Abstract
Cochleates have been of increasing interest in pharmaceutical research due to their extraordinary stability. However, the existing techniques used in the production of cochleates still need significant improvements to achieve sufficiently monodispersed formulations. In this study, we report a simple method for the production of spherical composite nanoparticles made of nanocochleates from phosphatidylserine and calcium (as binding agent). Formulations obtained from the proposed method were evaluated using electron microscopy and small angle X-ray scattering and were compared with conventional cochleate preparation techniques. In this new method, an ethanolic lipid solution 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 composite microparticles made of nanocochleates. This simple methodology eliminates elaborate preparation methods, while providing a monodisperse cochleate system with analogous quality.

Keywords
Cochleates, electron microscopy, microfluidics, phosphatidylserine, solvent effect

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). The term “cochleates” was introduced by Papahadjopoulos in 1975 while investigating cation-induced phospholipid membrane fusion in great detail (Papahadjopoulos & Poste, 1975; Papahadjopoulos et al., 1975; Wilschut et al., 1981, 1985). Cochleates represent a potential delivery system with remarkable advantages being 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 (Nagarsekar et al., 2014; Zarif, 2005). During the past few decades, cochleates were explored for a number of possible applications thanks to their peculiar properties. Cochleates have been employed to improve delivery of drugs (Syed et al., 2008; Zarif et al., 2000), genes (Zarif & Mannino, 2000), flavoring agents (Mannino & Gould-Fogerite, 2002), and antigens (Gould-Fogerite & 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 diverse if compared to lipid tubules or nanotubes (Garidel et al., 2001; Kulkarni et al., 1996, 1999; Sarig et al., 2011; Zarif, 2005). 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 (Papahadjopoulos et al., 1990; Sankar & Reddy, 2010). This is mainly due to the uncontrolled and continuous aggregation between the bilayer sheets. During the last decade, research was focused on production strategies to form nanocochleates mainly by isolating or limiting the number of building blocks that come in close contact with each other. The development of methods such as hydrogel isolation (Zarif et al., 2003) or emulsion–lyophilization (Wang et al., 2014) have opened up a new field of application-based research for various molecules 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 rarely received attention. These limitations can critically limit suitability of cochleates as drug delivery systems for 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; Papahadjopoulos et al., 1975; Zarif, 2002), which 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 uncontrollable 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 formed much smaller stacks during precipitation from ethanol compared to precipitation in water. In this study, we have utilized this solvent effect toward 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 a one-step, simple, reproducible, and scale up of various nanoparticles, where a final product is an attractive choice as the process parameters such as rate and scale 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, microfluidics have been explored for production 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 (Belliveau et al., 2012b; Kastner et al., 2014; Whitesides, 2006).

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-phosphatidylycerine (sodium salt) (DOPS) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Araldite® LY 564 used for embedding studies was obtained from Huntsman Advanced Materials GmbH (Basel, Switzerland). Absolute ethanol used to prepare lipid solutions was purchased from VWR International GmbH (Darmstadt, 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, Darmstadt, Germany).

Preparation of cochleates

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

Preparation of cochleate composites using microfluidics

A modified nanoprecipitation method was employed for the preparation of cochleate composites. Ethanolic solution of DOPS was prepared with the aid of bath sonication. 60 mM CaCl2 solution in 10 mM Tris buffer was used as source of binding agent. Formulations were prepared using a benchtop NanoAssemblr™ instrument (Precision NanoSystems Inc., Canada) employing poly(dimethyl siloxane) micromixer chips (Precision NanoSystems Inc., Vancouver, 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 CaCl2. 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 into a Falcon™ tube.

In the first set of experiments, for understanding the effect of solvent ratio on morphology of the particles, the ratio of the aqueous phase:organic phase was varied steadily from 1:1 to 1:9 (with constant flow rate of 12 ml/min). In the second set of experiments the flow rate was varied from 1 ml/min to...
18 ml/min (keeping the flow ratio of 1:9 constant). Concentration of lipid was kept constant at 1 mg/ml for both sets of experiments. Further, in the third set of experiments, to understand the effect of lipid concentration, the amount of DOPS in the organic phase was varied from 0.1 mg/ml to 1 mg/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).

Preparation of cochleate composites by modified trapping method

To understand the influence of microfluidic device on the morphology, cochleate composites were also prepared using a T-25 Ultra-Turrax® homogenizer (IKA Werke GmbH & Co. KG, Staufen, Germany) maintaining the 1:9 CaCl$_2$(aqueous):DOPS$_{\text{ethanolic}}$ 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 mixing speed of 13 500 rpm with T-25 Ultra-Turrax® homogenizer mixing was continued for another 5 min after complete addition of calcium chloride. Mixture was then stirred continuously up to 1 h using a magnetic stirrer (IKA Werke GmbH & Co. KG, Staufen, Germany). The molar ratio of lipid to calcium was 1:1. At the end of 1 h precipitated mixtures were collected and stored at 4°C. Samples were prepared in triplicate and evaluated by EM.

Preparation of conventional cochleates by trapping method

Conventional cochleates were prepared to evaluate the difference in SAXS patterns and size distribution with respect to cochleate composites prepared using methods described above. For the preparation of conventional cochleates, DOPS liposomes were prepared by thin-lipid film hydration method as described elsewhere (Nagarsekar et al., 2014). 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, Labortecnik GmbH, Essen, 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 nanocholeates by hydrogel isolation method

Nanocochleates were prepared by hydrogel isolation method as described previously by Jin et al. (2001). DOPS liposomes were formed as described earlier. The liposome suspension was further dispersed in 40% w/w dextran 50 000 solution in ratio of 1:2 v/v. This mixture was then added slowly to 15% w/w PEG 8000 solution with a syringe under magnetic stirring (1100 rpm). To this dispersion an aqueous CaCl$_2$ solution (60 mM) was added to reach the equinolar ratio of lipid and calcium. After 2 h of stirring, the resultant mixture was washed three times by mixing with equal volume of buffer (1 mM CaCl$_2$ 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, Großlöbichau, Germany). Excess liquid was removed with a lint-free filter paper and examined in a LEO 1530 Gemini (Carl Zeiss, GmbH, Jena 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 ImageJ 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 (%) = ($\sigma$/D) × 100 where $\sigma$ 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, Großlöbichau, 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., Pleasanton, CA) and inserted into the cryoelectron microscope Philips CM 120 cryo-TEM (Philips, Eindhoven, 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, the sample was redispersed in 100 mM cacodylate buffer (pH 7.2) containing 1% OsO$_4$ for 2 h. The 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, Wolftratshausen, Germany) at room temperature. The slices were transferred to copper grids (Quantifoil Micro Tools GmbH, Großlöbichau, Germany) and examined by Philips CM 120 TEM.

Small angle X-ray scattering

Small angle X-ray scattering (SAXS) measurements were performed at $\lambda = 1.54\text{Å}$ using a Bruker Nanostar (Bruker GmbH, Karlsruhe, Germany) equipped with a μ-focus X-ray source (μS, Incoatec GmbH, Geesthacht, Germany),
equipped with a 2D position sensitive detector Vantec 2000 (Bruker GmbH, Karlsruhe, 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 2h. 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 $2\theta =$ scattering angle and $\lambda =$ 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 a spontaneous 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 (Rao et al., 2007; Zarif et al., 2003). 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 (Figure 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 (Belliveau et al., 2012a; Yu et al., 2009). 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 EM. The conditions which allow most uniform formulations 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 (Jin, 2008). 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 1:1, 1:2, 1:3, 1:5 and 1:9. In the complete absence of ethanol, only conventional long cylindrical cochleates were obtained (see supplementary material, Figure S1). This observation did not change significantly when high ratio of CaCl$_2$(aqueous):DOPS(ethanolic) (3:1) was used (see supplementary material, Figure S2). However, when the ethanol fraction was increased beyond 75%, the morphology of the resulting particles changed from large cochleate aggregates to spherical nanocoachleate composites, as shown in Figure 1. At the ratio of 1:1 (Figure 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 \(\sim 6-12 \mu m\) in diameter consisting of large cochleates were formed as seen in Figure 1(b). At both ratios cochleates formed were in micron range. Flow ratio of 1:3 produced particles containing nanocochleates (Figure 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 is 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 (Figure 1d) which were more spherical and uniform than before (\(\sim 3-6 \mu m\) diameter). These cochleate composites also showed a tendency to aggregate and individual cochleate composites were made up of aggregated nanocochleates. The ratio with the minimal amount of water (1:9) produced cochleate composites (Figure 1e) which were spherical and made up of densely packed nanocochleates. When the precipitation was carried out in pure ethanol, ethanolic DOPS and ethanolic CaCl\(_2\), formation of stacks was observed (Figure 1f). However, these stacks did not form any cochleates even when the samples were observed for 8 months of storage. From these results, we suspect that the presence of at least a small amount of water is crucial for curling of bilayers into nanocochlate cylinders. However further investigation is necessary to understand the exact underlying mechanism.

**Influence of flow rate**

It is well known that particle size can be varied by changing the rate of mixing in a microfluidic reactor (Hung & Lee, 2007; Kastner et al., 2014). 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. The resulting lipid aggregates were analyzed using EM. Figure 2 shows the influence of flow rate on the morphology of resulting cochleate composites. In general, we found that lower flow rates formed particles, which were 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 Figure 2(a). These precipitates may consist of 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 (Figure 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 (Figure 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 (Figure 2d and e, 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.

![Figure 2](image-url)

**Figure 2.** SEM images of DOPS cochleate composites obtained from NanoAssembler\(^\text{TM} \) 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.
At 18 ml/min particles seemed to vary in size much more than that at 12 ml/min. Figure 2(e) 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 flow rate of 12 ml/min was chosen as most appropriate for further experiments.

Influence of lipid concentration

Figure 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)$:DOP$(ethanolic phase) at 1:9. All lipid concentrations produced spherical composites made of nanocoehlotes. 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 Figure 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 (Figure 3b). For smallest lipid concentration of 0.1 mg/ml the cochleate composites were extremely regular (Figure 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 lipid stacks along with few nanocoehlotes. In our opinion, lipid stacks may be the primary structures formed from nucleates when the 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 nanocoehlotes and highly curled stacks. This was probably because aggregation was relatively gentle and allowed time for the stacks to evolve in to nanocoehlotes.

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. Figure 4(a) shows a high magnification SEM micrograph of a cochleate composite showing many nanocoehlotes (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. Figure 4(b) shows a cryo-TEM image of cochleate composite at higher magnification where nanocochleates and curled stacks (dark arrow) can be clearly seen. Nanocochleates had 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 Figure 4(c). 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 (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. EM studies of these samples revealed formation of cochleate composites having irregular non-spherical shape (see supplementary material, Figure S3) which confirmed our anticipation that efficient mixing achieved in the NanoAssembler™ 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 the hydrogel isolation method and conventional trapping method are shown in Figure 5. The particle size distribution histograms of cochleates prepared by all three techniques are shown in Figure 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 (Figure 6a and b). The total range of particle size found for cochleates prepared by the trapping method ranged from 1.2 μm to 16.5 μm and for nanocochleates, from 0.1 μm 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 the 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 NanoAssembler™ and “modified trapping method” showed exactly the same pattern as conventional DOPS cochleates and nanocochleates (Figure 7). Diffractograms of cochleate composites, nanocochleates and conventional cochleates yielded an 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 agree well with EM results and confirm that cochleate composites are in fact aggregates of nanocochleates with high regularity.

Formation mechanism of cochleate composites

One probable formation mechanism for the cochleate composites could consist of two steps: (1) nucleation, where phosphatidylserine molecules bind calcium and grow longitudinally to form small lipid stacks which eventually roll into nanocochleates and (2) aggregation, where high mixing rates within the microfluidic device give rise to spherical cochleate composite formed by aggregation of nanocochleates. From our observation, the nucleation process was heavily influenced by the ethanol concentration. The present 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 the reaction mixture contained 50% ethanol (solvent ratio 1:1) and 50% aqueous calcium chloride, conventional long-cylindrical cochleates were formed. Only when the ethanol concentration was raised above 75% nanocochleates were formed. Ethanol forms hydrogen bonds with the phosphate in the phospholipid head group and when present in high concentrations, this can increase alkyl chain disorder leading to bending energy decrease in phospholipid bilayers (Chanturiya et al., 1999; Feller et al., 2002; Patra et al., 2006). 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 (Martin-Molina et al., 2012; Nagarsekar et al., 2014). 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. The morphology of the composites was dictated by the mixing stage. This stage 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. These ideal mixing conditions are difficult to achieve using conventional mixing devices as we found out when we tried to replicate the production of composites using a classic homogenizer. In the 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). It should be stressed that the mechanism described here for the formation of cochleate composites is of speculative nature and a thorough investigation of the formation mechanisms will be necessary.

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 a 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 the 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, release experiments and to study the morphology when these composites are manufactured from different cochleate-forming lipids. Also a detailed biophysical characterization of the system is warranted in order to understand the mechanism of formation of cochleate composite microparticles.

Declaration of interest

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