Cochleates: New insights into drug delivery system

seit 1558

Dissertation

To fulfill the requirements for the degree of

'*Doctor rerum naturalium*' (Dr. rer. nat.)

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Submitted by Kalpa Nagarsekar

Master of Pharmaceutical Sciences

Born on 25th December 1984 in Mumbai, India

Cochleates: New insights into drug delivery system

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Dissertation

zur Erlangung des akademischen Grades

***„Doctor rerum naturalium“* (Dr. rer. nat.)**

vorgelegt dem Rat der Biologisch-Pharmazeutischen Fakultät

der Friedrich-Schiller- Universität Jena

von Kalpa Nagarsekar

Master of Pharmaceutical Sciences

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1 Introduction

1.1 Discovery of cochleates:

The transition of negatively charged lipids from the liquid-crystalline to the gel state due to ionic interaction has been intensively investigated in past decades (Papahadjopoulos et al., 1973; Träuble and Eibl, 1974). This interaction leads to major morphological transformation into a rolled up cylindrical structure that were noted by Verkleij and coworkers during morphological evaluations in 1974 (Verkleij et al., 1974; Ververgaert et al., 1975). These cylindrical structures were also observed by Dr. D. Papahadjopoulos and co-workers while developing method for formulation of large vesicles (Papahadjopoulos and Kimelberg, 1974; Papahadjopoulos et al., 1975), at an intermediate stage, who named them 'cochleates' in 1975 (Papahadjopoulos, 1978). The term cochleate originates from Greek term for 'snail with spiral shell' which corresponds to the folding pattern of cylinders (Figure 1.1).

Cochleates are formed due to self-assembly of negatively charged lipid bilayers and cations. Self-assembly at molecular level can be described as a process in which spontaneous arrangement of molecular subunits results into formation of ordered structures with properties unlike their precursors (Zhang, 2003). During cochleate formation positively charged binding agent and lipid head group interacts in order to form hierarchical superstructures (Poste et al., 1976). The interactions involved in this process are usually of non-covalent nature (e.g. electrostatic interactions, Van der Waals forces, hydrogen bonds, hydrophobic interactions etc.). This process finally results in formation of highly ordered cochleates from structures with less ordered states.

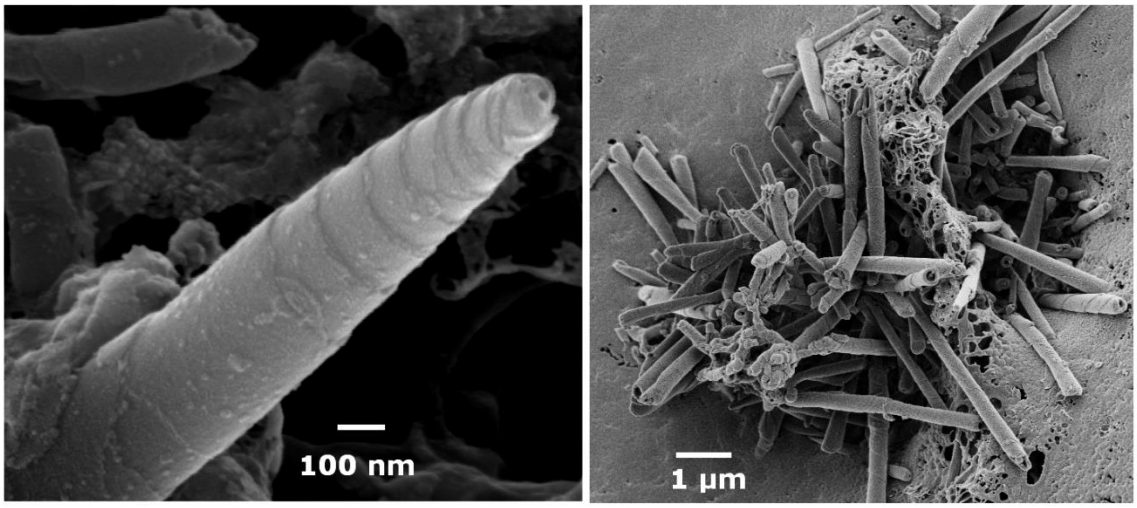

Figure 1.1: Cryo scanning electron micrograph of calcium cochleates of a) DMPS displaying folding pattern (figure from unpublished data) and b) DOPS showing cylindrical morphology (figure reported in article 1 of this thesis).

1.2 Materials for cochleate formation

Lipids: Naturally occurring phospholipids have often been used for preparation of cochleates. Most commonly used classes are anionic phospholipids e.g. Phosphatidic acid (Kouaouci et al., 1985), Phosphatidylethanolamine (Sarig et al., 2011), Phosphatidylinositol and Phosphatidylglycerol (Garidel et al., 2001), phosphatidylserines (PS) etc. Until the last two decades cochleate systems were mainly synthesized from mixtures of lipids viz bovine brain PS (Papahadjopoulos, 1978), Porcine brain PS, Soya PS (Zarif and Tan, 2003) etc. More recently pure synthetic lipids like 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (DOPS) have been used in studies to incorporate drugs (Zarif et al., 2000). There are also reports regarding formation of cochleates from neutral lipids. Galactosylceramides (Kulkarni et al., 1999), sphingosines (Archibald and Mann, 1993) have been evaluated in past for their ability to produce cochleates. In 2010 cholesterol cochleates were synthesized by Harris et al. using ethanol injection method (Harris et al., 2011).

Binding Agents: Metal cations are commonly used as binding agents for cochleate formation. Divalent cations like Ca^{+2}, Mg^{+2}, Ba^{+2} and Zn^{+2} have been used in the past as binding agents with negatively charged phospholipids (Loomba and Scarabelli, 2013). Amongst these calcium is reported as most potent cation for cochleate formation (Papahadjopoulos et al., 1978). Monovalent ions like Na^{+} have also served as binding agents for cochleate formation from phosphatidylglycerols (Garidel et al., 2001). Organic cations like drugs or antimicrobial agents e.g. Oligo-acyl-lycyls (Sarig et al., 2011), 2,3,5,6-tetraaminopyrimidine, tobramycin, and polylysine have been employed for cochleate formation from anionic phospholipids (Jin, 2004). Cochleate formation from lipids like galactocerebroside in absence of binding agents has also been reported. In these cases the conversion of acyl chains to gel state and cochleation were initiated due to use of non-aqueous solvents and temperature fluctuations (Archibald and Yager, 1992).

1.3 Basic structure of cochleates:

Cochleates have a peculiar multilamellar structure (Figure 1.2) which imparts them extraordinary stability. Interaction of negatively charged bilayers with cations result in substantial structural rearrangement that causes aggregation and fusion of bilayers. This interaction triggers conversion of aliphatic acyl chains in lipid bilayer into gel state (Jacobson and Papahadjopoulos, 1975). The resulting planar bilayer sheets consist of lipid and binding agent in molar ratio of 2:1 (Bangham and Papahadjopoulos, 1966). These crystallized planar sheets further tightly fold upon to form cylindrical carpet roll like structure. Hence a typical cochleate is made up of sheets consisting alternating layers of binding agent and lipid bilayer (Rao et al., 2007).

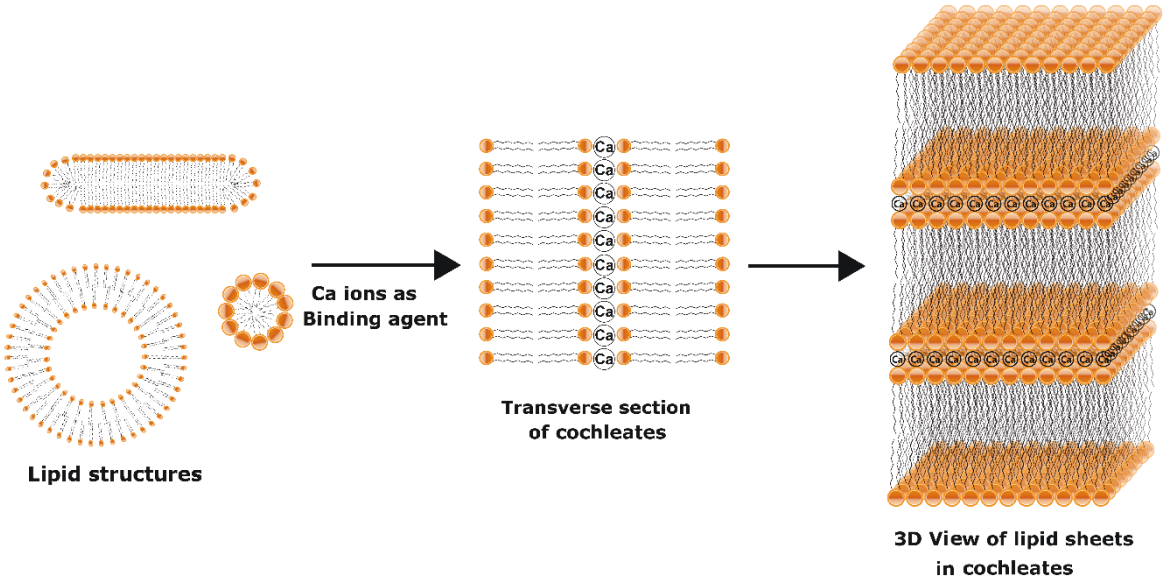

Figure 1.2: Diagrammatic representation of structure of cochleates, with close view of organization of lipid molecules before and after interaction with of calcium ions (binding agent)

Molecules with different properties viz. hydrophobic, hydrophilic, charged etc. are known to associate with cochleates and their incorporation into cochleates has been explained theoretically by Zarif et al. (2005). The schematic diagram in Figure 1.3 explains the possible embedding of molecules with different properties. As per this theory the hydrophobic molecules will get entrapped in lipid chains to minimize interaction with water (Figure 1.3a). Amphiphilic molecules may have higher probability to fit perfectly between bilayer with their hydrophobic and hydrophilic regions aligned with lipid molecules (Figure 1.3b). Whereas the charged molecules can partition into interbilayer domains (Figure 1.3c and d) since charged interactions with lipids and binding agent can promote the encapsulation.

Although entrapment of the molecule is highly defined by its nature the shape of molecule is also an important parameter. It has been observed that hydrophobic and linear molecules are best candidates to get incorporated in cochleates. Whereas small neutral hydrophilic molecules have lower chances of getting incorporated into cochleates considering hydrophobic nature of cochleates.

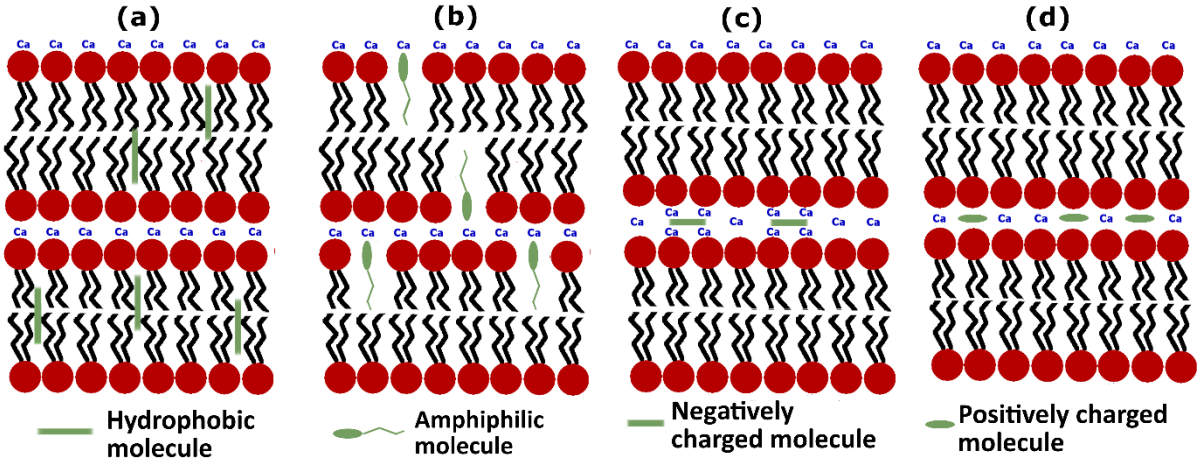

Figure 1.3: Diagrammatic representation of organization of A) hydrophobic B) amphiphilic C) negatively charged and D) positively charged drug molecules in cochleates based on their nature (reproduced from (Zarif, 2005)).

1.4 Advantages of cochleates

Cochleates are comprised of well tolerated and non-immunogenic excipients which are of natural origin (Ramasamy et al., 2009). The building blocks of cochleates such as phosphatidylserines impart additional benefits such as improved cognitive functions, better immune function and antiaging benefits (Sankar and Reddy, 2010). Repeated oral and intra-peritoneal doses of cochleates to mice have shown that cochleates are well tolerated, not toxic and non-inflammatory (Gibson et al., 2004; Zarif and Mannino, 2002). Condensed framework of cochleates can facilitate in protection of associated molecules from harsh conditions such as extreme pH, proteolytic enzymes etc. The extremely hydrophobic nature of cochleate surface and resistance to oxygen penetration protects the internalized moieties from oxidation. Multilamellar structure of cochleates imparts them potential for slow release of drugs. All these advantages are mainly favorable for establishing oral efficacy of drugs which are prone to degradation in gastric environment. Cochleates can easily be produced on large scale. They are very stable formulations with long shelf life. They can remain stable over a year at room temperature in absence of aqueous medium, or over 2 years when refrigerated at 4 °C (Rao et al., 2007). They can retain their structure, shape, and function even after being subjected to processes such as lyophilization. Hence solid structure of cochleates can provide chemical as well as mechanical stability (Bozo et al., 2015) and increase the shelf life of fragile susceptible molecules (Rao et al., 2007).

1.5 Theoretical aspects of mechanism of drug release from cochleates

Mechanism of action of cochleates is not completely understood at cellular or tissue level in biological systems. When administered in body, supramolecular and crystalline structure of the cochleates may

provide protection to the encochleated molecules from the hostile environments. This could facilitate the components within the interior of the cochleate to remain intact, even though the outer layers of the cochleate are exposed to degradation (Mannino and Gould-Fogerite, 1995). Hence, gradual digestion of cochleate may take place leading to slow release and enhanced absorption. In results of cell line studies, cochleates containing fluorescent probes have been shown to bind to the plasma membrane (Gibson et al., 2004). One of theories put forth for explanation of uptake studies considers phagocytic processes for cell internalization. Cochleates with smaller dimensions or their fragments may be taken up by endocytosis in active phagocytic cells and release the contents gradually in cell cytoplasm. In course of time concentration gradient between cochleate and endocytic vesicle may cause calcium to leach out facilitating release of the contents within the endocytic vesicle. Another theory for mechanism of action is based on the fact that calcium ions and phospholipids play pivotal roles in naturally occurring membrane fusion events. This probably increases chances of interaction between cell surface and calcium rich cochleates. During close encounter the outer layer, edge or end of the cochleate and the cell membrane might undergo fusion. As a result encochleated material may be transferred onto cell membranes and eventually be released into the cytoplasm of the cell. The cochleate could then separate from the cell and undergo another fusion event, with the same or another cell. Hence the contact of the cochleate system with the mucous membrane may facilitate in drug absorption (Rao et al., 2007).

1.6 Methods for preparation of cochleates:

1.6.1 Trapping method:

Papahadjopoulos reported preparation of PS-Ca cochleates by trapping method (Papahadjopoulos et al., 1975) i.e. simple mixing of aqueous suspension of lipid and binding agent in molar ratio of 2:1. Trapping method involves the formation of lipid dispersion viz liposomes, micelles etc. as initial step. This is followed by dropwise addition of a solution of binding agent like metal cations into lipid dispersion. Although it is a straight forward procedure it seldom offers narrow size distribution of the final product (Mannino et al., 2005; Zarif and Mannino, 2002). Some modifications such as solvent drip method have been used to improve the loading of active ingredients in cochleates. Briefly, in this modification the active ingredient is dissolved in solvents such as ethanol and added to liposomal suspension. This triggers transfer of active ingredient in higher concentration on liposomal bilayer and hence in cochleates precipitated from them (Mannino et al., 2014). In other modification, liposomes were prepared at higher pH by simple mixing of excipients and drug or by film hydration method in order to attain increase in entrapment. These liposomes were further subjected to cochleate formation by the trapping method (Zarif, 2005).

1.6.2 Dialysis method:

In this method cochleates are prepared by a process involving dialysis. First approach is called '*LC dialysis method*'. In this approach, small unilamellar vesicles are prepared from mixture of lipid and detergents using dialysis. These vesicles are then further subjected to a second dialysis step in presence of binding agent. The second approach is called '*DC dialysis method*'. In this approach, a mixture of detergent and lipids is directly dialyzed in the solution of binding agent. Both approaches necessitates use of detergent (e.g. Octyl β D glucopyranoside 2% w/v solution). Dialysis method forms cochleates with large dimensions (Gould-Fogerite and Mannino, 2000; Gould-Fogerite and Mannino, 1992; Papahadjopoulos, 1978; Zarif, 2002).

1.6.3 Hydrogel isolation method

Hydrogel isolation method utilizes a binary aqueous–aqueous emulsion system for producing small-sized cochleates from unilamellar vesicles. Liposomes are suspended in dispersed phase of an aqueous emulsion formed by immiscible polymer solution (e.g. Dextran-500000/PEG-8000). Further the solution of positively charged binding agent such as Ca^{2+} or Zn^{2+} is added to this emulsion. Slow diffusion of binding agent through polymer solution results in formation of cochleates with particle size in nano-range. The precipitate is then subjected to washing to get rid of polymers. This process can be used to produce cochleates containing drugs or biologically relevant molecules (Jin et al., 2001).

1.6.4 Emulsification-lyophilization method

This process involves formation of multiple emulsion. Briefly, lipid is dissolved in a solvent (viz. chloroform, cyclohexane) which is considered as the oil phase (O). A solution containing binding agent and cryoprotectant are used as the inner water phase (W1), and the buffer as the outer water phase (W2). At initial stage, a primary emulsion with submicron particle size is prepared with aid of probe sonicator using phase O: phase W1 in 3:1 ratio. This emulsion is further added as dispersed phase to five parts of aqueous phase (W2) and gently emulsified to form double emulsion of W1/O/W2. The resultant double emulsion is lyophilized and cochleates are formed on rehydration from lyophilized powder (Wang et al., 2014).

1.6.5 Solvent injection method:

Harris et al. and coworkers reported a method of preparation for cholesterol cochleates in 2011. In this study, ethanoic solution of cholesterol was rapidly injected and mixed with distilled water in ratio 1:9. Mixing was performed above room temperature (40°-75°C). Cochleates obtained by this procedure were hollow cylindrical structures of varying length. They were made up of tightly coiled

multi-bilayer cholesterol helices sometimes with one end closed. Some characteristic planar hexagonal microcrystals were also observed along with cylinders (Harris et al., 2011).

1.7 Characterization of cochleates:

1.7.1 Evaluation of morphology and dimensions of cochleates

Morphology of cochleates have often been evaluated by simple visualization (Miclea et al., 2007; Ramani and Balasubramanian, 2003; Zarif, 2005). Optical microscopy was used to evaluate success of cochleation by several working groups. This could be achieved by addition of EDTA solution to the cochleate suspension and observing formation of large vesicles arising from aggregated cochleates under optical microscope. Electron microscopy techniques such as TEM, SEM, AFM etc. have been mainly employed to study structural details of cochleates (Bozo et al., 2015). Amongst these freeze fracture TEM technique is argued to be most favorable to evaluate the morphology of cochleates (Papahadjopoulos-Sternberg, 2012; Zarif, 2005).

The dimensions of cochleates depend upon size of lipid particles (Liposomes, micelles etc.), final lipid concentration and rate of calcium addition (Zarif, 2005). Cochleates prepared by trapping method or dialysis method are in micron size. Images of cochleates in micron range acquired by electron microscopy (e.g. SEM) have been utilized for particle size determination (Zarif and Mannino, 2002). Such large cochleates have also been evaluated for particle size by incorporation of fluorescent probe and visualization using fluorescence microscope (Miclea et al., 2007). Particle size measurement of nanocochleate formulations prepared by techniques such as hydrogel isolation or emulsification-lyophilization have been reported based on photon correlation spectroscopy data (Pham et al., 2014; Wang et al., 2014).

1.7.2 Differential scanning calorimetry (DSC)

Differential scanning calorimetry has been used to measure a number of characteristic properties of cochleate samples especially in order to observe crystallization events during complexation of lipid and binding agent. The addition of binding agent to lipid dispersions induces the appearance of peak at higher temperature away from original transition peak of pure lipid (Portis et al., 1979; Silvius and Gagne, 1984; Takahashi et al., 1995). The ability of DSC to record transitions in heat capacity has been exploited in monitoring the effect of incorporation of various drugs in cochleate systems and the physicochemical properties of drug after incorporation (e.g. Ketoconazole cochleates)(Landge et al., 2013; Sarig et al., 2011).

1.7.3 Fourier transform infrared (FT-IR) spectroscopy

Fourier transform infrared spectroscopy was reported as effective technique to investigate the conformational changes that occur in lipid after conversion to cochleate phase by studying methylene vibrational bands. Methylene vibrational bands in spectral region 2919 and 2850 cm^{-1} correspond to antisymmetric and symmetric stretching CH_2 modes respectively. Cochleate phase showed symmetric stretching of CH_2 in acyl chains. Monitoring the frequencies in scissoring vibrations of the methylene and methyl groups can also shed some light on acyl chain packing. In this region cochleate phase with an orthorhombic subcell lattice (with molecules ordered all-*trans* acyl chains being arranged perpendicular to each other) gives rise to a splitting of band into two components at 1462 cm^{-1} and 1472 cm^{-1} (Garidel et al., 2000; Snyder, 1967). A marked narrowing of the band arising due to C=O ester stretching with band maximum to 1732 cm^{-1} is observed. Such narrow shape results from a reduced head group mobility, changes in the polarity or possible interfacial hydrogen bonding interactions (Garidel et al., 2000; Zhang et al., 1997). FT-IR studies of PS-Ca complex suggested that Ca^{2+} binds to the phosphate in head group and causes a dehydration of the phosphate ester (Dluhy et al., 1983; Flach and Mendelsohn, 1993). The data obtained from FT-IR supports observation that crystallization of the hydrocarbon chains takes place after complex formation (of lipid and binding agent) as explained in thermal analysis. The possibility of hydrogen bonding between the drug molecules and phospholipids in cochleate formulations have also been determined based on FT-IR studies (Chellampillai et al., 2014; Landge et al., 2013).

1.7.4 X-ray analysis:

X-ray analysis of cochleates revealed supporting data for DSC and FT-IR studies (Garidel et al., 2001; Hauser et al., 1977). The repeat distance obtained by small angle x ray scattering (SAXS) and wide angle x ray scattering (WAXS) indicate formation of a tight, anhydrous Ca-lipid chelate with crystalline hydrocarbon chains (Garidel et al., 2001; Hauser et al., 1977). The SAXS patterns of cochleates show multiple reflections suggesting the lamellar phase. The repeat distance of the cochleates can be calculated from the first reflection of highest intensity. This SAXS pattern remains constant below transition temperature of cochleate phase. When cochleates are heated above its transition temperature a hydrated lamellar phase is formed which exhibits completely different SAXS pattern with an increased lamellar repeat distance and broad diffraction peaks with almost similar intensities. Hydrated gel phase can also be differentiated from reflection pattern of cochleates. WAXS data reveals information about the packing of the acyl chains. The cochleate phase shows appearance of multiple sharp reflections in the wide angle scattering region which is indicative of the formation of a highly ordered chain lattice (Blaurock and McIntosh, 1986; Ruocco and Graham Shipley, 1982). Above transition of cochleate phases a very broad peak is observed, which is characteristic for the

liquid-crystalline phase with fluid acyl chains.(Blaurock, 1982; Tardieu et al., 1973). Hydrated gel state also shows a single broad WAXS band at lower repeat distance, indicating bilayer with tilted hydrocarbon chains (Ranck et al., 1974; Tardieu et al., 1973). Hence diffraction patterns of crystalline cochleate phase and other phases are characteristic and can be easily identified using x-ray analysis.

1.7.5 Nuclear magnetic resonance (NMR)

NMR studies on phosphatidylserine calcium cochleates were reported by Hauser et al. (1977). The ^{2}H-NMR spectra of aqueous lipid suspension in absence of binding agent consisted of a broad singlet. This can be explained by presence of excess free water giving rise to fast exchange between free and bound water of the hydration shells in bilayer lipids. In contrast, the complex of binding agent and lipid gave rise to sharper singlet in the ^{2}H-NMR spectra. This can be regarded to interaction of binding agent with lipid head group which can displace water molecules. The spectra remained constant even after freeze-drying. ^{31}P-NMR studies showed that the signal of lipid dispersion is completely broadened to the baseline on complex formation. This can be correlated with interaction of binding agent which leads to higher order and reduced segmental motion of the polar head group (Butler et al., 1970; Tocanne et al., 1974; Verkleij et al., 1974).

1.8 Applications of cochleates

1.8.1 Pharmaceutical industry

In last few decades considerable research has been devoted towards study of lipid based drug delivery systems amongst which cochleates are one of potential drug delivery system owing unique advantages. Cochleates have been investigated for the safe and effective delivery of number of therapeutically active molecules. Although hydrophobic or amphipathic molecules are usually preferred drug candidates, cochleates have been used as vehicles for different kinds of drugs (Zarif, 2005) as discussed below;

Cochleates showed potential to encapsulate number of antifungal agents. Many studies on oral delivery of cochleate-mediated amphotericin B (Amb), a potent antifungal drug have been reported. Amb cochleates proved efficient when orally administered in a murine model of systemic candidiasis (Santangelo et al., 2000). Delmas et al. investigated Amb cochleates in murine model of systemic aspergillosis which showed improved safety profile by oral route (Delmas et al., 2002). Other antifungal agents such as nystatin have also been incorporated into cochleate formulations (Mannino and Lu, 2014). Ketoconazole cochleates were characterized and evaluated for topical delivery (Landge et al., 2013).

Nanocochleates of Amb and miltefosine were developed for oral administration against visceral leishmaniasis (Pham et al., 2014). Efficacy of orally administered Amb cochleates against

leishmaniasis was confirmed by Wasan et al in hamster model (2009). Cochleates of aminoglycoside antibiotic amikacin have also shown positive results on oral and intra peritoneal administration in black mice infected by *Mycobacterium avium* complex (Lu and Mannino, 2014). Cochleate formulations of other antibiotics such as gentamicin, paromomycin have been evaluated to enhance their oral bioavailability. Clofazimine cochleates showed lower toxicity and improved bactericidal action during *in vitro* evaluation in anti-tubercular model (Popescu et al., 2001). Cochleates can be used to deliver antiviral drugs, such as acyclovir and nelfinavir(Gould-Fogerite and Mannino, 1999; Zarif et al., 2003). Bio Delivery Sciences Inc.(Morrisville, NC) has investigated the potential of cochleates to deliver anti-inflammatory agents (e.g. aspirin, ibuprofen, naproxen, acetaminophen)(Rao et al., 2007). Aspirin and acetaminophen cochleate formulations were found more effective than free drugs in inhibition of nitric oxide synthase in cell-culture studies. *In vivo* studies also showed cochleates to be beneficial in protecting the gastrointestinal tract from side effects such as gastric irritation, ulceration and bleeding (Delmarre et al., 2004b). Cochleates have been employed to deliver anticancer drugs such as paclitaxel, fisetin etc. (Chellampillai et al., 2014). Apart from this, cochleates can be used as a safe, non-viral approach in vaccine therapy. Cochleate have been proved efficacious mediators for induction of antigen-specific immune responses *in vivo* following intranasal (Del Campo et al., 2010), oral (Gould-Fogerite and Mannino, 1996) and intramuscular (Gould-Fogerite et al., 1998) administration. Evaluation of cochleate formulations containing antigens showed generation of strong, long-lasting, mucosal and circulating antibody responses in rodent models. Cochleates were also reported to be useful for delivery of plasmid DNA, gene and proteins (Rao et al., 2007).

1.8.2 Food industry

Cochleates can stabilize and protect an extended range of micronutrients. Hence nutraceutical cochleate formulations can be employed to increase the nutritional value of processed foods, baked goods, natural or artificial dairy products and ready to make products (Delmarre et al., 2004a). Domesticated animal chow, fish food, poultry feed can also be improved by using this approach. It has been evaluated as a strategy to deliver fragile nutrients which can be destroyed during food manufacturing or storage, e.g. quercetin, silibin, vitamins and lycopene. Using cochleates as vehicle such molecules could be efficiently added without affecting the color or taste of both nutraceutical and food (Jin et al., 2007). Cochleates of omega-3 fatty acids, beta carotene have been used in products such as cakes, muffins, pasta, soups and cookies without altering their taste or odor (Sankar and Reddy, 2010). Cochleates can be employed to incorporate one or more additional agents such as coloring agents, flavoring agents, edible acids, preservatives etc.

1.8.3 Cosmetic industry

Phospholipids are known to exhibit beneficial effects on the visual appearance of humans. The topical application of phospholipid lamellar systems offer a wide range of advantages such as increased moisturization, restoring action, biodegradability, biocompatibility and extended release. Their similarity with biological membranes allows better penetration into skin, compared with other delivery systems. Therefore, cochleates can be a favorable strategy to improve the topical delivery of cosmeceuticals (Lasic, 1997; Rahimpour and Hamishehkar, 2012).

1.8.4 Perfume industry

Perfumes are mixtures of fragrances which are chemicals with low molecular weight, lipophilic character and high vapor pressure. Their reactive functional groups (viz. ketones, aldehydes) make them susceptible to degradation by oxidation or hydrolysis (Cortial et al., 2015). For most perfumes as well as the essential oils used for insect repellants prolonged release of fragrance is more desirable than burst release (Nasir, 2010). Therefore, in order to improve the stability of fragrance, prevent evaporation and sustain hydrophilic environment the encapsulation in solid lipid delivery systems such as cochleates might be beneficial (Cortial et al., 2015; Delmarre et al., 2004a).

1.9 Aim of the study

Cochleates offer a high potential for several biomedical applications. Considering the advances of this system towards pharmaceutical industry many studies have focused on the feasibility of employing cochleates as drug delivery vehicles. However, further research is needed on relatively unexplored areas such as thermodynamic stability, formation pathway or structural features of cochleates as detailed understanding of this system may prove vital for formulation development. Another noteworthy challenge is polydispersity of cochleate formulations. Cochleate formation follows a continuous self-assembly process which includes unrestrained growth of particles (Kulkarni et al., 1999; Zarif, 2002). Hence controlling the morphology and the dimensions of individual particles in cochleate formulations is a difficult task. Also the coexistence of intermediate structures makes the purification of formulation a demanding affair. Traditional strategies for preparation of cochleates, such as simple mixing, dialysis etc. lack precise control over morphology of final product. Such structural diversity of formulation may cause unpredictable, non-reproducible and erratic drug release resulting in highly variable bio-availability of incorporated drugs. Therefore, there is a growing need for a strategy to address this issue. Although in past few decades improvements were made in cochleate formation protocols, these methods have limitations for large scale production. In view of all the above mentioned aspects our study was aimed at following goals:

- **To study formation mechanism of cochleates through detailed electron microscopy** in order to understand different intermediate and metastable structures. Motivation of this study arises from the fact that although formation of cochleate cylinders has been explained, specific aspects of formation process are not known.

- **To develop a theoretical model for estimation of dimensions of cochleates** and validate the model by comparing experimentally observed dimensions.

- **To develop simple, reproducible and economic procedure for formation of cochleates** which could be easily transformed to large scale production, reduce random particle aggregation and thereby decrease the size dispersity in cochleate systems.

2 Publication overview

2.1 Publication 1:

Understanding cochleate formation: Insights into structural evolution

Kalpa Nagarsekar, Mukul Ashtikar, Frank Steiniger, Jana Thamm, Felix Schacher, Alfred Fahr

Soft Matter, submitted on 12th June 2015, manuscript resubmitted after peer review

Abstract

Phosphatidylserines of different chain lengths (Viz. C8:0, C10:0, C14:0, C16:0, C18:1, C18:0) were evaluated for cochleate formation using calcium as binding agent. The chosen lipids in the present study exhibited transition temperatures in range of -11 to 68˚C. During cochleate formation, process temperature was decreased to decelerate the self-assembly. Detailed electron microscopy study of these samples was carried out to investigate formation of array of structures during self-assembly of cochleates. Our observations suggested that variation in phosphatidylserine chain length did not have remarkable influence on the type of structures that evolved during formation process. During progression of cochleate formation structures such as ribbons, stacks and networks were identified in all lipid samples. Based on these findings from microscopy studies we have proposed revision in probable pathway for cochleate formation.

Own contribution to manuscript:

1. Experimental design
2. Preparation and evaluation of cochleates except for FFTEM, SEM and SAXS.
3. Data evaluation, interpretation and presentation of the results.
4. Preparation of the manuscript.

2.2 Publication 2:

Electron microscopy and theoretical modeling of cochleates

Kalpa Nagarsekar, Mukul Ashtikar, Frank Steiniger, Jana Thamm, Felix Schacher, Alfred Fahr and Sylvio May

Langmuir, 2014, 30 (44), pp 13143–13151 Published on October 28, 2014

Abstract

Cochleates of phosphatidylserine C18:0 and C18:1 were prepared using calcium ions as binding agent under identical conditions. Detailed structural analysis of cochleates from both lipids was carried out using different microscopy techniques. Our investigations confirmed presence of a continuous hollow channel in rolled-up bilayers stating that most of the cochleate particles formed from lipids under investigation are not like cigars as explained in past. The structure of cochleate was found to be variant along its long axis and tend to exhibit a pattern like an involute in most of the cases. Also the estimated dimensions of the internal channels appeared to be characteristic for building blocks. This knowledge was used for building a thermodynamic model based on minimizing phenomenological free energy for estimating optimal dimensions of a cochleate. Our calculations suggest that membrane bending and calcium induced bilayer−bilayer adhesion alone are insufficient to reproduce the observed experimental dimensions.

Own contribution to manuscript:

1. Experimental design
2. Preparation and evaluation of cochleates except for SAXS, SEM and cryo-electron tomography.
3. Data evaluation, interpretation and presentation of the results except for the theoretical model.
4. Preparation of the manuscript except for theoretical model.

2.3 Publication 3:

Micro-spherical cochleate composites: method development for monodisperse system

Kalpa Nagarsekar, Mukul Ashtikar, Frank Steiniger, Jana Thamm, Felix Schacher, Alfred Fahr

Journal of Liposome Research, submitted on 8th September 2015, manuscript under review.

Abstract

A new method was developed to fabricate cochleate composites with a simple microfluidic setup, by employing solvent effect. Product obtained from the proposed method was characterized using electron microscopy, small angle X-ray scattering and compared with those obtained by other established methodologies. These microspheres (3−5 μm in diameter) made of nanocochleates retain the basic advantages of cochleates. Our simple strategy eliminates elaborate preparation methods, is convenient and could easily be programmed to transform for large scale production. The results of current investigation illustrate this approach to be promising for preparing monodisperse cochleate system with analogous quality.

Own contribution to manuscript:

1. Experiment design
2. Preparation and evaluation of cochleate formulations except for SEM and SAXS.
3. Data evaluation, interpretation and presentation of the results.
4. Preparation of the manuscript.

3 Publications

3.1 Publication 1:

Understanding cochleate formation: Insights into structural evolution

Kalpa Nagarsekar, Mukul Ashtikar, Frank Steiniger, Jana Thamm, Felix Schacher, Alfred Fahr

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Soft Matter

Understanding cochleate formation: Insights into structural evolution

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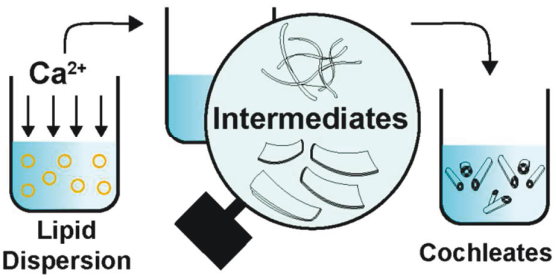
Abstract

Understanding of cochleates, their structure and self-assembly process has become increasingly necessary considering the advances of this drug delivery system towards pharmaceutical industry. It is well known that the addition of cations like calcium to a dispersion of anionic lipids such as phosphatidylserines results in stable, multilamellar cochleates through spontaneous assembly. In the current investigation we have studied the intermediate structures generated during this self-assembly of cochleates. To attempt this, we have varied the process temperature for altering the rate of cochleate formation. Our findings from electron microscopy studies showed formation of ribbonlike structures, which with proceeding interaction associate to form lipid stacks, networks and eventually cochleates. We also observed that variation in lipid acyl chains did not make a remarkable difference to the type of structures evolved during formation of cochleates. More generally, our observations provide a new insight into the self-assembly process of cochleates based on which we have proposed a pathway for cochleate formation from phosphatidylserine and calcium. This knowledge could be employed in using cochleates for a variety of possible biomedical applications in the future.

Keywords: Cochleates, phosphatidylserine, self-assembly, electron microscopy, intermediate structures

Graphical abstract



Introduction

Cochleates are lipid-based supramolecular assemblies which may show potential as advanced drug delivery system[1]. Cochleates have elongated shape and carpet roll like morphology invariably accompanied by tightly packed bilayers.[2] Apart from being biocompatible, cochleates exhibit high stability which is attributed to their unique compact structure[3]. Since discovery, there have been number of modifications in preparation methods of cochleates in order to yield suitable structures for specific purposes, albeit not much has been explored regarding the actual mechanism of cochleate formation. Hierarchical superstructures originating from unrestrained self-organization impart impressive complexity to the cochleate system but it is accompanied by higher degrees of polydispersity. This lack of homogeneity can be a prominent obstruction in large scale production of cochleates. Due to the higher polydispersity, a tendency for aggregation, and peculiar geometry cochleates have remained as one of the challenging systems for characterization by standard methods used for nanoparticles. In order to overcome this challenge, thorough knowledge of all metastable species arising from the involved building blocks is obligatory. Although the general concept for cochleate formation has been established, there may be a scope to elaborate further and such information can be of assistance in the future to predict properties of the resulting products or to maneuver the process towards an expected outcome. Hence, it is important to study formation of cochleates closely in order to gain understanding of this drug delivery system.

Many theories have been presented to explain formation of cochleate cylinders yet specific details remain hidden. Transformations of lipid membrane into tubular structures have been thoroughly investigated in theory, however, these theories are difficult to verify conclusively[4-6]. The most accepted theory describes that binding of bilayer sheets results in formation of high axial ratio microstructures like helices which in turn due to intrinsic bending of bilayers form structures like cochleates[7, 8]. For cochleate formation, interaction between phospholipid headgroups and the cationic bridging agent is a pivotal step which is essential to trigger the membrane fusion[9]. It is well documented that after addition of divalent cations the fusion between lipid dispersion gives rise to extraordinarily stable high melting[10], crystalline phases by dehydration and strong immobilization of the serine headgroups[11-13]. The resultant lipid complexes have aliphatic acyl chains in a crystalline state[14] which further results in stable cylindrical multilamellar superstructures[8]. Apart from this, chirality may play an essential role in order to form cochleates[15]. As per theories based on molecular chirality, chiral interactions result in packing of molecules with non-zero angle against neighboring molecules. This in turn contributes to the twisting of bilayers leading to formation of a cylinder[4, 16, 17].

In the last decades many detailed studies have addressed the mechanistic understanding of bilayer fusion. The most commonly encountered difficulty in such studies is to segregate the kinetics of bilayer fusion from consecutive stages of destabilization[18-20]. Characterization of such studies often depicts overlap of multiple simultaneous events. Acquiring experimental evidence to affirm various steps and their order in cochleate formation is a challenge as it is difficult to pace these stages during the course of the experiment. Conditions that induce aggregation in anionic lipids have been studied extensively. Along with the concentration of the bridging agent, temperature is a very important aspect affecting the rate of bilayer fusion[21, 22]. We attempted to use process temperature as a variable to observe gradual evolution of cochleates during the formation process. Therefore, synthetic phosphatidylserines (PS) with varying phase transition temperatures (Tm) were selected for this study.

PS are anionic phospholipids which have been repeatedly exploited for the preparation of cochleates. PS are negatively charged over a wide pH range. They consist of a serine headgroup with three ionizable sites, i.e. phosphate group, amino group and a carboxyl functionality. Both the carboxyl and phosphate groups in the serine headgroup have a high inclination to bind with positively charged moieties[23, 24]. This makes serine lipids

one of the most eligible phospholipids to form cochleates. Apart from calcium, cations such as magnesium, zinc etc have also been used to form PS cochleates in past[25,26]. It should be noted that there are reports of substances other than PS forming cochleates[27, 28]. However for the purpose of this article we will only discuss calcium PS cochleates. Further, by formulating cochleates using various PS with 8-18 carbon chains we attempted to assess the influence of building blocks over characteristics of this drug delivery system. It would be interesting to understand if alterations in chain length have any effect on the resulting structures.

In the current study, the basis of cochleate formation has been explored by investigating the observed peculiar metastable microstructures and their coexistence with cochleates. In order to fathom intermediate stages of cochleates we observed specimens multiple times during outset using electron microscopy as primary characterization technique. In the past most of the investigations related to structural features or formation of cochleates have relied strongly on freeze fracture techniques[29]. In the current study along with Freeze Fracture Transmission Electron Microscopy (FFTEM) we employed other EM techniques like cryo-SEM (Scanning electron microscopy) or cryo-TEM (Transmission electron microscopy) to visualize cochleates from different perspectives. Our morphological exploration was complemented by small angle X-ray scattering (SAXS). During imaging studies we came across different intermediate metastable structures which may shed some light on the underlying formation mechanism. We identified ribbonlike structures as an important subunit and report their formation as alternative first stage, which is an unrecognized facet in phosphatidylserine cochleate formation. Our results indicate that all selected PS displayed presence of similar intermediate structures some of which can coexist with cochleates. Based on our findings we present details for possible pathways for cochleate formation from phospholipid aggregate precursors.

Materials and Methods

Materials :

1,2-dioctanoyl-sn-glycero-3-phospho-L-serine [sodium salt] (DOctPS); 1,2-didecanoyl-sn-glycero-3-phospho-L-serine [sodium salt] (DDPS); 1,2-Dimyristoyl-sn-glycero-3- phospho-L-serine [sodium salt] (DMPS); 1,2-dipalmitoyl-sn-glycero-3-phospho-L-serine [sodium salt] (DPPS); 1,2-dioleoyl-sn-glycero-3-phospho-L-serine [sodium salt] (DOPS) and 1,2-distearoyl-sn-glycero-3-phospho-L-serine [sodium salt] (DSPS) were obtained from Avanti Polar Lipids Inc (Alabama, USA). Araldite LY 564 was obtained from HunTcan Advanced Materials GmbH (Switzerland). All other chemical reagents were of analytic grade and used as received without further purification. Ultrapure water (RiOs™ 8, Millipore, Eschborn, Germany) was used for all experiments.

Preparation of cochleates

Briefly, the lipid was dissolved in a mixture of chloroform:methanol (3:1) in a round bottom flask followed by removal of the organic solvents using a rotary evaporator (R-144 BÜCHI Labortechnik GmbH, Germany) to form a thin lipid film. The lipid film was further hydrated with 10 mM TRIZMA® [2-Amino-2-(hydroxymethyl)-1,3-propanediol] buffer above the Tm of the lipid. The dispersion was vortexed followed by extrusion through a polycarbonate membrane of 100 nm pore size to produce a homogenous dispersion of lipid. Cochleates were formed by slow (10 µl at a time) addition of calcium chloride (100 mM) to the lipid dispersion in TRIZMA® buffer (NaCl, 100 mM; TRIZMA®, 10 mM) under constant stirring above Tm of the lipid. The molar ratio of lipid to calcium was 1:1. Precipitated mixtures were then stored at 4 °C. Apart from these batches in order to study intermediate structures as explained above, batches of cochleates were also prepared at lower temperatures, see Table-1. All samples were evaluated immediately after preparation and periodically during incubation up to 12 months by electron microscopy (EM).

Table 1: Process temperatures used during preparation of various phosphatidylserine cochleates.

Phosphatidylserine	Transition Temperature	Process temperature
DOctPS (C8:0)	< 10 °C	4 °C and 20 °C
DDPS (C10:0)	< 10 °C	4 °C and 20 °C
DOPS (C18:1)	-11 °C	4 °C and 20 °C
DMPS (C14:0)	35 °C	20 °C and 40 °C
DPPS (C16:0)	54 °C	20 °C and 59 °C
DSPS (C18:0)	68 °C	20 °C, 50 °C and73 °C

Electron Microscopy:

Scanning Electron Microscopy: A droplet of the cochleate sample was adhered for 5 min onto a perforated Formvar-coated copper grid (300 mesh, Quantifoil, Germany). Excess liquid was removed with a lint-free filter paper and examined in a LEO 1530 Gemini (Carl Zeiss, Germany) scanning electron microscope (SEM) at 4 kV acceleration voltage.

Cryo-Scanning electron microscopy: 10 µL of sample was adhered onto a gold stub (3 mm, Zeiss, Germany). Further, grid was quickly plunged in liquid ethane (-180 °C) and the sample was cryo-transferred with the help of the vacuum cryo-transfer system BALTEC BAF 060 (Baltec, Switzerland) in liquid nitrogen in order to knife-fracture the specimen. Further, temperature of the sample was raised to −80 °C to allow controlled sublimation of water in order to expose the particles. To improve contrast sample was then coated by 1.5 nm of tungsten. Images were acquired using a Zeiss Auriga 60 (Carl Zeiss, Germany) using the SEM imaging routine at 3 kV acceleration voltage and a working distance of 6 mm using a secondary electron detector.

Freeze fracture-transmission electron microscopy: An aliquot of 5 µL of the dispersion was placed between copper profiles and subjected to plunge freezing immediately using liquid ethane/propane mixture. Fracturing and replication of the samples were carried out at –150˚C in a BAF 400T freeze-fracture device (BAL-TEC, Liechtenstein) equipped with electron guns and a film sheet thickness monitor. Samples were coated with 2 nm of Pt(C) under an angle of 35, followed by perpendicular evaporation of C. The replicas were cleaned with a chloroform-methanol mixture, and examined in an EM 902A (Carl Zeiss, Germany) electron microscope.

Transmission electron microscopy: An aliquot of 5 µL of the dispersion was placed onto a Formvar-coated copper grid (300 mesh, Plano, Germany) for 4 minutes. Excess of liquid was removed with a lint-free filter paper and the grid was air dried. TEM grids were investigated using Zeiss EM 902A (Carl Zeiss, Germany) operated at 80 KV. Images were taken with a 1k x 1k FastScan-F114 CCD Camera (TVIPS GmbH, Germany). To evaluate and compare dimensions of cochleates of high Tm lipids micrographs were analyzed using ImageJ version 1.47[30].

Cryo-Transmission electron microscopy: 5 µL of Dispersion of sample was placed on carbon-coated copper grid (R1.2/1.3 + 2 nm, Quantifoil Micro Tools GmbH, Germany). It was subjected to plunge freezing in liquid ethane at −180 °C. Grids were then transferred into a liquid nitrogen cooled (T = −196 °C) cryo-holder (Gatan Inc., USA) and inserted into Philips CM 120 cryo-TEM (Philips CM 120, Netherlands) for evaluation. The images were recorded with 1k CCD camera (FastScan F114 TVIPS, Germany).

Investigation of Cross Section of Resin-Embedded stacks: Embedding was carried out using same procedure as described by *Nagarsekar et al.*[2]. Briefly, sample was redispersed in 100 mM cacodylate buffer (pH 7.2) containing 1% OsO4 for 2 h. Sample was centrifuged again and rinsed in buffer before dehydration in ethanol (50% v/v) for 15 min. It was further subjected for 1 h to 1% uranyl acetate solution to improve contrast. The pellet was then redispersed in an epoxy resin Araldite (Agar Scientific, United Kingdom) used as embedding medium. The mixture was polymerized and the polymer blocks were ultramicrotomed into thin (70−100 nm) slices, using a diamond knife mounted on an Ultracut E device (Reichert Labtec, Germany) at room temperature. The slices were then transferred to copper grids (Quantifoil, Germany) and examined by TEM.

Small angle X-Ray scattering

All samples were dialyzed and freeze dried before evaluation. SAXS measurements were performed on a Bruker Nanostar (Bruker, Germany) equipped with a IµS (IncoaTec, Germany) X-ray source, operating at λ=1.54Å. The sample-to-detector distance was 107 cm. Samples were mounted on a metal rack and fixed using tape. All measurements were carried out at room temperature for 120 minutes, with a 2D position sensitive detector Vantec 2000 (Bruker GmbH, Germany). The scattering patterns were corrected for the background (Scotch Tape, Germany) prior to evaluations and radially integrated to obtain the scattering intensity as a function of the scattering vector, $q=(4\pi/\lambda)\sin\theta$, with 2θ being the scattering angle and λ the X-ray wavelength.

Results and Discussion

To decrease the polydispersity, all lipid dispersions were subjected to extrusion before cochleate formation. Amongst chosen lipids, DOPS formed liposomes. DOctPS and DDPS having strong surfactant properties formed micelles along with small vesicles. High Tm lipids like DSPS, DPPS and DMPS formed bilayer discs (Fig 1). Cochleates were formulated using simple trapping method contemplating the ease of control over process conditions. Considering the strong influence of process parameters on morphology of the final products, it was assured that during preparation all conditions were kept constant except the variable of interest i.e. temperature. Formulations were prepared at different temperatures to identify intermediates and determine the pathway of cochleate formation. Visually, during formulation all translucent lipid dispersions went turbid following precipitation upon addition of $CaCl_2$ solution. This observation was consistent for all process temperatures. White fluffy precipitate and a clear supernatant were formed in the samples which were prepared below the Tm of the respective lipid. However, the samples prepared above Tm showed a densely aggregated suspension. The change from opalescent to turbid suspension after addition of calcium was considered as an indication for binding processes taking place. The visual appearance of samples did not change with aging. The microstructures formed in all samples were explored using different electron microscopy techniques.

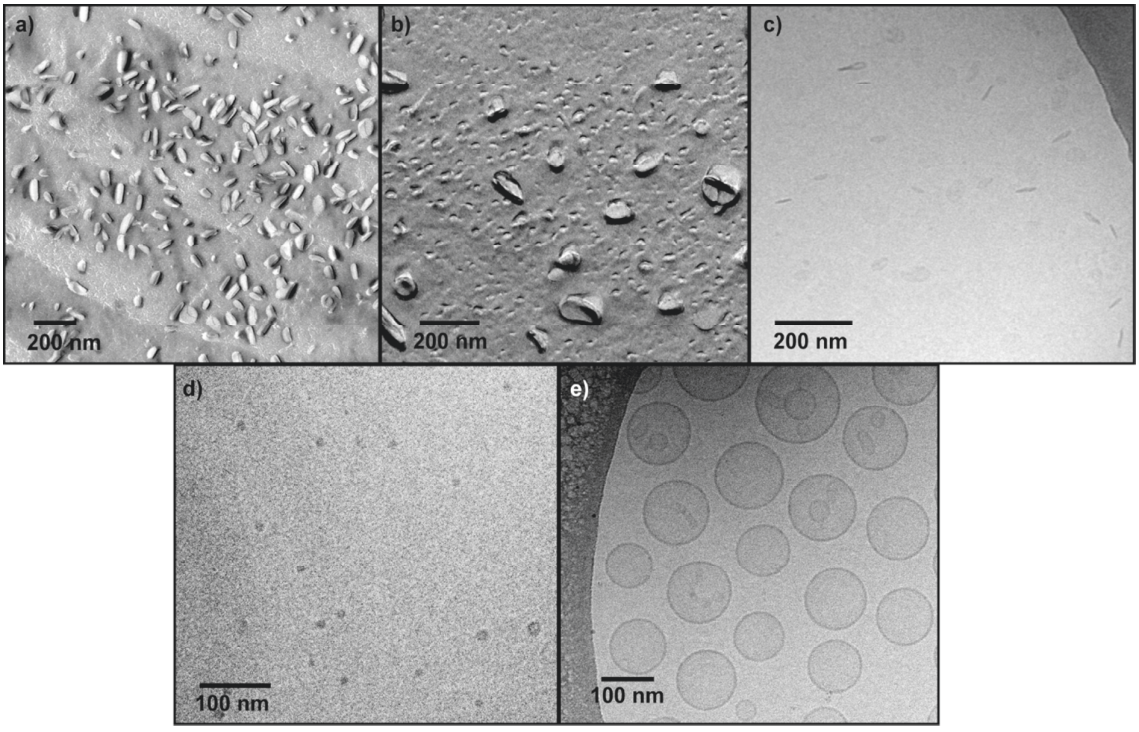


Figure 1: Electron micrographs showing discs of (a) DSPS and (b) DMPS by FFTEM. Cryo-TEM images of (c) DPPS discs, (d) DDPS micelles and (f) DOPS liposomes.

Although most cochleate samples observed under TEM and cryo-TEM could be visualized without staining, for better contrast aggregated bilayers and ribbons were visualized by negative staining with uranyl acetate in TEM. The drying of cochleate samples during electron microscopy did not affect the tubule structure, or cause collapsing of the internal hollow space. This was probably due to the rigid nature of the multilamellar system[31]. Nonetheless, no decomposition artifacts were generated as a result of electron dosage used to capture micrographs of the sample observed in the study. In order to confirm the findings multiple techniques such as SEM, cryo-SEM, cryo-TEM, FFTEM and resin-embedding were employed. Throughout the text, DOctPS, DDPS and DOPS are referred to as low Tm lipids while DMPS, DPPS and DSPS are referred to as high Tm lipids.

Aggregation and formation of calcium chelated bilayers

Aggregation is an inceptive step during cochleate formation which in turn leads to the generation of calcium chelated bilayers[32]. Literature suggests addition of calcium ions to PS vesicles triggers formation of array of intermediate structures such as discs, sheets and partially folded bilayers which finally give rise to cochleate cylinders[8]. Being nucleation-dependent, aggregation processes are usually characterized by a lag period, i.e. time required for the critical nuclei to form can be decelerated by reducing the process temperature[33]. Since fusion processes and rates of morphological transitions are very fast (in milliseconds) in substrates with smaller or unsaturated carbon chain length, it is not easy to trap these initial intermediate structures. Consequently, few early intermediate structures could be observed during evaluation of cochleate samples of low Tm lipids. Apart from this some higher order intermediate structures also appeared simultaneously in these samples. We found that among selected PSs high Tm lipids were ideal candidates to trap early intermediate structures by this method since, these transformations could be prolonged more efficiently in these lipids.

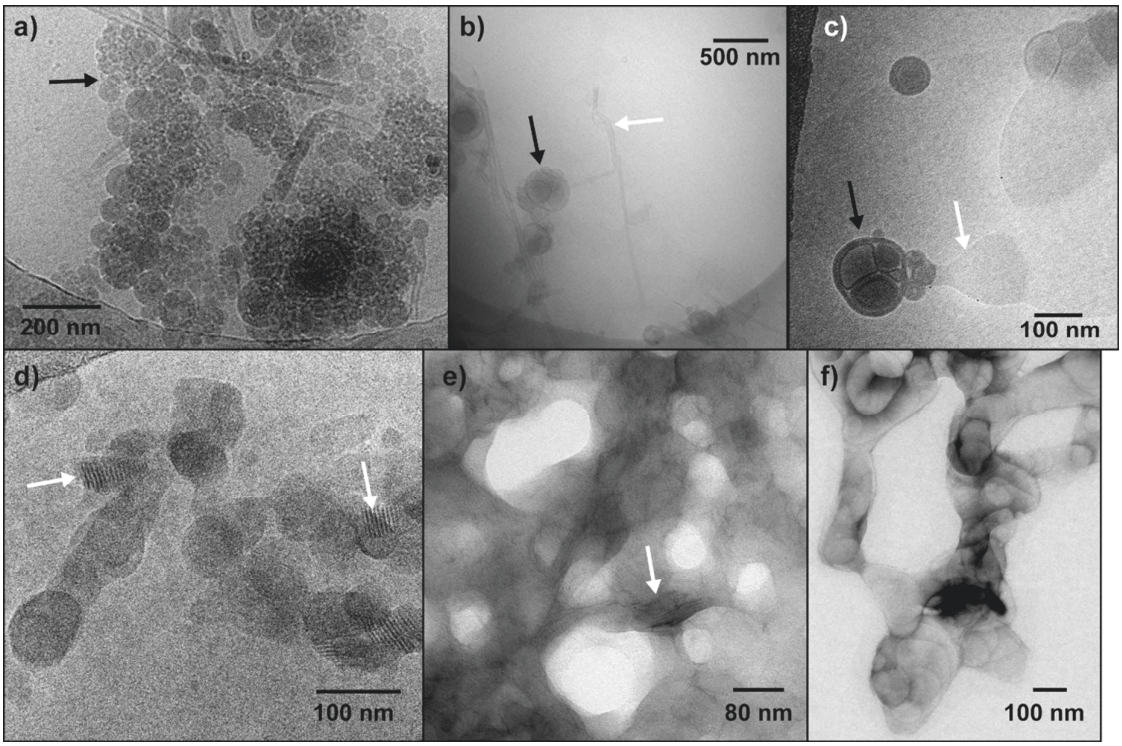


Figure 2: Aggregation observed in cryo-TEM images of (a) DOctPS (b) DDPS and (c) DOPS samples prepared at 4 °C. TEM images of samples prepared below Tm (d) DMPS, Cryo-TEM image (e) DSPS aggregates showing twisted bilayers and (f) DPPS aggregates. White arrows indicate formation of ribbonlike structures while dark arrows point at aggregated lipid bilayers. All micrographs were taken in 10 seconds after addition of calcium.

Samples prepared above the Tm of lipids mainly showed cochleates along with higher intermediate structures and did not show early intermediates e.g aggregates of liposomes, disc micelles etc. Cochleate samples prepared at lower process temperatures were evaluated immediately (10 seconds) by EM before incubation to visualize early intermediate structures. Samples of low Tm lipids prepared at 4 °C showed presence of aggregation structures along with ribbons or sheets. Aggregates observed in these samples varied in size and shape. These aggregates appeared to contain a large number of dense particles (as indicated by dark arrows in Fig. 2a-c). Multilamellar aggregates observed in samples of DOPS appeared largely dehydrated and revealed repeat distance of bilayer suggesting efficient binding of calcium ion. We presumed that such dense aggregates may be highly unstable as they displayed asymmetrical but closely packed lamellae. Such instability may result from destabilization of the outer monolayers during vesicle fusion as reported in the past[34].

Previous investigations on calcium interaction with PS vesicles also reported deformation of primary structures following aggregation and possibility of rupture or fusion of the multilamellar aggregates which was in line with our observations[34-36]. DDPS and DoctPS samples revealed several such aggregates showing formation of ribbonlike structures (Fig. 2b). Similar patterns were observed in samples of DOPS which showed aggregates radiating broad ribbonlike structures (Fig. 2c). Though rare, few cochleate cylinders were also observed in DoctPs samples at this stage.

In high Tm lipid samples prepared at 20 °C, large fraction of agglomerated bilayers and ribbons appeared to coexist at the aggregation stage. No traces of cochleate cylinders were evident. In high Tm lipids, edges of bilayer discs probably fuse with a specific orientation to form ribbonlike silhouette giving rise to three dimensional rigid network. Chain-like morphology of aggregated discs in micron length were also observed (Fig. 2f). These three dimensional aggregated structures finally appear to evolve into ribbons (Fig. 2d), which were formed in abundance and was in micron range. The edges of bilayer discs in aggregated structures often appeared twisted indicating instability, which may be responsible for such transformation or fragmentation. Nucleation at the edges may also be preferred due to better accessibility and probability of orientation.

Ribbons:

EM images of all samples prepared at lower process temperatures revealed presence of ribbonlike bilayer strips. Fig. 3 shows images of ribbons from different lipids. The length of ribbons observed in samples were quite divergent. Although highly transparent all ribbons had well-shaped edges with thickness varying from 2-3 bilayers as can be seen in cryo-TEM image of DSPS ribbons (Fig. 3e). Though few in number, ribbons were noticed in samples of low Tm lipids prepared at 4°C. DOctPS (Fig. 3d) and DOPS samples showed presence of ribbons within 10 seconds after addition of calcium. In case of DDPS, ribbons were observed in abundance within an hour after addition of calcium ions in (Fig. 3c).

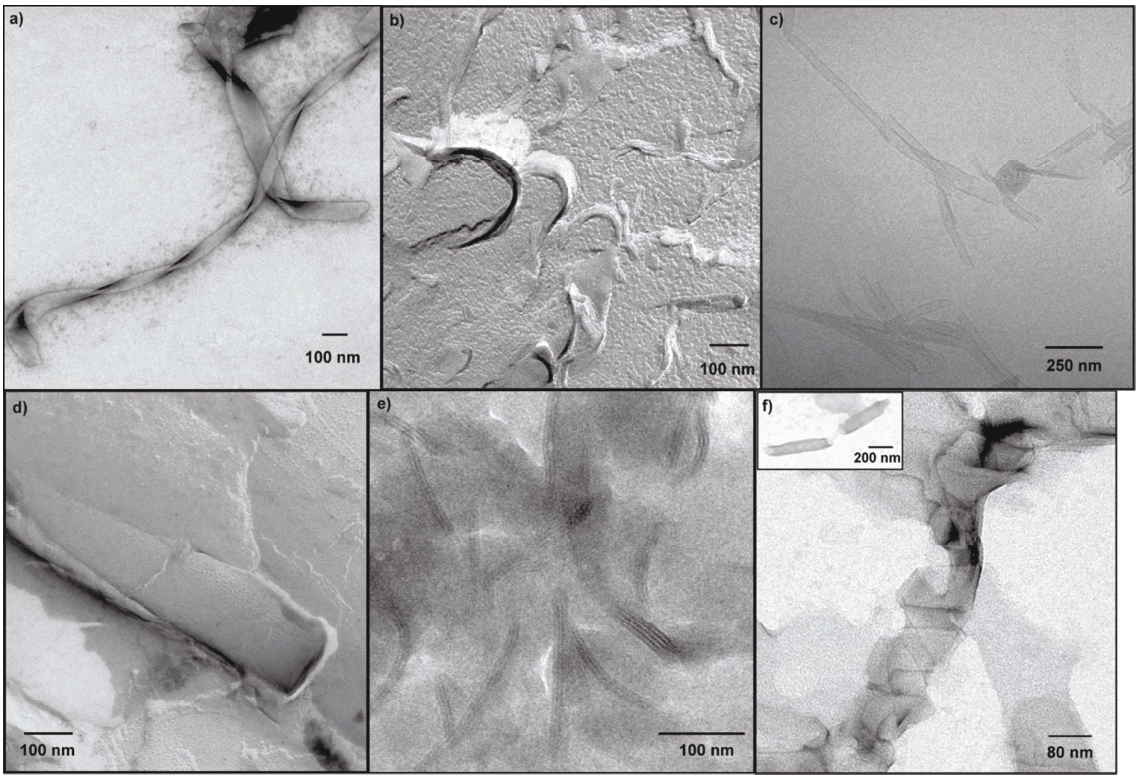


Figure 3: Ribbons observed in samples prepared below Tm (a) TEM image of DSPS (b) FFTEM image of DPPS (e) Cryo-TEM of DSPS and (f) TEM of DMPS with occasionally observed helical ribbon and tubules inset. Ribbons observed in samples prepared at 4°C (c) Cryo-TEM of DDPS (d) FFTEM of DOctPS.

The samples of high Tm lipids prepared at 20°C also consisted of a large amount of ribbons that partially overlapped (Fig. 3 a, b and e). Amongst these samples of DSPS and DPPS did not show any other higher order intermediate structures. The main feature of ribbons observed in DSPS and DPPS samples (Fig. 3a and b) prepared at 20 °C is that they did not evolve into closed aggregates or give rise to nanotubes. Even upon prolonged incubation up to twelve months both DSPS and DPPS samples prepared at 20 °C showed only the presence of aggregated ribbons. They formed structures that looked like loose networks (shown in Fig. 7b) however failed to form cochleates. In case of DMPS samples prepared at 20 °C few helical ribbons and tubule like structures formed from single ribbon were also observed (within 20 min after addition of calcium ions). These tubule like structures (Fig. 2f inset) resembled nanotubes and seemed to lack the multilamellar structure of a typical cochleate. As compared to other high Tm lipids the DMPS samples prepared at 20 °C showed higher inclination towards formation of higher order structures in the hierarchy of cochleate formation. In order to confirm that stack formation takes place after formation of ribbons, DSPS ribbons were heated up to 50 °C. EM evaluation of this sample showed a large number of stacks. Further heating of this DSPS sample, above Tm gave rise to cochleates (data not shown).

Literature advocates that large lipid sheets condense and curl up to form a cylindrical structure[37]. We presume from our findings that ribbonlike fragments are probably formed at early stage during cochleate formation. Initiation point for any supramolecular assembly would be the formation of a template by nucleation before subsequent growth. Ribbons are also most repeatedly observed precursors of tubular structures[38]. The Repeat distance observed for ribbonlike structures is smaller as compared to that of bilayers without calcium interaction (with reference to SAXS data). This suggest orientation and organization of lipid molecules governed by interactions of their polar headgroups[39]. Hence ribbon formation may be the outcome of the dehydrated headgroups of lipid molecules sealing in a zipper effect due to divalent cations[9].

Stack formation

The contrast between ribbons and stacks due to variation of the packing density assisted in identification of structures in EM analysis. No negative staining was required for evaluation of stacks in the samples unlike ribbons. Fig. 4 shows the morphology of stacks which have a strong tendency to associate into multilamellar aggregates (as highlighted in Fig. 4d). TEM images from embedding study (Fig. 4f) and cryo-SEM data (Fig. 5c) also reveal multilamellarity of these lipid stacks. Interestingly, most of the stacks showed an elongated trapezoidal shape. Some exhibited different morphology due to curling, aggregation, and possible fusion with other stacks. Although our results of characteristic shape and multilamellarity of stacks are in line with previous findings of the Blume workgroup[28] our data revealed that the dimensions of stacks varied considerably in width, thickness and length(Fig. 5a & b). We observed that stacks were inclined to attach to cochleates or fuse together to form larger stacks. Our EM data also indicated cochleate formation from helically coiled stacks, which may be the result of curvature arising from packing frustration of the terminal lipid molecules. In our previous work we have hypothesized that energy required for rolling of stacks comes from a combined contribution of bending, adhesion and a frustration energy[2]. Although formation of large planar multilamellar sheets is considered as critical step before the formation of cylindrical cochleate structures[8, 40], our findings suggested that it may not be essential. PS samples such as DOPS and DoctPS prepared above Tm displayed large planar sheets in abundance as compared to others which showed larger number of stacks and networks.

No stack formation was observed in the samples of DPPS and DSPS, which were prepared at 20 °C below the Tm of the respective lipids. These samples did not form stacks or any cylindrical structures even upon prolonged incubation for up to 12 months. In contrast, DMPS samples prepared below Tm showed presence of lipid stacks within 1 hour after addition of calcium and gave rise to cochleates along with large amount of networks after further incubation. This might be an indication that stack formation is an essential step in formation of cochleate cylinders without which the process of cochleate formation cannot reach completion.

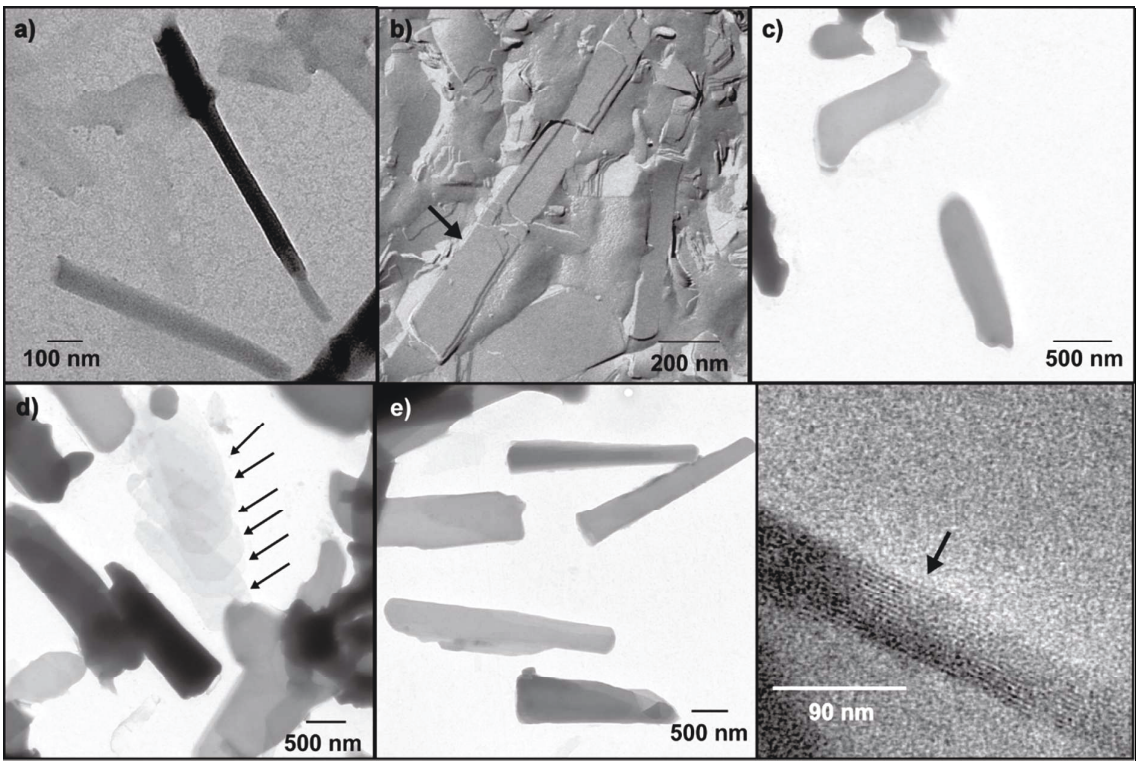


Figure 4: Electron micrographs showing stack formation (a) TEM image of DOctPS, (b) FFTEM image of DMPS, (c) and (d) TEM of DPPS; arrows indicate multiple plates with similar outline. TEM of (e) DOPS, (f) DDPS sample; cross section by resin embedding technique. Samples of low Tm lipids were prepared at 4˚C where as DPPS and DMPS are prepared above Tm

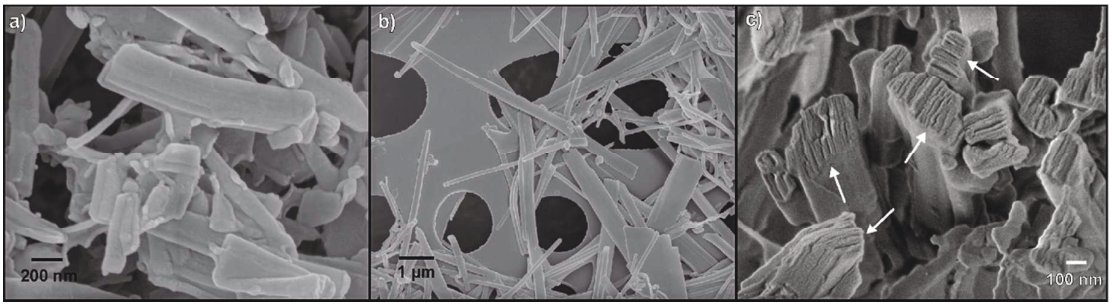


Figure 5: DDPS samples prepared at 4˚C showing stacks (a) adhered to network-like structure in SEM image b) with varying dimensions in SEM image and c) displaying multilamellarity at the edges in a cryo-SEM image.

A peculiar ring like structure was observed on multiple occasions at the terminal edges of stacks (Fig. 6). EM data of all PS samples displayed this structure with an approximate diameter of 200-300 nm. It was more prominently observed in high Tm lipids. Such formations were mainly observed at the ends of lipid stacks or sheets. Due to breaks or irregularities at the boundaries of sheets or stacks the probability of ring formation may be enhanced. Although its function is not completely clear up to now, it could be an initiation point for cochleate formation. It is difficult to comment about immergence of such structure. One could suspect that this is a result of curling starting at the most vulnerable end of the lipid sheet, which may initiate formation of cylindrical structure and this kind of structure may serve as a support for another sheet. However, for this to work one can imagine that the axis of the ring must be parallel to the sheet that will eventually roll around the ring structure. Nevertheless, all the ring like structures we observed had its axis perpendicular to the stack.

Hence, it is difficult to conclude based on our findings if such structures attach to a stack and lead to further curling or if they have any other significance in the formation of cochleates. Ring formation was never observed in DSPS and DPPS samples prepared below Tm of lipid which displayed ribbons. This hints that such structure may have been formed after formation of stacks. We would like to stress that the ring formation was not completely understood as it was not possible to isolate these structures and further studies are needed to understand them completely.

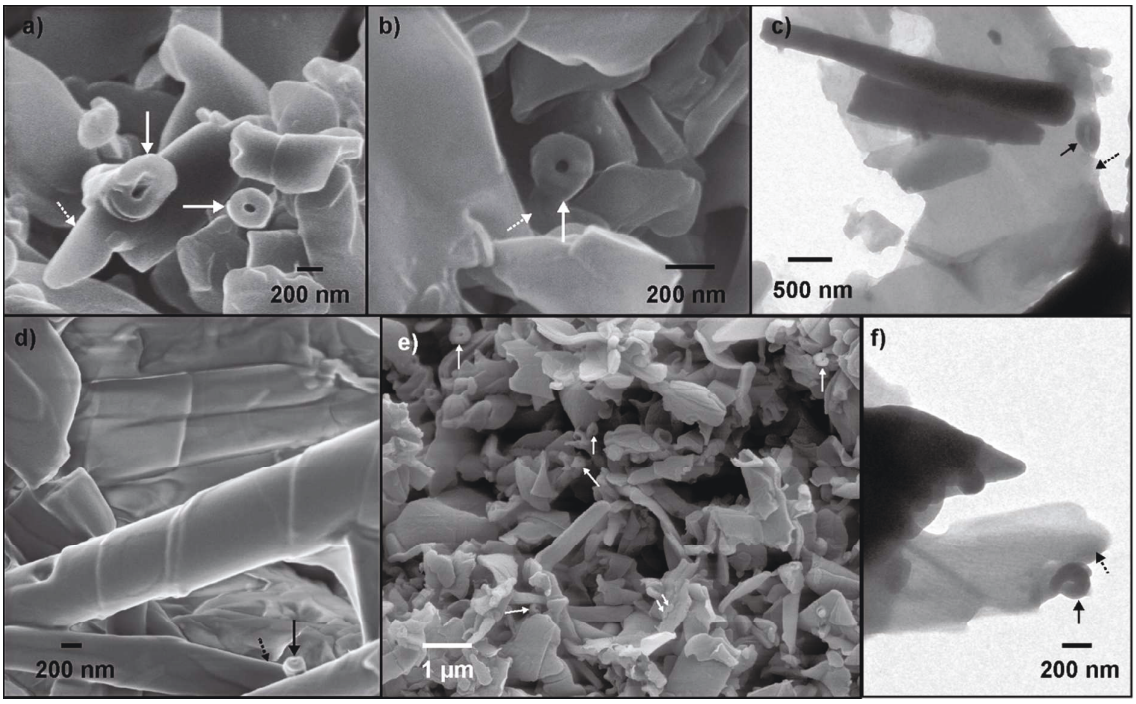


Figure 6: SEM images obtained from samples of (a) & (b) DPPS and (d) DOPS. (e) Overview of DPPS stacks with multiple sites showing ring formation in SEM image. TEM images of (c) DDPS and (f) DMPS, bilayer sheet attached to a ring. Dotted arrows indicate stacks/sheet and solid arrows indicate ring formation. All samples were prepared above Tm of respective lipids.

Networks

All samples prepared above Tm displayed the presence of networks of stacks as seen in Fig.7. The lateral dimensions of lamellae building network varied from a couple of bilayers to lipid stacks with coherent piling. Apparently, the process of network formation might be promoted due to unrestrained interactions between lipid stacks resulting in random attachment to one another (Fig. 7a). Occasionally, these convex–concave bilayers showed a continuous flattened regions. These regions in networks clearly showed outlines of lipid stacks at higher magnification (Fig. 7f). This suggests that formation of a nexus must be via continuous intercalation of bilayers on the host aggregate, which ultimately undergoes fusion in order to achieve thermodynamic equilibrium. However, for cochleate formation curling of stacks or sheets is a critical step, which may be hindered by uncontrolled formation of networks. The size and spatial distribution of these networks must have a strong influence on a fraction of stacks that undergo transformation into cylinders, as it directly correlates with the potential number of favorable interaction sites. Our observation suggests, samples of DMPS, DPPS and DSPS prepared above Tm had higher fraction of networks than low Tm lipids. These samples also exhibited a large number of cochleates attached to networks. TEM of these structures as seen in Fig. 7h shows fragments of curved lipid bilayers on what appears to be as pieces of incompletely formed cochleates. Although not abundant, presence of networks was also observed in samples of low Tm lipids (Fig. 7g).

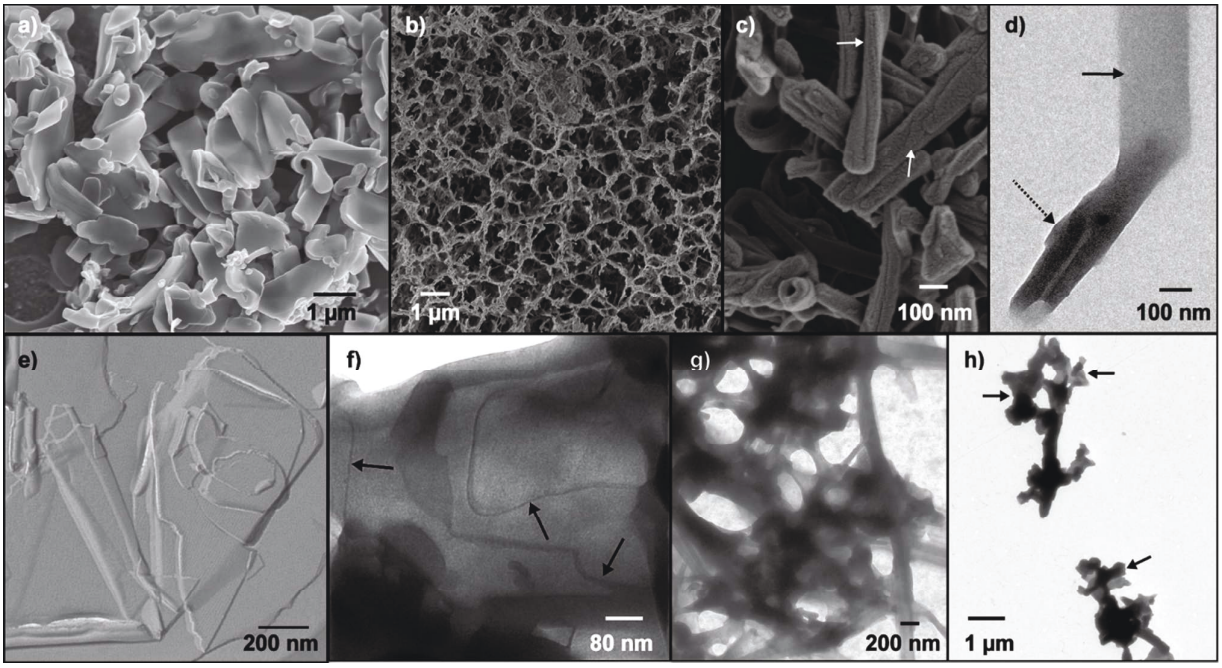


Figure 7: Networks observed in (a) SEM image of DPPS and (h) TEM image of DSPS sample, prepared above Tm. Networks observed samples prepared below the Tm b) Cryo-SEM image of DPPS (c) Cryo SEM image of DMPS. (g) TEM image of DDPS sample prepared at 4 °C. (d) TEM image of single stack forming a cochleate in sample of DMPS prepared below the Tm. Sheet formation observed in sample prepared above Tm in (e) FFTEM image of DPPS (f) TEM image of DSPS, image clearly shows outline of fused edges of lipid stacks. Solid arrows indicate stacks whereas dashed arrow indicates cochleate

Cochleate samples of DSPS, DPPS and DMPS prepared below Tm of lipids also showed formation of network like structures. Fig. 7b shows morphology of dense porous sponge like structures observed two month after formation of DPPS samples (prepared at 20 °C). This three-dimensional sponge like aggregates formed from the interaction of ribbons may be the result of physical gelation, a phenomenon often observed in concentrated lipid suspensions incubated for longer time[41]. From SEM images, the distribution of pores within the networks DSPS and DPPS samples appeared more homogeneous as compared to the networks observed in samples prepared above Tm. These network like structures never showed any presence of entangled cochleates. Morphology of these networks did not change significantly over the period except for DMPS samples prepared below Tm. In case of DMPS samples prepared at 20 °C presence of networks made of stacks was observed (Fig. 7c). These networks were different in appearance as compared to the one found in samples of DPPS and DSPS, prepared at 20 °C. In DMPS samples stacks varied in width and occasionally showed presence of cochleates (Fig. 7d) in detailed EM analysis. Fig. 7d shows cochleate forming from a single stack of DMPS in a sample prepared at 20 °C.

Cochleates

All the PSs used in this study formed cochleates when prepared above the respective Tm. Altering the length of acyl chains of PS significantly changed the aggregated structures. In samples prepared above Tm, DSPS, DPPS and DMPS showed the presence of intermediate structures along with the cochleates (Fig. 8) yielding to relatively higher polydispersity. Cochleates of high Tm lipids were often observed attached to stacks or networks (Fig. 8c). In contrast, DOPS, DOctPS and DDPS showed less intermediate structures along with cochleates and were less polydispersed. Fig. 8e shows a cochleate amidst a cluster of network and stacks.

Although the shape of cochleates is usually reported as cylindrical, the shape of many particles in samples of low Tm lipids was rather like a truncated cone where one end was tapering away from a broad end. Cochleates of high Tm lipids appeared more cylindrical in shape.

All samples of low Tm lipids prepared at 4 °C showed formation of cylindrical structures. DoctPS samples prepared at 4 °C showed cochleates within first 2 minutes after addition of calcium along with intermediate structures. However, samples observed 40 minutes after addition of calcium predominantly showed cochleates. In DDPS samples prepared at 4 °C cochleate formation was observed only one hour after addition of calcium.In case of DSPS and DPPS when cochleate formation was carried out below the Tm, the aggregation process seemed to stop at the formation of ribbons and there was no presence of any cochleates. This arrest may be attributed to low acyl chain mobility of these lipids below the Tm and hence highlights the importance of process temperature for the optimal cochleate formation[42]. One can anticipate the increased mobility of lipid molecules above Tm which would help the self-assembly into lamellar structures which would further enhance lamellar order by condensation. In case of DMPS few cochleates were observed when the samples were prepared at 20 °C

Table 2: Size Analysis of cochleates

Phosphatidylserine	Width (nm ± SE)	Length (nm ± SE)
DSPS (C18:0)	283.1±8.4 (n=352)	3190.7±106.3 (n=352)
DPPS (C16:0)	435.2±8.9 (n=490)	3137.4±84.8 (n=385)
DMPS (C14:0)	492.7±14.3 (n=385)	3294.7±98.9 (n=457)
DOPS (C18:1)	314.8±6.4 (n=224)	5156.8±156.4 (n=249)

To find out effect of acyl chains variation on the resulting cochleates, we studied dimensions of cochleates formed in samples prepared above Tm. We evaluated over 1500 TEM images to measure width and length of DSPS, DPPS, DMPS, and DOPS cochleates (Table 2). When plotted against number of carbon atoms in lipid chain, the width of cochleates of DMPS, DPPS and DSPS reveals a negative linear association with a fair value of the correlation coefficient (r^2= 0.9364). This suggests that cochleates get slender with increase in carbon chain length. It is well known that transition of the lipid molecules from the liquid crystalline to the gel state results in a decrease of area per molecule[43]. In the past we have proposed that the extent of this decrease may affect the size of the inner channel of cochleates when acyl chain length is constant[2]. This could explain difference in widths between DMPS, DPPS and DSPS cochleates. It is however of interest to note that the mean length in cochleates of high Tm lipids did not differ much with variation in carbon chain of saturated fatty acids. Samples of low Tm lipids showed inclination towards the production of cochleates with larger dimensions. It is noteworthy that an unsaturation in fatty acyl chain in case of DOPS (C18:1) led to formation of longer cochleates (5.1 µm) as compared to completely saturated DSPS (3.2 µm).

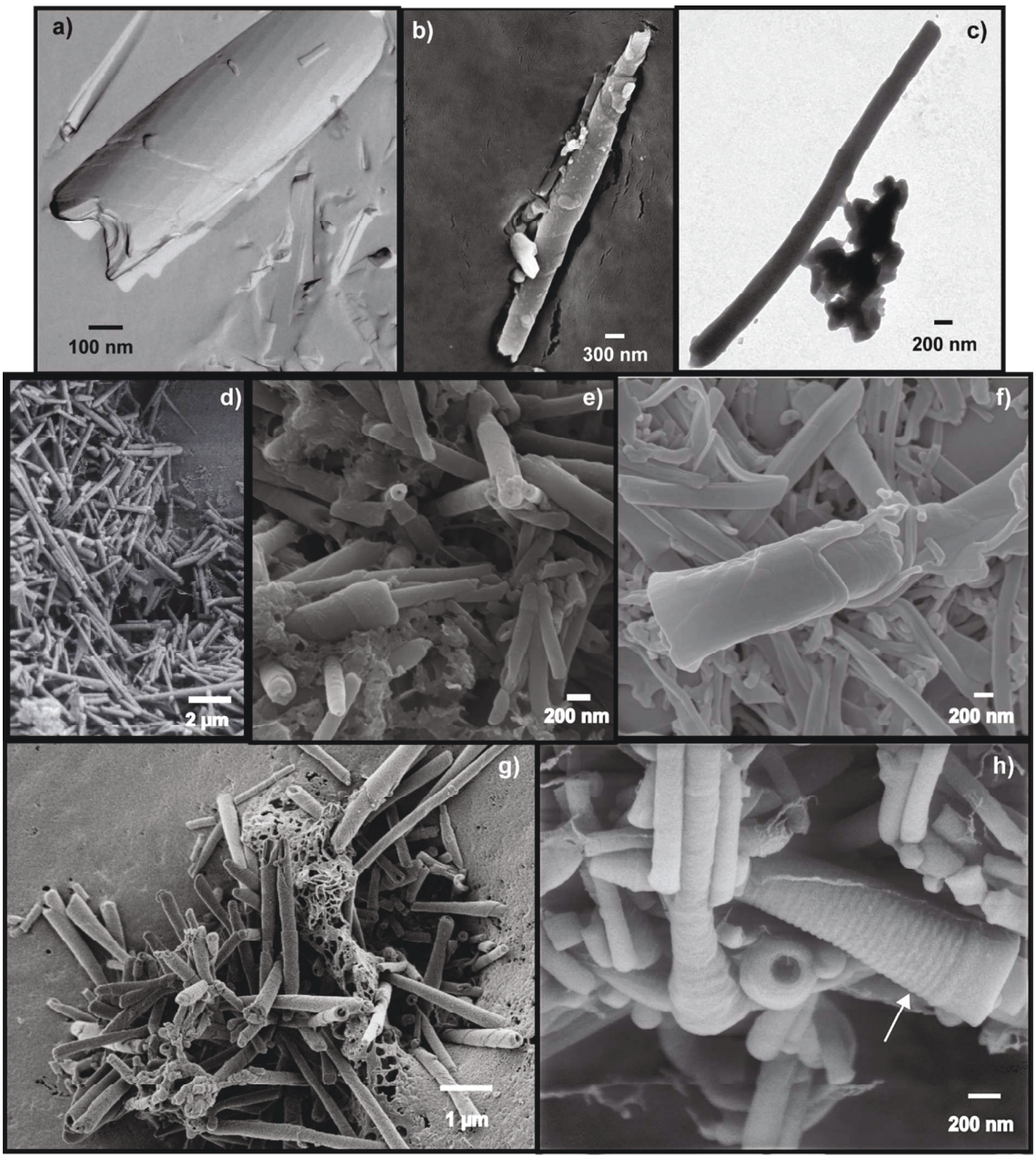


Figure 8: Cochleates observed in different samples prepared above their respective Tm. (a) FFTEM image of DDPS, (b) Cryo-SEM image of DPPS, (c) TEM image of DSPS; image showing cochleates tangled with network of lipid stacks, (d) SEM image of DOctPS, (e) cryo-SEM image of DMPS; showing attached networks (f) SEM image of DDPS, stacks building cochleate cylinder g) cryo-SEM image of DOPS and h) SEM image of DOctPS; arrow showing cochleate having a ridge like pattern.

In DOctPS samples SEM images of some cochleate particles revealed regular striations on the entire surface (Fig. 8h). These structures were observed only in DOctPS cochleate samples. We did not come across any reference of such structures in the literature for cochleates. The angle of the striations was approximately perpendicular as compared to the length of cochleates indicating the structures to be periodic decorations. These structures may result from self-assembly of free amphiphilic molecules on the hydrophobic surface as previously suggested by *Ke et al.*[44]. Adsorption of surfactants at interfaces in aqueous environment is commonly observed and is a phenomenon that can create a distribution of charges on particles[45]. According to

the model put forth by *Islam et al*., it is also possible that surfactant hemimicells are chemically adsorbed on the hydrophobic surfaces[46]. The fact that these structures were only observed for DOctPS, which is the most surfactant like PS in this study, points toward hydrophobic interactions between cochleate surface and surfactant molecules. At this moment, we can only speculate on these structures and a further detailed investigation would be required before origins of such structures can be explained in detail.

SAXS studies

Small angle X-ray scattering (SAXS) was employed as an alternative approach for further characterization and to understand distinguishing characteristics of the intermediate structures. DSPS was the choice of lipid for this study since it does not allow stack or cochleate formation when prepared at 20 °C allowing for evaluation of sample with single morphology. Prior to SAXS studies, the dialyzed-freeze dried samples were evaluated under electron microscope to make sure the samples contained the desired structures, which were of interest. The SAXS data of DSPS for four structures i.e. bilayers before calcium interaction (discs), bilayers after calcium interaction at 20 °C (ribbons), stacks prepared just below the Tm at 50 °C and cochleates formed above the Tm are shown in Fig. 9. According to SAXS, the inter-lamellar repeat distance for all four structures was calculated. DSPS bilayers without calcium revealed the repeat distance of 5.7 nm whereas the corresponding repeat distance for ribbons, stacks, and cochleates was 5.4 nm, 5.2 nm, and 5.1 nm, respectively. Dissimilar SAXS values for discs and ribbons are indicative of a strong interaction between calcium ions and the serine headgroups taking place even below Tm of the lipid. Gradual reduction in the repeat distances from discs to cochleates may be indicative of the increase in degree of condensation in different intermediate structures during cochleate formation. Despite the same basic molecular subunits the degree of disorder and polydispersity may also establish differences in SAXS patterns of different intermediates. Hence being organized molecular assemblies, physical properties of cochleates are not solely indicated by their molecular nature but are also strongly influenced by their ensemble.

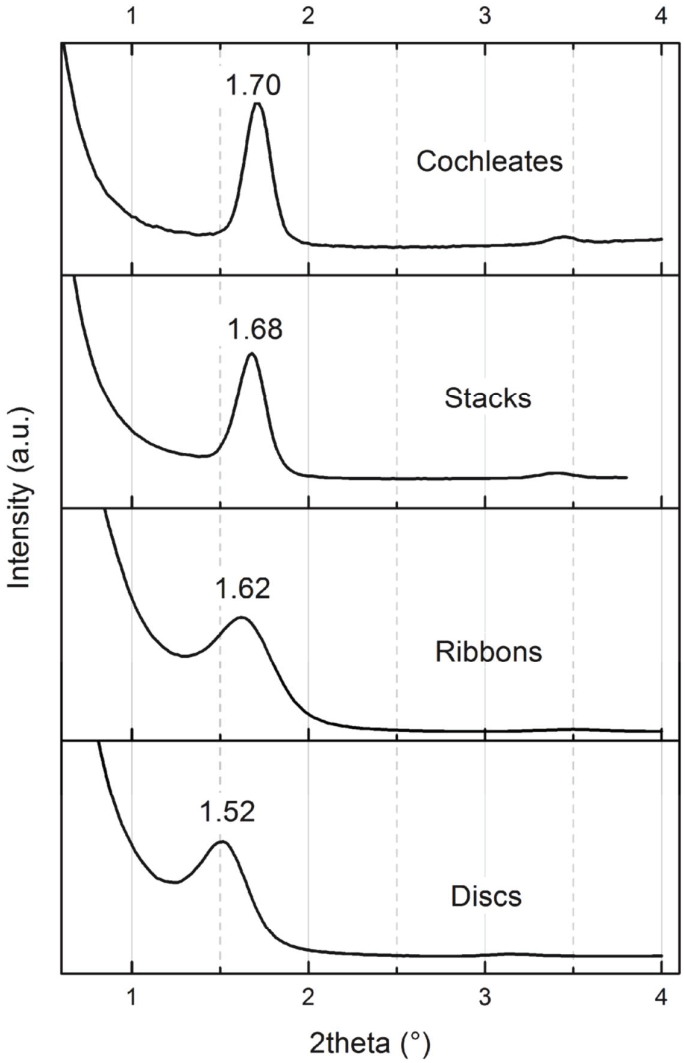


Figure 9: SAXS spectra for DSPS discs, ribbons, stacks and cochleates.

Possible mechanism of cochleate formation:

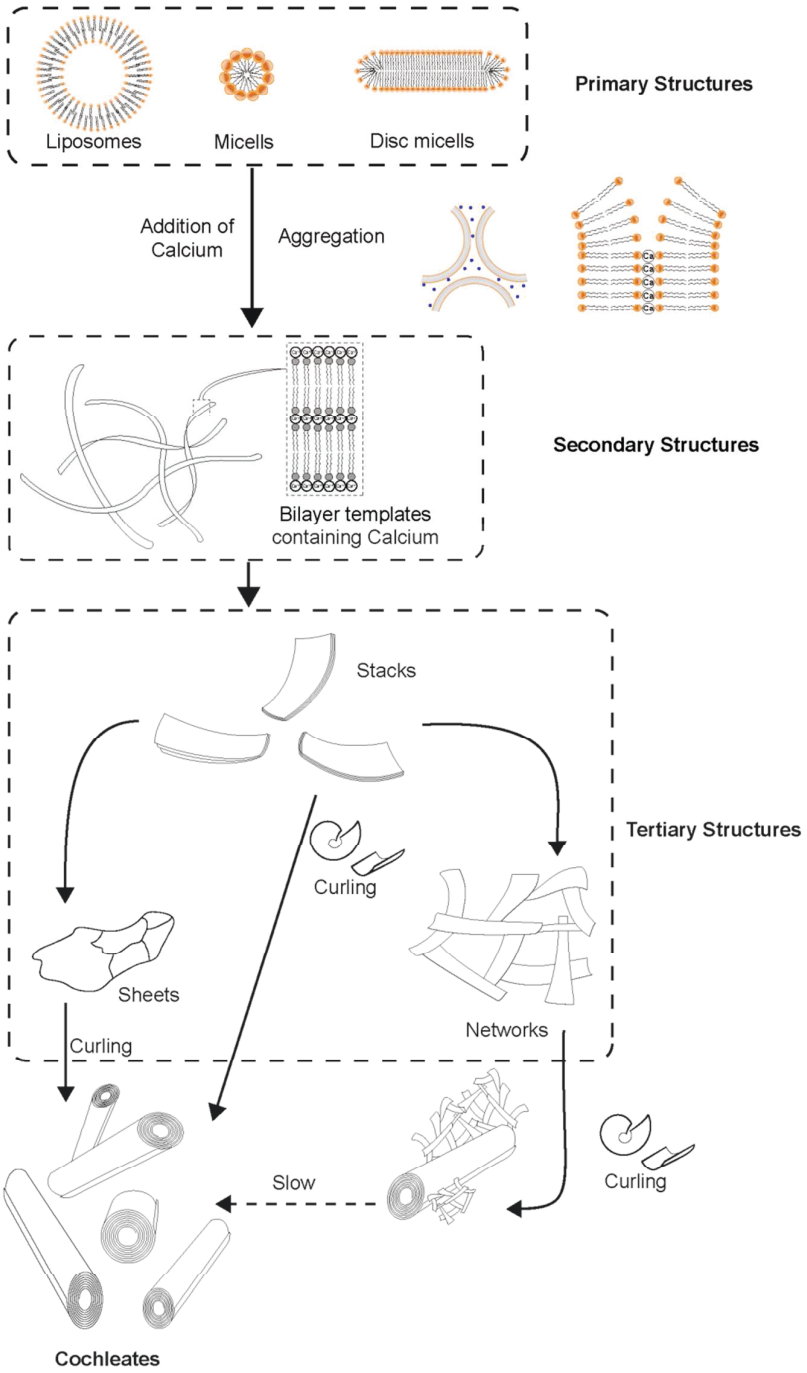


Figure 10: Schematic showing probable order for evolution of microstructures during formation of cochleates

In the current article we have mainly focused on understanding various intermediate structures involved in the formation mechanism of cochleates. The mechanism of cochleate formation from PS has been briefly discussed previously by Papahadjopolous[8]. Some further investigations on intermediates arising during interaction of PS vesicles with calcium ions were reported which mention fusion or violent rupture of vesicles and formation of flattened structures[35,36]. Studies on this scheme have exercised use of video-enhanced microscopy [35], rapid mixing [34]and freezing device [36] etc in past. In the current study we have focused on the

process temperature and effect of its variation on rate of cochleate formation. Cochleate formation being a polymorphous process may have more than one secondary pathways due to uncontrolled interaction of calcium and PS headgroups. In this section we have summerized the sequence of morphological changes obseved during this investigation to put forth the probable formation mechanism.

We have classified structures under primary, secondary and tertiary structures (Fig. 10). Cochleate formation involves two main stages: first, rapid nucleation which acts like a template followed by aggregation and association of templates to assist in growth of the final superstructures. During growth cochleate formation pathway most likely evolves through rearrangement and oriented attachment[47]. Primary structures are the starting materials and represent liposomes, discs or micelles of PS without calcium. Addition of calcium to primary structures triggers the mutual adhesion, deformation, fusion and formation of precipitates or aggregates of intrinsically ordered fragments[35]. Our observations showed that the multilamellar intermediate structures observed immediately after aggregation give rise to secondary bilayer fragments (which we named ribbons). We interpret that these structures may result due to bilayer destabilization or rupture due to high tension in intermediate multilamellar aggregates [48, 49]. These resulting ribbonlike templates of lipids containing cations are secondary structures. Tendency of a system to minimize free energy probably promotes further aggregation and particle growth. The two possible reasons may include reduction in surface area and regulation of high energy surfaces. Because ribbons would have larger energetically unfavorable surfaces they may undergo curling, adhesion or fusion comparatively faster in order to give rise to complex multilamellar structures.

We speculate that ribbons undergo fusion to form an array of tertiary structures most characteristic of which are stacks. These structures had also been identified by *Garidel et al.* as bands in DMPG cochleate samples and were suggested to form cochleates[28]. Stacks are multilamellar and generally have elongated trapezoidal shape. Our results highlight that stack formation may be an essential step in order to form cochleates. Stacks can form cochleates without undergoing further aggregation. It was observed that stacks can also undergo fusion to form broad sheets which are irregular in shape and roll up to form cochleates. Our study points to the possibility that generation of ring like structures may play a part in cochleate formation by initiating curling of stacks, sheets etc. Intermediates observed throughout the cochleate formation process are non-stoichiometric and occur due to transformations precursors undergo before forming cochleates. Cochleates are carpet-roll like structures with hollow central channel and open ends, which can further undergo aggregation. Complete absence of tertiary structures was seldom observed in samples of cochleates prepared from PS. Reason being various steps may take place simultaneously during cochleate formation such as curling, branching, agglomeration, rearrangement etc and these complex interactions may give rise to diverse tertiary structures which can further form networks of tertiary structures and/or cochleates. Complex architectures composed of fused lipid networks, sheets and cochleates may be the final result of thermodynamic equilibrium during formation process. Such tertiary structures appear to be stable and may coexist with cochleates. Hence rate of conversion of tertiary structures to cochleates is an important parameter which would influence polydispersity of the final cochleate formulation, which in turn would be of great interest to a formulation scientist.

Conclusion

Current research was undertaken on various pure PSs with a goal to understand fundamental mechanisms of cochleate formation. It was found that the samples prepared at lower process temperatures allowed a glimpse into intermediate structures as compared to those prepared above the Tm. This could probably be a result of decreased rate of cochleate formation at lower temperatures, however more studies with focus on kinetics and calorimetry are required for further understanding of this aspect. It is noteworthy that the inability to form stacks in samples prepared below Tm resulted in failure to form cochleate structures. Hence, the proposed formation mechanism has identified the generation of stacks as an essential stage in the cochleate formation. Current investigation also showed that saturation and chain length of PS fatty acids have a strong influence on final morphology of cochleates. Despite the variation in lipid acyl chains, similar intermediate structures were observed during formation of cochleates from all phosphatidylserines. Our findings ascertained that cochleate formation is not by simple accretion of monomers, but through evolution of well-defined hierarchical structures like ribbons, stacks, networks, sheets which leads to stable cochleates. Hence for the development of a desired cochleate system, monitoring reciprocity of several processes, whose relative rates determine the final outcome, may be a necessity. We believe our effort will provide better understanding regarding formation of cochleates which may present future scientists with ability to modulate formulation by making rational alterations and could eventually aid to design and characterize a product for pharmaceutical applications.

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3.2 Publication 2:

Electron microscopy and theoretical modeling of cochleates

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Electron Microscopy and Theoretical Modeling of Cochleates

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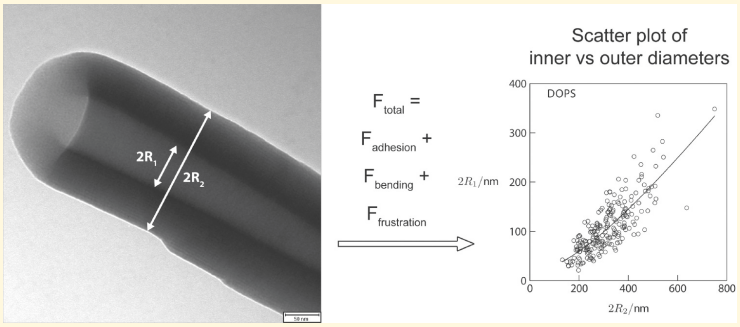

ABSTRACT: Cochleates are self-assembled cylindrical condensates that consist of large rolled-up lipid bilayer sheets and represent a novel platform for oral and systemic delivery of therapeutically active medicinal agents. With few preceding investigations, the physical basis of cochleate formation has remained largely unexplored. We address the structure and stability of cochleates in a combined experimental/theoretical approach. Employing different electron microscopy methods, we provide evidence for cochleates consisting of phosphatidylserine and calcium to be hollow tubelike structures with a well-defined constant lamellar repeat distance and statistically varying inner and outer radii. To rationalize the relation between inner and outer radii, we propose a theoretical model. Based on the minimization of a phenomenological free energy expression containing a bending, adhesion, and frustration contribution, we predict the optimal tube dimensions of a cochleate and estimate ratios of material constants for cochleates consisting of phosphatidylserines with varied hydrocarbon chain structures. Knowing and understanding these ratios will ultimately benefit the successful formulation of cochleates for drug delivery applications.

INTRODUCTION

Since their discovery in 1975 by Papahadjopoulos and co-workers,[1] cochleates have attracted considerable interest, both because of their remarkable structural properties[2] and because of their potential use as drug delivery vehicles. Cochleates are elongated spiral rolls that consist of phospholipid and divalent (or higher-valent) cation complexes. Their highly condensed solidlike structure imparts protection to incorporated molecules[3] and potential for slow release. This together with stability, ease of production, and biocompatibility renders cochleates promising candidates for the delivery of a wide range of drugs and fragile molecules such as proteins and peptides.[4,5] Until now, cochleates have been successfully utilized for the delivery of multiple active ingredients such as antifungal agents,[6] DNA, and vaccines.[7]

The building blocks of cochleates are negatively charged phospholipids with either saturated or unsaturated hydrocarbon chains. Several natural phospholipids such as phosphatidylserine,[8] phosphatidylglycerol,[9] and phosphatidic acid[10] have been used as precursors for cochleates. Their interactions with divalent or higher-valent cationic condensing agents result in the formation and precipitation of stacks of bilayer sheets. Interbilayer attraction and expulsion of water molecules are believed to originate from bridging of the condensing agent between opposed lipid layers, thus forming a compact aggregate with a concomitant increase in the value of the endothermic transition temperature.[11] Bending of the condensate into a cylindrical structure appears to be thermodynamically more favorable than the formation of other supramolecular structures, including a planar bilayer stack.

The past few decades have witnessed substantial improvements of protocols to form cochleates.[12,13] In addition, the usage of drug molecules as condensing agents instead of metal cations has been explored. One of the reported strategies employing multivalent cationic drugs utilizes tobramycine as the bridging agent.[14] We would like to point out that most previous studies have focused on the feasibility of using cochleates as drug delivery vehicles rather than on investigating

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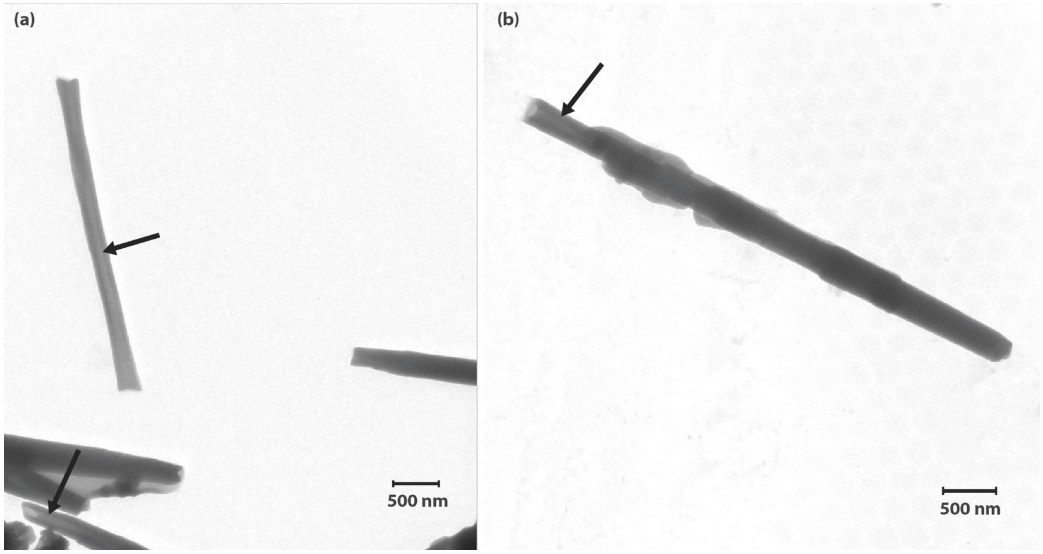

Figure 1. Transmission electron micrographs of (a) DOPS-Ca cochleates and (b) DSPS-Ca cochleates. Positions marked by arrows indicate the tubelike hollow cochleate structure.

their structure and stability. As a result, structural features, thermodynamic stability, and formation pathways of cochleates have remained largely unexplored. Our study addresses some of these aspects in a combined experimental and theoretical approach. By employing electron microscopy we demonstrate that both DOPS (dioleoylphosphatidylserine) and DSPS (distearoylphosphatidylserine) cochleates prepared with Ca^{2+} as bridging agent contain an aqueous inner channel. We quantify both the inner and outer radii of cochleate tubes consisting of calcium and phosphatidylserine with varying degree of chain saturation. In an attempt to rationalize the measured distribution of tube radii, we propose a theoretical model. Based on a phenomenological free energy expression, the model enables us to estimate the optimal dimensions of a cochleate. The model accounts for the degree to which cochleates are rolled up in thermal equilibrium and establishes a relation between the inner and outer radii. The model suggests that the sum of bilayer bending energy and calcium-mediated bilayer−bilayer adhesion energy alone is insufficient to explain the experimentally observed size distribution of cochleates. However, our data can be rationalized if we further add a mismatch contribution to the free energy that originates from an incomplete lipid relaxation during bending of an initially planar membrane stack into a tubular structure.

EXPERIMENTAL SECTION

Materials. Dioleoylphosphatidylserine and distearoylphosphatidylserine were obtained from Avanti Polar Lipids Inc. (USA). For embedding studies, Araldite LY 564 was obtained from Huntman Advanced Materials GmbH (Switzerland). All buffers and other chemical reagents were of analytical grade and were obtained from Sigma-Aldrich GmbH (Germany).

Preparation of Cochleates. Cochleates were prepared by a trapping method.[6] Briefly, lipid was dissolved in a mixture of chloroform:methanol (3:1) in a round-bottom flask followed by removal of the organic solvents using a rotary evaporator (R-144 BUCHI Labortechnik GmbH, Germany) to form a thin lipid film. The lipid film was further hydrated with 10 mM TRIZMA [2-amino-2-(hydroxymethyl)-1,3-propanediol] buffer above the transition temperature. The suspension was vortexed and extruded through a polycarbonate membrane of 100 nm pore size (Avestin, Canada) to produce a homogeneous lipid dispersion. Cochleates were formed by addition of calcium chloride (100 mM) to the lipid dispersion in buffer (NaCl, 100 mM; TRIZMA, 10 mM) under constant stirring above the lipid transition temperature. The molar ratio of lipid to calcium was 1:1. The precipitated dispersions were then stored at 4 °C.

Scanning Electron Microscopy. An aliquot of 5 μL of the cochleate dispersion was adhered for 5 min onto a perforated Formvar-coated copper grid (300 mesh, Quantifoil, Germany). Excess of liquid was removed with a lint-free filter paper. Further, the grid was quickly plunged in liquid propane−ethane (−180 °C), freeze-dried in a BAL-TEC BAF 060 (Baltec, Liechtenstein) and examined in a LEO 1350 Gemini (Carl Zeiss, Germany) scanning electron microscope (SEM) at 3 kV acceleration voltage and working distance of 6 mm using a secondary electron detector.

Transmission Electron Microscopy. Cochleate dispersion was air-dried on a Formvar-carbon coated copper grid (300 mesh, Plano, Germany). Grids were investigated using a Zeiss EM 902A (Carl Zeiss, Germany) transmission electron microscope (TEM) operated at 80 kV. Images were acquired with a 1k × 1k FastScan-F114 CCD Camera (TVIPS GmbH, Germany).

Cryoelectron Tomography. *Sample Preparation.* Cochleate dispersion was added to a carbon-coated copper grid (R1.2/1.3 + 2 nm, Quantifoil Micro Tools GmbH, Germany). After excess liquid was blotted from the sample, it was subjected to plunge freezing in liquid ethane at −180 °C as described by Dubochet et al.[15] The grid was freeze-dried at −90 °C for 1 h in a Philips CM 120 cryo-TEM (Philips, Netherlands). Further, the grid was gold labeled by placing 3 μL of 5 nm colloidal gold clusters (Sigma, USA) for subsequent alignment purposes. The grids were then transferred into a liquid nitrogen cooled (T = −196 °C) ±70° tilt cryo-holder (Gatan Inc., USA) and inserted into the cryoelectron microscope.

Preparation of Tomographic Tilt Series. Tomographic tilt series were collected under low-dose conditions with an increment of 3° within a tilt range from −65 to +65° using a TEM operating at 120 kV and a 1k × 1k FastScan-F114 CCD Camera (TVIPS GmbH, Germany). The defocus level was typically around −3 μm. Data acquisition was fully automated as described previously.[16] The pixel size at the specimen level was between 1.08 and 1.7 nm.

Image Processing and Visualization. The projection images were aligned and back-projected, resulting in a three-dimensional (3D)

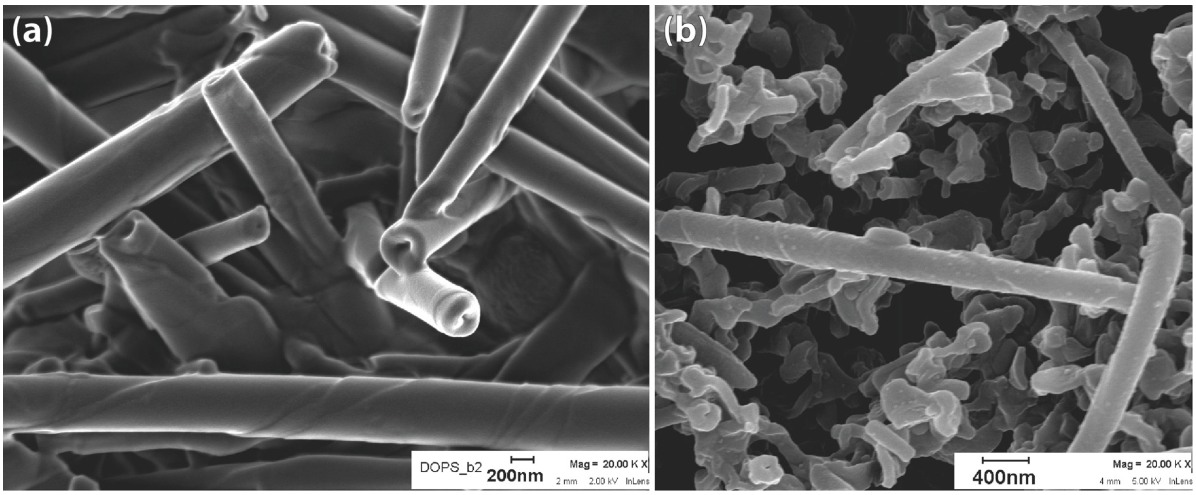

Figure 2. Scanning electron micrographs of (a) DOPS-Ca cochleates and (b) DSPS-Ca cochleates.

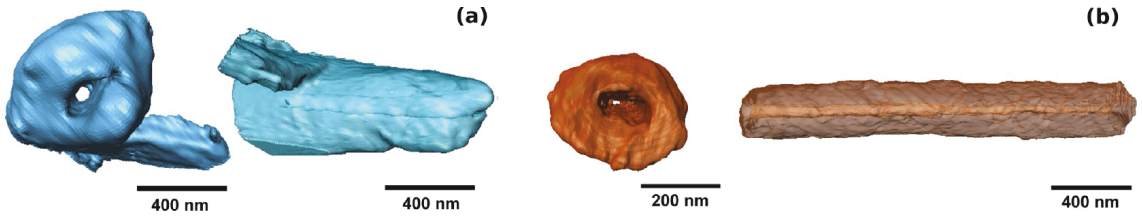

Figure 3. Three-dimensional reconstruction of (a) DOPS-Ca cochleates and (b) DSPS-Ca cochleates showing the inner channel from different perspectives.

reconstruction with a resolution of about 10 nm.[17] The IMOD software package (University of Colorado, USA) was used for 3D processing and visualization.

Investigation of Cross Section of Resin-Embedded Cochleates. Cochleates were centrifuged and the pellet was redispersed in 100 mM cacodylate buffer (pH 7.2) containing 1% OsO_4 for 2 h. Cochleates were centrifuged again and the pellet was rinsed in buffer and dehydrated in ethanol (50% v/v) for 15 min. It was further subjected for 1 h to 1% uranyl acetate solution to improve contrast. The pellet was then dispersed in an epoxy resin Araldite LY 564, used as the embedding medium. The mixture was polymerized in a closed mold for 2 days at 60 °C. The polymer blocks were microtomed into thin (70−100 nm) slices, using a diamond knife mounted on an Ultracut E device (Reichert Labtec, Germany) at room temperature. The slices were then transferred to copper grids (Quantifoil, Germany) and examined by TEM.

Small Angle X-ray Scattering (SAXS). Cochleate samples were dialyzed using Spectra/Por CE dialysis tubing, 10 000 molecular weight cutoff (Spectrum Medical Industries, USA) and freeze-dried (Christ Epsilon 2-4 LSCPlus, Martin Christ Gefriertrocknungsanlagen GmbH, Germany) before evaluation. SAXS measurements of cochleate samples were performed on a Bruker Nanostar (Bruker, Germany), equipped with a microfocus X-ray source (Incoatec IμS Cu E025, Incoatec, Germany), operating at λ = 1.54 Å. A pinhole setup with 750, 400, and 1000 μm (in the order from source to sample) was used, and the sample-to-detector distance was 107 cm. Samples were mounted on a metal rack and fixed using tape. All measurements were carried out at room temperature for 120 min, with a two-dimensional position-sensitive detector, Vantec 2000 (Bruker GmbH, Germany). The scattering patterns were corrected for the beam stop and the background prior to evaluations and radially integrated to obtain the scattering intensity as a function of the scattering vector $q = (4\pi/\lambda) \sin \theta$, with 2θ being the scattering angle and λ the X-ray wavelength.

RESULTS AND DISCUSSION

Assessment of Cochleate Formation. Following the procedure outlined in the Experimental Section, DOPS and DSPS cochleates were prepared with Ca^{2+} as bridging agent by the trapping method.[6] Samples were first observed by TEM and SEM. The presence of cylindrical structures indicated successful cochleate formation for both lipids. Figure 1 shows TEM micrographs of cochleate particles. In TEM analysis, cochleates appeared like elongated cylinders with the occasional appearance of bilayer patterns at higher magnification. Most importantly, at higher accelerating potentials, a hollow tube within the cochleates (highlighted in Figure 1 by arrows) was evident despite poor contrast due to the electron-dense nature of the system. SEM micrographs in Figure 2 also indicate channels inside the cylindrical particles, corroborating our TEM findings. All the samples were prepared in triplicate. The freeze-dried samples were also analyzed by electron microscopy to evaluate changes in their structure. The morphology of lyophilized cochleates was not altered, which can be attributed to the negligible water content associated with the bilayers.[4] All the samples were confirmed to have formed cochleates before they were subjected to any further characterization.

Cochleate Structure Studied by Cryoelectron Tomography. The self-assembly of cochleates is characterized by a compact packing of lipid bilayers. Consequently, electrons cannot pass through the cochleate easily thus resulting in an image with poor contrast. Hence, TEM is not the method of choice for obtaining specific information about the internal cochleate structure, as most of the particles appear dark against

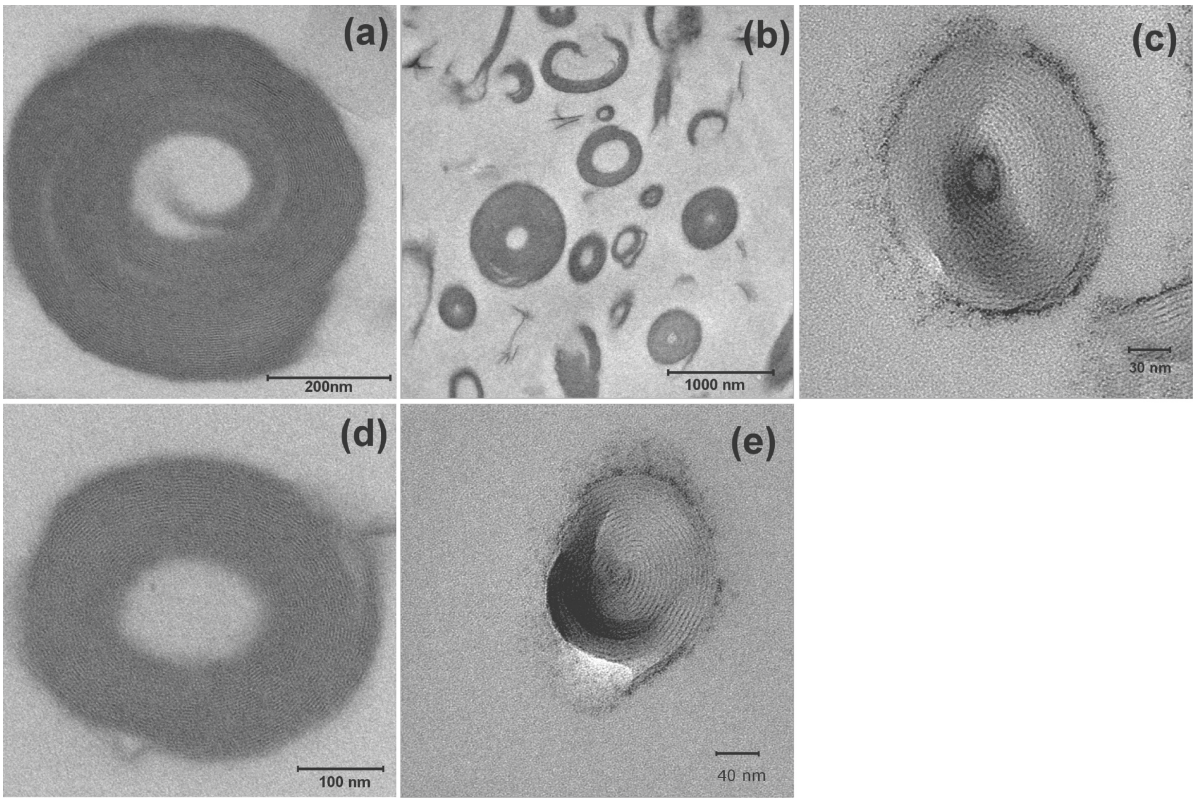

Figure 4. TEM of transverse sections of (a and b) DOPS-Ca and (c) DSPS-Ca cochleates obtained from resin-embedded samples showing inner cavities, (d) DOPS-Ca cochleate with concentric bilayers and inner cavity, and (e) DSPS-Ca cochleate with lack of inner cavity and bilayer representing a rolled-up structure.

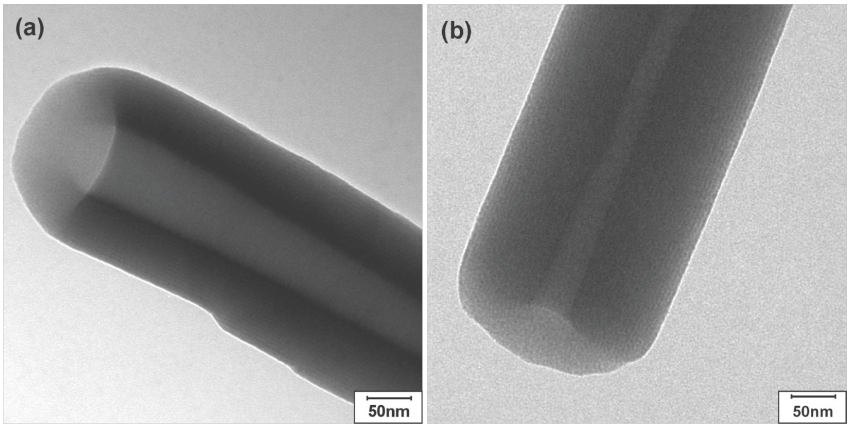

Figure 5. TEM images of (a) DOPS-Ca and (b) DSPS-Ca cochleates at medium magnification with clearly visible inner tubules.

a bright background. Electron tomographic techniques can be more useful in providing detailed information about the inner structure of cochleates. We performed a 3D reconstruction of a cochleate particle from acquisition and postacquisition processing of several TEM projection series for both DOPS-Ca and DSPS-Ca cochleates. Figure 3 shows the corresponding reconstructions viewed at two different angles for cochleate microstructures corresponding to each of the two lipids. The 3D reconstruction revealed a dense and uniformly packed bilayer stack adopting a cylindrical geometry with a channel spanning the entire structure. No sample damage was observed during measurements.

In the past, cochleates have been described employing predominantly freeze−fracture microscopy as cigar-shaped structures.[18−20] Some studies also suggested the possible presence of a luminal opening.[9,21] In our microscopic investigations we observed virtually all cochleate particles to exhibit a continuous central channel that spans the entire

structure, with an opening at each terminus of a cochleate. In aqueous dispersions the central channel would be filled with water. On the basis of the prevalence of a central channel in cochleates, we suggest referring to its morphology as "carpet roll" rather than "cigarlike", as cigars usually have a dense core. The central channel in a cochleate may be of relevance in pharmacokinetics due to its possible influence on the release kinetics of active ingredients trapped inside the particle.

Investigation of Cross Section of Resin-Embedded Cochleates. TEM micrographs of cross sections of resin-embedded DOPS-Ca and DSPS-Ca cochleates (Figure 4) display the presence of hollow tubules inside the particles. The majority of investigated particles exhibit cross sections of multilamellar scrolls folding into a compact, rolled-up structure. However, this is not discernible from all images as some micrographs displayed lipid bilayers arranged in a concentric manner. The tubules inside the particles were filled with epoxy resin, which most likely entered via the open ends of the central channel in the particle. Particles without any voids were also found, although their occurrence was very rare. During microscopy some fields also revealed bilayer stacks attached to the cochleate cylinders. The orientation of cochleate cylinders within the resin matrix could not be controlled in this technique, and as a result not all cross sections were perpendicular to the long axis of the cochleates.

Evaluation of Dimensions of the Cochleates. Unlike liposomes or microemulsions, cochleates cannot be adequately characterized with the help of dynamic light scattering due to their complex cylindrical shape. Hence we decided to analyze 479 TEM micrographs with the help of ImageJ version 1.45s[22] for determining dimensions of cochleates. Specifically, we measured the outer and inner diameters for an ensemble of cochleate tubes at randomly selected positions. In the TEM micrographs (for an example, see Figure 5) the boundaries of the inner channel can clearly be discerned at high accelerating potentials. For a given single tube, both outer and inner diameters were measured at the same point. The results for the average outer and inner diameters (and their ratio) are listed in Table 1. The difference between the ratios for DOPS-Ca and DSPS-Ca cochleates is apparent and statistically significant. Below, in Figure 7, we also show the individual diameters in a scatter plot. Data obtained from these measurements are used in the section Theoretical Modeling to motivate a theoretical model.

Table 1. Average Dimensions for DOPS-Ca and DSPS-Ca Cochleates[a]

lipid	mean outer diam (nm) ± SE	mean inner diam (nm) ± SE	ratio ± SE
DOPS (n = 224)	314.7 ± 6.4	108.1 ± 3.6	3.3 ± 0.07
DSPS (n = 255)	203.6 ± 4.3	35.5 ± 1.6	7.4 ± 0.2

[a]SE = standard error of the mean; n = number of samples.

According to the summary of our data in Table 1, unsaturation in the lipid chain resulted in the formation of cochleates with wider inner tubules as compared to the saturated lipid.

SAXS. The SAXS patterns of DOPS-Ca and DSPS-Ca cochleate samples (Figure 6) exhibit an intense reflection along with further weak reflections of higher order, characteristic of a lamellar structure.[23] The first reflection peak in the diffraction pattern corresponds to the characteristic domain spacing in the sample, i.e., the bilayer thickness together with an interbilayer space containing the bridging agent.[24] Both diffractograms of DOPS-Ca and DSPS-Ca cochleates yielded an identical interlamellar repeat distance of 5.1 nm. The magnitude of the repeat distance obtained from SAXS studies agrees well with that measured geometrically using TEM micrographs (data not shown).

Theoretical Modeling. Our electron microscopy results demonstrate cochleates to be tubelike structures with an inner aqueous channel running through the particle. Visual inspection of our TEM images suggests a statistical distribution of tube sizes with significant differences between DOPS and DSPS. To quantify these differences, we have plotted the inner diameter $2R_1$ and outer diameter $2R_2$ of more than 200 randomly selected cochleate tubes (Figure 7), where R_1 and R_2 denote the corresponding radii.

Despite the large scatter in the measured tube sizes, there appears to be a systematic lipid-dependent relation between the inner and outer diameters, $2R_1$ and $2R_2$. In the following we discuss possible mechanisms that may give rise to this relation. To this end, we propose a theoretical model in which we assume the cochleate to be a cylindrically symmetric multilamellar vesicle—of sufficiently large extension L so that end effects can be neglected—with inner and outer radii R_1 and R_2, respectively, and a bilayer repeat distance h. Because it is invariant along its axis of symmetry, we need to consider only the cochleate's free energy per unit length of the cylinder, F/L. We first account for two contributions to the total free energy $F = F_b + F_a$, a bending energy F_b of the lipid multilayer structure and a calcium-mediated bilayer−bilayer adhesion energy F_a between each pair of neighboring lipid bilayers in the cochleate.

The bending energy of a single cylindrically curved bilayer in a cochleate tube that is at distance r from the axis of cylindrical symmetry is $f_b = A_{bl}\kappa c^2/2$, where $A_{bl} = 2\pi rL$ is the lateral area of this bilayer and $c = 1/r$ is its curvature. The constant κ denotes the bending energy of a single bilayer. Integrating the result, $f_b = \pi L\kappa/r$, over all bilayers of the cochleate yields the total bending energy per unit length of the cochleate:

$$\frac{F_b}{L} = \frac{1}{Lh}\int_{R_1}^{R_2} \mathrm{d}r\, f_b(r) = \frac{\pi\kappa}{h}\int_{R_1}^{R_2}\frac{\mathrm{d}r}{r} = \frac{\pi\kappa}{h}\ln\frac{R_2}{R_1} \quad (1)$$

We point out that the integration in eq 1 treats the individual bilayers as a continuum. Yet this is an excellent approximation as becomes evident by comparing the continuum expression $F_b/(L\pi\kappa) = \ln(R_2/R_1)/h$ with the discrete version $F_b/(L\pi\kappa) = \sum_{i=1}^{n} 1/[R_1 + h(i - 1/2)]$, where $n = (R_2 - R_1)/h$ is the number of bilayers crossed along the radial direction of the cochleate. For example, choosing the representative case h = 5 nm, R_1 = 20 nm, and R_2 = 70 nm yields $F_b/(L\pi\kappa) = 0.2505$/nm for the continuum approximation and $F_b/(L\pi\kappa) = 0.2501$/nm for the discrete version.

The adhesion energy can be expressed by assigning an energy penalty to the water-exposed innermost and outermost bilayers. Denoting this free energy penalty per unit area by σ, we can write for the adhesion energy of the innermost bilayer $2\pi R_1\sigma L$, and analogously $2\pi R_2\sigma L$ for the outermost bilayer. Hence, the total adhesion energy penalty per unit length of the cochleate is $F_a/L = 2\pi\sigma(R_1 + R_2)$. Combining the expressions for F_b and F_a leads to the total free energy:

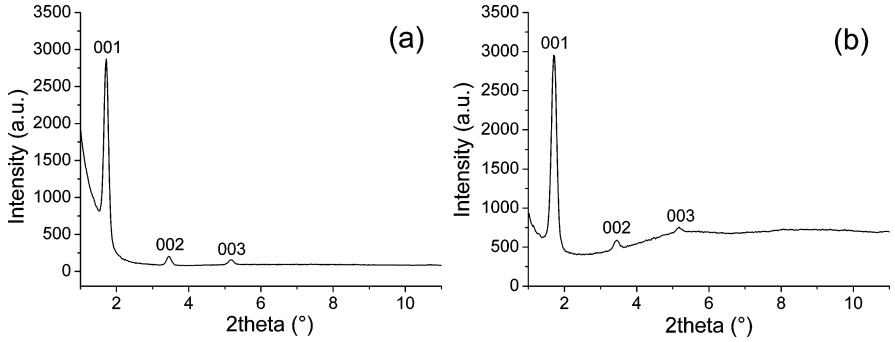

Figure 6. SAXS patterns of (a) DOPS-Ca and (b) DSPS-Ca cochleates exhibiting a primary peak (001) followed by higher order peaks (002) and (003).

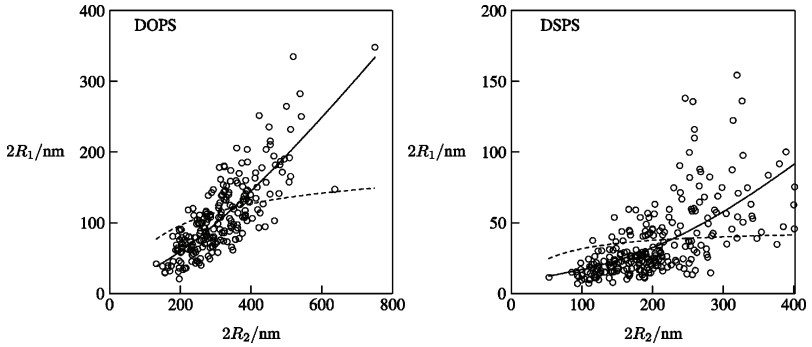

Figure 7. Scatter plots of inner and outer diameters, $2R_1$ and $2R_2$, of randomly selected cochleate tubes of DOPS (n = 255, left) and DSPS (n = 224, right). Dashed and solid lines are best fits of eqs 4 and 7, respectively.

$$\frac{F}{L} = \frac{F_b}{L} + \frac{F_a}{L} = \frac{\kappa\pi}{h}\ln\frac{R_2}{R_1} + 2\pi\sigma(R_1 + R_2) \tag{2}$$

Note that, for the model in eq 2 to apply, it is irrelevant if we assume the cylindrical cochleate to be multilamellar (that is, with $(R_2 - R_1)/h$ individual bilayers) or a rolled-up structure, which consists only of one single bilayer that is wound upon itself. The latter can be modeled, mathematically, as the involute of a circle $x(s) = (h/2s)[\cos(s) + s\sin(s)]$ and $y(s) = (h/2s)[\sin(s) + s\cos(s)]$, which maintains a fixed bilayer–bilayer distance everywhere. The rolled-up cochleate has a local curvature $c = 2\pi/(hs)$, which (as a simple calculation shows) leads to the same free energy as for the multilamellar structure as long as the inner diameter is significantly larger than the repeat distance h. We assume the cochleate consists of a fixed number of lipids, each with constant cross-sectional area. The cochleate then has a fixed total bilayer area $A = Ll$, written here as the product of the cochleate tube length L and the total contour length

$$l = \frac{2\pi}{h}\int_{R_1}^{R_2} dr\, r = \frac{\pi}{h}({R_2}^2 - {R_1}^2) \tag{3}$$

of all bilayers that appear in the cochleate's cross section. For any fixed l, the two radii R_1 and R_2 are no longer independent. Hence, the total free energy $F(R_1,R_2)$ adopts its minimum in thermodynamic equilibrium with respect to R_1 and R_2, subject to l being kept constant. The minimization can be carried out conveniently using the Langrangian multiplier method, which gives rises to the relation

$$R_1 = \frac{R_2}{1 + 2R_2 h\frac{\sigma}{\kappa}} \tag{4}$$

A least-squares fit of the relation in eq 4 to the data in Figure 7 yields $h\sigma/\kappa$ = 0.0054/nm for DOPS and $h\sigma/\kappa$ = 0.022/nm for DSPS. The corresponding relation between R_1 and R_2 according to eq 4 is shown in Figure 7 as a dashed line in each diagram. Note that eq 4 predicts a saturation of the inner cochleate radius R_1 with growing outer radius R_2. The predicted saturation is a result of the membrane's bending energy penalty which makes the decrease of the inner tube radius R_1 below a value of about $\kappa/(2h\sigma)$ very costly. Since no such penalty affects the outer radius R_2, eq 4 predicts the cochleate to increase radius R_2 with only minor concomitant changes of R_1. However, our experimental data in Figure 7 do not support the prediction of an essentially constant radius R_1. What they suggest, instead, is that R_1 continues to grow with R_2.

Membrane bending and calcium-mediated membrane–membrane adhesion alone cannot rationalize the data in Figure 7. The question arises, what other physical mechanism is able to keep R_1 and R_2 growing together? In the following, we discuss an additional free energy contribution that is able to account for the joint growth of R_1 and R_2. Consider the bending of a planar membrane stack into a multilamellar cylindrical vesicle. Note that this hypothetical bending scenario merely compares the two states prior and after bending; it does not imply that the actual formation of a cochleate proceeds this way. If all individual bilayers of the initially planar stack are of the same lateral area, then an elastic stretching/compression deformation of the bilayers is required for a multilamellar tube

to form. Specifically, the outer bilayers must stretch and the inner ones must compress in order to compensate for the changes in circumference of the bilayer's cross section after bending. The energy cost F_m per unit length L of the cochleate of this area mismatch-compensating deformation depends on the area compressibility modulus K of a bilayer (which typically is on the order[25] of $K = 50\ k_BT/nm^2$, where k_B is the Boltzmann constant and T is the absolute temperature) and can be expressed as

$$\frac{F_m}{L} = \frac{1}{h}\int_{R_1}^{R_2} dr\ 2\pi r \frac{K}{2}\left(1 - \frac{r}{\bar{r}}\right)^2 = \frac{K\pi}{6h}\frac{(R_2 - R_1)^3}{R_2 + R_1} \quad (5)$$

where $\bar{r} = (R_1 + R_2)/2$ is the mean radius of the cochleate tube. Note that eq 5 assumes there is no lipid exchange between the bilayers during the bending deformation. Indeed without lipid exchange bending deformations would imply large stretching and compression deformations, especially of the outer and inner bilayers, of the cochleate. For example, a cochleate with an outer radius twice the inner one (which is not an unusual case; see Figure 7) the relative stretching of the outer bilayer would be 33%, which would lead to membrane rupture. However, a large degree of lipid exchange will substantially reduce the degree of stretching. A reduction to about 0.3% of the stretching/compression values without any lipid relaxation is, in fact, what we find below from the analysis of our experimental data.

The presence of lipid exchange can approximately be accounted for by replacing $\bar{r}$ with $\bar{r} + \eta(r - \bar{r})$ in the integral of eq 5, where η is a relaxation parameter. The relaxation parameter adopts the value $\eta = 0$ if no lipid exchange takes place and $\eta = 1$ for complete lipid exchange. In the latter case lipids rearrange during the bending deformation such that all area mismatch is removed, implying $F_m = 0$. If η is only slightly smaller than 1, we can still use eq 5, yet with K replaced by the effective area compressibility modulus $\bar{K} = K(1 - \eta)$. We will use $\bar{K}$ instead of K in the following and justify this choice a posteriori. Adding F_m/L to the total free energy in eq 2 and performing the minimization, again subject to fixing l, yields

$$2h\frac{\sigma}{\kappa} = \frac{1}{R_1} - \frac{1}{R_2} + \frac{2}{3}\frac{\bar{K}}{\kappa}\frac{(R_2 - R_1)^3}{(R_2 + R_1)^2} \quad (6)$$

Equation 6 has two unknown parameters: $h\sigma/\kappa$ and $\bar{K}/\kappa$. For $\bar{K} = 0$ (or, equivalently, $\eta = 1$, where the bilayer stretching/compression deformation is absent and thus does not incur an energy penalty) the model reduces to that in eq 4. Note that eq 6 cannot conveniently be expressed explicitly as $R_1 = R_1(R_2)$. Still, we can calculate R_1 numerically and then find the best fit of eq 6 to the data in Figure 7. The result is shown as the solid line in each of the two diagrams of Figure 7. The fit yields $h\sigma/\kappa = 0.0318/nm$ and $\bar{K}/\kappa = 0.00295/nm^2$ for DOPS, as well as $h\sigma/\kappa = 0.0691/nm$ and $\bar{K}/\kappa = 0.00299/nm^2$ for DSPS. We first point out that the model in eq 6 is able to reproduce the joint growth of R_1 and R_2. The reason is that the energy penalty of the stretching/compression deformation energy in eq 5 grows quadratically with the ratio R_2/R_1 of the two radii. Hence, this free energy contribution tends to keep the difference $R_2 - R_1$ small. For a cochleate with a large number of lipids (and thus a large contour length l), it is favorable to form a larger tube (with a larger inner diameter), despite the less favorable membrane−membrane adhesion energy. More specifically, for a sufficiently large cochleate, the radii R_2 and R_1 become independent of the bending stiffness κ, and the relative difference of the two radii turns smaller with growing R_1.

$$\frac{R_2 - R_1}{R_1} = \left(\frac{6h\sigma}{R_1\bar{K}}\right)^{1/3} \quad (7)$$

Recall that the repeat distance $h = 5.1$ nm is known from our SAXS experiments and is the same for both lipids. Hence, we obtain an estimate for the ratio between the calcium-mediated adhesion energy σ and the bending stiffness, κ, namely $\sigma/\kappa = 0.0062/nm^2$ for DOPS and $\sigma/\kappa = 0.0135/nm^2$ for DSPS. Neither one of the individual material constants is known from experiment. Generally, the order of magnitude for the bending stiffness of lipid bilayers[26,27] in their fluid state is 10 k_BT. We only know of one single experimental determination of κ involving phosphatidylserine. It was reported for a DOPS/DOPE mixture residing in the inverse-hexagonal phase using X-ray diffraction and applying osmotic stress,[28] yielding $\kappa = 11\ k_BT$, largely independent of composition. However, cochleates involve millimolar calcium concentrations at which the lipids are condensed, with highly ordered alkyl tails and a loss of lipid-associated water,[29] and likely a significantly larger bending stiffness. Indeed κ can increase by an order of magnitude when transitioning from the liquid to the gel state.[30] Comparing DOPS with DSPS, it is likely that for DSPS κ is equally large or larger because its saturated lipid chains tend to be more condensed. It is thus remarkable that DSPS—a lipid with likely not a smaller bending stiffness than DOPS—forms cochleates with a smaller tube diameter. Let us assume, as an order-of-magnitude estimate, $\kappa = 50\ k_BT$ for the membranes in both cochleate types. This would give rise to $\sigma = 0.31\ k_BT/nm^2$ for DOPS and $\sigma = 0.67\ k_BT/nm^2$ for DSPS, amounting to an energy gain on the order of a few tenths of a k_BT per lipid associated with the calcium-mediated bilayer−bilayer adhesion. The fact that calcium mediates a bridging interaction between phosphatidylserine in apposed lipid bilayers was already proposed long ago[29] and is supported by recent molecular dynamics simulations[31] (using, however, phosphatidylcholine instead of phosphatidylserine). The 2 times larger value of σ/κ for DSPS as compared to DOPS likely reflects a stronger calcium-mediated bilayer−bilayer interaction. What is the reason for the difference, given that the headgroups of DOPS and DSPS are of identical chemical structure? Recent work that combines experimental investigations with atomistic computer simulations provides insights into the interactions of calcium with phosphatidylserine or mixed phosphatidylserine/phosphatidylcholine bilayers.[32,33] Specifically, Boettcher et al.[32] find calcium to order and rigidify the phosphatidylserine headgroup, thereby favoring two major headgroup conformations and exerting only a minor influence on the mobility of the acyl chains. A dehydrating effect of calcium as well as preferential binding to the phosphate and carboxyl moieties of the phosphatidylserine headgroup were identified by Martin-Molina et al.[33] Endothermic membrane binding of calcium is also an indication for the liberation of water molecules[34] and a concomitant membrane dehydration. All this evidence supports a calcium-induced ordering and dehydration of the phosphatidylserine headgroups in each bilayer. Spatially more ordered headgroups in apposed bilayers facilitate calcium to mediate a bridging interaction. Hence, we expect the calcium-mediated bilayer−bilayer interaction to depend sensitively on how hydrocarbon chain packing affects the degree of headgroup ordering. The more ordered DSPS chains can be expected to

allow for a larger degree of headgroup order as compared to DOPS, thus facilitating the bridging interaction and increasing σ.

The estimate for the parameter $\bar{K}/\kappa = (1 - \eta)K/\kappa = 0.003/\mathrm{nm}^2$ from the fit of eq 6 to the data in Figure 7 is the same for both DOPS and DSPS. Although neither K nor κ is known, their orders of magnitude are very roughly $K = 50\ k_BT/\mathrm{nm}^2$ and $\kappa = 50\ k_BT$, implying $\eta = 1 - (\kappa/K)(0.003)/\mathrm{nm}^2 = 0.997$. As we had assumed (see the discussion after eq 5), η is very close to 1. That is, our model prediction is consistent with a very minor degree of area mismatch for the lamellae in the cochleate. Yet, this small mismatch is still sufficient to cause the inner cochleate radius R_1 not to saturate as the model in eq 4 would predict. Varying degrees of η may also contribute to the large size variations of cochleates, namely the occurrence of a variety of outer radii R_2 at fixed R_1.

We finally discuss a possible structural implication of having a relaxation parameter η close to 1. Recall that $\eta = 1$ corresponds to complete lipid relaxation, thus removing any stretching/compression deformations upon bending a multilamellar sheet from an initially planar state to a cylinder-like cochleate. In fact, there is a simple structural mechanism that allows for complete lipid relaxation: the multilamellar sheet may close after bending such that sheet n connects with sheet $n + 1$, thus forming a cochleate that consists of a single rolled-up bilayer. Because a rolled-up cochleate consists of only one single bilayer, it can more easily relax internal stress as compared to a multilamellar cochleate, where the relaxation would require fission and fusion or lipid transfer from one bilayer to another. Hence, our estimate of η being very close to 1 suggests that the cochleates consist predominantly of rolled-up bilayers and not of a multilamellar sheet.

CONCLUSION

We have used electron microscopy to study the structure of cochleates that consist of calcium as binding agent and phosphatidylserines with either saturated or unsaturated hydrocarbon chains. For both lipid types we demonstrate cochleates to be tubules where a stack of cylindrically curved bilayers encloses an inner aqueous channel. The inner and outer radii of a cochleate vary statistically, yet with characteristic differences for the two lipids. Specifically, the unsaturated lipid tends to form cochleates with larger dimensions, and the diameter of the inner tube does not saturate as the outer diameter of the cochleate grows. To rationalize these findings, we have proposed a theoretical model that is based on minimizing a phenomenological free energy expression. Our calculations suggest that membrane bending and calcium-induced bilayer−bilayer adhesion alone are insufficient to reproduce the observed relation between inner and outer cochleate tube radii. Yet, we can reproduce it by considering an additional free energy contribution that accounts for an area compression/expansion of the lipid bilayers within the cochleate. Our modeling approach yields predictions for ratios of material constants, namely the ratio between the area compressibility and the bending modulus as well as the ratio between the bilayer adhesion energy and the bending modulus. Although our model contains approximations (such as the neglect of the end-cap energy of the cochleate), it is likely that it captures some of the underlying principles of cochleate stability and thus may prove valuable for the design and understanding of cochleates as drug delivery vehicles.

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Notes

The authors declare no competing financial interest.

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3.3 Publication 3

Micro-spherical cochleate composites: method development for monodisperse system

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Micro-spherical cochleate composites: method development for monodispersed cochleate system

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Cochleates, phosphatidylserine, microfluidics, solvent effect, electron microscopy

ABSTRACT

Cochleates have been of increasing interest in pharmaceutical research due to their extraordinary stability. However the existent techniques used in the synthesis of cochleates still need significant improvements to achieve monodispersed formulations. In this study, we report a simple method for the synthesis of spherical composites (3−5 μm in diameter) made up of nanocochleates from phosphatidylserine and calcium. Products obtained from the proposed method were evaluated with electron microscopy and small angle X-ray scattering and compared with those obtained by the established cochleate preparation techniques. In this method, a lipid solution in an organic solvent, ethanol and aqueous solution of a binding agent is subjected to rapid and uniform mixing with a microfluidic device. The presence of high concentration of organic solvent promotes the formation of composites made of nanocochleates. This simple methodology eliminates elaborate preparation methods, while providing a monodisperse cochleate system with analogous quality.

INTRODUCTION

Cochleates are cylindrical particles featuring a rolled up carpet-like morphology made up of multiple stacked layers of negatively charged phospholipids bound by a positively charged binding agent such as calcium (Nagarsekar et al., 2014). Cochleates represent a potential delivery system with remarkable advantages being non-immunogenic, highly stable and with the ability to impart safety to incorporated active ingredients (Zarif, 2002). Cochleates have a hollow central channel and little or no internal aqueous space in the lipid sheets (Zarif, 2005, Nagarsekar et al., 2014). In past few decades cochleates are being explored for a number of possible applications thanks to their exceptional properties. The know-how of cochleates has been employed to improve delivery of drugs (Zarif et al., 2000, Syed et al., 2008), genes (Zarif and Mannino, 2000), flavoring agents (Mannino and Gould-Fogerite, 2002), and antigens (Gould-Fogerite and Mannino, 2000). Cochleates of various lipids such as phosphatidylserines, sphingolipids, and phosphatidylglycerols have been synthesized by groups around the world. Cochleates are also found to be structurally more divergent if compared to lipid tubules or nanotubes (Zarif, 2005, Kulkarni et al., 1999, Sarig et al., 2011, Kulkarni et al., 1996, Garidel et al., 2001). Cochleates are accepted to be well suited for intramuscular (Gould-Fogerite et al., 2000) and oral delivery (Delmas et al., 2002) however, being prone to aggregation they are not ideally suitable for intravenous or ocular administration (Wang et al., 2014).

Different strategies have been developed for the preparation of cochleates. The conventional preparation strategies such as trapping method or dialysis method are known to form aggregated cochleates with variable and large particle sizes (Sankar and Reddy, 2010). This is mainly due to the uncontrolled and continuous aggregation between the bilayer sheets. During the last decade research was focused on synthesis strategies to form nanocochleates mainly by isolating or limiting the number of building blocks that come in close contact. The development of methods such as hydrogel isolation (Zarif et al., 2003) and emulsion-lyophilization (Wang et al., 2014) have opened up a new field of application based research for various biomolecules using cochleates with sizes in the sub-micrometer

range. However, these are multistep methods which commonly involve formation of colloidal structures such as liposomes or micelles as the first step, and subsequently form the cochleates (Huergo et al., 1997, Jin et al., 2001). These processes often involve complex and time consuming steps such as multiple emulsions, washing off polymers etc., which tend to be difficult to scale up and might also cause regulatory issues. Although these methods were successful in preparing sub-micron cochleates, they often form cochleates with a broad particle size distribution, a problem that has seldom received attention. These limitations can critically limit suitability of cochleates as drug delivery systems in the pharmaceutical market (Zayas et al., 2013). Hence, an economical method which could easily be translated into mass production needs to be developed for the formation of monodisperse cochleate systems.

Cochleate formation follows a continuous self-assembly process which includes unrestrained nucleation and growth of particles (Kulkarni et al., 1999, Zarif, 2002), this makes it difficult to control either morphology or the dimensions of individual particles. In conventional cochleate formation, bilayers or micelles of an acidic phospholipid in presence of a cationic binding agent undergo an aggregation step to form planar bilayer stacks. These stacks further aggregate to form larger sheets which eventually undergo curling to form cochleates with a carpet roll like morphology (Poste et al., 1976). Also the intermediate structures that could coexist with the cochleates make the purification of cochleate system a challenging task (reference: manuscript under review). Hence, the development of a formation strategy should mainly focus on limiting uncontrolled interactions after nucleation. Moreover, such a novel methodology for attaining monodispersity in case of natural lipids might also render a cost effective system.

Recently we came across a very interesting solvent effect where phosphatidylserines (PS) formed much smaller stacks during precipitation from ethanol compared to precipitation in water. In this study we have utilized this solvent effect towards formulation development. We tried to exploit this finding with the aim of reducing random particle aggregation and thereby decreasing size dispersity

in cochleate systems. We have developed one-step, simple, reproducible and economic procedure where emulsification or polymers are not required prior to the formation of cochleates. In this case, we exploit precipitation by solvent displacement. Nanoprecipitation is an attractive choice as the process parameters such as rate of mixing or the concentration of reactants allow the control of size, shape, and encapsulation efficiency of the final product. In order to precisely control the mixing rate and mixing ratios we decided to employ a microfluidic reactor. A reactor with fast and reproducible mixing has proven to be an excellent tool for creating a monodisperse system of multiple particles with varied morphologies (Nie et al., 2006). Recently, the area of microfluidics has been explored for synthesis and scale-up of various nanoparticles, where a final product with small and uniform dimensions can be easily achieved by precision controlled fast mixing, dominated by diffusion or convection (Whitesides, 2006, Kastner et al., 2014, Belliveau et al., 2012).

In this study, we report the formation of novel microparticles with spherical morphology made up of nano-sized cochleate subunits using a microfluidic device. Throughout this manuscript we have referred to these novel particles as 'cochleate composites'. We have demonstrated that controlled mixing provided by the microfluidic device enabled us to fine-tune the cochleate composites. Products obtained from the proposed method have been characterized and compared with those obtained from conventional methodologies. Our method eliminates elaborate preparation procedures, while providing a unique monodispersed cochleate system with analogous qualities.

MATERIALS AND METHODS

Materials

1, 2-dioleoyl-sn-glycero-3-phsphatidylserine (DOPS) was purchased from Avanti Polar Lipids, Inc (Alabama, USA). Araldite® LY 564 used for embedding studies was obtained from HunTcan Advanced Materials GmbH (Switzerland). Absolute ethanol used to prepare lipid solutions was purchased from VWR International GmbH (Germany). All other chemical reagents were of analytic grade and were

used as received. Buffers used in the study were prepared using ultrapure water (Milli-Q® Direct-Q™ System, Merck-Millipore, Germany).

Preparation of cochleates

To achieve spherical morphology with desired particle size and to obtain a stable dispersion of cochleate composites, the effect of different process variables was investigated during preparation. To optimize the organic phase, various organic solvents which could dissolve DOPS i.e. ethanol, methanol, chloroform, dichloromethane and DMSO were studied for preparation of cochleates. Ethanol was selected as organic phase based on preliminary screening for all further experiments.

Using microfluidics:

Modified nanoprecipitation method was employed for preparation of cochleate composites. Ethanolic solution of DOPS was prepared with the aid of bath sonication. 60 mM $CaCl_2$ solution in 10 mM Tris buffer was used as source of binding cations. Formulations were prepared using a benchtop NanoAssemblr™ instrument (Precision NanoSystems Inc., Canada) employing Poly (dimethyl siloxane) micromixer chips (Precision NanoSystems Inc., Canada). The micromixer chips had molded channels which were 200 µm in width and 79 µm in height and a series of herringbone features of 50 × 31 µm (Kastner et al., 2014). Disposable 1 mL syringes (B-D Disposable Syringes, Luer-Lock Tips) attached at the two inlet streams to the chip served as the reservoir of reactor solutions viz. organic lipid solution and aqueous $CaCl_2$ solution. The syringe pumps allowed for a precise control of the flow rates and the solvent phase ratios between the two inlet streams. The resulting suspensions were collected from the outlet stream. To understand the effect of mixing rate on morphology of the particles, the ratio of the aqueous phase : organic phase was varied from 1:1 to 1:9 (with constant flow rate of 12 ml/min). In a different set of experiments the flow rate was varied from 1 ml/min to 18 ml/min (with constant flow rate ratio of 1:9). Concentration of lipid was kept constant at 1 mg/ml for both experiments. Further, to understand the effect of lipid concentration, the amount of DOPS was varied from 0.1

mg/mL to 1mg/mL keeping the ratio of aqueous phase : organic phase (1:9) and flow rate (12 mL/min) constant. All samples were prepared in triplicate and the effect of changing parameters on the morphology of formulation was assessed using electron microscopy (EM).

Modified trapping method:

To understand the influence of microfluidic device on the morphology, cochleate composites were also prepared using T-25 Ultra-Turrax® homogenizer maintaining the 1:9 $CaCl2_{(aqueous)}$: $DOPS_{(ethanolic)}$ ratio. Cochleate composites were formed by slow (10 µl at a time) addition of aqueous calcium chloride (60 mM in 10 mM Tris) to an ethanolic DOPS solution under constant stirring. Addition of calcium chloride solution was carried out at a constant 13500 rpm with T-25 Ultra-Turrax® homogenizer (IKA, Germany) stirring was continued for another five minutes after complete addition of calcium chloride. Mixture was then stirred continuously up to 1 hour using a magnetic stirrer (RCT Basic-Ika, Staufen, Germany). The molar ratio of lipid to calcium was 1:1. At the end of 1 hours precipitated mixtures were collected and stored at 4 °C. All samples were prepared in triplicate and evaluated by EM.

Preparation of cochleates by conventional methods:

Conventional cochleates were prepared in order to evaluate the difference in SAXS patterns and size distribution with respect to cochleate composites. For the preparation of conventional cochleates, DOPS liposomes were prepared by thin-lipid film hydration method. In brief, DOPS was dissolved in a mixture of chloroform : methanol (3:1) in a round bottom flask and was subjected to a rotary evaporator (R-144 BÜCHI Labortechnik GmbH, Germany) to form a thin lipid film. The lipid film was hydrated with 10 mM Tris buffer and the resultant dispersion was subjected to extrusion through a polycarbonate membrane of 100 nm pore size to produce uniform dispersion of liposomes. Cochleates were formed by slow (10 µl at a time) addition of calcium chloride (60 mM) to the liposomes under

constant stirring using a magnetic stirrer at room temperature. The molar ratio of lipid to calcium was 1:1. Precipitated mixtures were then stored at 4°C. All samples were prepared in triplicate.

Preparation of nano cochleates by hydrogel isolation method:

Nano cochleates were prepared by hydrogel isolation method as described previously by Jin et al. (2001). DOPS liposomes were formed as described above in section 2.2.3. The liposome suspension was further dispersed in 40 %w/w dextran 500,000 solution in ratio of 1:2 v/v. This mixture was then slowly added to 15 %w/w PEG 8,000 solution with a syringe under magnetic stirring (1100 rpm). To this dispersion an aqueous $CaCl_2$ solution (60 mM) was added to reach the equimolar ratio of lipid and calcium. After 2 hours of stirring, the resultant mixture was washed thrice by mixing with equal volume of buffer (1 mM CaCl2 and 150 mM NaCl) and centrifuged at 3000 rpm for 30 min. The resultant pellet was resuspended with buffer and stored at 4˚C. All samples were prepared in triplicate.

Characterization of formulations

Scanning electron microscopy:

A droplet of the sample was adhered on a perforated Formvar -coated copper grid (300 mesh, Quantifoil Micro Tools GmbH, Germany). Excess liquid was removed with a lint-free filter paper and examined in a LEO 1530 Gemini (Carl Zeiss, Germany) scanning electron microscope (SEM) at 4 kV acceleration voltage. Images of randomly selected 110 particles of optimized formulation prepared using NanoAssemblr™ were measured using Image J to estimate the average size (Rasband, 1997-2014). The coefficient of variance or CV (%) corresponding to the particle size distribution was calculated as described by Perez et al. (2015) using formula CV (%)= (σ/ D)×100 where σ is the standard deviation (SD) of the particle size and D is their mean diameter.

Cryo-transmission electron microscopy:

A drop of formulation (2 µl) was placed on a carbon-coated copper grid (Quantifoil Micro Tools GmbH, Germany). The sample was subjected to plunge freezing in liquid ethane at −180 °C. The grids were then transferred into a liquid nitrogen cooled (T = −196 °C) ±70° tilt cryo-holder (Gatan Inc., USA) and inserted into the cryoelectron microscope Philips CM 120 cryo-TEM (Philips, Netherlands). Images were captured using a TEM operating at 120 kV.

Investigation of cross section of resin-embedded cochleate composites:

Embedding was carried out using the procedure described previously by *Nagarsekar et al*.(2014). Briefly, sample was redispersed in 100 mM cacodylate buffer (pH 7.2) containing 1% OsO4 for 2 h. Sample was centrifuged again and rinsed in buffer before dehydration in ethanol (50% v/v) for 15 min. It was further subjected to 1% uranyl acetate solution for 1 h to improve contrast. The pellet was then redispersed in an epoxy resin Araldite® used as an embedding medium. The mixture was polymerized and ultramicrotomed in to thin (70−100 nm) slices using a diamond knife mounted on an Ultracut E device (Reichert Labtec, Germany) at room temperature. The slices were transferred to copper grids (Quantifoil, Germany) and examined by cryo-TEM.

Small angle X-ray scattering:

Small angle X-ray scattering (SAXS) measurements were performed at λ=1.54 Å using a Bruker Nanostar (Bruker, Germany) equipped with a µ-focus X-ray source (IµS, Incoatec, Germany), equipped with a 2D position sensitive detector Vantec 2000 (Bruker GmbH, Germany). Sample-to-detector distance was maintained at 107 cm. Samples were mounted on a metal rack and fixed using tape. All measurements were carried out at room temperature and duration of measurement was 2 hours. Prior to evaluations the scattering patterns were corrected for the background (Scotch tape) and radially integrated to obtain the scattering intensity (using $q= (4\pi/\lambda) \sin \theta$, where 2θ =scattering angle

and λ = X-ray wavelength). All samples were centrifuged, washed and freeze dried before evaluation and measurements were undertaken in triplicates.

RESULTS AND DISCUSSION:

The cochleate formation is a result of an instantaneous self-assembly induced by aggregation of negatively charged lipid molecules interacting with a cationic binding agent such as calcium. So far water and aqueous buffers have been widely reported for the preparation of cochleates. In our preliminary studies, different solvent systems were compared for precipitation of cochleates to investigate lipid-calcium interactions. We could show that spherical cochleate-composites were only formed in aqueous-ethanolic mixtures (aqueous calcium chloride, ethanolic DOPS solutions). When ethanolic DOPS was precipitated with ethanolic calcium in water-free environment, SEM analysis revealed that the lipid precipitates formed did not exhibit any cochleates but showed aggregates of lipid sheets or stacks (Fig. 1f). When ethanol was replaced with non-polar solvents such as chloroform, dichloromethane and DMSO for dissolving DOPS, no cochleates or precipitates were formed. This might be due to the high solubility of the lipid in these solvents and, therefore, ethanol was selected as organic phase in our current study.

Preparation of cochleate composites using microfluidic device:

Microfluidic techniques provide a precise control over mixing of reactants and can be employed for reproducible mixing. The following studies were carried out with the aim to optimize parameters with respect to their effect on morphology and size of cochleate composites. Influence of solvent ratio, flow rate and lipid concentration on final product was assessed with electron microscopy. The conditions which allow most uniform formulation were identified based on the data obtained from these experiments.

Influence of ratio of aqueous phase to organic phase:

In nanoprecipitation, solvent concentration is an important parameter. This experiment was carried out in order to understand the influence of ratio of ethanol and water on the resulting morphologies of the cochleate composites. For the precipitation experiment, calcium chloride was dissolved in 10 mM Tris buffer and DOPS was dissolved in ethanol. Care was taken to maintain a constant molar ratio of calcium chloride : DOPS (1:1) for all experiments despite the changing flow ratios. Mixing was carried out at flow rate of 12 ml/min. We prepared cochleate composites to achieve aqueous: ethanolic phase ratios from 1:1, 1:2, 1:3, 1:5 and 1:9. In complete absence of ethanol, conventional long cylindrical cochleates were obtained in preliminary studies. This observation did not change significantly when high ratio of $CaCl2_{(aqueous)}$: $DOPS_{(ethanolic)}$ (3:1) was used (data not shown). However, when the ethanol fraction was increased beyond 75%, the morphology of the resulting particles changed from large cochleate aggregates to spherical nanocochleate composites, as shown in Fig. 1. At the ratio of 1:1 (Fig. 1a) large aggregated cylindrical cochleates were formed and their aggregation lacked structure. These samples also showed presence of unrolled sheets. Increasing the amount of ethanol (flow ratio 1:2) led to a big change in the morphology of aggregates. Agglomerates approximately 6-12 µm in diameter consisting of large cochleates were formed as seen in Fig. 1b. At both ratios cochleates formed were in micron range. Flow ratio of 1:3 produced particles containing nanocochleates (Fig. 1c) which were roughly spherical and showed high tendency of aggregation. Aggregates were usually made of at least 2-5 loosely packed cochleate-composites. It's important to note that at this ratio microspheres clearly showed presence of nanocochleates which seemed to indicate that increased ethanol content was important for the formation of cochleates with smaller dimensions. Further increase in amount of ethanol (ratio 1:5) resulted in particles (Fig. 1d) which were more spherical and uniform than before (approx. 3-6 µm diameter). These cochleate composites also showed the tendency to aggregate and individual cochleate composites were made up of aggregated nanocochleates. The ratio with minimum amount of water i.e. 1:9 produced cochleate composites (Fig. 1e) which were spherical and were made up of densely packed nanocochleates. When the

precipitation was carried out in pure ethanol, ethanolic DOPS and ethanolic $CaCl_2$, the formation of stacks was observed (Fig. 1f). However, these stacks did not form any cochleates even when the samples were observed after 8 months of storage. From these results we suspect that the presence of small amount of water is crucial for curling of bilayers into nanocochleate cylinders, however further investigation is necessary to understand the exact underlying mechanism.

Influence of flow rate:

It is well known that size and morphology can be varied easily by changing the rate of mixing in a microfluidic reactor. To evaluate this effect, we varied the flow rate from 1 ml/min to 18 ml/min keeping a constant flow ratio for $CaCl_{2(aqueous\ phase)}$: $DOPS_{(ethanolic\ phase)}$ at 1:9. Resulting lipid aggregates were analyzed using electron microscopy. Fig. 2 shows the influence of flow rate on the morphology of resulting cochleate composites. In general, we found that lower flow rates formed particles arrested and aggregated at intermediate stages of formation of cochleate composites. High flow rates due to faster mixing formed spherical cochleate composites. At the slowest flow rate of 1 ml/min long cylindrical cochleates were formed along with dense lipid precipitates as shown by arrows in Fig. 2a. These precipitates may be non-bilayer structures formed as a result of interfacial deposition of lipid due to displacement of ethanol. This result was an indication that not only high ethanol contents but also high mixing rates are a prerequisite for the formation of nano-sized cochleates. When the flow rate was increased to 3 ml/min, aggregates of 3-10 roughly spherical particles were observed (Fig. 2b). Particles seemed to exhibit a higher aggregation tendency and large conventional cochleates were also rarely observed. For samples prepared at 6 ml/min, dense isolated spherical particles with large diameter were observed (Fig. 2c). The surface of these particles appeared much denser compared to the cochleate composites. The dense nature of the particles was not completely clear and further evaluation is required, but it was observed repeatedly for this flow rate. It appeared as if the growth of particles was initiated before nucleation was completed. Which may result in competition between growth of new nuclei and grown particles and causes high polydispersity. Flow rate of 12 and 18

ml/min gave rise to much more spherical and regular particles (Fig. 2d and 2e respectively) as compared to those prepared at lower flow rates. Cochleate composites prepared at high flow rates were made up of aggregated nanocochleates and stacks. At 18 ml/min particles seemed to vary in size much more than that at 12 ml/min. Fig. 2e shows two particles, a completely formed particle (dark arrow) and an incompletely formed particle (white arrow) which was often observed at flow rate of 18 ml/min. This morphological evolution was probably due to the high flow rate which does not give enough time for aggregates to form spherical structures. Hence the flowrate of 12mL/min was chosen as most appropriate for further experiments.

Influence of Lipid Concentration:

Fig. 3 shows SEM images of cochleate composites at different lipid concentrations. We tested lipid concentrations ranging from 1 mg/ml to 0.1 mg/ml while maintaining a flow rate of 12 ml/min and flow ratio of $CaCl_{2(aqueous\ phase)}$: $DOPS_{(ethanolic\ phase)}$ at 1:9. All lipid concentrations produced spherical cochleate composites made of nanocochleates. The morphology of the composites became more regular and uniform in size with decreasing lipid concentration. For high lipid concentrations of 1 and 0.75 mg/ml, spherical cochleate composites were observed aggregated along with many small inconsistent non-spherical cochleate composites (shown by arrows in Fig. 3a). Such morphology was probably a result of very high nucleation and incomplete growth. Reducing the lipid concentration further resulted in much more uniform and spheroid particles and had almost no signs of attached aggregates (Fig. 3b). For smallest lipid concentration of 0.1 mg/mL the cochleate composites were extremely regular (Fig. 3c). Hence it was chosen as 'optimized formulation' for further experiments.

In samples with high lipid concentrations, cochleate composites were made of a large number of number of lipid stacks along with few nanocochleates. In our opinion, lipid stacks may be the primary structures formed from nucleates when lipid solution encounters the binding agent. High lipid concentrations probably gives rise to a large number of nucleates with small inter-particulate distances. This high density of nucleates might cause aggregation before 'stacks to cochleate'

transformation is complete. Therefore the cochleate formation or curling of the stacks in these particles seemed to be arrested at intermediate stages. For low lipid concentrations, cochleate composites showed nanocochleates and highly curled stacks. This was probably because aggregation was relatively gentle and allowed time for the stacks to evolve in to nanocochleates.

Morphological evaluation and size distribution of cochleate composites:

Optimized formulation of cochleate composites was further evaluated using SEM, cryo TEM and resin embedding technique to understand the morphology of the aggregated subunits. Fig. 4a shows a high magnification SEM micrograph of a cochleate composite showing many nanocochleates (white arrows) along with few curled stacks (dark arrows). Cochleates have a tube like appearance and can easily be identified by the hollow central channel. All of the aggregated nanocochleates and curled stacks had sub-micron dimensions. Fig. 4b shows a cryo-TEM image of cochleate composite at higher magnification where nanocochleates and curled stacks (dark arrow) can be clearly seen. Nanocochleates had approximate diameter of 100 - 200 nm and were made up of 8-20 lipid bilayers. Cross sections of cochleate composites obtained from the embedding study are as shown in Fig. 4c. The cross sections confirmed that cochleate composites were mainly made up of hollow nanocochleates which were aggregated to yield a spherical composite. Based on our EM studies, cochleate composites were mainly made up of (1) nanocochleates with hollow central channel (approx. length 400-600 nm) (2) curled bilayer stacks and (3) voids inside the cochleate composites (few hundred nanometers). To investigate whether the microfluidic device had any influence on the intrinsic structure of the nanocochleates, we also prepared cochleate composites using the 'modified trapping method' as explained in the method section 2.2.2. EM studies of these samples reveled formation of cochleate composites having irregular non-spherical shape (data not shown) which confirmed our anticipation that efficient mixing achieved in the NanoAssemblr™ was responsible for the spherical shape and uniformity of the cochleate composites.

The size distributions of cochleates obtained from trapping method and hydrogel isolation method were compared with cochleate composites. The representative SEM images corresponding to nanocochleates prepared by hydrogel isolation method and conventional trapping method are shown in Fig. 5. The particle size distribution histograms of cochleates prepared by all three techniques are shown in Fig. 6. The average diameter of optimized cochleate composites was about 4.4 ± 0.3 µm with CV (%) of 7.1. Compared to cochleates prepared by other methods, cochleate composites displayed a very narrow size distribution. The mean particle size obtained from both trapping method and hydrogel isolation method was 5.7 ± 3.06 µm and 1.1± 0.79 µm respectively. Their size distribution histograms indicated a skewed size distribution profile (Fig. 6a and 6b). The total range of particle size found for cochleates prepared by the trapping method ranged from 1.2 to 16.5 µm and for nanocochleates, from 0.1 to 6.6 µm. The CV (%) for cochleates prepared by trapping method was 53.3 and for nanocochleates it was 69.9, representing highly polydispersed samples. Considering CV (%) values, the samples of cochleate composites were quite monodispersed when compared to the conventional cochleates and nanocochleates. Hence the rate and homogeneity of mixing achieved using microfluidic technique has a marked effect on the particle size of final cochleate formulation. Similarly presence and amount of ethanol has a profound effect on the particle size distributions obtained by reducing the probability of aggregation.

Small angle X-ray scattering:

To understand differences in the lamellar order between conventional cochleates prepared by trapping method, hydrogel isolation method and cochleate composites presented in this study, SAXS experiments were carried out. The SAXS patterns of both cochleate composites, prepared using NanoAssemblr™ and 'modified trapping method' showed exactly the same pattern as conventional DOPS cochleates and nanocochleates (Fig. 7). Diffractograms of cochleate composites, nanocochleates and conventional cochleates yielded interlamellar repeat distance of 5.1 nm with sharp reflexes at 2Θ values of 1.7˚and 3.4˚. The SAXS data obtained for cochleate composites agrees

well with electron microscopy results and confirm that cochleate composites are in fact aggregates of nanocochleates with high regularity.

Formation mechanism of cochleate composites:

Probable formation mechanism for the cochleate composites consists two steps, 1) nucleation, where phosphatidylserine molecules bind calcium and grow longitudinally to form small lipid stacks which can eventually roll into nanocochleates and 2) aggregation, where high mixing rates within the microfluidic device gives rise to spherical cochleate composite formed by aggregation of nanocochleates. From our observation, the nucleation process was heavily influenced by ethanol concentration. Literature suggests that phospholipid bilayers can coexist in ethanol concentrations up to 45 – 50 % (Touitou et al., 2000) and from our experiments it could be seen that when reaction mixture had 50 % ethanol (solvent ratio 1:1) with 50 % aqueous calcium chloride, conventional long-cylindrical cochleates were formed. Only when ethanol concentration was raised above 75 % nanocochleates were formed. Ethanol forms hydrogen bonds with phosphate in the phospholipid head groups and when present in high concentrations can increase alkyl chain disorder and can lead to decrease in the bending energy in phospholipid bilayers (Patra et al., 2006, Feller et al., 2002, Chanturiya et al., 1999). When cochleates are prepared in aqueous environment, stack formation is caused by interaction between calcium and phosphatidylserine bilayers. This has a dehydrating effect on the bilayers which results in highly condensed alkyl chains with high degree of order and interior which is characterized by almost complete lack of water (Nagarsekar et al., 2014, Martin-Molina et al., 2012). The resulting stacks would aggregate rapidly due to their hydrophobic surfaces leading to larger planner sheets. These sheets further undergo curling to reduce their surface area to form cochleate cylinders. However in ethanol rich environment, hydrophobic surfaces of stacks would be stabilized by ethanol, resulting in smaller stacks. Stabilization coupled with lower bending energy could explain the nanoscale dimensions of the cochleates found within the spherical cochleate composites. Resulting nanocochleates and stacks further undergo aggregation to form cochleate composites.

Morphology of the composites was dictated by the mixing stage. This is a vital step and should be as uniform and as fast as possible since any fluctuations in mixing can result in asymmetrical particles with varying sizes. Poor mixing can also result in overlapping of nucleation and growth steps causing formation of heterogeneous system. Ideal mixing conditions were difficult to reach by using conventional mixing devices as we found out when we tried to replicate the composites using a homogenizer. Where as in case of micro mixer of microfluidic device the mixing time is very small (in milliseconds) which results in better control over process parameters and reproducibility (Kastner et al., 2014).

CONCLUSION:

Polydispersity has always been the 'Achilles heel' of a cochleate system. In this study we report on new monodisperse microparticulate system of nanocochleates. This method was developed to fabricate cochleate composites with a simple microfluidic setup, which could easily be programmed to transform for large scale production. We also propose strong influence of solvent conditions over formation of the cochleate structures. The morphology of the microspheres prepared using microfluidic device was strongly affected by flow rate and lipid concentration. Microfluidics could be used further to tailor particle diameter of the formulation. Cochleate composites being made of nanocochleates retain the basic advantages of cochleates. Hence the formulation of cochleate composites may emerge with potential uses for pharmaceutical applications. We believe that our study represents an important step forward to perceive processes governing the formation of monodispersed cochleate drug delivery systems. Future outlook for our work would involve drug loading and release experiments and to study the morphology when these composites are manufactured from different cochleate forming lipids.

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DECLARATION OF INTEREST

The authors report no declarations of interest.

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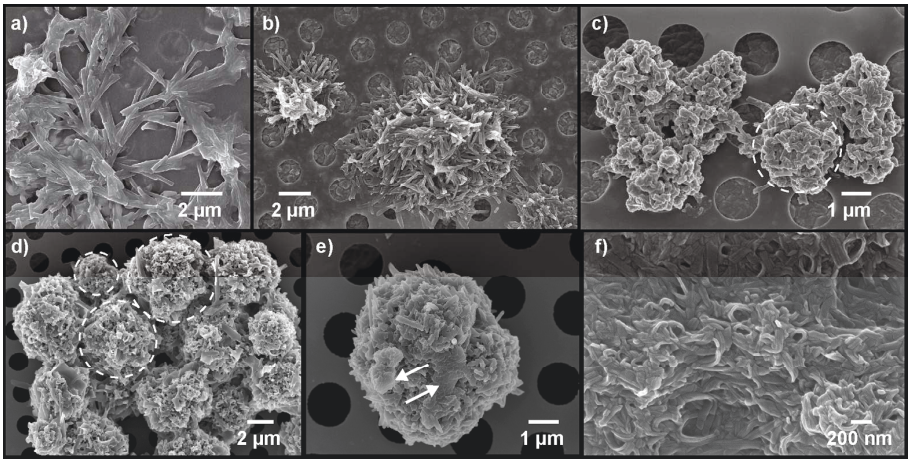

Figure 1: SEM images of DOPS cochleate composites obtained from NanoAssembler™ at $CaCl_{2\ (aqueous\ phase)}$: $DOPS_{(ethanolic\ phase)}$ ratios of (a) 1:1 (b) 1:2 (c) 1:3 and (d) 1:5; dashed circles marks the spherical morphology of the aggregated cochleate composites (e) 1:9 ; arrows indicates planar sheets aggregated on a cochleate composite, and (f) DOPS lipid sheets precipitated from mixing of ethanolic solution of DOPS and ethanolic calcium chloride. Flow ratio was maintained constant at 12 ml/min.
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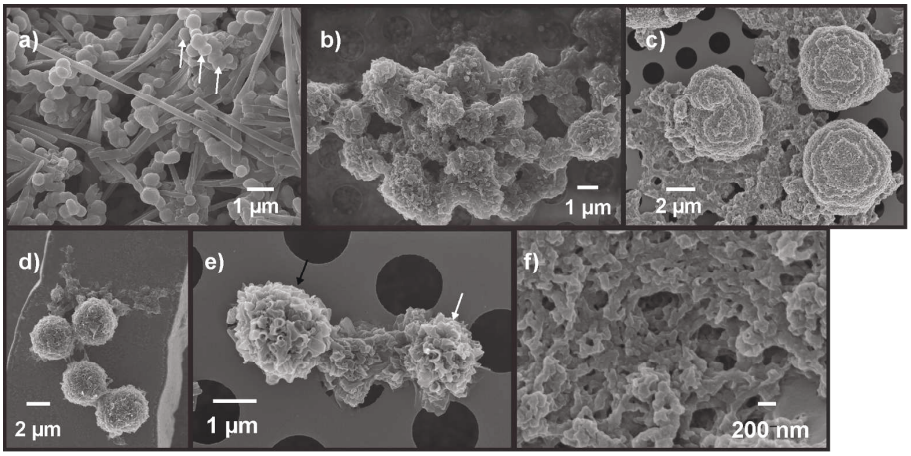

Figure 2: SEM images of DOPS cochleate composites obtained from NanoAssembler™ at different flow rates; (a) 1 ml/min; arrows showing non-bilayer structures (b) 3 ml/min (c) 6 ml/min (d) 12 ml/min (e) 18 ml/min and white arrow showing incomplete formation of particle (f) high magnification SEM image showing fused stacks formed in the dense particles obtained with flow rate of 6 ml/min. Constant flow ratio of 1:9 was maintained for all the samples.
168x83mm (300 x 300 DPI)

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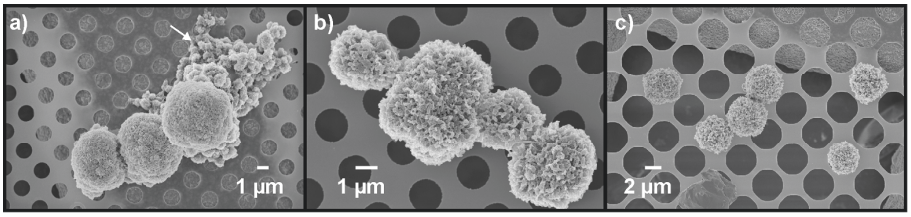

Figure 3: SEM images of DOPS cochleate composites obtained from NanoAssembler™ at lipid concentrations of (a) 0.75 mg/ml (b) 0.25 mg/ml (c) 0.1 mg/ml. Constant flow ratio of 1:9 and flow rate of 12 ml/min was maintained for all the samples.
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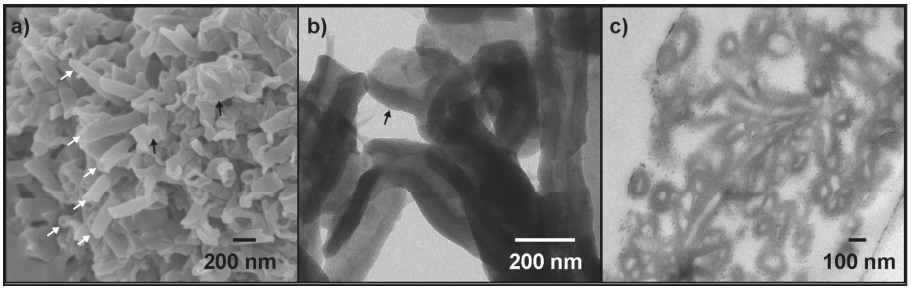

Figure 4: Morphology of optimized DOPS cochleate composites obtained from NanoAssembler™ a) SEM image showing nanocochleates (white arrows) and stacks (dark arrows) b) high magnification cryo-TEM image showing lamellar structure of the nanocochleates within the cochleate composites. Dark arrow shows a curling bilayer stack and c) TEM of cross section after resin embedding.
179x55mm (300 x 300 DPI)

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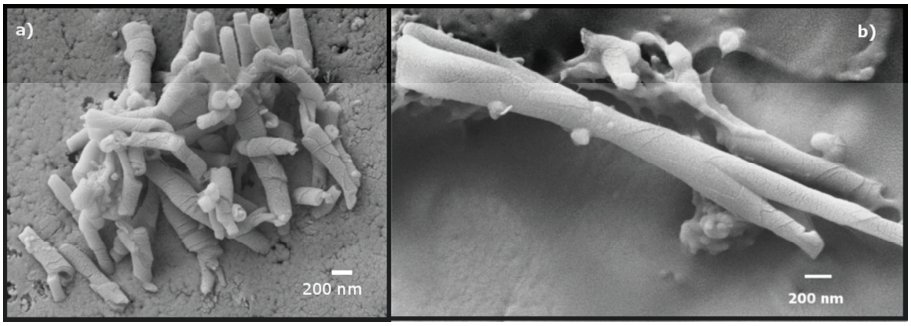

Figure 5: SEM images of DOPS, a) nanocochleates obtained by hydrogel isolation method b) conventional cochleates obtained by trapping method
86x30mm (300 x 300 DPI)

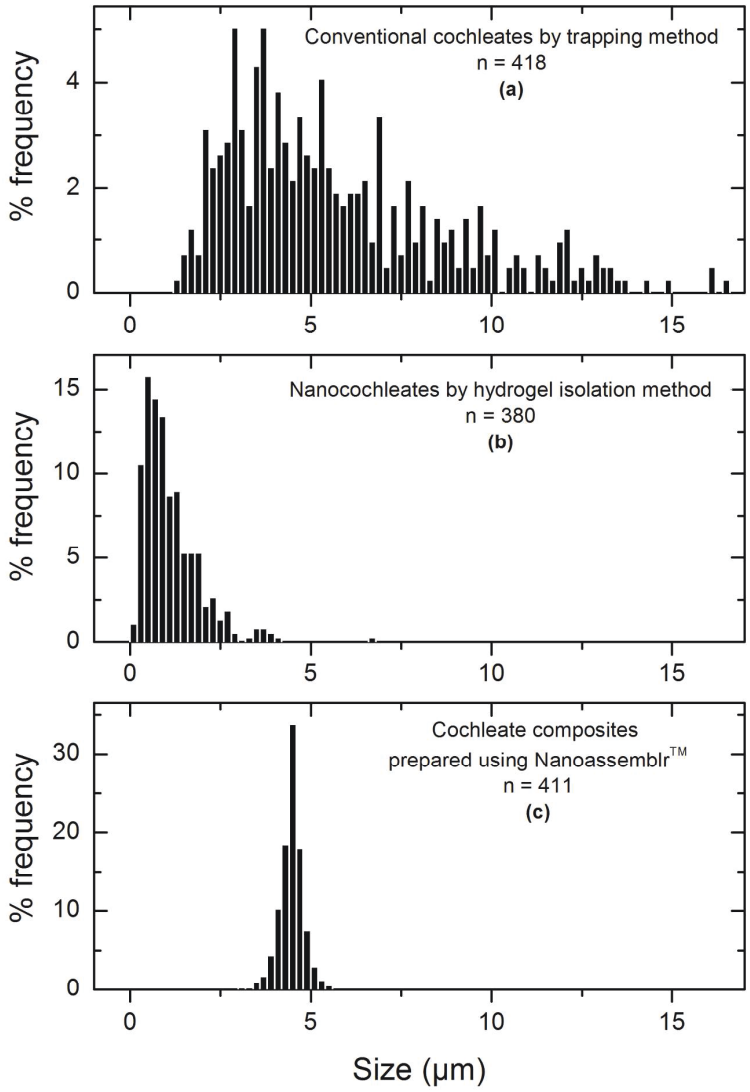

Figure 6: Particle size distribution determined from analysis of SEM images of a) conventional cochleates by trapping method b) nanocochleates by hydrogel isolation method c) optimized cochleate composites formulated using NanoAssembler™.
139x203mm (300 x 300 DPI)

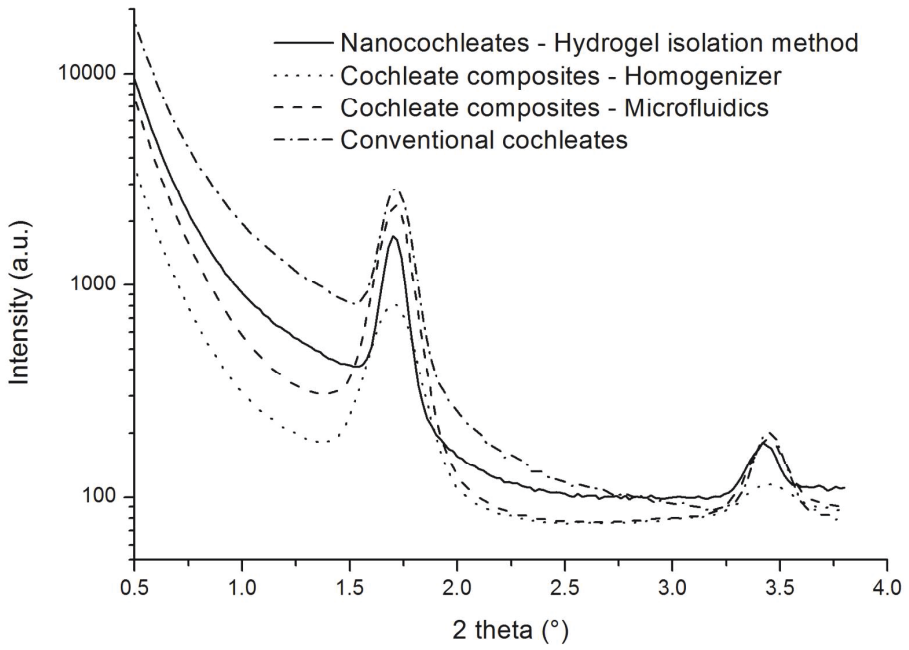

Figure 7: SAXS patterns of DOPS cochleates obtained by (a) conventional trapping method (- · - · -) (b) nanocochleates by hydrogel isolation method (_____) (c) cochleate composites prepared using NanoAssembler™ (- - -) and (d) cochleate composites prepared using a homogenizer (·····).
149x105mm (300 x 300 DPI)

4 Discussion

4.1 General discussion:

It is apparent that recent developments in preparation methods, characterization and evaluation have allowed considerable improvements of cochleate formulations for drug delivery. However, in order to reach the market there is still need to carry out further research. We believe that the detailed knowledge of cochleates may contribute to tailor them for optimal use *in vivo* and reflect on the challenges that lie in this way. Therefore, in this dissertation cochleates were evaluated for their structure and formation using different microscopy techniques. Apart from this, the challenge of polydispersity of cochleate formulations has also been addressed by successfully combining the knowledge of solvent effect with the concept of controlled mixing using microfluidics to prepare a monodispersed system. In this section, we intend to discuss the details regarding the pathway of cochleate formation, effect of the building block on structural features of cochleates, feasibility of our new method for formulation of cochleates and potential applications of these new developments.

4.1.1 Investigation on formation of cochleates:

The mechanism of cochleate formation from PS has been speculated in brief by Papahadjopoulos et al. (1975). Their theory was based on results from freeze fracture-TEM and explains that on the addition of high affinity cations to negatively charged lipids, a formation of array of structures takes place. Presumably, bilayer fusion is believed to advance though several semi-stable intermediate structures such as discs, sheets which finally are converted into cochleates. Some investigations on intermediates arising during cochleate formation have been reported previously. Kachar et al. reported that pure PS vesicles may undergo fusion or violent rupture on interaction with calcium ions (Kachar et al., 1986). In 1981 Miller et al. showed the presence of flattened structures in microscopy within 10 ms after addition of cations to PS. Most of these studies involved bovine brain extract PS rather than pure PS which have not been studied in detail. Our major objective in **(Manuscript 1)** was to provide a sequence of the morphological changes that bilayers of PS with different saturation and carbon chain length undergo after interaction with calcium using experimental evidence. A number of variations on this interaction scheme have been exercised such as video-enhanced microscopy (Kachar et al., 1986), rapid mixing (Rand et al., 1985) and using a freezing device (Miller and Dahl, 1982) etc. However in this study we have focused on the influence of the process temperature. When the process temperature is above the transition temperature of the phospholipid the intermediate steps occur at tremendous speed i.e. within milliseconds and an appearance of intermediate structures seems to be synchronous. To deal with this issue we hypothesized that decreasing the process temperature below or around transition temperature (Tm) of lipid might decrease the speed

of reaction allowing us a measurable window of time to have a look at different intermediate stages taking place. As noted in **(Manuscript 1)** we evaluated cochleate samples of different phosphatidylserines using various electron microscopy techniques. Our interpretation for the sequence of events during cochleate formation is based on the comparison between observed morphology in these samples. The experimental data from **(Manuscript 1)** shows that different chosen phosphatidylserines exhibited similar morphologies during cochleate formation. Our results agree with previously reported early morphological changes where immediately after addition of calcium, primary structures like liposomes, micelles undergo mutual adhesion and fusion (Kachar et al., 1986). The intermediate multilamellar structures with dense packing were often observed after aggregation. The flat areas of contact resulting due to aggregation indicated that interaction may be sufficiently strong to deform primary structures indicating high values of adhesion energy. Such deformation may further result in bilayer rupture due to increased tension (Evans and Kwok, 1982; Evans and Parsegian, 1983). Our observations of cryo TEM showed that these aggregated structures further give rise to secondary bilayer fragments (which we named ribbons). We interpret that these structures result due to bilayer destabilization in intermediate multilamellar aggregates. This loss of stability might evolve from fusion particularly between the outer monolayers of aggregated bilayer structures. It was demonstrated that the repeat distance of such ribbon like structures by SAXS appeared to be much different as compared to both cochleates and primary structures like liposomes or micelles. This may result due to presence of calcium in these bilayers and was considered as an indication for intermediate structure.

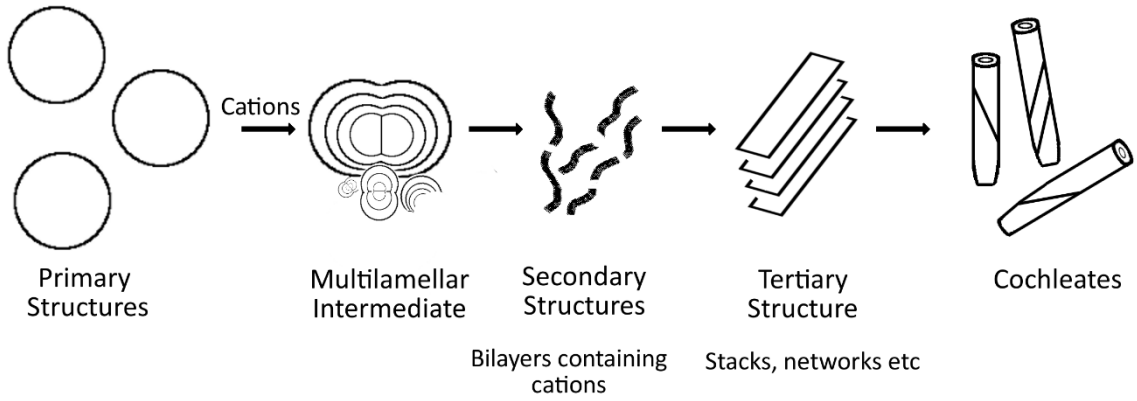

Figure 4.1: Diagrammatic representation of cochleate formation depicting sequence of intermediate structures during the process.

Our studies suggested that formation of cochleates directly from ribbons seems to be a demanding affair. This interpretation is based on the observation that no cochleate formation was observed in

DPPS and DSPS ribbon samples over long incubation times. This renders to possibility that formation of tertiary structures from ribbons requires energy. Our results show that ribbons further undergo aggregation to form an array of tertiary structures most characteristic of which are stacks. These structures had been previously also identified by Garidel et al. (2001) as bands in samples of DMPG cochleates. Stacks are multilamellar and generally have elongated trapezoidal shape (as depicted in Figure 4 of manuscript 1). Our results highlighted that stack formation may be essential in order to form cochleates. It was observed that stacks can also undergo fusion to form broad sheets or networks. Our SAXS experiments indicated that the tertiary structures do not reveal the same results for repeat distance as cochleates. Complete absence of tertiary structures was seldom observed in samples of cochleates prepared from PS. We interpret that these complex architectures composed of fused lipid networks, sheets and cochleates may be the final result of thermodynamic equilibrium during formation process. Such tertiary structures appear to be stable and may coexist with cochleates. The tertiary structures i.e. stacks or sheets further roll upon to form cochleates. Figure 4.1 displays diagrammatic representation of cochleate formation based on results from **(Manuscript 1)**. We presume that the rate of conversion of tertiary structures to cochleates is an important parameter which would influence polydispersity of the final cochleate formulation. This could be of interest from a pharmaceutical point of view considering the fact that for optimization of formulations, presence of intermediates can be an important issue as it can significantly influence physicochemical properties, drug loading, release properties etc.

Taken together **(Manuscript 1)** ascertained that cochleate formation is through evolution of well-defined hierarchical morphologies like ribbons, stacks, networks, sheets that leads to a stable cochleate structure. The most important conclusions from these studies are qualitative. Although cochleate formation may have more than one secondary pathway due to uncontrolled interactions, summary of our study in **(Manuscript 1)** provides enough support to speculate a fundamental order of probable intermediates arising during cochleate formation.

4.1.2 Structural features of cochleates:

Based on our microscopy results in **(Manuscript 1 and 2)**, the first point worth discussing about structure of cochleates is its central channel. As per Papahadjopoulos et al. cochleates are cigar like structures with folded morphology (1975) whereas Garidel et al. (2001) and Kodama et al. (1999) have mentioned the possibility of luminal opening in DMPG cochleates. All these arguments are based on freeze fracture TEM or TEM techniques, and need further research to support this observation completely. Hence to evaluate this issue the systematic observation of the internal structure of cochleates was carried out using 3D reconstruction from cryo electron tomography.

Figure 3 in **(Manuscript 2)** clearly depicts the presence of a continuous central channel that opens at both terminus of the cylindrical cochleate structure confirming the hypothesis of Garidel et al. (2001). Our observations suggested that unlike real cigars, cochleates do not possess a dense core. This observation was supported by other microscopy techniques such as SEM, TEM and evaluation of cross sections of resin embedded cochleates. Amongst all the evaluated cochleate particles, we very rarely came across some with absence of central channel as mentioned in **(Manuscript 2** Figure 4e**)**. Investigation of cross section of resin-embedded Cochleates in **(Manuscript 2** Figure 4**)** indicated that the majority of particles exhibited multilamellar scrolls folding into a compact, rolled-up structure as previously described by Papahadjopoulos et al. (1975). This study also confirmed the presence of central channel in cochleates. Based on these observations we deduced that cochleates have carpet roll like morphology and not cigar like structure. This structural feature might have a strong influence on physicochemical aspects of the drug delivery system. The parameters like mechanical stability, drug release pattern, area for surface chemistry modification or drug loading may change considerably depending upon presence or absence of the central cavity in the cochleate particle. Hence, one can anticipate that this knowledge can be casted to render an efficient drug carrier.

Evaluation of the effect of variation in acyl chains of phosphatidylserines on the structural features of cochleates was not carried out previously. We have used different phosphatidylserines to form cochleates in order to evaluate the effect of acyl chain length and saturation on structural features. In **(Manuscript 1 and 2)** we have analyzed electron micrographs with the help of the software Image J for determining dimensions of cochleates. We have specifically measured the width and length for a large ensemble of cochleate tubes. These measurements show apparent and statistically significant difference in dimensions with respect to the lipids used for preparation. Our microscopy studies showed that the width of inner channel of cochleates increased with presence of unsaturation. Changes in carbon chain length of lipids also resulted in change in dimensions of cochleates. Based on these results we suggest that the dimensions of cochleates are highly dependent upon their building blocks.

Considering the findings of electron microscopy studies, a theoretical model that was based on minimizing a phenomenological free energy expression was proposed. The calculations suggested that membrane bending and calcium induced bilayer−bilayer adhesion alone were insufficient to reproduce the observed relation between inner and outer cochleate tube radii. Yet, it could be reproduced by considering an additional free energy contribution that accounts for an area compression/expansion of the lipid bilayers within the cochleate. This modeling approach offers predictions for ratios of material constants, namely the ratio between the area compressibility and the bending modulus as well as the ratio between the bilayer adhesion energy and the bending

modulus. We believe that despite all approximations the theoretical model may succeed to capture some of the underlying principles of cochleate stability and may prove valuable for understanding of cochleates.

4.1.3 Effect of ethanol on formation of cochleates:

Since the preparation of cochleates by hydrogel-isolation method (Zarif et al., 2003), interest in their use for biomedical applications has been growing exponentially. In previously used preparation methods conventional techniques were used, for obtaining reaction conditions aimed at interaction of minimum number of bilayers with cations (Wang et al., 2014; Zarif et al., 2003). However cochleate formation is through process of continuous self-assembly which caters for coexistence of intermediates along with final product. Apart from this, the nature of cochleates to undergo continuous aggregation due to their hydrophobicity also makes control over final product a challenging task. Therefore, the approaches such as increasing viscosity of reaction medium or formulation of multiple emulsion did not help with issue of polydispersity even though they provided limited control over the size of final cochleate particles. Hence, we believe that although controlled mixing of reactants is a pivotal parameter for preparation of monodisperse cochleate system it is not the sole aspect that governs the final product.

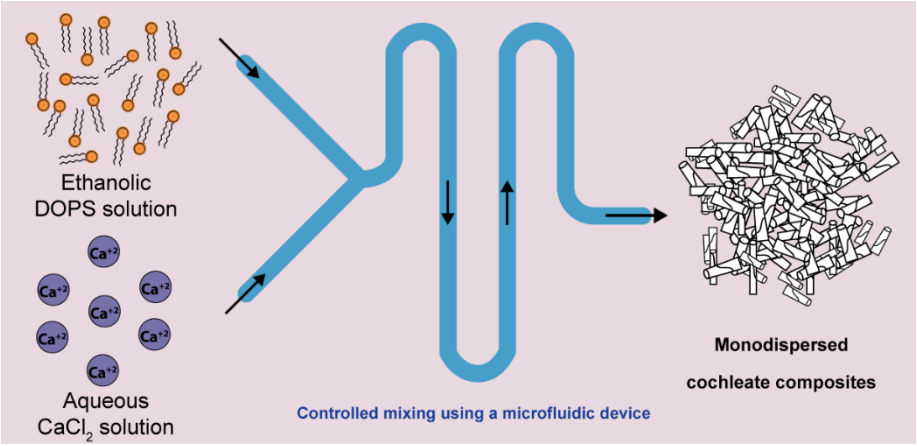

Figure 4.2: Diagrammatic representation of formation of monodisperse cochleate composites using microfluidic device after interaction of lipid solution with of calcium ions.

In **(Manuscript 3)** we have employed non-aqueous medium in combination with controlled mixing to formulate cochleates. The formation of lipid structures by nanoprecipitation in water-miscible organic solvents is not new. In this strategy using organic solvents along with aqueous phase aids in formation of nano-sized particles. Also as compared to aqueous environment it might aid in reducing surface tension at the interface of lipid particle and aggregation during preparation (Ramteke et al., 2012). Our dedication for this approach was supported by preliminary investigations which indicated

that the presence of water miscible organic solvent (>50% v/v) during precipitation gave rise to spheres made of nano-sized cochleates. To produce cochleates consistently, using microfluidic device was also a preferred choice. Microfluidics is a platform that has been known for its versatility regarding formulation of wide range of drug delivery systems (Zhang et al., 2013) and could exercise far greater control on the particle size than is possible by conventional methods.

In **(Manuscript 3)**, we have presented a microfluidics-based high through-put method for formation of microspheres (3-5 µm in diameter) made up of nano-cochleates. The formation of cochleates in a micromixer chip of microfluidic device is probably mediated by the diffusion of organic solvent into the miscible aqueous phase which causes nucleation, forming the lipid stacks. The high ethanol concentration during formulation of cochleates might stabilize the hydrophobic surfaces of stacks and assists in decreasing their bending energy (Feller et al., 2002; Patra et al., 2006). This may explain the formation of nano-scaled cochleates which further undergo aggregation to form microspheres. The process parameters such as flow rate and ratio of organic phase were optimized to produce monodispersed particles. Our results revealed that the particle size could be varied by changing the flow rate during preparation. The intermediates arising from overlapping of nucleation and growth phase could be minimized using optimized parameters. Particles obtained by this method were considerably uniform in size as compared to those obtained from conventional methods. This could be attributed to the fact that microfluidic device allowed for precise and reproducible mixing over micrometer length scales with controlled mechanical conditions. In comparison with conventional techniques, our approach offered easy handling and short formulation time. Microscopy studies and SAXS data confirmed the reproducibility of this technique and homogeneity of the products. Hence, development of this process could improve the quality attributes of cochleate formulations.

4.2 Conclusions and potential applications of this study

During current investigations of cochleates, findings regarding morphology and sequence of evolution of hierarchical structures have been made. The conclusions of this study may aid in finding missing links in previous literature on the formation mechanism of cochleates. Improved understanding of the formation mechanism could also allow for rectification of currently available formulation methods and the development new methods with higher reproducibility. The knowledge of different hierarchical structures and their possibility of coexistence might be of great interest considering their direct influence on physicochemical characteristics, stability, release kinetics etc. The high resolutions techniques used during this investigation availed gathering of additional information regarding peculiar structural features such as the hollow central channel. The detailed elucidation of structure of cochleates could help to establish a better perception regarding their

possible applications. This, in turn, would contribute to the design of cochleates for specific tasks. Developing a simple protocol to prepare monodisperse formulation of cochleates is a step ahead towards pharmaceutical industry and has several practical advantages. The described methodology availed reproducibility to the cochleate formulations. This would enable reproducibility of physicochemical characteristics of drug delivery system. Further a microfluidics device may allow for improved assistance in retaining the properties of sensitive drugs by providing controlled environment during preparation of cochleates as compared to conventional methods. The conventional approaches generate polydisperse aggregates of cochleates along with the intermediate morphologies. Given that stages like milling, homogenization, purification, washing of the final product cause an additional expense, using our strategy could prove cost effective. Cochleate microparticles synthesized using the microfluidic device can be easily modulated to produce higher yields. This can be of major advantage for expensive drugs considering the minimization of losses during production. So we postulate that our methodology described formation of monodisperse system that could widen the range of applications of cochleates. However, further studies are needed in order to reach the goal of incorporation of drug in formulation and further evaluation for efficiency. Nevertheless we hope that our study would contribute to advance the knowledge of cochleates and facilitate future research.

4.3 Future prospects of cochleates:

Cochleates are promising vehicles and are under investigation by companies like Matinas BioPharma in order to improve efficacy of some orally ineffective therapeutic agents. Considering medical applications, we can expect several drugs such as amphotericin B, ibuprofen etc. formulated into cochleate formulations in coming decades. Immunotherapy is another area in which researchers may expect progress. However to exercise potential of cochleates as drug delivery system *in vivo*, clear understanding of their mechanism of action at tissue and cellular level is required. More attention needs to be paid in understanding the effect of different building materials on systems efficiency *in vivo*. To design cochleates for successful drug delivery it is essential to carry out fundamental research to understand complex interactions such as drug release rates in different pathologies, effect of biological interactions like opsonization, and the barriers subjected while approaching target site etc. Given that the efficiency of cochleates is so far mainly gauged based on cell culture assays and murine models, the priority for future research should be to conduct bioavailability studies in humans. Apart from pharmaceutical industry cochleates may also prove to be useful in other areas such as food and nutrition industry, agricultural industry etc. Although during pharmaceutical investigations cochleates are made from pure and expensive raw materials using high technology, the low-purity

lipid extracts may also be used for preparation. The growth of lipid based novel technologies in cosmetic products like skin creams, sunscreen lotions etc. have always helped in capturing the attention of customers making cochleates one of the favorable strategy.

5 Summary

Cochleates represent a unique and attractive platform for biomedical applications. Interest of pharmaceutical industries towards phospholipid based biodegradable drug delivery systems have propelled investigations on cochleates in recent years. While, evaluating versatility and efficiency of cochleates as a drug delivery vehicle remains the main focus of current research, cochleates still need to overcome some hurdles which stand in the way to reach the other side of a product pipeline. Better understanding of fundamental details such as structure and formation mechanisms will assist in improving performance of cochleates as a drug delivery vehicle. In this thesis we have carried out investigations on structural features and formation pathways of cochleates. Furthermore, we have employed our knowledge in developing novel methodology to produce improved cochleate formulation.

In the past most of the investigations related to structural features or formation of cochleates have relied heavily on freeze fracture techniques. In this study, we have investigated cochleates of phosphatidylserines using different microscopy techniques for searching metastable intermediates and their coexistence with cochleates. Based on our morphological exploration, we speculate a probable order of well-defined hierarchical structures in the pathway for cochleate formation. This mechanism may be through evolution of unstable intermediate aggregates that form ribbonlike structures containing calcium. Ribbons further aggregate to form stacks or sheets, which leads to the formation of stable cochleates. It was found that the structures such as stacks, sheets and their networks can coexist with cochleates up to 12 months. The highlight of our study was the observation that, stack formation was an essential stage in the formation mechanism, as in its absence cochleate formation was hindered. Data from tomographic evaluation demonstrated cochleates to be cylindrically curved bilayers enclosing an inner channel. The dimensions of a cochleate vary statistically with changes in building lipids. Specifically, the unsaturated lipid tends to form cochleates with larger dimensions as compared to the saturated lipid. We proposed a theoretical model based on these findings in order to predict dimensions of cochleates.

All the observations of microscopy studies pointed towards the fact that for achievement of desired cochleate formulation, controlled environment allowing a single morphology to prevail is very important. Hence, in the current investigation we have developed a simple method for preparation of monodisperse cochleate formulations. We used a combined approach employing solvent effect along with controlled mixing by a microfluidic device. For this purpose, the lipid solution in an organic solvent was subjected to fast mixing with very small amount of aqueous solution of binding agent. The novel formulation obtained by this method consisted of

microspheres (~4 µm in diameter) made up of nanocochleates and was suitable for large scale production. Our studies suggest that during controlled mixing, lipid stacks are generated within milliseconds. These stacks probably entangle and fuse into a micro spherical composite. We presumed that excess of ethanol reduced the bending energy and might have stabilized the interface of lipid stacks reducing the aggregation. This might aid in both, formation of nanocochleates and regular microspheres. The microspheres obtained were characterized using SAXS and electron microscopy and compared with cochleates obtained from preexisting methodologies. EM data confirmed that the cochleate composites were clustered matrices of nanoscale cochleates. Findings from characterization studies confirmed that our methodology eliminated elaborate preparation methods, while providing a monodispersed cochleate system with analogous qualities to nanocochleates.

In conclusion our project has managed to capture new information regarding the structural details and formation mechanisms of cochleates. Furthermore a simple, fast, efficient and highly reproducible method with strong control over process parameters was successfully developed for formation of monodispersed cochleate system. We believe our effort will provide a better understanding of formation of cochleates, which may help scientists to tailor their formulations by making rational alterations.

Zusammenfassung

Cochleate stellen ein einzigartiges und attraktives Konzept für biomedizinische Anwendungen dar. Das steigende Interesse der pharmazeutischen Industrie an bioabbaubaren Arzneistoffträgersystemen auf Basis von Phospholipiden hat in den letzten Jahren die Forschung über Cochleate vorangetrieben. Aktuell ist das Hauptanliegen der Forschung die Ermittlung der Leistungsfähigkeit und Vielseitigkeit der Cochleate als Arzneistoffträgersysteme. Allerdings müssen noch einige Hürden überwunden werden, bevor Cochleate den Sprung aus der Produktpipeline auf den Markt schaffen können. Ein tieferes Verständnis grundlegender Eigenschaften wie die Struktur und der Entstehungsmechanismus sollen helfen, die Leistung von Cochleaten als Arzneistoffträgersystemen zu verbessern. In dieser Arbeit haben wir Untersuchungen zu strukturellen Merkmalen und Entstehungsmechanismen von Cochleaten durchgeführt. Zusätzlich haben wir unsere Erfahrung in der Methodenentwicklung genutzt, um verbesserte Cochleatformulierungen herzustellen. In der Vergangenheit basierte die Mehrheit der Untersuchungen zu strukturellen Eigenschaften oder der Entstehung von Cochleaten auf Gefrierbruchtechniken. In dieser Arbeit haben wir Cochleate aus Phosphatidylserinen mit Hilfe verschiedener Mikroskopietechniken in Hinblick auf metastabile Zwischenzustände und deren Co-Existenz mit Cochleaten untersucht. Basierend auf unseren morphologischen Untersuchungen beschreiben wir die Entstehung der Cochleate als eine mögliche Abfolge klar abgegrenzter, hierarchischer Strukturen. Der beschriebene Mechanismus verläuft über die Entstehung instabiler Zwischenprodukte, die bandartige Strukturen mit Calcium darstellen. Diese bandartigen Strukturen verbinden sich weiter zu Folien oder Stapeln, was schließlich zur Bildung stabiler Cochleate führt. Es konnte gezeigt werden, dass die Strukturen wie Stapel und Folien bis zu 12 Monate mit Cochleaten co-existieren können. Der Höhepunkt dieser Studie war die Beobachtung, dass die Stapelbildung ein essentieller Schritt in der Entstehung der Cochleate ist, da in ihrer Abwesenheit die Cochleatbildung verhindert wurde. Tomographische Untersuchungen zeigten, dass Cochleate aus zylindrisch gekrümmten Bilayern bestehen, die einen inneren Kanal umschließen. Die Größe von Cochleaten variiert statistisch und hängt vom verwendeten Lipid ab. Besonders ungesättigte Phosphatidylserine tendieren im Vergleich zu ihren gesättigten Analoga dazu, größere Cochleate zu bilden. Auf Grundlage dieser Beobachtungen stellen wir ein theoretisches Modell vor, mit dessen Hilfe sich die Größe der Cochleate vorhersagen lässt. Unsere Beobachtungen belegen die Tatsache, dass für die Produktion einer speziellen Cochleatformulierung kontrollierte Bedingungen, die das Auftreten einer einzigen Morphologie begünstigen, sehr wichtig sind. Somit haben wir mit der vorliegenden Arbeit eine einfache Methode zur Herstellung monodisperser Cochleate entwickelt. Wir haben hierfür

Lösungsmitteleffekte, die durch Ethanol auf Cochleate[1] verursacht werden, mit einem kontrollierten Mischen durch einen Microfluidizer[2] kombiniert. Zu diesem Zweck wurden die Phosphatidylserine in einem organischen Lösungsmittel gelöst und schnell mit einer kleinen Menge einer wässrigen Lösung des Bindemittels gemischt. Die durch diese Methode erhaltene neue Formulierung besteht aus Mikrokugeln[3] (~4 µm Durchmesser), die aus Nanocochleaten zusammengesetzt sind, und eignet sich für die Großproduktion. Unsere Studie legt nahe, dass während des kontrollierten Mischens Lipidstapel innerhalb von Millisekunden entstehen. Diese Stapel verfangen sich und fusionieren zu einem kugelförmigen Konstrukt im µm Bereich. Wir nehmen an, dass ein Überschuss an Ethanol die Biegeenergie reduziert und so möglicherweise die Grenzflächen der Lipidstapel stabilisiert und die Aggregation behindert. Dies kann sowohl bei der Entstehung der Nanocochleate als auch der regelmäßigen Mikrokugeln helfen. Die entstandenen Mikrokugeln wurden mit Hilfe von SAXS und Elektronenmikroskopie charakterisiert und mit Ergebnissen vorhandener Methoden verglichen, um die Bildung der Cochleate zu bestätigen. Durch Elektronenmikroskopie konnte belegt werden, dass die Cochleatverbände ein gebündeltes Netzwerk nanoskaliger Cochleate sind. Die Erkenntnisse aus den Charakterisierungsstudien bestätigen, dass unsere Methode etablierte Herstellmethoden verdrängt, indem eine monodisperse Cochleatformulierung mit vergleichbarer Qualität zu Nanocochleaten entsteht.

Zusammenfassend konnte unser Projekt einige neue Informationen in Bezug auf die strukturellen Eigenschaften und den Entstehungsmechanismus der Cochleate aufdecken. Weiterhin wurde eine einfache, schnelle, effiziente und reproduzierbare Methode mit streng kontrollierten Prozessparametern zur Herstellung monodisperser Cochleate etabliert. Diese Arbeit trägt zu einem besseren Verständnis der Entstehung von Cochleaten bei und gibt Wissenschaftlern die Möglichkeit, ihre Formulierungen durch rationale Änderungen anzupassen.

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List of Abbreviations

AFM	→	Atomic force microscopy
Amb	→	Amphotericin B
DDPS	→	1,2-didecanoyl-*sn*-glycero-3-phospho-L-serine
DMPG	→	1,2-dimyristoyl-*sn*-glycero-3-phospho-(1'-rac-glycerol)
DMPS	→	1,2-dimyristoyl-*sn*-glycero-3-phospho-L-serine
DOctPS	→	1,2-dioctanoyl-*sn*-glycero-3-phospho-L-serine
DOPS	→	1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine
DPPS	→	1,2-dipalmitoyl-*sn*-glycero-3-phospho-L-serine
DSC	→	Differential scanning calorimetry
DSPS	→	1,2-distearoyl-*sn*-glycero-3-phospho-L-serine
FTIR	→	Fourier transform infrared spectroscopy
Ms	→	Milliseconds
NMR	→	Nuclear magnetic resonance
PS	→	Phosphatidylserine
SAXS	→	Small-angle X-ray scattering
SEM	→	Scanning electron microscopy
WAXS	→	Wide-angle X-ray scattering
TEM	→	Transmission electron microscopy
Tm	→	Transition temperature

Curriculum Vitae

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Education

• PhD candidate (December. 2011 – Present)	Friedrich-Schiller-University Jena, Germany Thesis title: Cochleates: New insights into drug delivery system
• Masters of Pharmaceutical Sciences **(**October 2006 - October 2008**)**	University of Mumbai, Mumbai, India Thesis title: Preparation and Evaluation of leaf extract of *Vitex negundo* Linn ,
• Bachelor of Pharmaceutical Sciences **(**October 2002 - July 2006**)**	University of Mumbai, Mumbai, India

Research Experience

Doctoral Research:

- Investigations on structural features of cochleates.
- Evaluation of intermediate morphologies generating during formation pathway of cochleates
- Developing novel methodology to produce improved cochleate formulation.

Master Research:

- Preparation and characterization of the supercritical fluid extract (SCF) and ethanolic extract of leaves of *Vitex negundo* Linn
- In-vivo and in-vitro evaluation for anti-lipid peroxidation activity of the extracts.

Other projects:

- Preparation, optimization and characterization of polymer-lipid composite nanoparticulate system of Amphotericin B for splenic targeting.
- In-vivo and in-vitro evaluation of Amphotericin B formulation for toxicity.

Publications

- **Nagarsekar K**, Ashtikar M, Steiniger F, Thamm J, Schacher F, Fahr A. Understanding cochleate formation: Insights into structural evolution *Soft matter* (2015) being revised.
- **Nagarsekar K**, Ashtikar M, Steiniger F, Thamm J, Schacher F, Fahr A. Micro-spherical cochleate composites: method development for monodisperse system *Liposome research* (2015) being revised.
- **Nagarsekar K**, Ashtikar M, Thamm J, Steiniger F, Schacher F, Fahr A, May S. Electron microscopy and theoretical modeling of Cochleates. *Langmuir* (2014) 30: 13143-51
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- **Nagarsekar K**, Nagarsenker MS, Kulkarni SR. Evaluation of composition and antimicrobial activity of supercritical fluid extract of leaves of Vitex negundo. *Indian J Pharm Sci* (2010) 72:641-3.

Presentations

1. **Oral presentations**

- Insight in Morphology of cochleates at 23rd Liposome workshop, Oberjoch, Germany, March 2014.
- Cochleates: A novel drug delivery platform at 22nd Liposome workshop at Ameland, Netherlands, September 2012.

2. **Poster presentations**

- Insights into the morphology of cochleates, poster presentation at Liposome Research Days, Copenhagen, Denmark (August 2014).
- Insights into the morphology of cochleates, Phospholipids in pharmaceutical research symposium; Heidelberg, Germany (September 2013).
- Amphotericin B cholesterol LIPOMER: Molecular aggregation and Characterization, Controlled Release Society German Chapter Annual Meeting & International Symposium of the Thuringian ProExcellence Initiative NanoConSens, Jena, Germany (March 2011).
- Formulation and in-vitro evaluation of amphotericin B LIPOMER. , Advances in Technology and Business Potential of New Drug Delivery Systems Organized By Controlled Release Society-Indian Chapter Mumbai, India (Feb 2010).
- Ethanolic extract of leaves of *Vitex negundo*: evaluation and feasibility of formulation development. Advances in Technology and Business Potential of New Drug Delivery Systems' Organized By Controlled Release Society-Indian Chapter Mumbai, India (Feb 2009).
- Composition and anti-lipid peroxidation activity of ethanolic extract and supercritical fluid extract of leaves of *Vitex negundo* Linn, 2nd international conference on drug discovery and development – south Asian perspective Mumbai, India (Oct 2008).
- Microscopy of Nirgundi and Nutmeg, National conference in Medicinal Botany Mumbai, India (Sept 2007).

Declaration

I would like to acknowledge that I am familiar with the 'Promotionsordnung' of the Faculty of Biology and Pharmacy in the Friedrich-Schiller-University Jena. All chapters of this dissertation were produced by myself. This dissertation does not contain any material previously submitted for any degree or diploma at any other University. Also this dissertation does not contain any material previously written or published by any other person, except where due acknowledgement or reference has been made in the text. I would also like to declare that I did not enlist the assistance of any 'dissertation-counseling agent' and that I did not provide any direct or indirect monetary benefit to any third party for work connected to my dissertation.

Kalpa Nagarsekar

Jena, 23.10.2015