuge tube, it is allowed to spin at 4000 rotations per minute. During this process centrifugal force is applied to the liposomal suspension, leading to an increase in the input of kinetic energy on the system (liposomal suspension) this leads to mechanical dispersion which causes formation of smaller liposomes. It is important to note that smaller liposomes tend to be more stable than their larger counterparts. The hydrodynamic size can be obtained using dynamic light scattering. It can be described as the size of a hypothetical hard sphere that diffuses in the same speed as the particle or molecule being measured. The average hydrodynamic diameter of liposomes in the sample before and after purification was measured to assess the effect of the purification process on the stability of liposomes.

Effect of the different purification methods on liposomal response to thermal elevation, stability and release kinetics

The release kinetics of calcein from three different samples of temperature-sensitive liposomes prepared using the purification methods were assessed. The efficiency of the purification methods was determined by the extent of calcein release which was detected by the change in fluorescence intensity after heating the temperature-sensitive liposomes to 45°C as displayed in Figure 3. Calcein is a fluorescent molecule with an excitation and emission wavelength of 495/515 nm. Calcein has been utilized widely as fluorescent indicators to examine liposome stability and release profiles of liposomes. Calcein has self-quenching properties at higher concentrations. Liposomal encapsulation of a high concentration of calcein renders its fluorescence negligible by the phenomenon called quenching. Only if the calcein is released to the external medium in which the liposomes are suspended does the molecule regain its strong fluorescence property. Calcein molecules in the internal aqueous vacuum of the liposomes showed induced self-quenching.[26] On application of mild hyperthermia (45°C, 10 minutes), there is a release of encapsulated calcein from temperature-sensitive liposomes into the surrounding buffer solution giving an increase in fluorescence intensity of the calcein molecules. It is therefore necessary that the surrounding external buffer solution be free of un-encapsulated calcein, for a significant increase in fluorescence intensity to be detected. The detection of change in fluorescence intensity largely depends on the purity of the liposomal solution. The change in fluorescence intensity was detected using a fluorescence microplate reader (Tecan, Switzerland). The concentration of calcein was calculated from the fluorescence intensity using a calibration plot. The release ratio was also calculated from the fluorescence intensities using the formula for release ratio.[27]

From the results, temperature-sensitive liposomes, purified by size exclusion chromatography showed low fluorescence intensity before heating, and a slight increase was observed from 4.5± 0.11µg/ml to 4.51± 0.20µg/ml on heating of temperature sensitive liposomes to 45°C indicating that the method was not very effective in its purification process.

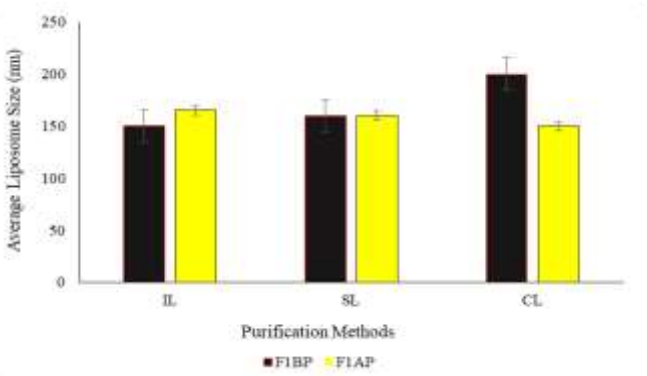

Figure 2: Change in liposome size due to the purification. F1BP represents the size of liposomes before purification and F1AP represents the size of liposomes after purification (n= 3 ± S.D).

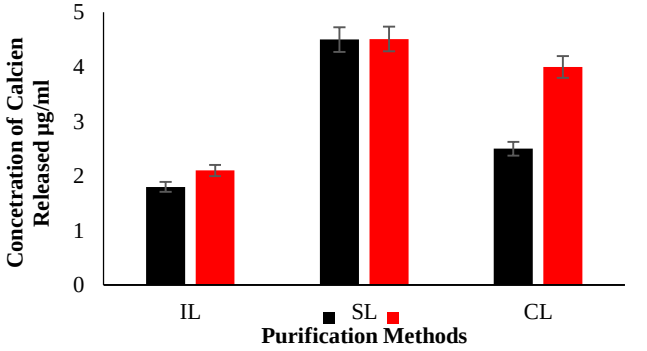

Figure 3: The effect of the different purification methods on the increase in the concentration of calcein in the liposomal solution of unheated and heated liposomes.
The black bars represent unheated liposomes (21°C at room temperature) whilst the red bars liposomes heated to 45°C. All bars represent (n=3 ± S. D), error bars show the standard deviation.

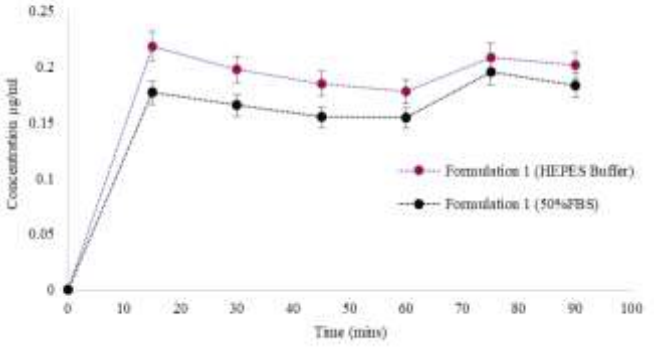

Figure 4: Stability profile of temperature-sensitive liposomes in HEPES buffer (pH 7.4) and 5% Foetal Bovine Serum at 37°C. The black markers represent liposomal formulation in the plain HEPES buffer while the red markers represent liposome formulation in 5% Foetal Bovine Serum. The leakage of calcein from temperature-sensitive liposomes overtime was measured from the change in fluorescence intensity of the liposomal suspension, at seven-time points 0, 15, 30, 45, 60, 75, and 90 minutes, the concentration of calcein in solution at each time point was then calculated using a fluorescence intensity against concentration calibration plot. All data points represent (n=3 ± S. D).

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Temperature sensitive liposomes purified using ion-exchange beads already showed a large concentration of calcein in buffer therefore high fluorescence intensity before heat triggering, so no significant increase was observed on heating. It was most likely that the liposomes were destroyed on interaction with the anionic beads, the calculated release ratio; 0.9, was exceptionally low indicating extremely poor efficacy of the purification method.

Liposomes purified by centrifugation showed a large increase in calcein fluorescence intensity and concentration with a release ratio of 1.489. Stability of temperature sensitive liposomes was least affected by this method. Therefore, centrifugation of temperature sensitive liposomes resulted in the removal of nearly all the excess free calcein allowing accurate detection of fluorescence increase on release by the micro-plate reader. Therefore, centrifugation method (ultrafiltration by centrifugation) was found to be the most suitable method and therefore applied in the purification of temperature sensitive liposomes after preparation.[26] To evaluate the stability of the temperature sensitive liposomes formulation, the leakage of calcein from temperature sensitive liposomes into surrounding solution was monitored over time at 37 °C in two different media, HEPES buffer (pH7.4) and 5% Foetal bovine serum. The stability profile of the liposomal formulation was analysed; from the stability profile in Figure 4, it was observed that initially there was rapid leakage of calcein in the first 15 minutes of incubation. However, a gradual decline in leakage was observed from 20-60 minutes. The percentage release of calcein at the initial peak concentrations which is at 15 minutes was calculated.[27] The peak concentrations and percentage release of calcein at 15 minutes were 0.18 ± 0.10µg/ml, 3.93% in serum, and 0.22± 0.17µg/ml, 4.84% in the HEPES buffer. Both showed a release lower than 5% which shows that the temperature-sensitive liposomes were stable in both media. The release profiles of calcein-loaded temperature-sensitive liposomes in both media were similar so a statistical t-test was carried out to check for the significant difference, and there was no statistically significant difference between the two release profiles. This shows that the stability of temperature-sensitive liposomes in 5% Foetal Bovine Serum was not affected by the change in media or by the presence of antibodies (gama globulin), protein, and growth factors. The inclusion of 7% mol PEG_{2000} to the liposomal membrane sterically stabilizes the liposomes and prevents proteins from binding to their surface. The use of phospholipids with a long chain saturated hydrocarbon tail contributes to the rigidity of the bilayers and reduces leakage.[28] Approximately 30% mol of doxylstearic acid was included as an antioxidant to prevent hydrolysis or lipid oxidation of the phospholipids and enhance the long-term stability of the temperature-sensitive liposomes. Figure 5 shows the amount of encapsulated calcein released at three thermal points, namely, room temperature (21ºC), physiological body temperature (37ºC), and mild hyperthermia (45°C). Calcein release at these thermal points were evaluated to determine the temperature sensitivity and release efficiency of the liposomes. At room temperature (21°C) the concentration of calcein in HEPES buffer was 2.25 ± 0.43µg/ml, the concentration at (37°C) was 2.30 ± 0.16µg/ml, at these temperatures the liposomes were stable therefore minimal change in concentration occurred; this shows that there was no release of the encapsulated calcein.[29] On the application of heat, (temperature-sensitive liposomes at 45°C), a significant increase in released calcein concentration from 2.30 ± 0.16µg/ml to 4.70 ± 0.22µg/ml was observed. This shows that liposomes formulated were not only stable but had good temperature sensitivity and release efficiency. The release of encapsulated calcein on heating indicates optimal temperature sensitivity of the liposomes. Sink conditions do not necessarily apply in this case as there is controlled release (and not dissolution) of calcein also liposomes will eventually be intended for intravenous use. The presence of lyso-lipid and its inclusion in the temperature-sensitive liposome bilayer allows the release of liposome content at higher temperatures (39-42°C) by the formation of pores at the bilayer due to destabilization of the lyso-lipid. This promotes an instant and efficient release of the loaded calcein.[28]

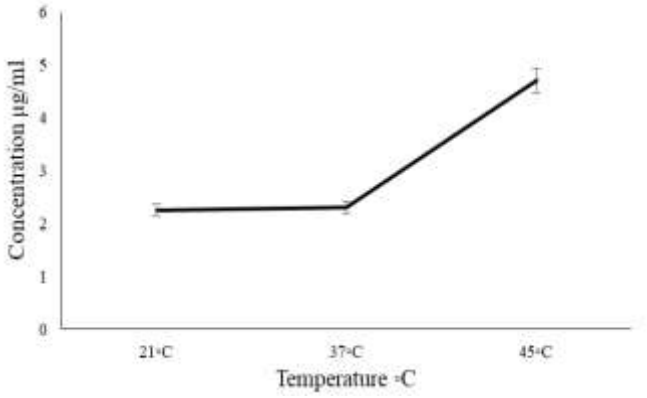

Figure 5: Concentration of calcein in HEPES buffer solution at room temperature, at physiological body temperature, and on the application of mild hyperthermia. All data points represent (n = 3 ± S. D). Concentrations of calcein in suspension were calculated from the calcein fluorescence intensity measurements in each sample using a calibration curve.

Conclusion

In this study, temperature-sensitive liposomes were prepared using the thin lipid film method and purification carried out by centrifugation was the most efficient. After centrifugation of liposomes, major outcomes observed include a reduction in liposome size from 200 nm to 150 nm with no reduction in the count per ml. Stability studies showed a minimal release of calcein from liposomes at room temperature and showed good physical characteristics: size and poly disparity index and minimal leakage of encapsulated content when incubated at 37°C in plain buffer serum and 50% foetal bovine serum. It also showed a sharp response to hyperthermia, with a significant increase in the concentration of calcein when heated to 45°C. A release ratio of 1.489 which was close to the target release ratio of 1.5 was obtained indicating a functional temperature-sensitive liposome formulation with optimal release kinetics. [24, 30]

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

References

1. Zhao Y, Cai F, Shen X, Su H. A high stable pH-temperature dual-sensitive liposome for tuning anticancer drug release. Synth. Syst. Biotechnol. 2020; 5(2):103-10.
2. Motamarry A, Asemani D, Haemmerich D. Thermosensitive Liposomes. [Online]. 2017 [cited 2022 May 24] Available from: https://www.intechopen.com/chapters/55377
3. Amin M, Huang W, Seynhaeve A, ten Hagen T. Hyperthermia and Temperature-Sensitive Nanomaterials for Spatiotemporal Drug Delivery to Solid Tumors. Pharmaceutics. 2020; 12(11):2892-2900.
4. Nkanga C, Bapolisi A, Okafor N, Krause R. General perception of liposomes: Formation Manufacturing and Applications [Online]. 2019 [cited 2022 May 24]. Available from; https://www.intechopen.com/chapters/66369
5. Rossmann C, Mc Cracklin A, Armeson E, Haemmerich D. Temperature sensitive liposomes combined with thermal

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ablation: Effects of duration and timing of heating in mathematical models and in vivo. PLOS ONE. 2017; 12(6):1-15.
6. Xin Y, Haung Q, Tang J, Hou X, Zhang P, Zhang L, Jiang G. Nanoscale delivery for targeted chemotherapy. Cancer Lett. 2016; 379(1):24-31.
7. Neguishi Y, Hamono N, Omata D, Fujisawa A, Manandhar M, Nomizu M, Aramaki, Y. Effects of Doxorubicin encapsulating AG73 peptide modified liposomes on tumour selectivity and cytotoxicity. Results Pharma Sci. 2011; 1(1):68-75.
8. Cheung C, Ma G, Ruiz A, Al Jamal W. Microfluidic production of lysolipid-containing temperature-sensitive liposomes. J. Vis. Exp. 2020; 3(157):10.3791/60907.
9. Jose A, Ninave M, Karnam S, Venuganti V. Temperature-sensitive liposomes for codelivery of tamoxifen and imatimib for synergistic breast cancer treatment. J Liposome Res. 2019; 29:153-62.
10. Du C, Li S, Li Y, Galons H, Guo N, Teng Y. F7 and topotecan co-loaded thermosensitive liposome as a nano-drug delivery system for tumour hyperthermia. Drug Deliv. 2020; 27(1):836-847.
11. Zagana P, Mourtas S, Basta A, Antimisiaris S. Preparation, Physicochemical Properties, and In Vitro Toxicity towards Cancer Cells of Novel Types of Arsonoliposomes. Pharmaceutics. 2020; 12(4):327-346.
12. Dimov N, Kastner E, Hussain M, Perrie Y, Szita N. Formation and purification of tailored liposomes for drug delivery using a module-based micro continuous-flow system. Sci Rep. 2017; 8(1):1-13.
13. Abatchev G, Bogard A, Hutchinson Z, Ward J, Fologea D. Rapid production and purification of dye-loaded liposomes by Electrodialysis-Driven Depletion. Membranes (Basel). 2021; 11(6):417-430.
14. Vyas J, Daxini K, Patel J. Formulation and Characterization of Moxifloxacin Nanoparticles with Ion Exchange Resin. J. drug deliv. ther. 2020; 10(1-s):51-61.
15. Zhao Y, Cai F, Shen X, Su H. A high stable pH-temperature dual-sensitive liposome for tuning anticancer drug release. Syst. Biotechnol. 2020; 5(2):103-110.
16. Lin M, Qi X, Purification Method of Drug-Loaded Liposome.[Online] 2018 [cited 2022 May 24] Available from: https://link.springer.com/referenceworkentry/10.1007/978-3-662-49320-5_24#citeas
17. Roberts S, Parikh N, Blower R, Agrawal N. SPIN: rapid synthesis, purification, and concentration of small drug-loaded liposomes. J Liposome Res. 2017; 28(4):331-340.
18. Sopyan I, Sunan I, Gozali D. A Review: A Novel of Efforts to enhance liposome stability as Drug Delivery Approach. Sys Rev Pharm. 2020; 11(6):555-562.
19. Burke C, Dreher M, Negussie A, Mikhail A, Yarmolenko P, Patel A. Drug release kinetics of temperature sensitive liposomes measured at high-temporal resolution with a milli fluidic device. Int J Hyperthermia. 2017; 34(6):786-794.
20. Facts at your Fingertips: Ion Exchange Resins in Water Treatment [Internet]. Chemical Engineering. 2020 [cited 2021 May 26]. Available from: https://www.chemengonline.com/water-treatment-ion-exchange-resins/
21. Lane R, Korbie D, Anderson W, Vaidyanathan R, Trau M. Analysis of exosome purification methods using a model liposome system and tunable-resistive pulse sensing. Sci. Rep. 2015; 5(1):7639.
22. Hall M. Size Exclusion Chromatography (SEC). In: Jagschies G, Lindskog E, Łącki K, Galliher P, (Eds.). Biopharmaceutical Processing, Elsevier, 2018; 421-432.
23. Trucillo P, Campardelli R, Reverchon E. Liposomes: From Bangham to Supercritical Fluids. Processes. 2020; 8(9):1022-1035.
24. Ahmed K, Hussein S, Ali A, Korma S, Lipeng Q, Jinghua C. Liposome: composition, characterisation, preparation, and recent innovation in clinical applications. Journal of Drug Targeting. 2018; 27(7):742–61.
25. Massing U, Ingebrigtsen S, Škalko-Basnet N, Holsæter M. Dual Centrifugation - A Novel "in-vial" Liposome Processing Technique [Online] 2017[cited 2022 May 24]. Available from: https://www.intechopen.com/chapters/55431
26. Miles F, Lynch J, Sikes R. Cell-based assays using calcein acetoxymethyl ester show variation in fluorescence with treatment conditions. J. Biol. Methods. 2015; 2(3):1-12.
27. Pitt W. Cytosolic Delivery of Doxorubicin from Liposomes to Multidrug-Resistant Cancer Cells via Vaporization of Perfluorocarbon Droplets. JNMR. 2017; 5(4):11-12.
28. Bi H, Xue J, Jiang H, Gao S, Yang D, Fang Y et al. Current developments in drug delivery with thermosensitive liposomes. Asian J. Pharm. 2019; 14(4):365-379.
29. Ruiz A, Ma G, Seitsonen J, Pereira S, Ruokolainen J, Al-Jamal W. Encapsulated doxorubicin crystals influence lysolipid temperature-sensitive liposomes release and therapeutic efficacy in vitro and in vivo. JCR. 2020; 328(10):665-678.
30. Cardoso-Daodu M, Ilomuanya M, Amenaghawon N, Azubuike P. Artificial neural network for optimizing the formulation of curcumin-loaded liposomes from statistically designed experiments. Prog Biomater. 2022; 11(1):55-65.

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