



Preparation of in vivo cleavable agglomerated liposomes suitable for modulated pulmonary drug delivery

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Abstract

In an attempt to achieve post-inhalation modulation of drug release rate, Bhavane et al. have recently proposed a microparticle agglomerate of nano-sized liposomal particles, with the agglomeration process consisting of chemical cross-linkages that are capable of cleavage [Bhavane et al. *J. Cont. Rel* 93 (2003) 15–28.]. There, the in vitro modulation of release from agglomerated liposomes encapsulating the antibiotic ciprofloxacin was demonstrated. However, the cleaving agents used in the previous studies are not acceptable for in vivo use. In the present work therefore, a new generation of in vivo compatible agglomerated liposomes has been developed. The release rate of encapsulated compounds from these carriers can be modulated by the addition of mild thiolytic cleaving agents such as cysteine. Specifically, an amino terminated PEG conjugate has been successfully synthesized, similar to the conjugate proposed by Zalipsky [Bioconjugate Chemistry, 10 (5) (1999) 703–707.]. This conjugate contains a dithiobenzyl urethane linkage between the lipid and the PEG, cleavable by the addition of cysteine. The amines at the distal ends of the PEG are used to cross-link the liposomes into agglomerates by the addition of a suitable cross-linking agent reactive towards amines. The cross-linkages were cleaved by cysteine at the DTB sites, resulting in changes in the size distribution of the agglomerates, as well as changes in the release rate of the encapsulated drug.

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1. Introduction

1.1. Pulmonary drug delivery

During the past decade, the lungs have been considered a promising route for the administration of therapeutics not only for the treatment of local pulmonary diseases (e.g. asthma) but also for the treatment of systemic conditions (e.g. diabetes). This increased interest in pulmonary drug delivery is based on (1) the high bioavailability of protein and peptide drugs when delivered to the respiratory system, (2) the large surface area (140 m^2) of the adult lung, in intimate contact with the circulatory system via the alveoli, (3) the equivalence of inhalation to arterial injection, and (4) the consequent avoidance of first pass hepatic and renal effects. Compounds with high potential for delivery by the respiratory route include insulin, cyclosporin, interferon, antitrypsin, protease inhibitors, deoxyribonucleases, recombinant adenoviruses and many more [1]. Further, in the wake of recent terrorist attacks on the United States, there has been a renewed interest in protection against biological agents, many of which infect via the respiratory route, and are therefore best protected against by the respiratory delivery of anti-infectives and antibiotics [2].

The state of the art in pulmonary delivery of small molecules or proteins and peptides has dramatically changed within the last 10 years. The goal for a systemically active, inhaled drug is to reach the alveolar region in order to be transported into the blood stream. In order to reach the deep lung, particles must have an aerodynamic diameter (D_{aer}) of less than about $5 \mu\text{m}$. The aerodynamic diameter is related to the geometric diameter (D_{geo}) as $D_{\text{aer}} = D_{\text{geo}} \rho^{0.5}$, where ρ is the specific gravity of the particle. For particles with specific gravity=1 (the case for most *solid* pharmaceutical powders), the geometric diameter and the aerodynamic diameter are identical. Therefore, particles that are optimal for deep lung delivery will often also have a geometric diameter of less than $5 \mu\text{m}$, putting them in the size range where pulmonary macrophage activity is the highest (this is not surprising, since the macrophage system was probably optimized by evolutionary processes to eliminate those contaminant particles that arrive at the deep lung). *Solid* drug particles optimized for drug

delivery to the deep lung would therefore meet the same fate, and be cleared by macrophage uptake and likely pass via the pores of Kohn into the circulatory system and thence to the reticulo-endothelial system. Only the portion of the particles that were not thus affected would contribute to drug delivery. A secondary consequence of this rapid macrophage uptake and clearance is the short duration of delivery for an inhaled drug, since the delivery must take place before macrophage mediated clearance of the particles takes place. Typically, this time frame is around an hour.

In an attempt to reduce the macrophage uptake and increase both the bioavailability as well as the duration of action, Edwards et al. [3] made *porous* particles with very low ρ , thus enabling very large D_{geo} , but keeping D_{aer} in the respirable range. It was recognized that the macrophage uptake system relied on the geometric diameter of particles to recognize and clear them, while the deposition of the particles in the lung depended on the aerodynamic diameter. In the breakthrough publication that first described this approach, Edwards et al. were able to demonstrate a 24–72 h duration of release of insulin in the lungs of rats. A number of inhaled insulin programs are underway today, in various stages of clinical trials. This is an indication of the feasibility of extended release of therapeutics and antibiotics from the lung.

In subsequent work, Edwards et al. [4] prepared *nanoparticles* of lipid and other excipients by spray drying, and then physically agglomerated them into large porous nanoparticles that once again provided the advantages of large geometric diameter ($\sim 10 \mu\text{m}$) with aerodynamic diameter in the respirable range (2–4 μm).

1.2. Agglomerated vesicle technology

While these advances permit controlled release of the drug at some pre-programmed rate, *they do not permit modulation of this rate on demand*. To address this need, a new technology [5,6] based on chemically agglomerated vesicles (the AVT technology) that allows the modulation of the release rate from long-residence time particles deposited in the lung was advanced. These agglomerates are fundamentally different from all previous work in that they are designed from the outset with the intent of modulating the release rate of encapsulated drug, after admin-

istration to a patient. Such post-administration modulation, while somewhat futuristic, promises improved control over the available drug level at the site of action.

The AVT technology is illustrated in Fig. 1. In this figure, core particles with spacer arms, bearing ligands on their ends are shown as the starting particles. The core particles could be polymeric nanoparticles with drug incorporated into them, or even drug nanoparticles to which the spacer arms have been conjugated. A particularly versatile choice of core nanoparticle is a liposome that can conveniently be fabricated to present a variety of ligands at the distal ends of spacer PEG chains [7]. The ligands at the distal ends of the spacer arms are then used to cross-link the nanoparticles into larger clusters or agglomerates, by using a suitable chemical reaction. For example, by choosing nanoparticles with $-\text{COOH}$ and $-\text{NH}_2$ ligands, one could cross-link them using the well-known carbodiimide reaction [8]. Depending on the conditions of the reaction mixture, one would expect the resultant agglomerates to have different

densities. For example, under diffusion-limited conditions, where the overall concentration of the core nanoparticles and linker are low, the so-called diffusion-limited-aggregation (DLA) condition would apply, resulting in a limiting fractal dimension of 1.5–2.5 [9–12]. Most chemical reactions used for cross-linking are expected to be rapid compared to the diffusion of relatively large (100–400 nm) liposomes, resulting in DLA clusters being formed.

The standard techniques of drug encapsulation in liposomes are used to incorporate drug into AVT particles as well. It is most convenient to perform the encapsulation before the agglomeration is performed. There are some limitations to the range of drugs that can be encapsulated in AVT particles—since the highest payload capacity of the core liposomes is in the internal aqueous compartment, the technique is best suited to hydrophilic drugs. Lipophilic drugs can also be accommodated in the bilayer, but the available volume is very low, and high levels of lipophilic drug can easily disrupt the bilayer. However, there are other liposome-like structures that are well suited to

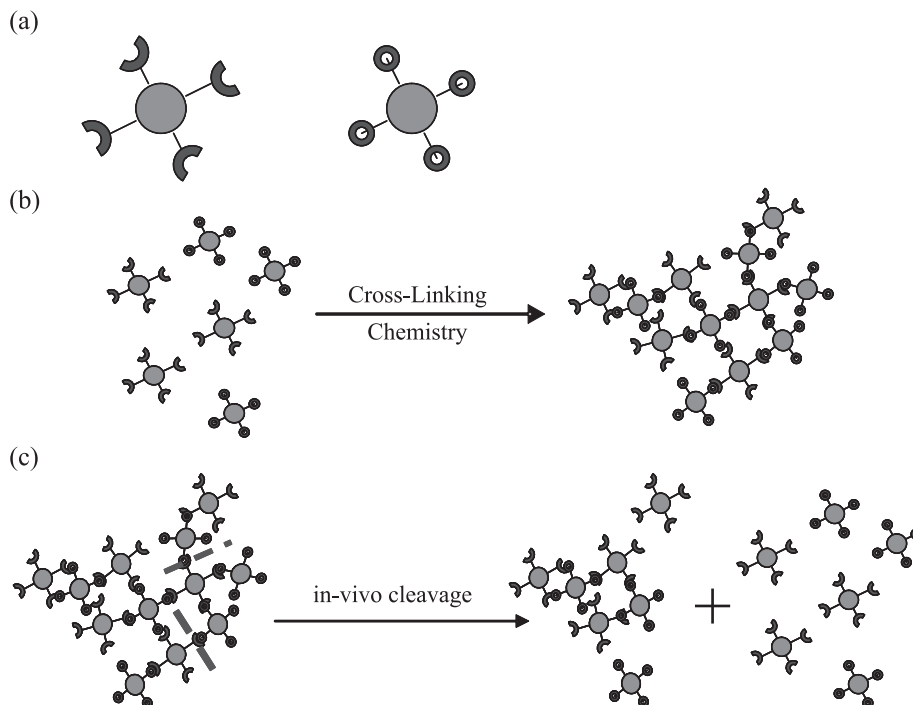


Fig. 1. Schematic of core particles bearing ligands (a); agglomeration of core particles by linkers to form agglomerates (b); based on the linker chemistry some can be cleaved in-vivo by components of lung fluid (c).

carrying lipophilic drugs (e.g. the liposphere concept proposed by Domb [13]) that can be easily adapted to the AVT platform. Therefore, while the currently demonstrated AVT particles are restricted to hydrophilic drugs, we believe that adaptations to lipophilic drugs are certainly feasible.

In previously published studies [5], Bhavane et al. have demonstrated (1) the aerodynamic and geometric diameters of AVT particles, showing they are large enough to evade macrophage uptake while being aerodynamically in the respirable range, (2) the stability of AVT particles upon nebulization, (3) controlled and modulated release of the antibiotic ciprofloxacin from AVT particles. Liposomes encapsulating ciprofloxacin, with spacer arms (PEG chains) bearing amine groups on their ends were the parent particles. The amine groups at the distal ends of the spacer arms were then used to cross-link the parent liposomes into larger clusters or agglomerates, by using a suitable chemical linker reactive towards amines. Depending on the conditions of the reaction mixture, the resultant agglomerates had different densities. The choice of the linker affected the release patterns of the encapsulated compound. Cleavable linkers were used to make breakable linkages among the parent liposomes. Introduction of a cleaving agent caused cleavage of the links resulting in release of core parent liposomes and accelerated drug release. *However, the linkers used required the use of toxic cleaving agents that are not suitable for in vivo use. In this work therefore, the development of linkers and agglomerates that are safe in vivo is sought.* Therefore, the synthesis of a linkage system that is

cleavable by physiologically acceptable levels of cysteine is demonstrated. Liposomes and AVT particles are constructed using this linkage system, and the release of ciprofloxacin from the particles is examined. The modulation of release rate by cleavage of the links using cysteine is demonstrated in vitro.

1.3. In vivo compatible AVT drug carriers

A strategy was proposed by Zalipsky et al. [14], who synthesized an mPEG-DSPE conjugate containing an *o*- or *p*-dithiobenzyl carbamate (DTB-urethane) linkage located between the lipid and the mPEG. It was shown that the DTB-urethane linkage can be cleaved by a relatively mild thiolytic agent (e.g. cysteine). Specifically, a 1.13 mM suspension of liposomes bearing 3 mol% PEG chains conjugated to lipids on the surface had 90% of the PEG cleaved from the surface by the presence of 0.150 mM cysteine within 30 min. The cleavage of this disulfide-linked conjugate resulted in regeneration of the lipid in its natural form.

Therefore, a new approach for the development of biocompatible cleavable agglomerates is based on utilizing the cysteine-cleavable conjugate proposed by Zalipsky et al. The conjugate includes the lipid (DSPE) and the PEG connected by the DTB urethane linkage. The structure of such a conjugate is shown in Fig. 2. The PEG chains bear amine groups on their distal ends available to the cross-linking reactions during the agglomeration. The cleavage of the *p*-DTB urethane-linked mPEG-DSPE conjugate was described by Zalipsky [14]. The *o*-DTB urethane

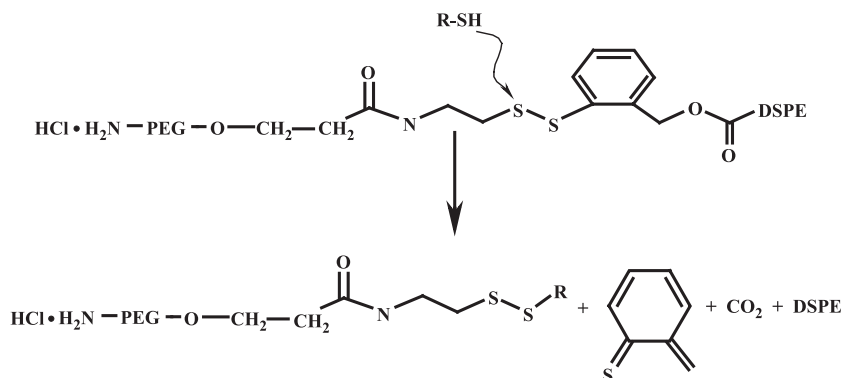


Fig. 2. Molecular structure of the DTB urethane-linked amine-PEG-DSPE conjugate and breakage of the conjugate upon exposure to a free thiol.

linkage of our conjugate is expected to be cleaved by thiols following a similar mechanism shown in Fig. 2. The thiol reacts with the disulfide bond causing a cascade of electron transfer from the disulfide bridge, through the aromatic ring, and finally to the amide bond. The cleavage results in the breakdown of the conjugate into smaller parts including the regeneration of the lipid. The incorporation of the cysteine-cleavable conjugate in the agglomerates is shown in Fig. 3. The linker carries a disulfide bond cleavable by the addition of free thiols. While there are low levels of cysteine and glutathione in the lung, capable of causing some cleavage of disulfide links between nanoparticles, the further addition of a free thiol as a “cleaver” dose would trigger the accelerated release of drug by cleavage of the inter-particle links and liberation of free nanoparticles.

In order for cysteine to act effectively as a cleaving agent to the AVT particle, the required concentration of cysteine in the lung surfactant should be below 300 mM representing a total amount of about 1 g. L-Cysteine and its derivative *N*-acetyl-L-cysteine (NAC) are available as commercial dietary supplements from numerous manufacturers. Cysteine is an amino acid which is commonly found in food and synthesized by the body. NAC helps break down mucus (for that reason, inhaled NAC is used in hospitals to treat bronchitis), increase levels of the antioxidant glutathione and detoxify harmful substances in the body.

Therefore, cysteine and NAC offer a variety of potential therapeutic uses, particularly in the prevention of acetaminophen poisoning, heart disease, respiratory illness, HIV/AIDS and many more [15]. Mucomyst® brand of acetyl-cysteine (Bristol Laboratories), a mucolytic agent for inhalation, requires a maintenance dose of 6 g for an adult with a body weight of 80 kg. Hence, the needed dose of cysteine as a cleaving agent is not considered forbidden for in vivo use at this preliminary stage of this work.

2. Materials and methods

2.1. Materials

The phospholipids 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphatidylcholine (DPPC) and 1,2-Distearoyl-*sn*-Glycero-3-Phosphatidylethanolamine (DSPE) were purchased from Genzyme Pharmaceuticals (Cambridge, MA). Cholesterol was purchased from Sigma (St. Louis, MO). Boc-PEG₃₄₀₀-SPA was obtained from Nektar (San Carlos, CA). The cross-linker Dithiobis[succinimidyl]propionate] (DTSSP) was purchased from Pierce (Rockford, IL). Extra dry chloroform, methanol and acetone (water <50 ppm) were obtained from Fisher Scientific (Houston, TX) and the rest of the minor reagents were also purchased from Fisher Scientific.

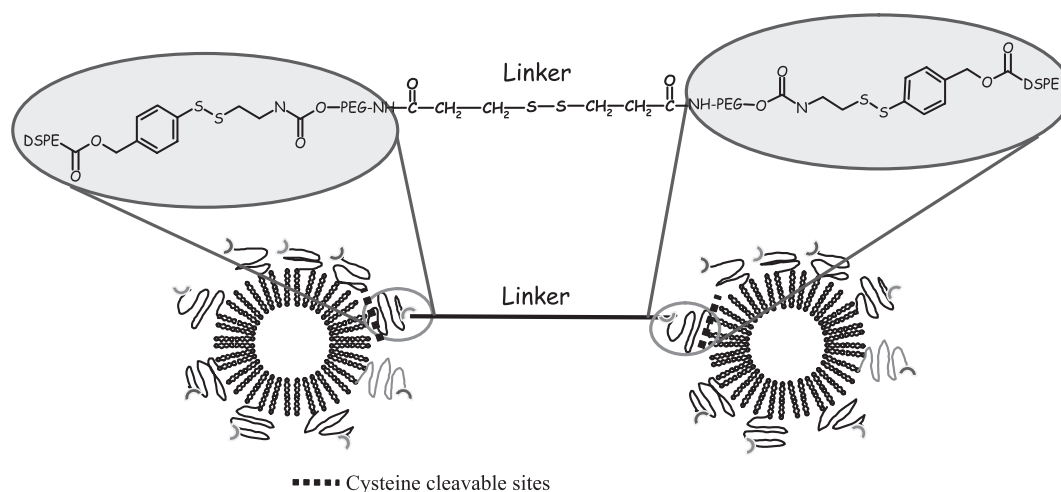


Fig. 3. Cysteine-cleavage of cross-linked liposomes incorporating the amine-PEG-DTB-DSPE conjugate.

2.2. Synthesis of the conjugate

Fig. 4 describes the synthetic sequence for the preparation of amino-PEG-DTB-DSPE. The scheme is similar to one described by Zalipsky et al. [14] with minor difference. The synthesis starts with preparation of the intermediate **1**, the DTB linker, which was prepared according to the published procedure [16]. The coupling of activated Boc-PEG₃₄₀₀-SPA with intermediate **1** yielded the intermediate boc-PEG₃₄₀₀-DTB (**2**). The introduction of

active carbonate was achieved by reacting the intermediate **2** with *p*-nitrophenyl chloroformate resulting in intermediate **3**. This was followed by treating intermediate **3** with DSPE resulting in the boc-PEG₃₄₀₀-DTB-DSPE (**4**). Deprotection of boc group with acid yielded the desired conjugate amino-PEG₃₄₀₀-DTB-DSPE.

Each intermediate product as well as the final conjugate was characterized by ¹HNMR and TLC. ¹HNMR was recorded on a Varian Inova 400 MHz spectrometer. Thin layer chromatography (TLC) was

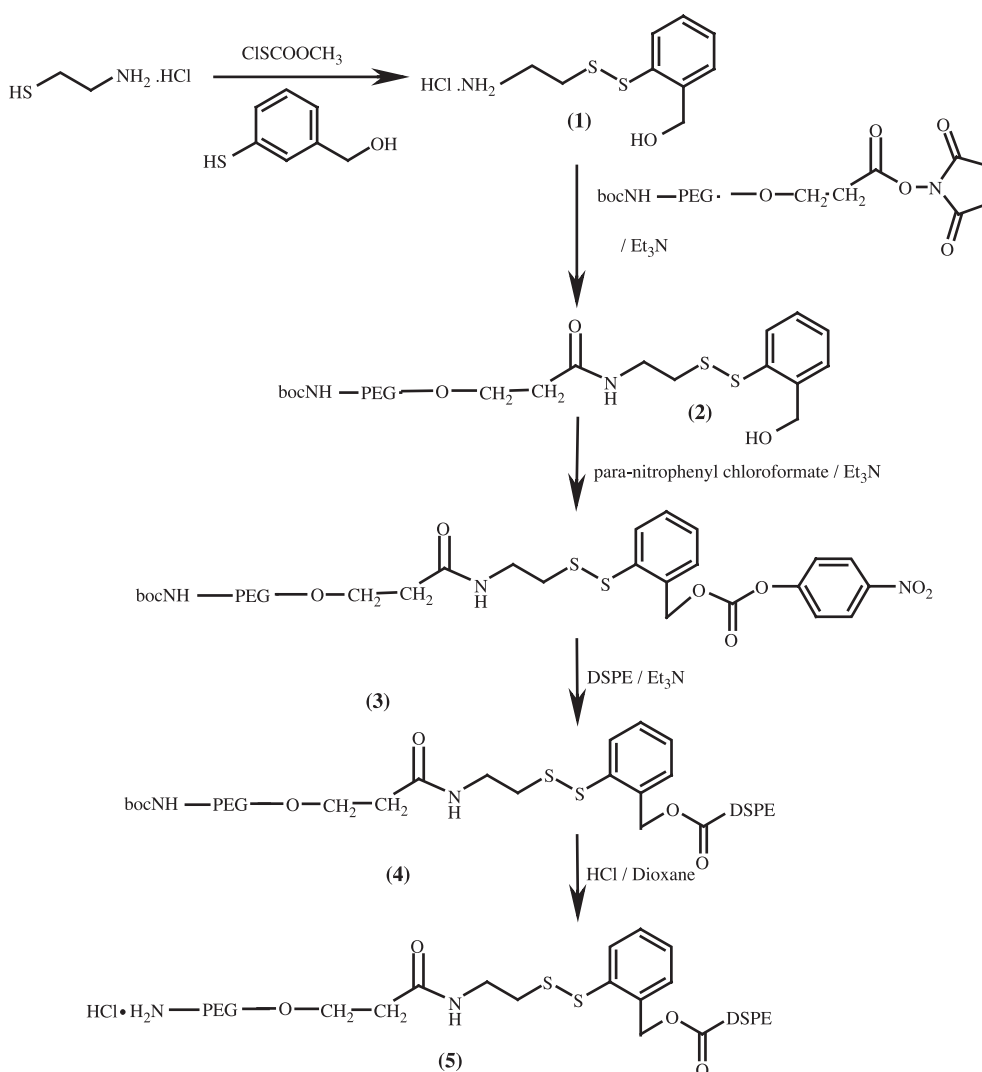


Fig. 4. Synthetic process of the DSPE-DTB-PEG-NH₂ conjugate which contains a disulfide bond cleavable by cysteine.

carried out on pre-coated plates (silica gel 60 F254). MALDI-TOFMS was acquired on Applied Biosystems 4700 proteomics analyzer in order to confirm the molecular mass of the final conjugate. The spectrum was obtained in negative ion mode in a α -cyano-4-hydroxycinnamic acid (CHCA) matrix.

2.2.1. 2-(2-Dithiobenzyl alcohol)ethanamine hydrochloride (**1**)

The procedure followed was as previously reported [16]. An ice cold solution of methoxycarbonylsulfonyl chloride (1.113 g, 8.8 mmol) in dry methanol (under nitrogen) was treated dropwise with a solution of 2-aminoethanethiol hydrochloride (1 g, 8.8 mmol) in dry methanol (10 ml). The reaction mixture was stirred at room temperature for 2 h. The solvent was then evaporated and the residual oil was crystallized from acetone to yield a solid (536 mg). This solid was dissolved in dry methanol (10 ml) and treated drop-wise with a solution of 2-mercaptobenzyl alcohol (368 mg, 2.6 mmol) in methanol (5 ml) at 0 °C under nitrogen. The solution was stirred for 1 h at room temperature and concentrated to a small volume and diluted slowly with acetone until crystallization occurred. The crystals were collected by filtration to yield compound **1** (522 mg). Rf=0.25 in CHCl₃:MeOH=90:10. ¹HNMR (CD₃OD solvent): δ 3.0 (t, CH₂S), 3.15 (t, CH₂N), 4.9 (s, benzyl, CH₂), 7.3 (d, 1H), 7.45 (m, 2H), 7.8 (d, 1H).

2.2.2. Boc-PEG₃₄₀₀-dithiobenzyl alcohol (**2**)

A mixture of boc-PEG₃₄₀₀-SPA (500 mg, 0.14 mmol) and triethylamine (59 mg, 0.58 mmol) in dry chloroform (2 ml) under nitrogen at 0 °C was treated dropwise with a solution of compound **1** (50 mg, 0.17 mmol) in dry methanol (1 ml). The reaction was stirred overnight at room temperature. Solvents were evaporated and column chromatography (silica gel, 63–200 mesh size) of residue with 92% CHCl₃ and 8% MeOH as eluant resulted in compound **2** (214 mg, with a 39% yield based on the initial amount of boc-PEG₃₄₀₀-SPA). Rf=0.6 in CHCl₃:MeOH=90:10. ¹HNMR (CDCl₃ solvent): δ 1.44 (s, ^tBu, 9H), 3.1 (t, CH₂S, 2H), 3.3 (b, bocNH-CH₂, 2H), 3.5 (m, O-CH₂CH₂CONH, 2H), 3.6 (s, PEG, 308H), 3.77 (t, PEG-CH₂CONH, 2H), 4.93 (s, benzyl, CH₂), 5.03 (b, NH), aromatic protons at 7.3 (d, 1H), 7.45 (m, 2H) and 7.8 (d, 1H).

2.2.3. Boc-PEG₃₄₀₀-dithiobenzyl nitrophenyl carbonate (**3**)

To an ice cold solution of compound **2** (214 mg, 0.061 mmol) and triethylamine (22 mg, 0.22 mmol) in dry chloroform (2 ml), *p*-nitrophenyl chloroformate (31 mg, 0.15 mmol) in dry chloroform (1 ml) was added drop-wise and reaction was stirred overnight under nitrogen at room temperature. The solvent was evaporated and to the residue cold diethyl ether was added. The white precipitate formed was washed three times with cold ether. The solid was then dried under vacuum yielding 200 mg of compound **3**. Rf=0.53 in CHCl₃:MeOH=90:10. ¹HNMR (CDCl₃ solvent): δ 1.44 (s, ^tBu, 9H), 3.1 (t, CH₂S, 2H), 3.3 (b, bocNH-CH₂, 2H), 3.5 (m, O-CH₂CH₂CONH, 2H), 3.6 (s, PEG, 308H), 3.77 (t, PEG-CH₂CONH, 2H), 4.93 (s, benzyl, CH₂), 5.03 (b, NH), aromatic protons at 7.3 (d, 1H), 7.45 (m, 2H) and 7.8 (d, 1H), 8.2–8.3 (m, nitrophenyl, 4H).

2.2.4. Boc-PEG₃₄₀₀-DTB-DSPE (**4**)

DSPE (55 mg, 0.073 mmol) was added to a stirred solution of compound **3** (200 mg, 0.054 mmol) in chloroform (2 ml) followed by triethylamine (25 mg, 0.024 mmol). The resulting suspension was maintained at 45 °C for about 1 h. The solvent was evaporated and the residue was taken in acetone to separate insoluble unreacted DSPE. The residue was then filtered and filtrate was evaporated. The gummy solid obtained was dried under vacuum yielding 178 mg of compound **4**. Rf=0.4 in CHCl₃:MeOH=90:10. ¹HNMR (CDCl₃ solvent): δ 0.86 (t, CH₃, 6H), 1.22–1.40 (m, CH₂ of lipid, 56 H), 1.44 (s, ^tBu, 9H), 1.6 (b, CH₂CH₂CO₂, 4H), 2.3 (t, CH₂CO₂, 4H), 3.1 (t, CH₂S, 2H), 3.6 (s, PEG, 308H), 3.77 (t, PEG-CH₂CONH, 2H), 3.87 (m, CH₂-CH₂-N), 4.12–4.2 (m, CH₂ of glycerol, 4H), 4.35 (m, PO CH₂-CH₂-N, 2H), 5.05 (m, CH of glycerol, 1H), 5.1 (s, benzyl), aromatic protons at 7.3 (d, 1H), 7.45 (m, 2H) and 7.8 (d, 1H).

2.2.5. Amino PEG₃₄₀₀-DTB-DSPE (**5**)

Cleavage of the boc group of compound **4** was carried out in 1 ml of 4 M HCl in dioxane for 2 h. After removal of the solvent and all the volatiles by drying under vacuum, the product was quantitatively recovered in the form of a white gummy solid (116 mg). The spot on TLC (Rf=0.3 in CHCl₃:MeOH=90:10) was

ninhydrin positive. ^1H NMR (DMSO- d_6 solvent): δ 0.86 (t, CH_3 , 6H), 1.22–1.40 (m, CH_2 of lipid, 56 H), 1.6 (b, $\text{CH}_2\text{CH}_2\text{CO}_2$, 4H), 2.3 (t, CH_2CO_2 , 4H), 3.1 (m, CH_2S , 4H), 3.6 (s, PEG, 308H), 3.77 (t, PEG- CH_2CONH , 2H), 3.87 (m, $\text{CH}_2\text{-CH}_2\text{-N}$), 4.12–4.2 (m, CH_2 of glycerol, 4H), 4.35 (m, PO $\text{CH}_2\text{-CH}_2\text{-N}$, 2H), 5.05 (m, CH of glycerol, 1H), 5.1 (s, benzyl), aromatic protons at 7.3 (d, 1H), 7.45 (m, 2H) and 7.8 (d, 1H).

2.3. Preparation of cysteine-cleavable agglomerated liposomes

2.3.1. Fabrication of parent liposomes

The liposomes were made by extruding a suspension of dissolved hydrated lipids through a single 400 nm Whatman Nuclepore polycarbonate track-etch membrane in a Lipex Biomembranes Extruder. A lipid composition of 58.5 mol% DPPC, 40 mol% cholesterol, and 1.5 mol% DSPE-DTB-PEG $_{3400}$ -NH $_2$ conjugate was used. The lipids were dissolved in ethanol at 60 °C and then hydrated with ammonium sulfate solution (ethanol volume not exceeding more than 10% of the final volume), the lipid concentration in the final mixture being 40 mM. The suspension was then passed seven times through the extruder at 60 °C and a pressure of approximately 100 psi. The liposomes were dialyzed (using 300,000 MWCO dialysis tubing) for 2 h against 400 mL of saline at pH of 5.3 (adjusted by HCl) in order to remove ethanol and ammonium sulfate from the external phase of the liposomes.

The liposomes were characterized by Dynamic Light Scattering (DLS) using a Brookhaven Instruments BI-9000AT Digital Autocorrelator, a BI-200SM goniometer and a Hamamatsu photomultiplier. The light source was a 532 nm, Ti-sapphire, frequency doubled laser. For the measurement, the liposomal suspension was appropriately diluted in saline.

2.3.2. Loading ciprofloxacin into the liposomes

Ciprofloxacin was remotely loaded into liposomes of 40 mM lipid content [17]. Blank liposomes were prepared in a 400 mM ammonium sulfate unbuffered solution. 5 mL of the liposomes were dialyzed (using 300,000 MWCO dialysis tubing) for 2 h against 400 mL of saline at pH of 5.3 (adjusted by HCl) in order to remove ethanol and ammonium sulfate from the external phase of the liposomes. Ciprofloxacin was

dissolved in saline at pH 4.2 and 55 °C. The solution of ciprofloxacin (1 mL) was added gradually into 3 mL of the liposomal suspension and the temperature was maintained at 55 °C. The remote loading procedure was terminated after 1 h by rapidly dropping the temperature using an ice bath. Finally, the suspension was separated from untrapped ciprofloxacin by dialyzing the liposomal suspension for 2 h against 300 mL of saline at pH 5.3. The final lipid content of the ciprofloxacin-loaded liposomes was 30 mM.

2.3.3. Determination of ciprofloxacin concentration

The concentration of ciprofloxacin was determined by HPLC. The HPLC system consisted of a Shimadzu SCL-10Avp liquid chromatograph, SPD-10Avp UV-Vis detector (278 nm) and SIL-10Advp auto-injector. The chromatographic conditions were as follows: Chromatography was carried out with a Waters Symmetry 5 μm C18 column (150 \times 4.6 mm); The column temperature was maintained at 28 °C and the flow rate was 1.0 mL/min; The mobile phase was a mixture of 15% (v/v) of acetonitrile and 85% (v/v) of 25 mM sodium phosphate buffer; The sample injection volume was 10 μL . Under these assay conditions, ciprofloxacin eluted in 5 min, allowing rapid multiple assays.

2.3.4. Determination of entrapped ciprofloxacin

Dialysis (using 300,000 Dalton MWCO tubing) was used to evaluate the encapsulated fraction of drug. The ciprofloxacin-loaded formulations were dialyzed against saline at pH 5.3. The volume of the external buffer was 100 times the volume of the liposomal formulation. Samples were taken from the external phase and were assayed. Complete clearance of the untrapped drug was validated when equilibrium was established for more than 6 h. Then, the formulation was removed from the dialysis tubing and was lysed with methanol (30% total volume). The measured drug by HPLC was the encapsulated fraction inside the formulation.

2.4. Preparation and characterization of agglomerated liposomes

2.4.1. Agglomeration process

The ciprofloxacin-loaded liposomes were agglomerated with the cross-linker DTSSP. DTSSP is a

water-soluble, homobifunctional *N*-hydroxysuccinimide (NHS) ester [8]. These cross-linkers are active towards the primary amines on the distal end of the synthesized conjugate resulting in a covalent amide bond and the release of *N*-hydroxysuccinimide (Fig. 5). The coupling reaction of the PEG-amines with DTSSP was carried out at pH of 7.2 in saline. The amount of DTSSP used was 100-fold molar excess of the NH₂ groups on the PEG. The pH was kept at 7.2 for 45 min and then was returned to 5.3, where ciprofloxacin was stably encapsulated. This pH range is optimal for the activity of the cross-linker resulting in fully agglomerated liposomes. The 100 fold molar excess is a stoichiometric excess, provided to accelerate the reaction and insure complete agglomeration.

2.4.2. Size characterization of the agglomerates before and upon cleavage

The size distribution of the agglomerates was determined using Fraunhofer diffraction technique (Malvern Mastersizer with 100 mm lens). The agglomerates were measured at different time points in a period of 3 days.

The cleavage of the DTSSP agglomerates can be accomplished with the thiolytic agent dithiothreitol (DTT) at 37 °C. Although, cysteine is also a thiolytic cleaving agent, which cleaves the dithiobenzyl carbamate (DTB-urethane) linkage of the conjugate, has no

effect on the DTSSP cross-linker due to the mild thiolytic behavior of cysteine.

2.4.3. In vitro release studies

The release of ciprofloxacin from each formulation was evaluated in-vitro. The commercial pulmonary surfactant replacement Survanta[®] (Abbott Laboratories) was used to simulate the lung environment. About 0.35 mL of the liposomal formulation and 0.35 mL of Survanta[®] (1:1 volumetric ratio respectively) were incubated at 37 °C in a dialysis bag (300,000 MWCO) immersed in 30 mL of PBS at a pH of 7.4. In order to investigate the effect of the cysteine on the release of ciprofloxacin, cysteine was added into the suspension. Even though the physiological level of cysteine is 0.015 mM, we also used amounts much higher than that (e.g. 200 mM) to clearly observe the results upon rapid cleavage. Samples from the external phase were taken and were assayed for ciprofloxacin in the HPLC. In the end of the experiment the content of the dialysis bag was also assayed.

3. Results and discussion

3.1. Characterization of the conjugate

The cysteine-cleavable conjugate was successfully synthesized with a quantitative yield of 18.5% (based

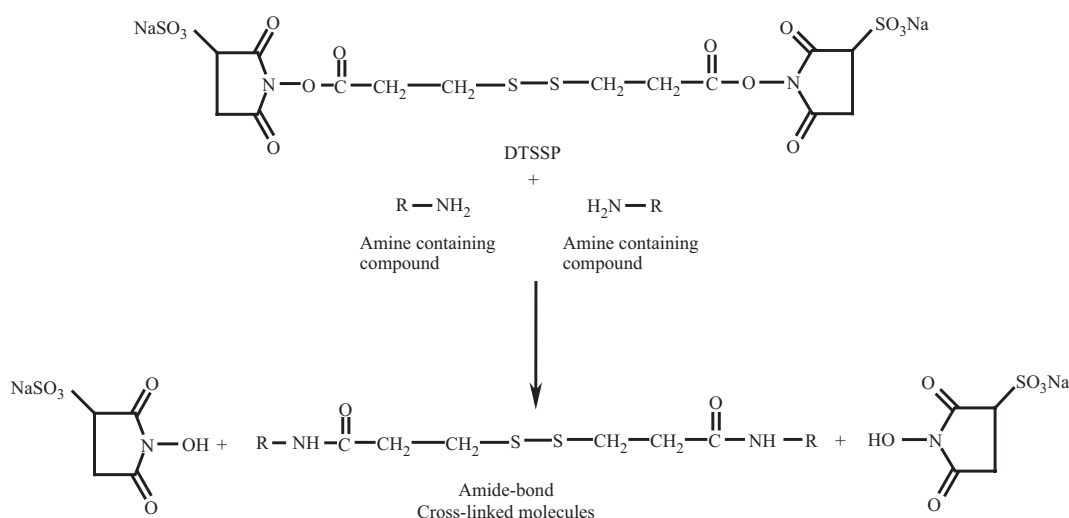


Fig. 5. Reaction scheme of DTSSP agglomeration [7].

on the initial 500 mg of boc-PEG₃₄₀₀-SPA). The ¹HNMR spectrum of the conjugate verified the structure of the molecule and showed unreacted DSPE (<10%). In addition, the MALDI-TOFMS confirmed the molecular mass of the conjugate. The mass spectrum shown in Fig. 6 has a bell-shaped distribution of 44 Da-spaced lines indicating a singly charged PEG conjugate. The distribution has its mode at 4274 Da, which is in good agreement with the theoretical molecular mass (~4200 Da).

3.2. Characterization of parent liposomes

The liposomes, extruded through a 400 nm polycarbonate membrane, were in the range of 180–210 nm exhibiting a mean diameter of 195 nm. This is consistent with the diameters reported by Sood [18] who showed that extrusion of liposomes containing 44.3 mol% cholesterol through a 400 nm membrane resulted in liposomes having diameters of around 200 nm. Traditionally, liposomes are prepared having sizes of about 100 nm due to the fact that their main use is as injectable formulations. Larger liposomes would result in increased encapsulation volume. On the other hand larger liposomes are also susceptible to the formation of multiple bilayers. Therefore, a size of 200 nm was chosen, yielding twice the internal volume per unit area as the 100 nm liposomes, while not causing a significant number of multilamellar vesicles to be formed.

After removal of the free ciprofloxacin, the concentration of the encapsulated drug, determined by HPLC analysis, was 12 mg/mL, representing 45% of the initial drug. In previous studies [5], Bhavane et al. have reported a much higher loading efficiency (90% of the initial amount added). However, the overall ciprofloxacin concentration in the previous studies was lower (10.5 mg/mL) than the current 12 mg/ml in an identical liposomal formulation. The additional loading is achieved as a result of increasing the total amount of ciprofloxacin present in the loading phase, albeit at the cost of overall loading efficiency. After loading, the encapsulation was stable during the period of experimental study, exhibiting negligible leakage.

3.3. Size of the agglomerates before and upon cleavage

Fig. 7a shows the size distributions measured by Fraunhofer diffraction, during the formation of AVT particles by the agglomeration of –NH₂ terminated liposomes using the DTSSP cross linker. The large agglomerates exhibited sizes between 2 and 31 μm and a mode at 8 μm was seen. The DTSSP linkage reaction proceeds optimally around pH 7.2 and therefore particles <1 μm were not seen indicating that all the parent liposomes were consumed in the cross-linking reaction. In comparison, the size distribution of the parent liposomes has its mode at 0.8 μm

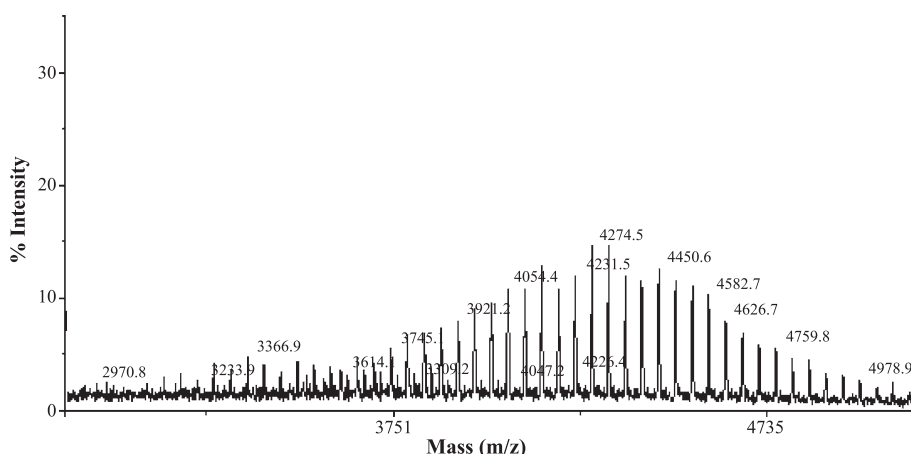


Fig. 6. MALDI-TOF mass spectrum of the DSPE-DTB-PEG-NH₂ conjugate.

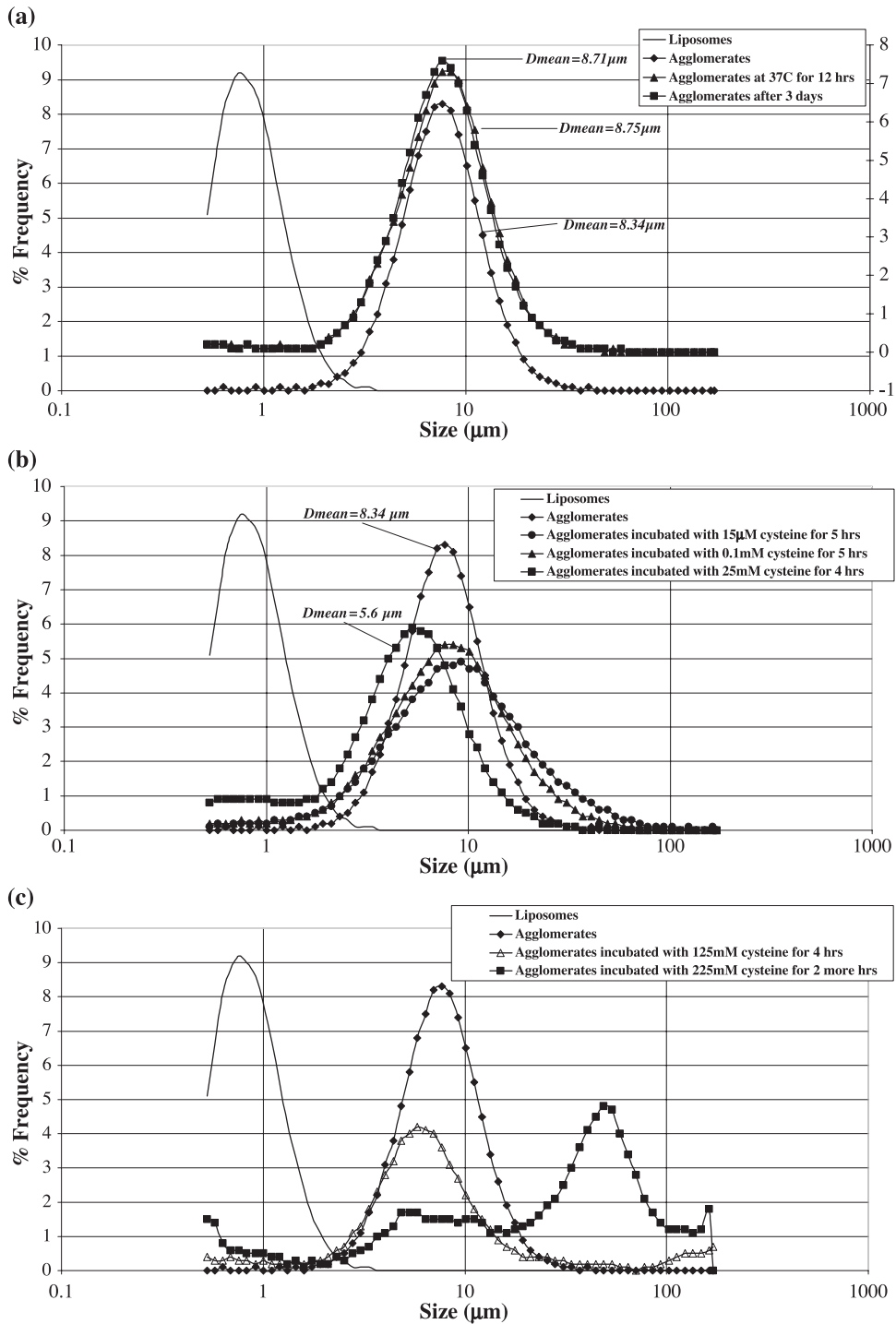


Fig. 7. (a) Comparison of size distributions of liposomes, agglomerates, agglomerates after 3 days of storage and agglomerates heated at 37 °C for 12 h; (b) Size distributions of agglomerates cleaved with different amounts of cysteine for different durations; (c) Size distributions of agglomerates cleaved by sequential dosing of cysteine over the period of 6 h.

(shown as an overlay in the same figure). It is noted that the diameter of parent liposomes, indicated by Fraunhofer diffraction is substantially different from that indicated by dynamic light scattering (DLS). This is due to the inaccuracy of Fraunhofer diffraction in this low size range. Therefore, this peak is used only as an indication of the presence of small particles, such as parent liposomes, but separate DLS measurements were performed to determine the size of the parent liposomes accurately.

3.3.1. Factors affecting the ultimate agglomerate size

A detailed study of the control of agglomerate size is reported elsewhere [19,20]. Several factors affect the size of the agglomerates formed during the AVT formation process. These include pH, the linker:lipid ratio, the lipid molarity and the number of linking ligands present on the surface of the parent liposomes. Each of these parameters affects the reaction rate of the linking ligands with the linker and thus controls the rate of formation of the interliposome cross-links. The linker itself, however, is subject to hydrolytic degradation, and therefore the reaction stops in a finite time after addition of the reactants. The higher the reaction rate is, the greater the number of cross links that can be formed in this time interval. Therefore, factors that increase the reaction rate are found to increase the size of agglomerates. Generally, increasing the number of linking ligands or the overall lipid concentration causes an increase in the reaction rate, and therefore the ultimate agglomerate size. Similarly, working at the optimal pH for the reaction also causes an increase in the agglomerate size. Interestingly, the increase in linking ligand concentration on the bilayer also causes an increase in intraliposomal links, but this is generally compensated by the increase in inter-liposomal links that are also formed as a result. In the present work, with 1.5 mol% of linking ligand in the bilayer, the polymer is close to its pancake configuration, and the likelihood of intraliposomal links forming is quite low. The fact that such intraliposomal links are not predominantly formed is confirmed by the disappearance of the parent liposome peak from the size distributions of the agglomerates, suggesting that complete interliposome link formation, and integration of all liposomes into agglomerates has indeed taken place.

Therefore, the addition of large excess of the DTSSP cross-linker to the liposomal suspension at the optimal for this linker pH of 7.2 resulted in “fully agglomerated” liposomes, evidenced by the disappearance of the parent liposome peak in the size distribution. Even though the cross-linking reaction was carried out at the optimal pH (7.2) for complete agglomeration whereas the drug loading required a lower pH of 5.3, no leakage of the encapsulated ciprofloxacin was expected to take place due to the pH difference. A disturbance of the equilibrium of the remotely loaded molecule would be expected if proton equilibrium was established between the two sides of the liposomal bilayer. However, the time scale for such equilibrium to occur is in the order of hours whereas the pH difference in this study was maintained for 45 min. Nonetheless, a leakage of 11% of the encapsulated ciprofloxacin from the agglomerated liposomes did take place during the duration of the agglomeration reaction. This low level of leakage indicates that the agglomeration process caused acceptable losses for the purposes of our experiments since the final concentration of liposomal ciprofloxacin was still above 10 mg/mL. It is hypothesized that the leakage occurred during the cross-linkages formation and the orientation of the agglomerate under agitation. Since the cross-linking reaction was rapid compared to the diffusion of relatively large particles such as liposomes with diameters of 200 nm, the diffusion-limited agglomeration system required well-mixedness provided by agitation. It is suspected that some of the cross-linkages were pulled out from the liposomal bilayer under the shear stresses applied by agitation. This is supported by Wong et al. [21] who have shown that the force required to pull out a biotin-PEG lipid (similar to conjugate PEG-DTB-DSPE in our study) from a liposome is lower than the force needed to break a streptavidin-biotin bond (analogous to the cross-linkages in our study). Such pullout could result in leakage from the liposomes, such as observed in this study. In earlier studies [5], the leakage of the remotely loaded ciprofloxacin under similar conditions was seven times greater. This substantially higher leakage was due to the fact that the cross-linking reaction was carried out for a longer duration of 3 h compared to the current study's duration of 45 min. Hence, a greater number of linkages was pulled out during the longer duration of agitation.

3.3.2. Cleavage of the agglomerates

Stability of the agglomerate structure is important since the release rate is dependent on the structure of the agglomerates. It has been previously demonstrated that once the agglomerate structure begins to disintegrate, release of the contents beyond that expected from unagglomerated liposomes occurs [5]. Therefore, the cleavage of the cysteine-cleavable agglomerates was studied carefully. Note that the cleavable link lies between the DSPE anchor and the PEG, thus agglomerates' formation is expected to be identical to the previous work [5]. However, upon cleavage, the present construct liberate non-pegylated liposomes while the previous work's construct liberated pegylated liposomes. This has implications to the release of drug upon cleavage.

The size distribution after a 3-day storage period at 4 °C was not altered as shown in Fig. 7a. The size distribution of the agglomerates 3 h after preparation and 3 days later exhibited a mean diameter of 8.34 and 8.71 μm , respectively. The stability of the remotely loaded ciprofloxacin also remained unchanged. Additionally, the size of the agglomerate remained unchanged after incubation at 37 °C for 12 h showing a mean diameter of 8.75 μm (Fig. 7a). This is consistent with Zalipsky et al. [14] who reported that a similar conjugate, mPEG-DTB-DSPE, remained stable when incubated at 37 °C. Since the liposomal bilayer and the linkage themselves remain stable at 37 °C, the maintenance of the agglomerate integrity under these conditions was expected.

Upon induction of cleavage however, the agglomerates cleaved rapidly. A comparison of size distributions of cleaved and non-cleaved agglomerates is shown in Fig. 7b. Agglomerates of 30 mM lipid content were incubated with different amounts of cysteine at 37 °C and measured by Fraunhofer Diffraction at different time points after the addition of the cleaver. The results show that the extent of cleavage increased with the concentration of cysteine. The incubation of the agglomerates with 0.015 mM (physiological level) and 0.1 mM cysteine for 5 h resulted in a broadened size distribution, suggesting a loose-jointed agglomerate being formed. However, the agglomerate maintained its integrity, evidenced by the absence of small (parent liposomal) particles in the size distribution. Further increases in the concentration of the cleaving agent (25 mM cysteine) caused an

obvious size reduction as indicated by the decrease of the mean diameter to 5.6 μm and the appearance of parent liposomes.

Zalipsky et al. [14] reported in a previous publication that about 80% of micellar or liposomal mPEG-DTB-DSPE conjugate with a 0.033 mM concentration was cleaved by 0.015 mM cysteine within 30 min. In the current study, a significant cleavage of the agglomerate by low concentrations of cysteine (0.015 and 0.1 mM) was not observed. Even though cleavage of the conjugate was expected and took place as evidenced by the broadened size distribution in Fig. 7b, the overall concentration of the cleavable conjugate in the agglomerate was high (0.45 mM) and therefore the remaining large number of non-cleaved linkages still maintained the integrity of the agglomerate. The use of the higher cysteine levels (25 mM) did indeed result in cleavage of sufficient links to release parent liposomes, as evidenced by the appearance of parent liposomes. However, even in this case, with a 50-fold excess of cleaving agent, the cleavage was not complete, and significant agglomerate structures remained. While one could speculate that in the cross linking process, links could have been formed from the free DSPE present in the lipid bilayer as an impurity from the synthesis of the DTB conjugate, it is unlikely this is the case, since the PEG layer on the DTB conjugate effectively shields the bilayer surface from chemical reaction. Further, the length of the PEG tether forms a spacer between liposomes, preventing them from ever approaching close enough for a DSPE based linkage to take place. Therefore, it is not apparent why the cleavage of the agglomerates is not complete, even at such high concentrations of the cleaving, beyond attributing it to a possible kinetic effect. Future experiments will probe the structure of the agglomerates further, and answer the question of why the cleavage is not complete at these high cleaver doses.

The effect of sequential doses of the cleaving agent at different time points is shown in Fig. 7c. Incubation of agglomerates of 3 mM lipid content (0.045 mM conjugate content) with 125 mM cysteine for 4 h at 37 °C resulted in the appearance of small (<1 μm) as well as large (>100 μm) particles. A multi-modal size distribution was observed, when further cysteine resulting in a final concentration of 225 mM was added and incubated for two more hours. This

suggests that as the cross-linkages of the agglomerate were continuously cleaved, the structure of the particle changed from a compact, sphere-like morphology to a looser, diffuse one. At the same time free liposomes were liberated from the agglomerate.

These results demonstrate that cysteine-mediated cleavage of the DTB-urethane conjugate broke the inter-liposomal linkages resulting in looser agglomerate structure as well as liberation of free liposomes.

3.3.3. Location of the cleavage sites

In contrast to previous work [5] the present cleavable DTB-urethane linkage provides for a cysteine cleavable disulfide between the DSPE and PEG-NH₂ components. Liposomes are prepared with this conjugate present in the bilayer, and are agglomerated using a bifunctional linker (DTSSP) that links two amine groups together. It is speculated based on the low level of conjugate in the bilayer that intra-liposomal links are few in number, consistent with the disappearance of parent liposomes from the size distribution of the agglomerates. This DTSSP linker itself, bears a disulfide, however, this disulfide is not cleavable by cysteine. Indeed, in previous work, cleavage of this link was accomplished by strong thiols such as DTT, which are not compatible with *in vivo* experiments, thus motivating the current work. The use of a DTSSP linker between the amine terminii assures agglomeration behavior consistent with that

observed earlier [5]. However, DTSSP does not participate in the cleavage. The disulfide that is cleaved by cysteine is the thiobenzyl group, between the DSPE and the PEG components. Two such disulfides are present on each inter-liposome cross-link. While there is no need for two such links to be present, we use this system again out of convenience. An alternative would have been to synthesize a thiobenzyl cross linker, and cross link liposomes presenting -PEG-NH₂ groups. Such an approach would have had the potential downside of not having agglomerate characteristics comparable to that already known from our previous work. The current approach however, equally serves the purpose of *in vivo* cleavability of the AVT particles, while allowing the use of well understood agglomeration conditions.

3.4. Release profiles of ciprofloxacin

Since each of the drug release experiments lasted for one or more days at 37 °C, loss of the surfactant activity was expected [22]. A possible degradation of ciprofloxacin was also expected due to the aggressive environment in the surfactant. At the end of each of the experiments, the contents of the dialysis bag were assayed for ciprofloxacin content remaining in the bag and compared with the amount of ciprofloxacin released into the external phase of the dialysis bag. The release experiments reported here were therefore

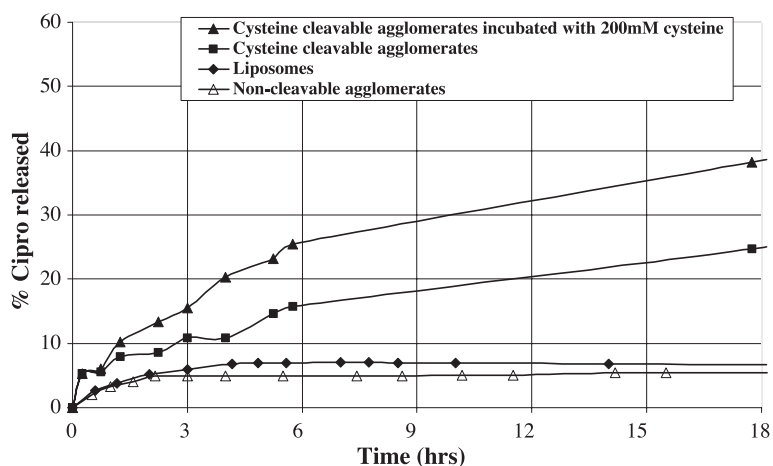


Fig. 8. *In vitro* release profiles of ciprofloxacin from cysteine cleavable agglomerates incubated in lung surfactant with 200 mM cysteine or no cysteine and from liposomes and non-cysteine cleavable agglomerates obtained from our previously published studies [4].

terminated after 1 day and the mass balances verified. In all cases, it was possible to account for over 99% of the ciprofloxacin.

Fig. 8 shows a comparison between the release profiles of ciprofloxacin from intact agglomerates, and agglomerates cleaved with cysteine. The release from both these formulations showed an initial burst in the first 30 min and thereafter the drug was released with a constant rate. However, the incubation with 200 mM cysteine present in the external and internal phase of the dialysis tubing clearly caused a higher rate of release showing 13% more ciprofloxacin being released at the end of 18 h (the majority of this increase occurred in the first 2–3 h after cysteine addition). It is interesting that the release pattern never reached a plateau even in the absence of cysteine.

Fig. 8 also shows the release profiles from the non-cleaved agglomerates and their parent liposomes reported in our previous publication [5]. In the past studies, the release pattern from both liposomes and agglomerates exhibited an initial burst which reached a plateau. However, the agglomerates reached a lower plateau than the liposomes suggesting that the agglomerate structure plays a role in protecting the liposomes in the inner core of the AVT particle. Comparing the level of drug released between the cysteine-cleaved and the non-cleaved formulations, the DTB-urethane based agglomerates released four to five times higher amounts of their content and more importantly the release did not reach a plateau. There are two possible explanations for this behavior:

1. It has been shown that the release of doxorubicin (loaded against ammonium sulfate, identical to the current procedure for ciprofloxacin) from Stealth liposomes is slower than that from conventional (non-PEGylated) liposomes [23]. This is attributed to the additional diffusional barrier provided by the PEG layer. While the amount of PEG in our bilayer is quite small (1.5 mol%) it is known that at this level, with a PEG molecular weight of 3400, the polymer chain is transitioning from the pancake to the mushroom configuration, providing a continuous layer around the core liposome. In the case of the cysteine cleavable conjugates, the cleavage occurs between the DSPE and the PEG components of the conjugate, thus revealing non-PEGylated liposomes when the cleavage is complete. The release of a remote-loaded drug from the cysteine cleavable agglomerate is therefore

expected to gradually trend towards that from non-PEGylated liposomes, as the cleavage proceeds. In contrast, earlier work on DTT cleavable AVT particles positioned the cleavage site between two PEG chains from adjoining liposomes. Thus, even after cleavage, the remaining liposomes would be PEGylated, and thus release drug at a lower rate.

2. Alternatively, it is also possible that the continuous release is due to the continuous cleavage of the inter-liposomal cross-linkages by the free thiols present in the lung surfactant. Note that the present work uses a higher surfactant: drug carrier ratio than the past work. It is not believed this had a significant effect on the results however, since in both cases the surfactant was in excess over the drug preparation.

The exact mechanism of release from the AVT particles is not understood at this stage. In previous work, it has been also speculated about the possibility of rearrangement of the agglomerate structure upon partial cleavage and the application of shear to the agglomerates [5]. A concerted effort to understand the correlation of the agglomerate structure at various levels of cleavage, to the release rates as a function of cleavage, is indicated, and is the subject of future work. Of particular interest is the “mix-and-match” capabilities that arise from using a mixture of cysteine cleavable and non-cleavable linkers, leading to partially cleaved structures.

4. Conclusions

In previous work, the controlled and modulated release capabilities of the AVT technology have been demonstrated [5]. In addition upon aerosolization, the AVT drug carriers exhibited aerodynamic diameters within the respirable range and maintained structural integrity and encapsulated drug. All these were evidence of a promising platform for administering drugs in the lungs.

With the goal of *in vivo* post-inhalation modulation of drug release rate, a DTB-urethane conjugate was successfully synthesized cleavable by an *in vivo* acceptable compound such as cysteine. The DSPE-DTB-PEG-NH₂ conjugate was successfully incorporated into the AVT particles and the preparation of micron-sized particles was achieved. The release of the antibiotic ciprofloxacin from the agglomerate showed

a controlled rate predetermined by the formulation. However, the addition of cysteine cleaved the disulfide bond of the DTB-urethane linkages. Hence, cysteine or any other free thiols (e.g. glutathione) can serve as a cleaving agent triggering the acceleration of the release. This is consistent with the size reduction observed upon the exposure of the agglomerate to cysteine.

It was observed that the cysteine-cleavable agglomerates released their content continuously, while the release from the non-cysteine-cleavable agglomerates reached a plateau after the acceleration due to cleavage. The reason for this behavior is not clear, and could be due either to (1) the difference in release rate from non-PEGylated liposomes generated in the cysteine-cleavage process, or (2) due to the continuous cleavage of agglomerates due to excess cysteine. In either case, this cysteine cleavable capability of the agglomerate provides an attractive platform for designing different release patterns. Different ratios of cleavable and permanent cross-linkages in the agglomerate could result in different modifications of the structure by cysteine and consequently different release patterns.

The aerodynamic properties of these particles have not been tested yet. However, particles made with non-cysteine-cleavable linkers have been previously tested, and were found to be acceptable for nebulization and pulmonary delivery. Therefore, it is anticipated that the current particles will also be suitable. Further, *the in vivo performance of cleavable and non-cleavable AVT particles is currently being investigated in rabbit models and is a subject of a future publication.*

The results of this work are the first demonstration of an in vivo compatible, post-inhalation modifiable drug release platform. Clearly, there are many factors controlling the behavior of these AVT particles that are not yet understood, and their optimization is the subject of ongoing investigation. While light scattering provides information on the overall size of the agglomerates, and is an excellent tool for the monitoring of the agglomeration process, it provides little information about the nanostructure of the agglomerates themselves. Obviously, the nanostructure of these agglomerates is of critical importance in determining the release of drug from them. Future work characterizing the structure of AVT agglomerates using atomic force microscopy or other structural tools and examining the correlation of the release to the structure is indicated.

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