Development of cochleate technology towards functional design and continuous production

Shuddhodana

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**NANYANG
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SINGAPORE

**DEVELOPMENT OF COCHLEATE TECHNOLOGY
TOWARDS FUNCTIONAL DESIGN AND
CONTINUOUS PRODUCTION**

SHUDDHODANA

**SCHOOL OF CHEMICAL AND BIOMEDICAL
ENGINEERING**

2021

**DEVELOPMENT OF COCHLEATE TECHNOLOGY
TOWARDS FUNCTIONAL DESIGN AND
CONTINUOUS PRODUCTION**

SHUDDHODANA

School of Chemical and Biomedical Engineering

A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Doctor of Philosophy

2021

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

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Assoc. Prof. Zaher Judeh

Authorship Attribution Statement

This thesis contains material from 4 papers published in the following peer-reviewed journals in which I am listed as an author.

Chapter 1 is published in parts as Shuddhodana, K. Nagarsekar and Z. Judeh. Recent Advances and Developments in Cochleate Technology. Nanomedicine & Nanotechnology Open Access 2(2), 000119 (2017). DOI: 10.23880/nnoa-16000119

The contributions of the co-authors are as follows:

- Assoc. Prof. Zaher Judeh provided the initial direction and edited the manuscript drafts.
- I wrote the manuscript draft.
- The manuscript was edited by Dr. Kalpa Nagarsekar.

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The contributions of the co-authors are as follows:

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- The manuscript draft was written by Dr. Lakshmi Madhuri Pappu.

- I edited the manuscript draft.
- Preparation of empty cochleates and their characterization were done by me at the School of Chemical and Biomedical Engineering.
- Preparation of liquid crystal cell doped with cochleates, characterization of switchable window and data analyses were done by Dr. Lakshmi Madhuri Pappu at Ben-Gurion University of the Negev, Beer-Sheva, Israel.

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- I wrote the manuscript draft and revised the manuscript.
- I co-designed the study with Assoc. Prof. Zaher Judeh and performed all the laboratory work at the School of Chemical and Biomedical Engineering. I also analysed the data.
- The small angle X-ray scattering was performed by me at the Facility for Analysis, Characterization, Testing and Simulation. The collection, evaluation and analyses of data were conducted by me.

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- The design and construction of off-the-shelf flow device, the continuous-flow experiments, computational fluid dynamics simulation, data collection, and analyses were performed by me.
- Dr. Pooi Wen Kathy Wong and I designed the in-vitro cytotoxicity and in-vitro intestinal absorption studies. Dr. Pooi Wen Kathy Wong helped in performing the experiments. The data collection, evaluation and analyses were done by me.

05/02/2021

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Abstract

Cochleates are supramolecular system of solid particulates comprised of large and continuous bilayer sheets of phospholipids, rolled-up to form scroll-like cylinders with little or no aqueous phase incorporated within the core. Lipid cochleates have emerged as a promising scaffold for the encapsulation of active molecules with applications in drug delivery and material science. However, research efforts focused on their formation, functional design, and reliable, controlled production, especially in a continuous fashion have remained largely unexplored and are essential for their successful translation.

The design aspects of drug delivery systems are crucial for their functional outcome. Hence, the mechanism of formation, design, and its consequences on the functional properties of cochleates formed using amikacin as a non-metallic bridging agent, with and without Ca^{2+} in sub-threshold concentration, in comparison to conventional Ca^{2+} -bridged cochleates were investigated. Based on the observations, a synergistic binding model involving both amikacin and Ca^{2+} for the bridging of phospholipid headgroups to form rigidly packed multiple bilayer cochleates was proposed. Additionally, tuneable amikacin release by simply varying the sub-threshold Ca^{2+} concentration with respect to amikacin was unravelled by studying the drug release kinetics. This study demonstrated the possibility of controlling the functional outcome by choosing specific type and concentration of the bridging agents used for the preparation of cochleates.

A cochleate-based formulation was then developed for the effective oral delivery of artemisinin, a life-saving antimalarial drug characterized by poor

bioavailability. The importance of lipid composition for the successful formation of cochleates with optimal encapsulation and loading of artemisinin was presented. A facile method for the coating of cochleates with alginate was developed to avoid their rapid dissociation under low pH gastric conditions. The alginate-coated cochleates showed better in-vitro controlled-release of artemisinin under simulated gastro-intestinal conditions than uncoated cochleates. While this study demonstrates the benefits of surface coating of cochleates with functional agents like alginate for enhanced oral delivery of artemisinin and similar drugs, it also opens attractive prospects for active targeting.

In addition to effectively designing a cochleate-based system to suit controlled-delivery of encapsulated drugs, it is important to develop a novel and efficient process for the preparation of cochleates that can overcome the challenges associated with existing conventional batch processes. Hence, a continuous flow-based process for the preparation of drug-loaded cochleates was developed. An inexpensive off-the-shelf flow focusing device was used to successfully prepare artemisinin-loaded cochleates with high throughput. By carefully controlling the flow focusing parameters, cochleates of uniform and tuneable size were obtained with low dispersity, narrow size distribution, and high reproducibility as compared to the batch process. These cochleates were non-toxic and showed effective transepithelial permeability of artemisinin across intestinal Caco-2 monolayer indicating better in-vitro bioavailability. Therefore, the off-the-shelf flow focusing device is envisioned to be a highly promising platform for continuous and high-throughput production of drug-loaded cochleates in a controlled and reproducible manner.

Overall, this thesis addresses several aspects such as the mechanistic understanding of cochleate formation to achieve a desired functional outcome, the effect of lipid composition on cochleate formation and drug encapsulation, their surface modification towards better controlled-release in gastro-intestinal milieu using functional coating with alginate, and a robust, reliable continuous flow-based process for their preparation. Therefore, this dissertation is believed not only to help and guide the future design of cochleates with specific functions in drug delivery and formulation, but also to potentially enable their smooth clinical translation with predictable drug release, absorption, and bioavailability.

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List of Abbreviations

ACB – Artemisinin-loaded cochleates prepared using batch method

ACF - Artemisinin-loaded cochleates prepared using continuous method

ACT – Artemisinin combination therapy

AE – Amikacin encapsulation efficiency

AL – Amikacin loading

ALG – Alginate/sodium alginate

ALG-Cochleates – Alginate-coated cochleates

ALIS – Amikacin liposomal inhalation suspension

AMK – Amikacin/amikacin sulphate

AMK-Ca-Coch – Cochleates prepared with both amikacin and sub-threshold concentration of Ca^{2+} as bridging agents

AMK-Coch – Cochleates prepared with amikacin alone as bridging agent

ANOVA – Analysis of variance

ART – Artemisinin

BSA – Bovine serum albumin

Ca-Coch – Cochleates prepared with Ca^{2+} (or CaCl_2) as bridging agent

CFD – Computational fluid dynamics

Cho – Cholesterol

COC – Cyclo-olefin copolymer

D – Diffusivity of the fluid

d_h – Hydraulic diameter

DL – Drug loading

DLS – Dynamic light scattering

DMEM – Dulbecco’s modified Eagle’s medium

DMPS – Dimyristoyl phosphatidylserine

DMSO – Dimethyl sulfoxide

DNA – Deoxyribose nucleic acid

DOPS – Dioleoyl phosphatidylserine

EC – Empty cochleates prepared without any encapsulated drug

EDS – Energy-dispersive X-ray spectroscopy

EDTA – Ethylenediaminetetraacetic acid sodium salt

EE – Encapsulation efficiency

FBS – Foetal bovine serum

FDA – Food and drug administration

FRR – Flow rate ratio

FTIR/IR – Fourier transformed infrared spectroscopy

HBSS – Hanks’ balanced salt solution

HFF – Hydrodynamic flow focusing

HPLC – High performance liquid chromatography

i.d. – Inner diameter

k_0 – Zero-order rate constant

k_1 – First-order rate constant

k_{deg} – Degradation rate constant

k_H – Higuchi rate constant

k_{KP} – Korsmeyer–Peppas rate constant

λ – X-ray wavelength

LC – Liquid crystal(s)

μ – Dynamic viscosity
 NTM – Non-tuberculous mycobacteria
 o.d. – Outer diameter
 P_{app} – Apparent permeability
 PBS – Phosphate buffered saline
 PC – Phosphatidylcholine
 PDI – Poly dispersity index
 PDMS – Polydimethylsiloxane
 PEEK – Polyether ether ketone
 PEG – Polyethylene glycol
 PS – Phosphatidylserine
 PTFE – Polytetrafluoroethylene
 q – Scattering vector
 Q_b – Buffer volumetric flow rate
 Q_m – Methanol volumetric flow rate
 Q_t – Total volumetric flow rate
 Re – Reynold's number
 ρ – Fluid density
 SAXS – Small angle X-ray scattering
 SEL-Cochleates – Artemisinin-loaded cochleates selected for alginate coating
 SEM/FE-SEM – Scanning electron microscopy (Field emission SEM)
 SGF – Simulated gastric fluid
 SIF – Simulated intestinal fluid
 SUV(s) – Small unilamellar vesicle(s)
 $t_{1/2}$ – Half-time

T_m – Melting/phase transition temperature

t_{res} – Residence time

θ – Scattering angle

τ_{mix} – Mixing time

TEER – Transepithelial electrical resistance

TEM – Transmission electron microscopy

USP – United States pharmacopoeia

v_f – Average flow velocity

w – Channel width or diameter

w_f – Width of the focused stream

WHO – World health organisation

CHAPTER – 1

BACKGROUND AND OBJECTIVES

1.1 Cochleate drug delivery system

The drug discovery and development programmes in the recent years have been driven towards finding prospective drugs with superior potency, specificity, reduced physicochemical liability, reasonable deliverability, and higher bioavailability. Despite the use of technologies such as high-throughput activity screening and computational modelling, a majority of about 70% of the pipeline drug candidates prove to be highly lipophilic with low solubility and low permeability.¹⁻³ These drugs often present several challenges such as poor bioavailability, variable pharmacokinetics, and drug precipitation.¹ This not only limits the possibility of effective oral administration but also increases the risk of patient non-compliance.¹ Hence, the development of new drug delivery systems that offer benefits such as sustained release and targeted delivery of drugs with minimal toxic effects is necessary to tackle these issues. Moreover, oral delivery, among various routes is considered as the most convenient and simplest method for administration of drugs.^{4,5} Many drug delivery systems have emerged over the years with strategies to attain efficient oral absorption of these drugs by increasing their dissolution rate and/or apparent solubility.¹ In view of this, a more recent and relatively successful strategy is to use micro- and nano-scale colloidal carriers as drug delivery systems.³

Micro- and nano-scale colloidal systems comprised of amphiphile assemblies with applications in the fields of drug delivery, tissue engineering, regenerative medicine, and biosensing have drawn great interest from researchers.⁶⁻⁹ For these nanoscale drug delivery systems to be successful, they should enhance the solubility of the drug, provide a means of interaction with cells to maximize drug uptake and minimize drug elimination or degradation before reaching its target site. Additionally, these systems should be biocompatible i.e., readily degrade in the body while producing less- or non-toxic metabolites.¹⁰ In this regard, natural amphiphiles like lipids have gained tremendous attention as a material of choice for the development of improved drug delivery vehicles in the recent past.^{1,11} For example, several formulations based on bilayer assemblies of phospholipids (such as liposomes) have been approved for clinical use and many are under development.^{3,12,13} In fact, liposomes are one of the first nanoparticle drug delivery systems to have seen successful clinical translation.¹⁴ However, the use of liposomal formulations for oral administration of drugs is often limited by their intrinsic properties like poor mechanical stability, short half-life, low entrapment efficiency, leakage, fusion, and phospholipid oxidation or hydrolysis.¹⁴ Owing to this, another lipid bilayer-based supramolecular system known as cochleates has recently gained increasing momentum as a potential drug delivery vehicle.^{15,16}

1.1.1 Definition and discovery

Cochleates are supramolecular system of solid particulates comprised of large and continuous bilayer sheets of charged amphiphilic molecules such as phospholipids, rolled-up to form scroll-like cylinders with little or no aqueous phase incorporated within the core.¹⁷⁻¹⁹ These cylindrical structures were first

reported by Papahadjopoulos and co-workers as ‘cochleates’, a Greek term for ‘spiral shell of snails’ corresponding to their rolled-up structure.¹⁷ Figure 1.1 shows a schematic and a representative scanning electron micrograph of cochleate particles. Due to their unique multilamellar structure, biocompatibility, and non-toxicity, cochleates are studied as delivery vehicle for a wide range of drugs including antifungals, anti-cancer agents, and fragile molecules including proteins, peptides, DNA, and vaccines.^{15,16,20} In addition, several other applications of cochleate-like assemblies as photocatalysts and optical metamaterials were also reported recently.^{21–23} Further, they exhibit high stability in solutions preserved for long periods and are known to retain their structural integrity when lyophilized and stored in powder form, enabling convenient storage and handling.¹⁶ These unique attributes provide a real opportunity for cochleates to be explored as an advanced drug delivery vehicle.

Figure 1.1 (A) Schematic (reproduced from Pawar et al.,¹⁶ with the permission of the Royal Society of Chemistry) and (B) A representative scanning electron micrograph of cochleate particles (Scale 1 μm)

1.1.2 Composition and chemistry

Cochleates are supramolecular structures generally made up of three major components: negatively charged lipid bilayers, cationic bridging molecules, and the drugs to be encapsulated or delivered (Table 1.1). The commonly used negatively charged phospholipids to obtain cochleates are phosphatidylserine (PS), phosphatidylcholine (PC), and/or a mixture of one or more of these lipids with other lipids.²⁴ The rolling-up of bilayer lipid sheets into cochleates is generally induced by the addition of divalent cations such as Ca^{2+} , Mg^{2+} , Ba^{2+} , Zn^{2+} or Fe^{2+} .¹⁶ Cations play a critical role in the formation of cochleates; they destabilize the negatively charged phospholipid bilayer, which in order to minimize the interaction with water, tend to roll-up, giving rise to cylindrical cochleates.^{18,19} Among the divalent cations, Ca^{2+} is known to form more tightly packed structures with less water of hydration at a lower concentration.²⁵ Additionally, Ca^{2+} plays a critical role in natural membrane fusion processes. In cochleates it is believed to help in facilitating the fusion of lipid bilayer edges with cell membranes, making it an important counterpart of the cochleate structure.^{16,26} Further, certain non-metallic, cationic peptides and drugs such as oligo-acyl-lysine, tobramycin, chitobiose etc., have been reported to interact with negatively charged lipids and act as inter-bilayer bridges instead of divalent cationic metal ions, leading to their encapsulation into inter-bilayer sheets of cochleates.^{26–28}

Table 1.1 List of common components required to form cochleates

Types of lipids	Bridging molecules	Types of drug molecules
Phosphatidylserine, phosphotidylcholine, phosphatidic acid, phosphatidylinositol, phosphotidyl glycerol, phosphotidylethanolamine, cholesterol	Ca^{2+} , Mg^{2+} , Zn^{2+} , Ba^{2+} , some cationic oligopeptides, cationic drug molecules	Peptides and proteins, polynucleotides, antiviral agents, anticancer agents, immunosuppressants, anti-inflammatory agents, tranquilizers, nutritional supplements

1.1.3 Mechanism of self-assembly of cochleates

Cochleates are formed by nucleation-dependent continuous aggregation and self-assembly process leading to the growth of rolled-up cylindrical particulate structures.^{18,19} Although the general mechanism of cochleate self-assembly was proposed a few decades ago, it is only recently that the studies to elucidate the underlying principles involved in their formation are being conducted. The principle that governs the process of cochleate formation includes fusion of bilayer vesicles followed by dehydration of lipid head-group and subsequent rearrangements.¹⁹ It is believed that in the presence of cationic bridging molecules, bilayer vesicles are ruptured and undergo fusion to form large planar bilayer stacks. These stacks further aggregate to form larger sheets which eventually coil to form cochleate cylinders.^{17,19,29} For the fusion to occur, a

barrier of hydration forces must be overcome and the interactions between phospholipid bilayers should be strong enough to allow the close contact of bilayer vesicles.^{25,30} This hydration barrier which commands a positive or negative work done to remove water is often determined by water–water, water–lipid, and lipid–lipid interactions and varies with the physical state of lipids such as hydrocarbon chains, polar group identity, polar group methylation, and mixing of different lipid classes.³¹ In the case of charged lipids (such as phosphatidylserine), strong cations such as Ca^{2+} are necessary to overcome the additional electrostatic repulsion and to induce the fusion of bilayers.²⁵ Upon addition of Ca^{2+} , close apposition of the lipid vesicles occurs resulting in the formation of anhydrous contacts between bilayers with crystallization of hydrocarbon chains followed by their precipitation.^{17,19,29} During the formation of anhydrous complex, vesicles fuse to form large sheets with highly ordered crystalline acyl chains with short lamellar spacing, indicating direct apposition of the bilayers with no significant amount of free water in between.^{29,32} In addition, the formation of this complex is characterized by a large release of heat (5.5 kcal/mol).³² The close apposition of membranes allows the Ca^{2+} ions to interact with two lipid head groups from the apposed bilayers, subsequently promoting spiral formation. This results in the formation of lipid cochleate cylinders when a lipid to Ca^{2+} mole ratio of 2:1 is used.^{17,29}

Recently, a step-by-step self-assembly of phospholipid cochleates (bridged by Ca^{2+}) by trapping and imaging the intermediate structures and morphologies during their formation at reduced temperatures was proposed (Figure 1.2).¹⁹ Studies conducted using lipids with different phase transition temperatures (T_m) revealed that the formation of critical nuclei can be decelerated by reducing the

process temperature below T_m .¹⁹ It was also reported that high- T_m lipids are ideal for studying the early intermediate structures as they can be trapped easily by prolonging the morphological transformation more efficiently.¹⁹ The presence of several early intermediate structures such as liposome aggregates, disc micelles or ribbons were reported. Further, some higher order intermediate structures like stacks and sheets were also reported using low- T_m lipids.¹⁹ Most recently, peptide amphiphiles and other amphiphilic molecules were also reported to show cochleate-like self-assembly.²² It was suggested that the transition from planar nanoribbon to cochleate structures occurred due to the electrostatic interactions facilitated by intrinsic curvature of the charged bilayers.²² In addition, theoretical basis for the formation of these cochleates along with mathematical models to determine their structure and dimensions were also proposed recently.^{22,30} However, there exists a critical gap in research to correlate the effects of these underlying mechanisms of cochleate formation and their resulting functional properties.

Figure 1.2 Schematic of possible mechanism of cochleate self-assembly
 (Reproduced from Nagarsekar et al.,¹⁹ with the permission of the Royal
 Society of Chemistry)

1.1.4 Methods of preparation and recent advances

Over the past years, several methods have been developed to prepare cochleates. Cochleates essentially are stacked phospholipid bilayers formed by the fusion of small unilamellar vesicles (SUVs) upon the addition of positively charged bridging molecules (like Ca^{2+}). Hence, a general method to prepare cochleates would be to first obtain uniform liposomes and then expose them to divalent cations to form cochleates. It is only by the beginning of year 2000 that research was focused on developing strategies to prepare cochleates with suitable structures and sizes for specific applications.^{33,34} The conventional strategies for preparation of cochleates include trapping and dialysis methods. These methods are known to form cochleate structures of large and varied particle sizes, mainly due to the continuous uncontrolled aggregation of lipid sheets.¹⁶ Further, the development of methods such as hydrogel isolation, emulsion-lyophilization, and microfluidics-based micro-spherical cochleate composites have opened application-based research approach to obtain cochleates in the nano- and sub-micrometre size ranges.^{35–37}

Trapping method utilizes either high pH for the formation of liposomes with simultaneous addition of divalent cations, called high pH trapping or addition of water to dried phospholipid film followed by sonication to form liposomes and then treating the suspension with divalent cations, called trapping film method.²⁴ The trapping film method is the most straightforward and most commonly used method for cochleate preparation. A schematic of this method is shown in Figure 1.3. Dialysis can be incorporated in two ways to prepare cochleates namely, direct cochleate dialysis and liposome before cochleate dialysis methods.³⁴ In the former method, a mixture of lipids with detergent is directly dialyzed against

a solution of divalent cations. This does not involve liposome formation, but the lipid bilayers are condensed by cations, resulting in large cochleate structures. Whereas the latter method is a two-step process involving removal of detergents by dialysis to first form liposomes and these liposomes are further dialyzed against a solution of divalent cations to form cochleates. The cochleates formed by this method are usually smaller compared to direct cochleate dialysis method.³⁴

Figure 1.3 Schematics of the two common preparation methods (A) trapping film method and (B) hydrogel isolation method

Further, the methods such as binary aqueous-aqueous emulsion, hydrogel isolation, and emulsification-lyophilization have enabled the formation of more uniform nano-sized cochleates by isolating and restricting the number of building blocks that fuse to form them.^{35,36} These methods use different

immiscible polymers in two-phase systems. The emulsion and hydrogel isolation process require uniform small liposomes to be suspended in a polymer solution A and addition of polymer A-liposome suspension into second polymer solution B, giving rise to a two-phase system followed by the addition of divalent cations to form nano-sized cochleate particles (Figure 1.3). The polymers are then separated from the formed nanocochleates by washing with buffer.³⁵ Emulsification-lyophilization method requires the formation of W/O/W double emulsion system with lipids dissolved in a solvent/oil phase followed by lyophilization. The lyophilized system is rehydrated with water to obtain nanocochleates.³⁶

Most recently, a technique of nanoprecipitation using a microfluidics device for preparation of micro-spherical particles made up of nanocochleate subunits called as “micro-spherical cochleate composites” was reported.³⁷ This technique exploits the combined benefits of nanoprecipitation such as elimination of use of polymer emulsions and advantages of microfluidic devices such as precise control over concentration ratios, flow, and mixing rates. It also facilitates control of size and shape by avoiding random particle aggregation and helps in forming spherical cochleate composites.³⁷ Despite the advances, these existing methods used for the preparation of cochleates, suffer from certain practical drawbacks which are discussed in further detail under Section 1.7 of this thesis. In addition, the need for the development of an inexpensive, robust, and reproducible method is also discussed.

1.1.5 In-vitro release and mechanism of drug delivery

Cochleates are capable of controlled and sustained release due to their multilamellar structure. Techniques like dialysis and ultrafiltration have been previously used for the determination of in-vitro release profiles from cochleates.³⁸ Release of drugs from cochleates depends on several aspects related to the multiple bi-layered structure of these particles. However, the actual mechanism of drug release from cochleates is not completely known and more studies need to be conducted. Cochleates show extreme mechanical resilience due to their tightly packed structure.³⁹ This helps them to provide unparalleled protection to the encapsulated drug molecules and deliver to the site of action. Since the drug molecules are entrapped between the repeating lipid bilayers without binding chemically, they remain protected from the harsh acidic environments and digestive enzymes in the gastrointestinal tract when administered orally.^{15,38} It has been suggested that the membrane fusion capability of cochleates is responsible for their enhanced activity.¹⁶ While calcium present in cochleates promotes membrane-cochleate interaction, the high tension at the bilayer edges of cochleates acts as the driving force for their fusion with cell membranes.^{20,26} As a result, fusion of internal bilayers takes place leading to the encapsulated material to be released into the cytoplasm of the cell. Hence, prolonging these interactions of cochleates with cells using muco-adhesive materials may be expected to facilitate an increased drug absorption.⁴⁰ In some cases, uptake of cochleates is believed to occur through phagocytic processes for cell internalization where they are taken up by endocytosis in active phagocytic cells and release the contents gradually in cell cytoplasm.¹⁶ When cochleates are inside the cell, the lower cytosolic calcium

concentration results in the opening of the cochleate structure, in turn releasing the encapsulated drug.²⁰ Further, when administered orally, absorption of cochleates takes place from intestinal epithelium where the drug molecule is delivered into the blood stream by transcytosis.^{20,26}

1.2 Techniques used for the characterization of cochleates

Analysis of cochleate structure and morphology have often been carried out by simple visualization. Several earlier studies have reported cochleate structures using optical microscopy, usually with the phase contrast technique.^{27,33} However, the newer preparative methods used for cochleates often produce particles in nano-scale size range for which high resolution techniques are necessary. Electron microscopy techniques such as scanning electron microscopy (SEM) and transmission electron microscopy (TEM) have been most widely incorporated for visualizing the structural features of cochleates.³⁰ Although cochleates are stable, the dryness of the sample required for these techniques may result in changes in the delicate structural features of cochleates. Freeze-fracture is another technique used for imaging cochleates which enables the visualization of the internal structural features of cochleate particles.^{15,24} Recently, advanced techniques such as cryo-SEM and cryo-TEM have shown to be the most suited techniques to visualize these particles as they preserve their structure under their native hydrated conditions.^{19,25} These advanced techniques have also helped in uncovering the formation mechanism of cochleates by capturing intermediate structures.¹⁹ Further, atomic force microscopy (AFM) has been used to characterize not only the hidden topological structural features of

cocleates, but also to study their mechanical properties which has helped to demonstrate the resilient nature of cocleate particles.^{39,41}

Particle size is one of the important parameters for nanomaterials to be used as drug delivery vehicles.⁴² Generally used techniques for the determination of particle size and dispersity of cocleates are photon correlation spectroscopy (PCS), dynamic light scattering (DLS), and electron microscopy.¹⁶ Techniques like PCS and/or DLS are rather suitable for cocleate particles in nano-scale size range and are considered as direct quantitative methods. However, due to their complex cylindrical shape, cocleates cannot be adequately characterized directly with the help of these techniques.³⁰ Hence, images of cocleates acquired by electron microscopy (both SEM and TEM) have been utilized for particle size determination, which involves measurement of individual particles for size and size distribution.³⁰ Size of cocleate particles was also measured using fluorescence microscopy by labelling liposomes with fluorophores and measuring the major axes of cocleate particles.¹⁶ Surface charge is another important parameter of nanoparticles that determines their behaviour in the biological environment as well as their interaction with drugs. Surface charge of cocleates measured as zeta potential is generally determined by laser doppler electrophoretic mobility measurements which is a fast and high-resolution technique.¹⁶

Earlier studies on cocleates have used Laurdan spectroscopy using fluorescent probe to study the membrane dynamics of the lipids in liposome and cocleate phases.⁴³ Fourier transform infrared spectroscopy (FTIR) was reported as an effective technique to investigate the conformational changes, membrane dynamics, physical and chemical interactions between lipid molecules, cations,

and drugs both in vesicles and cochleates.^{41,44} Additionally, differential scanning calorimetry (DSC) has been used to observe crystallization events during complexation of lipid and binding agent in cochleate samples using the change in transition temperatures of free lipid and cochleate lipid phase.⁴⁵ In some cases, DSC has also been used to record transitions in heat capacity to monitor the effect of incorporation of drugs in cochleate systems.^{45,46} Further, techniques like small angle X-ray scattering (SAXS) have also been used to study the lamellar architecture of cochleate particles.³⁴ SAXS technique is often complemented with FTIR and/or DSC data to correlate the structural transformation of lipids in different phases during cochleation process. Several parameters estimated by SAXS like bilayer repeat distance, bilayer thickness, and inter-bilayer space have helped better understand the formation and self-assembly of these supramolecular structures.¹⁹ SAXS has also been used to differentiate between several intermediate structures observed during the formation of cochleates.¹⁹

1.3 Existing applications of cochleates

1.3.1 Applications in drug encapsulation and delivery

Cochleates, due to their nature are preferably used for hydrophobic and amphipathic drugs as they can be easily inserted into the membrane bilayers.¹⁵ However, several hydrophilic drug molecules such as nucleotides, peptides and aminoglycosides can also be embedded into cochleates using their pH-induced charge-based properties. A schematic of encapsulation and/or localization of drug molecules based on their properties within the cochleate bilayers is shown in Figure 1.4. Cochleates are considered as well suited for oral administration due to their rigid and tightly packed multilamellar structure.^{15,24} They are also

non-toxic and show high stability when stored at room temperature both in solution and as lyophilized powder. In addition, they are known to enhance the solubility and intestinal permeability of encapsulated drugs.^{15,16,47} These unique properties render cochleates as a potential platform suitable for the delivery of drugs with poor/low bioavailability.

Figure 1.4 Drug–lipid bilayer architecture in cochleates as a function of nature of the drug (A) hydrophobic (B) amphipathic (C) hydrophilic cationic and (D) hydrophilic anionic drug

Over the last few years, substantial research dedicated towards the development of lipid-based cochleates as potential drug delivery system has been done. They have been investigated as safe and effective delivery vehicles for wide range of therapeutic agents such as antifungals, anti-cancer agents, and fragile molecules including proteins, peptides, DNA, and vaccines.^{15,16,20} Table 1.2 shows selected examples of cochleate system used for the encapsulation and delivery of certain drugs. In addition, cochleates have also gained considerable interest from industries owing to their unique advantages.

Table 1.2 Selected examples of cochleate systems used for delivery of drugs

Sl. No.	Drugs	Lipids/Composition	EE (%)	DL (mg/g)	Yield (%)	Application	Reference
1	Thymol, Carvacrol	PC + Cholesterol	46.3, 50.9	-	87	Preservation of antioxidant activity of thyme essential oils	48
2	Raloxifene, Raloxifene + dimethyl- β - cyclodextrin, Raloxifene + sodium taurocholate	DOPS	43.4, 36.2, 39.4	-	-	Breast cancer therapy	49
3	Insulin	Soy PS + Ferrite NPs	90	-	-	Oral delivery of insulin	45
4	Cyclosporine A	DOPS	81.4	-	-	Enhanced gastrointestinal absorption of cyclosporine A	28

5	Amphotericin B, Miltefosine	DOPS + Cholesterol	54, 59	29.9, 14.6	75	Oral formulation for treatment of visceral leishmaniasis	38
6	Monophosphoryl lipid A, BSA	Soy PS	38.2	-	-	Oral mucosal delivery of vaccine- adjuvants	50
7	Lipopolysaccharide, Amphotericin B	Soy PS	98- 99.6 for AmB	40, 0.5 $\mu\text{g/ml}$	-	Formulation of immunomodulatory antifungal delivery vehicle for treatment of sporotrichosis	51
8	Recombinant factor VIII	Brain PS	75	-	-	Intravenous delivery factor VIII for haemophilia A therapy	52
9	Fisetin	DMPC + Cholesterol	84.3	-	-	Improved bioavailability and in vitro anticancer activity towards human breast cancer cells	53
10	Doxorubicin	DMPC + Cholesterol	73.8	-	-	Biofunctionalized chitosan decorated nanocochleates for cancer targeted drug delivery	54

A cochleate-based formulation for oral administration of antifungal drug, amphotericin B to treat invasive fungal infections in patients on immunosuppressive therapy is currently reported to be under phase-2 clinical trials.⁵⁵ This encochleated amphotericin B was reported to be safe at all doses studied, with no observed renal or hepatic toxicity.⁵⁵ Another oral cochleate formulation that is being developed for gram-negative antibiotic, amikacin is currently under phase-1 clinical trials and has shown less toxicity and high efficacy against non-tuberculosis mycobacterial lung disease.⁵⁶ Also reported are preclinical animal studies as a proof of concept for the potential efficacy of orally administered amikacin cochleates for the treatment of multi-drug resistant gram-negative bacterial infections.⁵⁶ In addition, the efficacy of cochleates as topical drug delivery vehicles for broad spectrum antifungal drug ketoconazole was studied and cochleates were reported to be more stable than liposomes, with dermal and transdermal release capabilities.⁴⁶ Further, cochleates have been used for the encapsulation of bioactive natural compounds such as essential oils for applications in food industry such as preservation, flavouring, etc., due to their stable nature.⁴⁸ This has opened new avenues for cochleates in food and nutraceutical industries where it could be used to encapsulate and deliver extended range of fragile micronutrients.

1.3.2 Applications beyond drug delivery

Due to their unique high aspect ratio (10:1 or greater) bilayer structural feature, cochleates and cochleate-like assemblies have gained attention from several other areas beyond their applications in drug delivery. In a collaborative study, we recently reported the use of lipid cochleates as a tuneable photonic metamaterial (Figure 1.5).²³

Figure 1.5 a) Images obtained from the switchable cell made with 12 wt.% cochleate-BL036 LC composite in the absence of any polarizers, scattering state obtained with 0 V, translucent states at 30 V, and the transparent state at 90 V. b) and c) Schematic of LC surrounding a cochleate particle at zero voltage and when the voltage is turned on, respectively. d) Schematic showing the refractive indices seen by the light propagating perpendicular to the substrate (along Z) at the different sides of cochleate particle. Reproduced from Madhuri et al.²³ (2020) with permission.

A small concentration of cochleate cylinders were embedded into liquid crystals (LC) to form a new switchable composite capable of controlling light scattering by varying voltage and temperature. A switching device as thin as 6 μm , constructed with 12% (w/w) cochleates concentration, was adequate to reveal a switchable privacy window. This study shows cochleates as high potential

candidates not only for drug delivery but also as appropriate building blocks for the development of tuneable metamaterials with fascinating optical and electrical properties for applications in the field of liquid crystal-based photonic devices.²³

Several other studies have shown cochleate-like self-assembly in synthetic peptide amphiphiles and other amphiphilic molecules.²² Kazantsev et al., revealed the solution's ionic strength dependent morphological transformation of charged amphiphilic chromophores from ribbons to cochleates.²¹ These scroll-like supramolecular cochleate scaffolds were further shown to serve as efficient charge-transfer agents for photocatalysts in hydrogen production.⁵⁷

1.4 Research gaps and rationale behind the dissertation

Despite the advances in cochleates, certain aspects of this system such as, understanding of their self-assembly/formation, functional designing, surface modification/functionalisation, and new methods of production are seldom addressed. These aspects are also vital for the successful development of cochleate-based drug formulations. Herein, the existing gaps in research w.r.t, the said aspects of cochleates and rationales behind the different studies conducted in this thesis are discussed.

1.4.1 Significance of amikacin and its use in forming cochleates

Amikacin (Figure 1.6 A) is a class III, semi-synthetic aminoglycoside drug used as a wide spectrum antibiotic. It is an important antibiotic due to its effectiveness against several aminoglycoside modifying enzymes produced by multidrug-resistant gram-negative bacteria.^{58,59} It is often used to treat patients with

extensive or disseminated pulmonary non-tuberculous mycobacterial (NTM) disease. NTM lung disease is a serious infection caused by a group of widespread environmental bacteria particularly in individuals with impaired immunity.⁶⁰ Infections caused by NTM can affect a range of sites throughout the body but are usually manifested as chronic, progressive, and potentially life-threatening lung disease.^{61,62} However, the risk of its occurrence is limited to individuals with underlying lung conditions such as bronchiectasis, chronic obstructive pulmonary disease, asthma, cystic fibrosis, or prior tuberculosis. Clinical symptoms of NTM lung disease are like other lung conditions which include chronic cough, sputum production, fatigue, and weight loss and hence may remain undetected for months or sometimes years.⁶³ The treatment of NTM lung disease requires a multidrug antibiotic regimen over a prolonged period of time to achieve eradication of bacteria, which is challenging. Further, some treatment guidelines advise courses of i.v. aminoglycoside (specifically amikacin) for patients with advanced or relapsed disease conditions.⁶¹

Owing to its short half-life (~120 min) and poor intestinal absorption, amikacin formulations available in clinics are limited to intravenous and intramuscular administration.⁶⁴ Additionally, the high risk of nephrotoxicity and ototoxicity related adverse effects due to progressive dosing of amikacin limits their use in hospitalized patients alone.^{59,63} However, the encapsulation of amikacin in nano-carriers were reported to show reduced toxicity and increased intestinal absorption.^{59,60,64} Table 1.3 shows some examples of amikacin formulations based on nano-scale colloidal drug carriers. Despite the advances, none of the non-parenteral formulations are currently being used in the clinics. This has led to an extensive ongoing research for the development of oral amikacin

formulations both in academia and in industries alike.^{60,64–66} One such cochleate-based oral formulation of amikacin which has shown low toxicity and high efficacy against NTM lung disease is currently under phase-1 clinical trials.⁵⁶ In addition, proof of concept preclinical animal studies for the efficacy of orally administered amikacin cochleates for the treatment of multi-drug resistant gram-negative bacterial infections are also currently underway.⁵⁶

Figure 1.6 Chemical structures of (A) Amikacin sulphate and (B) Artemisinin

In addition to its clinical importance, Amikacin is a multivalent cationic drug molecule in the salt form (Amikacin sulphate Figure 1.6 A) which makes it a suitable candidate for use in preparing cochleates. Although the formation of lipid cochleates by multivalent cationic drug/organic molecules without the use of any metallic cations as bridging agents was previously described, the mechanistic understanding of the effects of these non-conventional bridging agents on the formation, structural features, and functional properties of cochleates have remained largely unexplored. Hence in this dissertation, one of the studies uses amikacin as both an active ingredient and a non-metallic bridging agent to prepare cochleates and to study the mechanism of formation and its implications on the resulting functional properties of cochleates.

1.4.2 Artemisinin and the need for its encapsulation in cochleates

Malaria is a life-threatening infectious disease prevalent in tropical and sub-tropical countries caused by parasitic protozoans belonging to the *Plasmodium* type. Amongst the different species of these parasites, *Plasmodium falciparum* is known to be the cause of most severe malaria infections and highest number of deaths.⁶⁷ There are only a limited number of clinically effective antimalarial agents existing in practice.⁶⁷ Artemisinin (ART) is currently one of the most effective drugs used for the treatment of malaria caused by multidrug resistant *Plasmodium* species. It is the World Health Organization (WHO) recommended norm for treatment of falciparum malaria in the form of artemisinin combination therapy (ACT) with companion drugs like lumefantrine, mefloquine, amodiaquine, sulfadoxine/pyrimethamine, piperaquine and chlorproguanil/dapsone.⁶⁸ ART is a sesquiterpene lactone containing endoperoxide bridge, derived from *Artemisia annua* (Figure 1.6 B).⁶⁹ Several semisynthetic derivatives based on ART have also been developed over years such as artemether, artesunate, and dihydroartemisinin, etc.⁷⁰ In addition to their anti-malarial activity, ART and its derivatives have shown effectiveness against several other parasitic diseases. They also exhibit antitumor and anticancer activities against multiple cell lines derived from various types of cancer.⁷⁰ ART acts by heme-mediated decomposition of the endoperoxide group upon reacting with iron, which produces carbon-centered free radicals.⁷¹ When formed inside a malaria parasite, these radicals cause cellular damage and cell death. In a similar way, it has been found that artemisinin is able to affect cancer cells due to their elevated iron concentration.⁷² Despite the effectiveness of ART and its derivatives, its usage is limited in the treatment of malaria due to the imbalance

between demand and supply, comparatively high cost of treatment, dosing complexity, and the lack of clinical experience.^{73,74} Besides, ART suffers from technological and biopharmaceutical issues such as its poor bioavailability due to low solubility in water (48 µg/mL at 37 °C) and oils, short half-life (~2.5 h), and risk of toxicity leading to low patient compliance.⁷⁵ In addition, it is not very stable and easily decomposes, most probably by the opening of the lactone ring.^{70,75} Commercially, ART is available as oral, rectal, and intramuscular (i.m.) dosage forms. Intramuscular injections are associated with low patient compliance and are not suitable for conditions where rapid suppression of parasites is required.⁷² Whereas, rectal 'relative' bioavailability of ART is poor and the inter-individual variability is higher after rectal administration.⁷⁴ Oral delivery is the most convenient route of drug administration with high patient compliance.^{4,5} Several formulation strategies to enable oral administration of ART and its derivatives have been reported. Table 1.4 shows a list of selected ART delivery approaches employed for different applications. However, these systems are far behind in meeting the requirements of a successful drug delivery system.⁷⁶ In addition, ART has an excellent permeability across intestinal membranes. Hence, it shows high peak plasma concentration after an initial burst effect when there is improvement in its solubility. But it metabolizes too quickly in vivo. This combined with the short half-life and rapid first pass metabolism may result in an incomplete clearance of malaria parasites, increasing the risk of drug resistance.⁷⁶ Therefore, to solve these issues, better delivery systems suited for oral administration of ART with controlled release properties such as cochleates are of high interest.

Table 1.3 Selected examples of nano-scale delivery systems used for amikacin

Sl. No.	Vehicle/composition	EE (%)	DL (mg/g)	Application	Reference
1	Liposomes, DPPC + Cholesterol	88	-	Enhance penetration of amikacin into NTM biofilms, efficacy against intra- and extra cellular NTM infections	58,77–79
2	Poly-lactic-co-glycolic acid (PLGA) nanoparticles	-	40	Oral permeability enhancement to facilitate reduced dosage and dosing frequency thereby, mitigating adverse effects	64,80
3	PLGA nanoparticles	76	26	Antibacterial and antibiofilm activity against <i>Pseudomonas aeruginosa</i>	59,81
4	Eudragit® RS 100/RL 100 polymeric nanoparticles	80	-	Ocular delivery of amikacin targeted against <i>Staphylococcus aureus</i>	82
5	Synthetic mesoporous silica and aluminosilicate supports	36	-	Amikacin encapsulation by adsorption and in-vitro release for oral delivery	83

	including MCM-41 and					
	AlMCM-41					
6	Cholesterol solid lipid nanoparticles (SLNs)	89	-	biodistribution study of amikacin SLNs after pulmonary delivery for the treatment of cystic fibrosis lung infections		84

Table 1.4 Selected examples of artemisinin-based drug delivery systems used for different applications

Sl. No.	Vehicle/composition	Drug	EE (%)	DL (mg/g)	Application	Reference
Liposomes						
1	PC (Egg) + Cholesterol	Dihydroartemisinin	71	-	Anticancer activity towards MCF-7 breast cancer cells	85
2	Soy PC + Cholesterol + Vitamin E	Artemether	76	-	Cancer therapy by sonodynamic activation of artemether liposomes	86

3	Soy PC + Cholesterol, DPPC + DSPC + Cholesterol	Artemisinin	89, 83	-	Anticancer targeting with transferrin and magnetic NP	87
4	PC (Egg) + Cholesterol, PEG + PC + Cholesterol	Artemisinin	78, 70	-	In vivo pharmacokinetics for antimalarial activity of artemisinin	88,89
5	PC (Egg) + Cholesterol + TPGS1000-MAN + DQA-PEG2000-DSPE	Paclitaxel + Artemether	80	-	Anticancer activity against brain glioma	90
6	Soy PC + Cholesterol	Artemether + lumefantrine	66	1.2	Parenteral delivery as ACT for malaria	91
Ethosome						
7	Soy PC	Artesunate	71	-	Antimalarial activity	92
Lipid nanocapsule						
8	Labrafil + PC + Chitosan	Artesunate	88-93	17	Anticancer activity against breast cancer	93
Protein based NPs						
9	Human Serum Albumin	Artemisinin	97	90	Antimalarial activity	74

Polymer based NPs						
10	Chitosan + Magnetite NPs	Artemisinin	55-62	200-250	Anticancer against breast cancer	94
11	Transferrin-8arm-PEG-NPs	Dihydroartemisinin	-	150	Anticancer activity	95
12	PU	Arteether	92	-	Anticancer activity	96
13	Core-shell/PLGA-DPPC	Dihydroartemisinin + Doxorubixin	-	190	Anticancer activity	97
14	PLGA + Tween 80, PLGA + SLS, PLGA + PVA	Artesunate	77, 60, 41	230, 190, 140	Anticancer activity	93
Electro-spun fibres						
15	PVP + HB	Artemisinin	78	200	Antimalarial and anticancer activities	72
Inclusion complexation						
16	β - and γ -cyclodextrin	Artemisinin	-	200, 179	Improved oral bioavailability for treatment of malaria	98

1.4.3 Requirement for alginate coating of cochleates

Achieving controlled drug release from nanoparticles with high surface area such as cochleates is desirable and challenging at the same time.⁹⁹ Cochleates are considered as well suited for oral administration of drugs with poor bioavailability. Although cochleates are highly stable in aqueous solutions due to their tightly packed multilamellar structure, they are reported to undergo dissociation at an increased rate in the low pH gastric conditions.³⁸ This might result in the rapid release of the encapsulated drugs, leading to a shorter gastrointestinal transit time, a major limitation faced in the oral delivery of drugs with poor bioavailability.¹⁰⁰ In this regard, modification of the nanoparticle surface by means of coating with functional agents (such as polymers) is an attractive option not only to achieve effective controlled release but also to enable possibilities like active targeting from systems like cochleates.^{54,101}

Alginate is a natural anionic intracellular biopolymer found in brown algae. It is highly biocompatible, has low toxicity, relatively low cost, and shows gelation properties on addition of divalent cations (such as Ca^{2+}) (Figure 1.7). Alginate has been extensively investigated and used in many biomedical applications.¹⁰² In addition, alginate gels tend to shrink at acidic pH, swell at neutral pH, and most importantly, show great potential towards controlled-release of drugs, especially in gastro-intestinal milieu making it suitable for oral administration.¹⁰² They also show mucoadhesive properties.^{103,104} In several studies, alginate has been successfully used to coat or entrap drug containing nanoparticles to achieve colon targeted delivery following oral administration.^{105,106} Further, the interaction of alginate with Ca^{2+} was described by the 'egg-box' model where the electronegative cavities on alginate are involved in both unidentate and

bidentate coordination modes leading to crosslinking.^{107–109} This presents alginate as a suitable material of choice to coat cochleate particles. Further, as divalent cations (such as Ca^{2+}) are an integral part of cochleates and are also exposed on their surfaces, alginate was selected to coat cochleates using a simple method.

Figure 1.7 Schematics of (A) composition and chemical structure of alginate, and (B) alginate crosslinking in the presence of Ca^{2+}

1.4.4 Need for the preparation of cochleate using continuous flow-based process

The conventional methods for the preparation of cochleates (discussed in detail under Section 1.1.4 of the thesis) such as trapping and dialysis methods are known to form cochleate structures of large and varied particle sizes due to the uncontrolled continuous aggregation of lipid sheets.¹⁶ The large particle size and a wide particle size distribution limits their suitability for the pharmaceutical and drug delivery applications.^{110,111} Alternatively, methods such as binary aqueous-aqueous emulsion, hydrogel isolation, and emulsification-lyophilization have enabled the formation of more uniform cochleates in the nano- and sub-micrometre size by isolating and restricting the number of building blocks that fuse to form them.^{35,36} However, these methods suffer from several inherent problems. They are based on batch processes and involve formation of colloidal structures in multiple steps that often require handling of emulsions, dialysis, washing of polymers, etc. Such steps are complex, tedious, time-consuming, and are difficult to scale up. Inter-batch reproducibility is also a major issue. Most recently, nanoprecipitation using a microfluidic device was employed to prepare micro-spherical particles of nano-cochleates.³⁷ Despite several advantages, this method, however, works with very low lipid concentrations (0.1 mg/mL or 0.12 mM)³⁷ and gives low throughput due to limited volumetric flow rates imposed by the small channel dimensions. Hence, the development of novel preparation methods that support robust and reproducible preparation of drug-loaded cochleates across a range of production scales is necessary. In view of this, a simple cross-junction based hydrodynamic flow focusing (HFF) was chosen for the preparation of cochleates. HFF is a simple technique that provides several

advantages compared to both conventional nanomanufacturing and other microfluidic methods.^{112,113} It is an attractive method widely used to produce lipid nanoparticles (such as liposomes, and ethosomes) and has gained much attention recently.^{13,110} Selected examples of different approaches used for in-flow production of nano-scale drug delivery systems are shown in Table 1.5. However, HFF is seldom used to produce solid supramolecular systems such as lipid cochleates. In flow focusing, a central stream of alcohol containing lipids is sheathed between two adjacent streams of aqueous buffer. This forms a hydrodynamically focused narrow central stream in which mixing occurs by rapid diffusion of molecules across the width of the stream.^{112,114} Due to the micrometre-scale laminar flow regime, the fluids would flow as parallel layered streams after they converge at the hydrodynamic focusing region (the cross-junction), enabling precise control of mixing by varying the ratio of the volumetric flow rates of the two streams.^{114,115}

Further, we used an inexpensive ‘off-the-shelf’ device to achieve flow focusing which was previously reported by several studies for the production of simple emulsions and liposomes.^{13,116} The use of an ‘off-the-shelf’ device configuration instead of the commonly used polydimethylsiloxane (PDMS), cyclic olefin copolymer (COC), or glass 2D chips not only helps to reduce the fabrication cost of microfluidic chips but also, its ease of availability in the market as standard HPLC components and the simple device assembly makes it an appealing alternative.^{13,116} In addition, off-the-shelf devices can withstand high-pressure operations. This allows the use of high flow velocities and in turn achieve high throughputs. While there is an upper limit to the flow velocity based on the flow regimes that could be employed during flow focusing (i.e., transition from

laminar to turbulent flow), pressure limitations of the device practically restrict the use of higher velocity. The build-up of high inlet pressures due to an increasing flow velocity can cause a substantial deformation at the channel walls of devices fabricated with materials such as PDMS and such deformations could lead to leakage or perturbations in flow-focusing.^{113,117} Whereas, the polyether ether ketone (PEEK) device components and polytetrafluoroethylene (PTFE) microchannels used to fabricate off-the-shelf device make them suitable for such operations. Additionally, the simple construction, modular nature, inexpensive and readily available components used in the off-the-shelf device further promotes paralleling this device on a single microfluidic platform to achieve enhanced production rates. Hence, the off-the-shelf device could be a highly promising platform to enhance the production rate of drug-loaded cochleates and to achieve high throughputs with an excellent control over the particle properties.

Table 1.5 Selected examples of continuous flow-based devices used to produce nano- and micro-scale drug delivery systems

Sl. No.	Purpose	Device type	Schematic of the device*	Reference
1	Liposome production using controlled microfluidic mixing and nanoparticle determination	Etched silicon wafer sealed with borosilicate glass wafer. Hydrodynamic flow focusing. Cross-junction with (a) oblique side channels and (b) orthogonal side channels	<p>Adapted with permission from Jahn et al. Copyright 2010 American Chemical Society.</p>	112
2	Liposome production with remote drug loading and in-line purification	PDMS/cellulose hybrid microfluidic device with a channel for buffer counterflow, remote drug loading, and patterned nano porous cellulose dialysis membrane.	<p>Reproduced from Hood et al., with the permission of the Royal Society of Chemistry</p>	118

3 High-throughput
continuous production of
liposomes

(i) Vertical flow focusing using
COC device

113,117,119

Reproduced from Hood et al. (2015) with permission

(ii) Hydrodynamic flow focusing in
high aspect ratio PDMS device

Adapted from Michelon et al. Copyright (2017), with permission from
Elsevier

(iii) Chaotic advection-based

microfluidic device with two or
three inlets

(iii)

Adapted from Ismail et al. Copyright (2020), with permission from

Elsevier

- 4 Production of liposomes Off-the-shelf T-junction and cross-junction flow focusing device

Adapted from Bottaro et al. Copyright (2016), with permission from Elsevier

- 5 Niosomes of non-ionic surfactants Hydrodynamic flow focusing in PDMS-glass device

Adapted with permission from Lo et al. Copyright 2010 American Chemical Society.

6	Nanoparticles of block copolymers (i)	PDMS-glass slide chip.
	Poly(lactide-co-glycolide)-b-poly(ethylene glycol) (PLGA-b-PEG)	Hydrodynamic flow focusing with orthogonal cross-junction geometry.
	(ii) Poly(o-nitrobenzyl acrylate)-b-poly(dimethylacrylamide) (PNBA-b-PDMA)	
	(iii) Poly(ϵ -caprolactone)-block-poly(ethylene oxide) (PCL-b-PEO)	

114,121–123

Adapted with permission from Karnik et al. Copyright 2008 American Chemical Society

Adapted from Bains et al. Copyright (2016), with permission from Elsevier

7	Drug loaded PLGA nanoparticles	Valco Internal cross stainless-steel union bore with stainless steel capillary tubing

Adapted from Bramosanti et al. Copyright (2017), with permission from Elsevier

8	Alginate core-shell microparticles for protein encapsulation	Flow focusing using PDMS/glass device

Adapted from Yu et al. Copyright (2019), with permission from Elsevier

1.5 Objectives

Lipid cochleates have emerged as a promising scaffold for applications in drug encapsulation and delivery. Despite the advances, certain aspects of cochleate systems are seldom reported by most of the research studies. These include the areas such as mechanistic understanding, functional designing, surface modification/functionalisation, and practical preparatory methods that are vital for the successful development of cochleate-based drug formulations. Owing to the current shortcomings, this dissertation aims to develop several strategies to aid in the further advancement of cochleate systems.

Firstly, studies on the underlying principles involved in the cochleate self-assembly and their effects on the functional properties towards achieving a desired outcome was deemed necessary to enable the effective functional designing of cochleates. Secondly, to aid in the effective oral delivery of drugs with poor bioavailability, a study aimed at surface modification of cochleates by coating them with a polymer having functional characteristics especially in gastro-intestinal milieu, was considered. Finally, to support the demand of efficient and reproducible production, development of a novel and reliable continuous flow-based process for the preparation of drug-loaded cochleates across a range of production scales was also conceptualised. We believe that an elaborate study on these aspects is crucial not only because it provides important insights into the functional designing of cochleates but also aids in the smooth translation of this system into successful clinical use.

The specific objectives of this dissertation are as listed below:

1. To study the design, mechanism of formation, and functional properties of amikacin-bridged cochleates with and without the presence of Ca^{2+} in sub-threshold concentrations in comparison to conventional Ca^{2+} -bridged cochleates.
2. To develop a cochleate-based formulation with controlled-release characteristics for the effective oral administration of artemisinin, an anti-malarial drug with poor bioavailability.
3. To establish a robust, reproducible, continuous flow-based process for the high-throughput preparation of drug-loaded cochleates using a simple, cost effective off-the-shelf flow focusing device.

CHAPTER – 2

UNDERSTANDING THE MECHANISM OF FORMATION AND ITS IMPLICATIONS ON THE FUNCTIONAL PROPERTIES OF COCHLEATES

2.1 Synopsis

Cochleates are tightly packed, multiple bilayer assemblies of phospholipids, bridged by divalent cations. They have emerged as an important colloidal system with applications in drug delivery and other related areas. The consequences of design of lipid cochleates are crucial on their functional outcome. Additionally, the functional designing of cochleates to achieve a desired outcome like tuneable and/or controlled release, surface charge, surface functionality, targeted delivery, etc., has always been challenging due to their complex nature and requires in-depth knowledge of the mechanism of their formation. This study describes the design and mechanism of the formation of non-conventional, amikacin-bridged cochleates with and without the presence of sub-threshold concentration of Ca^{2+} in comparison to conventional Ca^{2+} -bridged cochleates. Further, the impact of these bridging agents on the structural features and other functional properties like drug release and stability of these cochleates was also investigated. The DOPS cochleates were successfully prepared by using Ca^{2+} , amikacin, and amikacin+sub-threshold Ca^{2+} separately, as bridging agents. Synergistic bridging of the DOPS headgroups occurred in the presence of amikacin+sub-threshold Ca^{2+} resulting in rigidly packed, multiple bilayers with

uniform lamellar repeat distance of 51 Å. The rigidity of the internal structure of the cochleates increased with increasing concentration of Ca^{2+} . As a consequence, an increase in amikacin encapsulation ($71.63 \pm 8.98\%$) and loading (266.70 ± 12.01 mg AMK/g cochleates) was obtained. Additionally, the in-vitro amikacin release rate decreased as the concentration of Ca^{2+} was increased reflecting the tightly packed structures and suggesting prospects for controlled release. Drug release kinetics studied using the Higuchi model suggested that tuneable amikacin release could be achieved by simply varying the Ca^{2+} concentrations in sub-threshold range. The use of higher Ca^{2+} concentration positively affected the long-term stability of the cochleates and showed increased half-lives during 12 weeks of storage. The proposed mechanistic model of the formation of non-conventional cochleates promotes the synergistic effect of the bridging agents. This study reveals the importance of the type and concentration of the bridging agent to achieve a specific functional outcome and has the benefit to guide future design of cochleates with specific functions in drug delivery and formulation.

2.2 Materials and Methods

2.2.1 Chemicals and materials

Lipid 1,2-dioleoyl-sn-glycero-3-phospho-L-serine [sodium salt] (DOPS) powder was purchased from Avanti Polar Lipids Inc. Amikacin sulphate, calcium chloride, phosphate buffered saline (PBS, pH 7.4) and Sanger reagent (1-fluoro-2,4-dinitrobenzene) were purchased from Sigma-Aldrich. All other solvents, chemicals and reagents used in the study were of analytical grade.

2.2.2 Preparation of cochleates

Both amikacin-bridged and Ca^{2+} -bridged cochleates were prepared by the trapping film method. Briefly, lipid (DOPS) was dissolved in chloroform in a round bottom flask. The solution was evaporated under vacuum (using Buchi R-210 Rotovap) to give a thin film. The flask was placed under vacuum for 1 h to remove traces of solvents. The dried lipid film was then hydrated with PBS (pH 7.0) to a final lipid concentration of 5 mg/mL (~ 6.2 mM). The resulting suspension was sonicated for 10 min followed by extrusion through polycarbonate membrane of 100 nm pore size using a mini extruder (Avanti Polar Lipids Inc.) to obtain uniform small unilamellar vesicles (SUVs). At this stage, amikacin (100 mM in PBS; pH 7.0) or CaCl_2 (100 mM in PBS; pH 7.0) stock solution was added slowly to the suspension of SUVs at a rate of 10 $\mu\text{L}/\text{min}$ using a syringe pump under vigorous stirring until final DOPS:AMK or DOPS: Ca^{2+} molar ratio of 10:5 (i.e., amikacin concentration of 3.1 mM) or 10:10 (i.e., CaCl_2 concentration of 6.2 mM), respectively, was reached. Stirring was continued for 3 h. The resulting cochleate suspensions were stored at 4 °C for immediate use, and for long-term use, they were stored as a freeze-dried powder at 4 °C. In this chapter, the amikacin-bridged cochleates are referred to as “AMK-Coch” and the control, Ca^{2+} -bridged cochleates are referred to as “Ca-Coch”. In addition, cochleates bridged with amikacin and varying concentrations of Ca^{2+} prepared similarly with DOPS:AMK: Ca^{2+} molar ratios of 10:5:1, 10:5:2.5 and 10:5:5 (i.e., CaCl_2 concentration of 0.6, 1.5 and 3.1 mM, respectively) are referred to as “AMK-Ca-Coch1”, “AMK-Ca-Coch2” and “AMK-Ca-Coch3”, respectively.

2.2.3 Analysis of structure and morphology of cochleates by SEM

A droplet of dialyzed suspension of cochleates in de-ionized water was placed on Si-wafer, excess water was removed, and the sample was vacuum dried at room temperature. Once fully dried, the sample was sputter-coated with platinum, using JFC-1600 Auto Fine Coater. Imaging was done using JEOL JSM 6390LA scanning electron microscope (SEM) and/or JEOL JSM 6700F field emission scanning electron microscope (FE-SEM). The cochleate structure was visualized using this method throughout this thesis.

2.2.4 Particle size and zeta potential measurement of SUVs and cochleates

Dynamic light scattering (DLS) by Malvern Zetasizer-Nano ZS was used to determine the mean particle size, polydispersity index (PDI) of the SUVs at 25 °C. Cochleates, unlike vesicles, have a complex cylindrical shape and hence cannot be adequately characterized by DLS. Therefore, we analysed the images of randomly selected particles (n>200) using image analysis software ImageJ v1.05i to estimate the average size of the cochleates (both width and length).^{30,126} Further, to minimize the error in measurement of particle size, analysis of sample-to-sample variability and measurement repeatability was performed by making three repeat measurements on each of the different sample w.r.t, width and length of cochleates. In addition, the dispersity in size was estimated by measuring the coefficient of variation as described elsewhere using Eq. (2.1),¹²⁷

$$Dispersity = \frac{\text{Standard deviation of particle size (nm)}}{\text{Mean of particle size (nm)}} \dots \text{(Eq. 2.1)}$$

Zeta potential of the SUVs and the cochleates were determined by laser doppler micro-electrophoresis using Malvern Zetasizer-Nano ZS at 25 °C. Throughout

this thesis, the particle size and zeta potential measurements were performed in a similar manner as described here.

2.2.5 Determination of amikacin encapsulation and its loading in the cochleates

The cochleate suspension was centrifuged at 6000×g for 10 min to separate the cochleates from the supernatant. The amount of amikacin remaining in the supernatant was determined by spectrophotometry (Thermo Evolution™ 300 UV-Vis Spectrophotometer) against pure amikacin as the external standard reference according to the procedure reported elsewhere using Sanger reagent (1-fluoro-2,4-dinitrobenzene).¹²⁸ Further, the pellets containing cochleates were resuspended in de-ionized water and freeze-dried under vacuum to obtain cochleate powder (buffer-free). Accurately weighed freeze-dried cochleates (about 2 mg) were disrupted by adding EDTA (100 mM) prepared in 2% NaHCO₃ (pH>10). The resulting suspension of lipid vesicles was extracted with chloroform and the aqueous phase was then analysed to determine the amount of amikacin loaded in the cochleates by spectrophotometry as described above. The amikacin encapsulation (AE) in percentage and amikacin loading (AL) in mg AMK/g of cochleates were calculated as follows,

$$AE = \frac{\text{Total of AMK added (mg)} - \text{AMK in supernatant (mg)}}{\text{Total of AMK added (mg)}} \times 100 \% \dots (\text{Eq. 2.2})$$

$$AL = \frac{\text{AMK encapsulated in cochleates (mg)}}{\text{Amount of cochleates taken (mg)}} \times 10^{-3} \dots (\text{Eq. 2.3})$$

2.2.6 Turbidity measurement for the analysis of fusion of DOPS SUVs

The turbidity of the DOPS SUV suspensions as a function of added amikacin and Ca²⁺ concentrations were measured using a spectrophotometer (Thermo

Evolution™ 300 UV-Vis Spectrophotometer). The change in absorbance of vesicle suspension at 400 nm was measured after each stepwise addition of the respective bridging agent. The procedure was repeated until no change in the absorbance was observed. All experiments were performed at room temperature.

2.2.7 Detection of calcium in the cochleates

The calcium content in different cochleates prepared using varying Ca^{2+} concentrations as a bridging agent was determined by the energy dispersive X-ray spectroscopy (EDS) using FE-SEM (JEOL JSM 6700F) equipped with an EDS detector (Oxford EDS X-Max^N 80). Samples in the form of pellets with uniform thickness prepared using an appropriate amount of freeze-dried, buffer-free cochleate powder were sputter-coated with platinum (JFC-1600 Auto Fine Coater) and the data of elemental ratios were collected.

2.2.8 Fourier transform infrared (FTIR) spectroscopy

An appropriate amount of buffer-free, freeze-dried cochleate powder was mixed with dry potassium bromide and compressed into discs under 10 kN force at room temperature. The IR spectra were recorded using PerkinElmer Spectrum-One FTIR Spectrometer and/or Thermo Scientific™ Nicolet™ iS50 FTIR Spectrometer for a scanning range of 4000 to 400 cm^{-1} wavenumber was used. IR spectroscopy was performed in the similar manner throughout this thesis.

2.2.9 Small angle X-ray scattering (SAXS)

SAXS analysis of the cochleate suspension filled in sealed borosilicate capillaries of ~1.5 mm outer diameter and 0.01 mm wall thickness was performed in transmission mode using Nano-inXider vertical SAXS/WAXS

system (Xenocs SA) equipped with a microfocus source generating Cu-K α X-ray (Genix3D, Xenocs SA) with operating wavelength of $\lambda=1.54$ Å and hybrid pixel detectors (Pilatus 200K, Dectris, Switzerland). All measurements were carried out at room temperature, under vacuum for 60 min in several exposures of 5 min each. The recorded 2D scattering patterns were azimuthally integrated to 1D background-corrected patterns of scattering intensity as a function of the scattering vector (q) shown below (Eq. 2.4) using Foxtrot software (Xenocs SA).

$$q = \frac{4\pi}{\lambda} \sin \theta \dots \text{(Eq. 2.4)}$$

where, 2θ is the scattering angle and λ is the X-ray wavelength. Similar method was followed for SAXS studies throughout this thesis.

2.2.10 In-vitro amikacin release from the cochleates

The in-vitro release profile of amikacin from different cochleate samples was studied using dialysis bag diffusion technique in PBS (physiological pH of 7.4) at the physiological temperature of 37 °C. Freeze-dried cochleate powders encapsulating an equivalent of 8 mg of amikacin were resuspended in 5 ml PBS and introduced in a dialysis bag (cellulose membrane, molecular weight cut off 14,000 Da) and immersed in 45 ml of release medium with continuous stirring at 100 rpm. Amikacin release was monitored for 96 h by withdrawing samples at pre-determined time points and the volume of samples withdrawn was replaced by fresh PBS to maintain sink conditions. A separate sample of pure amikacin powder was used as a control to ensure that the diffusion of amikacin across the membrane was not the rate-limiting step. The samples were analysed for amikacin by spectrophotometry as described earlier (under Section 2.2.5) and the percentage amikacin release was calculated as below,

$$\% \text{ AMK release} = \frac{\text{Cumulative amount of AMK in a release sample (mg)}}{\text{Total amount of AMK taken as cochleates (mg)}} \times 100 \dots$$

(Eq. 2.5)

Further, the obtained amikacin release profiles were analysed by different kinetic models such as zero-order kinetics (Eq. 2.6), first-order kinetics (Eq. 2.7), Higuchi model (Eq. 2.8) and Korsmeyer-Peppas model (Eq. 2.9).¹²⁹

$$A_t = A_b + k_0 t \dots (\text{Eq. 2.6})$$

$$\ln \left(\frac{A_t}{A_0} \right) = k_1 t \dots (\text{Eq. 2.7})$$

$$A_t = k_H t^{1/2} \dots (\text{Eq. 2.8})$$

$$\log \left(\frac{A_t}{A_\infty} \right) = \log(k_{KP}) + n \log(t) \dots (\text{Eq. 2.9})$$

where, A_t is the amount of amikacin released after time t , A_b is the amount of amikacin in release medium at $t = 0$ (i.e., ideally $A_b = 0$), A_0 is the total amount of amikacin taken for release (i.e., encapsulated in cochleates), A_∞ is the amount of amikacin released after an infinite time, k_0 is the zero-order rate constant, k_1 is the first-order rate constant, k_H is the Higuchi rate constant, k_{KP} is the Korsmeyer–Peppas rate constant and n is the release exponent.

2.2.11 Accelerated stability studies of cochleates

To study the stability of different cochleate samples, they were stored in the form of freeze-dried powders at 45 °C. The degradation of cochleates was analysed by measuring amikacin loading and zeta potential by taking samples at weekly intervals over 12 weeks. Further, the degradation kinetics of different cochleate samples was studied by analysing residual amikacin loading using first-order

rate equation (Eq. 2.10) and half-life for AL of each sample was determined using equation (Eq. 2.11),

$$\ln \left(\frac{(AL)_t}{(AL)_0} \right) = -k_{deg} t \dots \text{(Eq. 2.10)}$$

$$t_{1/2} = \frac{-0.693}{k_{deg}} \dots \text{(Eq. 2.11)}$$

where, $(AL)_t$ is the amikacin loading after a storage time of t weeks, $(AL)_0$ is the initial amikacin loading, k_{deg} is the degradation rate constant and $t_{1/2}$ is the time (in weeks) required for AL to reduce by 50 % of its initial value called half-life.

2.2.12 Statistical analysis of data

All experiments were performed at least in triplicates with data represented as calculated means and standard deviations. The statistical significance was assessed by performing one-way ANOVA with Bonferroni's correction for multiple comparison followed by t-test for paired comparisons. The p values lower than 0.05 were recognized as significant. All data analysis and calculations were performed using Excel Office 365 (Microsoft Corp.) and/or Origin Pro 2017 (Origin Lab Corp.) software. The similar criteria were employed throughout this thesis for the statistical analysis.

2.3 Results and Discussions

2.3.1 Amikacin-bridged cochleates and the effect of sub-threshold Ca^{2+} on their formation

The general principle underlying the formation of cochleates includes several intermediate processes such as the fusion of bilayer vesicles, dehydration of lipid headgroups, the formation of large planar bilayer stacks, aggregation of these

stacks to form larger bilayer sheets with highly ordered DOPS acyl chains in crystalline form leading to rolling up of the sheets, and finally the precipitation of rolled-up cochleate cylinders.^{17,19} A general scheme of the conventional cochleate formation using metallic Ca^{2+} as bridging agent is shown in Figure 2.1.

At first, conventional DOPS cochleates were prepared with Ca^{2+} as bridging agent (referred to as “Ca-Coch” with DOPS: Ca^{2+} molar ratio of 10:10) to be used as benchmark and control for comparison. The “Ca-Coch” showed typical cochleate structure and were 212 ± 52 nm in width with a dispersity of 0.25 ± 0.01 and 1229 ± 565 nm in length with a dispersity of 0.46 ± 0.02 (Table 2.1). To obtain optimum amikacin loading in cochleates, amikacin-bridged cochleates were initially prepared using varying amikacin concentrations with DOPS:AMK in 10:10, 10:9, 10:5 and 10:2.5 molar ratios. In all the cases, clear cochleate structures were observed (Figure 2.2). The particle size (both in width and length of cochleates), as well as AE and AL of these cochleates, were analysed (Table 2.1). At 10:5 molar ratio of DOPS:AMK, best AE of 45.76 ± 2.06 % and a corresponding AL of 182.84 ± 8.23 mg AMK/g of cochleates were obtained. Hereon, this cochleate sample will be referred to as “AMK-Coch” throughout this chapter and selected for comparison. Amikacin-bridged cochleates in the presence of sub-threshold Ca^{2+} concentrations were prepared with DOPS:AMK: Ca^{2+} molar ratios of 10:5:1, 10:5:2.5 and 10:5:5 to study the impact of Ca^{2+} on their structural and functional properties.

Figure 2.1 Schematic of DOPS cochleate formation bridged by calcium, amikacin and amikacin in the presence of sub-threshold Ca²⁺ concentrations with proposed intermediate structures, also shown are chemical structures of DOPS and amikacin

Figure 2.2 SEM images showing cochleates prepared with different DOPS:AMK molar ratios of (A) 10:10, (B)10:9, (C) 10:5 and (D) 10:2.5

SEM images of all the prepared samples showed rolled-up cylindrical structure typical of cochleates, with clearly visible scroll-like rolled-up structure of cochleate particles (Figure 2.3). The control sample “Ca-Coch” (Figure 2.3 A) showed well-formed cochleate structures with clearly defined individual particles. However, the “AMK-Coch” (Figure 2.3 B) showed a higher degree of aggregation where many individual cochleate particles appeared as extending out from a matrix of large, fused lipid bilayers. While similar morphological features were also observed amongst “AMK-Ca-Coch1”, “AMK-Ca-Coch2” and “AMK-Ca-Coch3” (Figure 2.3 C, D and E), the individual cochleate particles showed increasingly well-defined structures as Ca^{2+} concentration was increased. Unlike “AMK-Coch”, the formation of increasingly well-defined particles also resulted in lesser particles being embedded in the aggregated lipid bilayer matrix (Figure 2.3 B vs C, D and E). These results suggest that, although

amikacin alone can form the cochleates, a combination of amikacin and low concentrations of Ca^{2+} results in better cochleation with an increased number of well-formed cochleates and lower aggregated sheet-like structures.

Figure 2.3 Scanning electron micrograph of (A) “Ca-Coch” prepared with DOPS: Ca^{2+} of 10:10; (B) “AMK-Coch” prepared with DOPS:AMK of 10:5; (C) and (D) “AMK-Ca-Coch1” with DOPS:AMK: Ca^{2+} of 10:5:1; (E) “AMK-Ca-Coch2” with DOPS:AMK: Ca^{2+} of 10:5:2.5; (F) and (G) “AMK-Ca-Coch3” with DOPS:AMK: Ca^{2+} of 10:5:5. Arrows indicate the clearly visible scroll-like rolled-up structure of cochleate particles

Among the samples prepared, both “AMK-Ca-Coch1” and “AMK-Ca-Coch2” showed maximum and similar AE and AL which were roughly 30% higher than “AMK-Coch” and “AMK-Ca-Coch3”. Interestingly, both “AMK-Coch” and “AMK-Ca-Coch3” showed similar AE and AL (Table 2.2). Analysis of the particle size revealed that “AMK-Coch” showed the largest size with a width of 265 ± 95 nm (dispersity of 0.36 ± 0.01 and $p<0.05$) and length of 1500 ± 930 nm (dispersity of 0.62 ± 0.03 and $p<0.05$) compared to other cochleate samples containing added Ca^{2+} (Table 2.2). Again, both “AMK-Ca-Coch1” and “AMK-Ca-Coch2” showed similarity in length and width as well as zeta potential (Table 2.2). The higher AE and AL observed in the case of “AMK-Ca-Coch1” and “AMK-Ca-Coch2” compared to “AMK-Coch” may be attributed to the presence of more and well-defined cochleate particles (as seen in Figure 2.3) with smaller size and no bilayer sheet structures (Table 2.2). However, it is important to note that this enhancement in AE and AL was observed only at lower Ca^{2+} threshold concentrations (with 10:5:1 and 10:5:2.5 of DOPS:AMK: Ca^{2+}). This was evident by the lower AE and AL of “AMK-Ca-Coch3” (10:5:5 of DOPS:AMK: Ca^{2+}) (Table 2.2). We attribute this to competitive binding, where a higher concentration of Ca^{2+} causes favourable bridging leaving lesser amikacin being incorporated in the cochleates as was seen in the case of inhibition of Polymyxin B binding with negatively charged phospholipids in the presence of Ca^{2+} .¹³⁰ Therefore, at a lower Ca^{2+} concentration, amikacin and Ca^{2+} act synergistically which leads to well-formed cochleate particles (Figure 2.3) with higher AE and AL (Table 2.2). Similar synergistic effects between Ca^{2+} and Mg^{2+} for inducing cochleation was reported earlier.³² Zeta potential measurements performed on different cochleate samples are shown in Table 2.2. All samples showed negative

zeta potentials as expected due to the negatively charged DOPS lipids. The change in zeta potential from -72 mV for DOPS SUVs to -38 mV and -24 mV for “Ca-Coch” and “AMK-Coch”, respectively, showed charge neutralization of DOPS during cochleation. Based on the above observation, a proposed scheme of the non-conventional cochleate formation using non-metallic cationic amikacin as well as amikacin with sub-threshold Ca^{2+} as the bridging cations is shown in Figure 2.1.

2.3.2 Analysis of fusion of SUVs upon addition of Ca^{2+} and amikacin

The fusion of bilayer SUVs is an important prerequisite for cochleation. For this fusion to occur, a barrier of hydration forces must be overcome, and the interactions between phospholipid bilayers should be strong enough to allow their close contact to form the stacks. In the case of cochleation involving negatively charged phosphatidylserine (DOPS), strong cations such as Ca^{2+} are necessary to induce fusion. To compare the minimum amikacin and Ca^{2+} concentrations required to initiate fusion of SUVs, the turbidity of the suspension was measured after the gradual addition of these bridging agents (Figure 2.4 A). Besides, the size of the SUVs and their zeta potential as a function of the concentration of the bridging agents were monitored by dynamic light scattering and laser doppler micro-electrophoresis, respectively (Figure 2.4).

Table 2.1 The AE, AL, particle size and dispersity of amikacin-bridged cochleates prepared by different molar ratios of DOPS:AMK

Sample (DOPS:AMK)	AE (%)	AL (mg AMK/g cochleates)	Width (nm)	Dispersity in width	Length (nm)	Dispersity in length
10:10	17.2±0.8	143.8±6.5	331±124	0.38±0.02	1744±1175	0.67±0.03
10:9	26.5±1.2	188.6±8.5	360±135	0.37±0.02	1776±952	0.54±0.03
10:5	45.8±2.1	182.8±8.2	266±99	0.37±0.02	1554±846	0.55±0.03
10:2.5	48.5±2.2	105.7±4.8	294±97	0.33±0.01	1678±801	0.48±0.02

Table 2.2 The AE, AL, particle size, dispersity, and zeta potential values of different samples

Sample name	AE (%)	AL (mg AMK/g cochleates)	Width (nm)	Dispersity in width	Length (nm)	Dispersity in length	Zeta potential (mV)
DOPS SUVs	N.A.	N.A.	124±1*	0.10±0.02 [#]	N.A.	N.A.	-71.6±1.7
Ca-Coch	N.A.	N.A.	212±52	0.25±0.01	1229±565	0.46±0.02	-37.7±3.2
AMK-Coch	44.4±4.2	178.3±5.4	265±95	0.36±0.01	1500±930	0.62±0.02	-24.2±2.0
AMK-Ca-Coch1	69.7±6.9	255.1±9.2	202±60	0.30±0.01	1353±803	0.59±0.02	-25.4±3.2
AMK-Ca-Coch2	71.6±9.0	266.7±12.0	201±55	0.27±0.02	1406±711	0.51±0.01	-25.3±0.9
AMK-Ca-Coch3	48.1±4.5	193.5±11.3	182±55	0.30±0.01	1332±613	0.46±0.01	-28.6±1.9

N.A.: Not applicable. In the case of DOPS SUVs, * the size was represented by hydrodynamic diameter (nm) and # the dispersity was represented as PDI, determined by DLS

An increase in the absorbance with increased concentration of bridging agents was observed indicating aggregation of SUVs. Both Ca^{2+} and amikacin showed a similar trend of increase in absorbance until a maximum plateau was reached. In the case of Ca^{2+} , the fusion started after addition of >1 mM and plateaued around 3 mM of Ca^{2+} concentration. This was similar to previously reported values.¹³¹ Therefore, these results confirmed that the Ca^{2+} concentrations (i.e., 0.6, 1.5 and 3.1 mM) used for the preparation of “AMK-Ca-Coch1”, “AMK-Ca-Coch2” and “AMK-Ca-Coch3” at molar ratios of DOPS:AMK: Ca^{2+} being 10:5:1, 10:5:2.5 and 10:5:5, respectively, were indeed in the desired sub-threshold range. However, amikacin showed vesicle fusion and plateau at a much lower concentration of about 0.08 mM and 0.19 mM, respectively. This was attributed to the tetravalent cationic nature of amikacin with four ionizable amine groups (Figure 2.1).^{130,132} The size measurement data showed an increase in SUV size as the concentration of the bridging agents increased (Figure 2.4 B and C). Representative distribution results showed unimodal size distributions of DOPS SUVs with hydrodynamic diameters centred near 120 nm (Figure 2.5). In the case of Ca^{2+} , the fusion was only induced above 1 mM Ca^{2+} which was manifested by particle growth, distribution broadening with the appearance of very large and polydisperse particles (of size >1000 nm at 1.6 mM Ca^{2+}). Whereas the addition of amikacin showed a fusion of SUVs by a gradual increase in size and broadening of distribution even at concentrations as low as 0.08 mM (Figure 2.4 C and Figure 2.5). Further addition of amikacin (>0.1 mM) showed multimodal size distribution of SUVs with size ranging from 170 nm to 950 nm with higher polydispersity attributed to the tetravalent nature of amikacin (Figure 2.5).

Figure 2.4 (A) Fusion of SUVs measured by suspension turbidity; (B) and (C) Size of SUVs as a function of added Ca²⁺ and amikacin concentration, respectively; and (D) Zeta potentials of SUVs as a function of bridging agent concentrations

In addition, the zeta potential of DOPS SUVs in the absence of bridging agents was about -72 mV. Upon increasing the Ca²⁺ concentration up to 2 mM, a stable zeta potential was observed at about -48 mV. Similar results were observed in the case of amikacin, but at a much lower amikacin concentration of 0.26 mM where a stable zeta potential of about -40 mV was observed (Figure 2.4). This indicated that bridging of vesicles by the cations lead to charge neutralization of the headgroups of DOPS. Further, our results showed consistent values for the

minimum bridging agent concentrations (or threshold) that was required to initiate fusion of SUVs as analysed by all the three methods (Figure 2.4).

Figure 2.5 Size distribution of SUVs to analyse vesicle fusion after addition of different concentrations of (A) Ca^{2+} and (B) AMK as measured by DLS method

2.3.3 Detection of calcium in cochleates samples

We determined the relative calcium content incorporated in “Ca-Coch”, “AMK-Ca-Coch1”, “AMK-Ca-Coch2 and AMK-Ca-Coch3” by energy dispersive X-ray spectroscopy (EDS) (Figure 2.6). As expected, “Ca-Coch” showed the highest calcium content, “AMK-Ca-Coch2 and AMK-Ca-Coch3” showed intermediate, and “AMK-Ca-Coch1” showed lowest calcium content representing the relative amounts of Ca^{2+} used for their formation Figure 2.6 (A).

Figure 2.6 (A) Calcium content in different cochleate samples; (B) Representative SEM image used for EDS elemental mapping of “Ca-Coch” with inset figure specifically showing calcium distribution in the sample and (C) Representative X-ray elemental analysis spectrum for the sample “Ca-Coch” with inset figure showing EDS elemental mapping

The insignificant calcium content shown by EDS for “AMK-Coch” was perhaps due to cross-contamination during sample preparation. However, it is unclear how much of the Ca^{2+} that was used to prepare these samples were incorporated into these cochleate samples. Further, a representative SEM image of “Ca-Coch” sample showing an elemental map specifically for calcium was shown in Figure 2.6 (B). Further, an overlay of a full elemental map with different coloured dots

representing different elements along with a representative X-ray emission spectrum showing unique peaks of various elements present in this sample is shown in Figure 2.6 (C).

2.3.4 FTIR spectroscopy analysis of lipid phases in the cochleate samples

To further confirm cochleation as a result of amikacin bridging, FTIR spectra of pure Amikacin sulphate, “Ca-Coch”, “AMK-Coch”, “AMK-Ca-Coc1”, “AMK-Ca-Coch2” and “AMK-Ca-Coch3” cochleates were measured (Figure 2.7 A). The IR spectrum of pure amikacin showed C–O stretching bands at 1050 cm^{-1} and 1120 cm^{-1} and N–H bend, C=O stretching corresponding to CONH group at 1540 cm^{-1} and 1635 cm^{-1} , respectively. Additionally, the C–H aliphatic stretching vibrations were observed at 2928 cm^{-1} and a very broad and strong band indicating NH, NH_2 and O–H stretching vibrations was seen around 3430 cm^{-1} .⁶⁴ The spectral analysis of different cochleate samples showed the presence of characteristic peaks at 1063 cm^{-1} and 1236 cm^{-1} attributed to the $-\text{PO}_2$ symmetric and asymmetric stretching, respectively, an ester $-\text{C}=\text{O}$ peak at 1730 cm^{-1} and the symmetric and asymmetric stretching corresponding to the CH_2 of the alkyl chains at 2852 cm^{-1} and 2923 cm^{-1} , respectively. Further, the presence of O–H stretching vibrations as a broad band at 3430 cm^{-1} in all amikacin-bridged cochleates (“AMK-Coch”, “AMK-Ca-Coc1”, “AMK-Ca-Coch2” and “AMK-Ca-Coch3”) and its absence in “Ca-Coch” sample confirmed the encapsulation of amikacin in the cochleates (Figure 2.7 A).

To reveal the effects of sub-threshold Ca^{2+} concentrations on the formation of “AMK-Ca-Coc1”, “AMK-Ca-Coch2” and “AMK-Ca-Coch3”, the IR spectra of all the cochleate samples as well as DOPS lipid (in hydrated state) were

compared. For this, we focused our study on the phosphate (1000 cm^{-1} to 1250 cm^{-1} , Figure 2.7 B), ester carbonyl (1690 cm^{-1} to 1760 cm^{-1} , Figure 2.7 C) and the alkyl chain (2800 cm^{-1} to 3000 cm^{-1} , Figure 2.7 D) stretching vibrations which expose the features of the lipid phase such as phosphate headgroup hydration, glycerol-group hydration, and acyl chain order, respectively. The IR spectrum of DOPS lipid showed featureless symmetric and asymmetric bands for phosphate stretching at 1065 cm^{-1} and 1216 cm^{-1} , respectively (Figure 2.7 B). In the “Ca-Coch” sample, the symmetric phosphate stretching band was split into four distinct peaks at 1063 cm^{-1} , 1075 cm^{-1} , 1102 cm^{-1} and 1112 cm^{-1} , and the asymmetric phosphate stretching band was shifted from 1216 cm^{-1} to 1238 cm^{-1} , indicating bidentate complexation of phosphate with Ca^{2+} and dehydration of the phosphate headgroups. While we observed similar features for phosphate headgroup region of “AMK-Ca-Coch1”, “AMK-Ca-Coch2” and “AMK-Ca-Coch3”, these regions showed broader stretching bands in the case of “AMK-Coch”. This may be attributed to the hydrogen bonding of phosphate headgroup with either water molecules causing partial dehydration or with the O–H groups in amikacin.¹³³ However, the presence of well-formed cochleates in all the samples (including “AMK-Coch”) confirms dehydration of phosphate headgroup.⁴⁴ Hence, we believe that amikacin forms hydrogen bonds with phosphate headgroup while displacing the existing hydrogen bound water leading to complete dehydration while showing the broader phosphate stretching bands (Figure 2.7 B).¹³³ At lower Ca^{2+} concentrations (“AMK-Ca-Coch1” and “AMK-Ca-Coch2”) the symmetric phosphate stretching bands were more prominent than “AMK-Coch”. We attribute this to the preferential binding of Ca^{2+} to phosphate head groups in the presence of amikacin resulting in strong

bidentate complexation which decreases hydrogen bonding to amikacin. This preferential binding of Ca^{2+} to phosphate head groups was similar to the previously reported synergistic effect of Mg^{2+} on Ca^{2+} binding with phospholipid bilayers where, the presence of Mg^{2+} reduces the concentration of Ca^{2+} required to bind to the bilayer head groups.³² On the other hand, at slightly higher Ca^{2+} concentrations (i.e. DOPS:AMK: Ca^{2+} of 10:5:5 molar ratio in “AMK-Ca-Coch3”) the IR spectra was largely similar to that of “Ca-Coch”. This could be attributed to the competitive binding of Ca^{2+} to phosphate head group leading to lesser amikacin being incorporated in the cochleates (Table 2.2).¹³⁰

The DOPS ester carbonyl groups showed broad vibrational stretching bands at 1730 cm^{-1} and 1740 cm^{-1} representing the two differently hydrated carbonyl groups (hydrated lipid, Figure 2.7 C). “Ca-Coch” showed three distinct narrow stretching bands at 1703 cm^{-1} , 1719 cm^{-1} and 1732 cm^{-1} , as well as a shoulder at 1747 cm^{-1} due to complexation of the headgroups with Ca^{2+} resulting in restricted motional freedom.¹³⁴ Similar observations, were made in the cases of “AMK-Ca-Coch1”, “AMK-Ca-Coch2” and “AMK-Ca-Coch3” (Figure 2.7 C), and were attributed to preferential or stronger binding of Ca^{2+} to headgroups over amikacin as seen in the case of phosphate headgroup binding in Figure 2.7 (B). However, “AMK-Coch” showed two narrow stretching bands at 1703 cm^{-1} and 1719 cm^{-1} , and a broader band centred at 1732 cm^{-1} which appeared to include the usual shoulder band at 1747 cm^{-1} (Figure 2.7 C). This points to differences in binding modes of amikacin to DOPS headgroup as compared to that of Ca^{2+} , which may result in the formation of hydrogen bonds between the carbonyl groups and amikacin (it's O–H and/or N–H groups).¹³⁴ Further, the alkyl group stretching vibrational bands pertaining to acyl side chain of DOPS (hydrated

lipid), “Ca-Coch”, “AMK-Coch1”, “AMK-Coch2” and “AMK-Coch3” were shown in Figure 2.7 (D). These results confirm that “AMK-Coch” shows slightly less rigid lipid packaging probably due weaker complexation of lipid headgroups with amikacin as compared to Ca^{2+} .

Based on the FTIR studies, and in support of the SEM images (Figure 2.3), amikacin causes the dehydration of DOPS headgroups by effectively bridging bilayer vesicles to form rolled up cochleates. However, there are several features in the lipid phase of “AMK-Coch” that retains some of the characteristics of DOPS SUVs. On the other hand, when small concentrations of Ca^{2+} (sub-threshold) were used with amikacin for bridging, cochleates with more spectral features resembling that of “Ca-Coch” were formed showing an increased number of well-formed cochleate particles as shown in Figure 2.3. Additionally, certain features of the samples with comparatively lower Ca^{2+} content, “AMK-Ca-Coch1” and “AMK-Ca-Coch2” (Figure 2.6 A) showed the existence of some degree of synergy between Ca^{2+} and amikacin for DOPS binding. This, in addition to the obvious presence of more well-defined cochleate particles, is the possible cause for increased AE and AL in these samples (Table 2.2). Whereas with “AMK-Ca-Coch3”, despite the lower AE and AL due to the competitive binding of Ca^{2+} over amikacin, a significant amount of amikacin was encapsulated in cochleates (Table 2.2). The IR results support our proposed competitive and synergistic concept during the fusion of the vesicles in the presence of both Ca^{2+} and amikacin as discussed above and also support our proposed model of cochleation in Figure 2.1. The cochleates are homogenous, meaning that they are not a physical mixture of amikacin-bridged and Ca^{2+} -bridged cochleates. Based on these observations we think that, there may be

other important implications to these interactions between DOPS and amikacin in the presence of sub-threshold Ca^{2+} concentrations.

Figure 2.7 FTIR spectra of different cochleate samples (A) indicating the encapsulation of amikacin; specific for (B) phosphate; (C) ester carbonyl and (D) acyl chain stretching regions showing the difference in lipid phases. Where, a – pure AMK, b – AMK-Coch, c – AMK-Ca-Coch1, d – AMK-Ca-Coch2, e – AMK-Ca-Coch3, f – Ca-Coch, and g – hydrated DOPS SUVs

2.3.5 SAXS analysis of cochleate samples

All cochleate samples, DOPS SUVs, “Ca-Coch”, “AMK-Coch”, “AMK-Coch1”, “AMK-Coch2”, and “AMK-Coch3”, were subjected to SAXS analysis and the SAXS patterns are shown in Figure 2.8. The hydrated DOPS SUVs,

showed a very broad signal in the range of $0.04 \text{ \AA}^{-1} < q < 0.25 \text{ \AA}^{-1}$ without any of Bragg peaks or undulations confirming that the suspension consists of uniform SUVs (Figure 2.8 A). This was due to the absence of long-range ordering caused by the electrostatic repulsion of the neighbouring bilayers due to the net negative charge of the DOPS.¹³⁵ The “Ca-Coch” formed by the addition of Ca^{2+} to DOPS SUVs showed a drastic change in the SAXS pattern with a sharp Bragg reflection followed by higher-order weak reflections. This was due to the highly ordered, periodic lamellar organization which was represented by equidistant Bragg-reflections at 0.12, 0.24 and $0.36 \text{ \AA}^{-1}$ (Figure 2.8 A). The first reflection peak in the diffraction pattern of “Ca-Coch” showed a corresponding characteristic domain spacing (or the interlamellar repeat distance) of $51 \text{ \AA}$ which was characteristic value for DOPS cochleates. The SAXS patterns of “AMK-Coch” showed similar features of ordered multilamellarity and tight packaging of lipid bilayers as that of “Ca-Coch”. However, “AMK-Coch” showed broad Bragg reflection peaks instead of sharp and intense peaks like “Ca-Coch”. Further, we observed the gradual sharpening of Bragg peaks in samples “AMK-Ca-Coch1”, “AMK-Ca-Coch2” and “AMK-Ca-Coch3” (Figure 2.8 A). To explain this, SAXS analysis of DOPS SUVs was performed (Figure 2.8 B) after the addition of bridging agents Ca^{2+} (1.6 mM) and amikacin (0.08 mM and 0.13 mM) at concentrations just sufficient to induce the fusion of SUVs but not complete cochleation as seen from Figure 2.4 and 2.5. The SAXS patterns of the sample with low Ca^{2+} concentration of 1.6 mM showed the coexistence of tightly packed multilamellar phase with some degree of vesicular bilayer phase schematically shown in Figure 2.1. This was evidenced by the emerging Bragg peaks from the broader signal of DOPS SUVs (Figure 2.8 B). Similar results were previously

reported with DOPS vesicles at low Ca^{2+} concentrations and such a SAXS pattern was attributed to the existence of dominant repulsive electrostatic interactions between the like-charged bilayer DOPS SUVs.¹³⁶ On the contrary, SAXS patterns of DOPS SUVs upon the addition of amikacin at low concentrations showed a rather compact line shape instead of Bragg peaks at both 0.08 mM and 0.13 mM (Figure 2.8 B). This might suggest certain structure factor modulations in DOPS SUVs due to their stable adhesion states as proposed in Figure 2.1.¹³⁵ This adhesion of vesicles upon addition of low concentrations of amikacin was seen in the form of a systematic increase in hydrodynamic radii of vesicles by the DLS studies (Figure 2.5). At 0.08 mM, the size distribution was still monodisperse indicating that strong vesicle aggregation has not occurred. However, at 0.13 mM of amikacin, the size distribution becomes bimodal and shifts to significantly higher hydrodynamic radii in agreement with previous studies.¹³⁵ Despite vesicle fusion (or adhesion), much higher amikacin concentration is required to obtain well-formed cochleates as seen from our results (Figure 2.3). These results combined with our FTIR analysis explains the Bragg peaks sharpening in “AMK-Ca-Coch1”, “AMK-Ca-Coch2” and “AMK-Ca-Coch3” samples which we think was due to the synergistic and/or competitive binding of Ca^{2+} to DOPS headgroups over amikacin. Additionally, all the cochleate samples containing amikacin (“AMK-Coch”, “AMK-Ca-Coch1”, “AMK-Ca-Coch2” and “AMK-CaCoch3”) showed interlamellar repeat distance of 51 Å same as that of the domain characteristics of “Ca-Coch” due to its planar structure while interacting with bilayer surface.¹³⁴

Figure 2.8 SAXS (A) patterns of different cochleate samples and (B) patterns after addition of bridging agents (Ca^{2+} and AMK) at concentrations just enough to induce fusion of SUVs but not complete cochleation. Where, a – DOPS SUVs, b – Ca-Coch, c – AMK-Coch, d – AMK-Ca-Coch1, e – AMK-Ca-Coch2, f – AMK-Ca-Coch3, g – DOPS SUVs with 1.6 mM Ca^{2+} , h and i – DOPS SUVs with 0.08 mM and 0.13 mM AMK, respectively

2.3.6 Effect of sub-threshold Ca^{2+} on the in-vitro drug release and stability

The in-vitro release of amikacin from cochleates samples “AMK-Coch”, “AMK-Ca-Coch1”, “AMK-Ca-Coch2” and “AMK-CaCoch3” was studied by dialysis method using phosphate buffered saline (PBS, pH 7.4). A separate sample of pure amikacin powder was used as a control reference to compare with different cochleate samples as shown in the amikacin release profile (Figure 2.9 A). Since, amikacin is a water-soluble drug, all of the pure amikacin was diffused from the dialysis bag into the release medium within the first 2 h. Whereas, all the four samples of cochleates showed similar biphasic amikacin release profile characterized by a relatively steeper release until 14 h with about 30 % of

amikacin released from “AMK-Coch”, about 20 % released from “AMK-Ca-Coch1” and “AMK-Ca-Coch2” alike, and about 24 % from “AMK-Ca-Coch3”. This was followed by a slower sustained release phase over the next 96 h with 84, 76, 63 and 54 % amikacin released by “AMK-Coch”, “AMK-Ca-Coch1” “AMK-Ca-Coch2” and “AMK-Ca-Coch3”, respectively (Figure 2.9 A). Such a release profile is attributed to the strong interaction of amikacin with the polar lipid headgroups and the multiple bilayer structure of cochleates.^{28,65} Further, the relatively faster release during the first 14 h could be attributed to small amounts of amikacin physically trapped on the outermost bilayer sheets of the cochleates. Nevertheless, all three “AMK-Ca-Coch1”, “AMK-Ca-Coch2” and “AMK-Ca-Coch3” showed slower and much sustained-release compared to “AMK-Coch” in the second phase which may be attributed to tighter encapsulation of amikacin within the tightly packed lipid phase of the cochleates due to the presence of low Ca^{2+} concentrations as a bridging agent. Additionally, the variable amikacin release observed from these cochleates could be attributed to the use of variable Ca^{2+} concentrations during their preparation. The increasing rigidity in cochleate lipid phases of “AMK-Ca-Coch1”, “AMK-Ca-Coch2” and “AMK-Ca-Coch3”, respectively, compared to “AMK-Coch” as evident from our FTIR and SXAS studies, led to the difference in amikacin release rates from these samples.

These *in-vitro* amikacin release profiles were further subjected to theoretical analysis using mathematical models for drug release. The amikacin release data was fit to four different models: zero-order, first-order, Higuchi and Korsmeyer-Peppas.¹²⁹ The correlation coefficient of each fit was considered to determine the best model. The release profile of pure amikacin was best fit to zero-order kinetics (Table 2.3). The data was fit into Korsmeyer–Peppas model since the

release mechanism of the cochleate systems is not well-known. Additionally, this model helps to determine whether more than one type of release mechanisms is involved which could be inferred from the release exponent 'n' obtained from the fit equation.¹²⁹ We observed that the pure amikacin release profile also showed a linear fit for the Korsmeyer-Peppas model with a release exponent value of ~ 1.0 which corresponds to Case-II transport with zero-order rate function of time. However, as the release exponents of cochleate samples lie in the range of $0.5 < n < 1.0$, the transport falls within the anomalous regime of Korsmeyer-Peppas release model and such release can be neglected.¹²⁹ Whereas, the Higuchi model governed by Fick's law of diffusion resulted in the best linear fit for all the cochleate samples and was considered for further analysis. Besides, there is evidence from the literature that for multi-layered systems (similar to that of cochleates), the Higuchi model is regarded as the most appropriate model to describe the drug release kinetics, when compared to zero-order, first-order and Korsmeyer-Peppas models.¹³⁷ We observed a significant decrease in the Higuchi rate constants for amikacin release from $0.65 \text{ mg.h}^{-0.5}$ for "AMK-Coch" to 0.55 , 0.49 and $0.45 \text{ mg.h}^{-0.5}$ for "AMK-Ca-Coch1", "AMK-Ca-Coch2" and "AMK-Ca-Coch3", respectively, which confirmed slower and more sustained amikacin release. Further, this decrease in Higuchi rate constants showed a linear correlation (R^2 value of 0.9922) with the square root of Ca^{2+} concentrations in the sub-threshold range used to prepare these cochleates (Figure 2.9 B). Therefore, the addition of Ca^{2+} in sub-threshold concentrations along with the main bridging agent amikacin not only affects the formation of the cochleates but also has greater implications towards tuneability of amikacin release which

aids in designing and modulating better cochleate formulations to cater for the patients' needs.

Figure 2.9 (A) The in-vitro amikacin release profile of different cochleate samples; (B) Variation of Higuchi rate constants obtained for amikacin release as a function of sub-threshold Ca^{2+} concentration used for the preparation of respective cochleate samples; (C) Stability of cochleates w.r.t amikacin loading studied as per first-order degradation (D) Changes in zeta potential of cochleates over the storage period

The long-term stability of all four cochleate samples “AMK-Coch”, “AMK-Ca-Coch1”, “AMK-Ca-Coch2” and “AMK-Ca-Coch3” was studied by storing them as a freeze-dried powder at 45 °C for over 12 weeks. The samples were assessed by measuring the change in amikacin loading and zeta potential values over this

duration every week. The degradation of cochleates was studied as a function of different sub-threshold Ca^{2+} concentrations by monitoring AL according to first-order decay kinetics (Figure 2.9 C and Table 2.4). While “AMK-Coch” was the least stable, “AMK-Ca-Coch3” having the highest Ca^{2+} content was the most stable with “AMK-Ca-Coch1” and “AMK-Ca-Coch2” showing intermediate stability. The calculated kinetic parameters showed that “AMK-Ca-Coch1”, “AMK-Ca-Coch2” and “AMK-Ca-Coch3” took 1.6-, 2.4- and 3.7-folds more time, respectively, than “AMK-Coch” to lose 50% of the initial AL upon storage (Table 2.4). Further, zeta potential analysis showed a similar trend of increasing zeta potentials in all the samples (Figure 2.9 D). While “AMK-Coch” showed the highest increase from -23 mV to -58 mV, “AMK-Ca-Coch3” showed the most stable zeta potentials values (-28 mV to -35 mV) over 12 weeks. These observations of change in amikacin loading and zeta potentials could be attributed to the dissociation of outermost bilayer sheets of cochleates by releasing the bridging agents (AMK and/or Ca^{2+}) resulting in a gradual increase of free or loosened bilayers which presents higher negative zeta-potential compared to cochleates. However, we noted that, with the increasing Ca^{2+} content (although at sub-threshold concentrations) in the amikacin cochleates, the stability also increased as expected due to the highly rigid lipid phase nature of Ca^{2+} bridged cochleates compared to “AMK-Coch”. It also pointed to stronger binding and interactions between Ca^{2+} and the lipid headgroups.

Table 2.3 Kinetic parameters estimated by fitting in-vitro release data to various models

Samples	Parameters	Zero-order	First-order	Higuchi	Korsmeyer–Peppas
Pure AMK	k	4.19 mg.h ⁻¹	1.49 h ⁻¹	4.79 mg.h ^{-0.5}	k=1.10 h ⁻ⁿ ; n=1.01
	R ²	0.9915	0.7815	0.9320	0.9983
AMK-Coch	k	0.08 mg.h ⁻¹	0.04 h ⁻¹	0.65 mg.h ^{-0.5}	k=0.06 h ⁻ⁿ ; n=0.84
	R ²	0.9085	0.5422	0.9963	0.9488
AMK-Ca-Coch1	k	0.07 mg.h ⁻¹	0.04 h ⁻¹	0.55 mg.h ^{-0.5}	k=0.02 h ⁻ⁿ ; n=0.98
	R ²	0.9600	0.5996	0.9832	0.9828
AMK-Ca-Coch2	k	0.06 mg.h ⁻¹	0.04 h ⁻¹	0.49 mg.h ^{-0.5}	k=0.03 h ⁻ⁿ ; n=0.90
	R ²	0.9100	0.5670	0.9932	0.9612
AMK-Ca-Coch3	k	0.05 mg.h ⁻¹	0.04 h ⁻¹	0.45 mg.h ^{-0.5}	k=0.04 h ⁻ⁿ ; n=0.80
	R ²	0.8165	0.4374	0.9910	0.9283

Table 2.4 Kinetic parameters of the first-order degradation in amikacin loading of cochleates

Sample	k _{deg} (week ⁻¹)	R ²	t _{1/2} (weeks)
AMK-Coch	-0.026	0.988	26
AMK-Ca-Coch1	-0.017	0.983	41
AMK-Ca-Coch2	-0.011	0.991	62
AMK-Ca-Coch3	-0.007	0.979	96

2.4 Conclusions

We studied the mechanism of formation and functional properties of amikacin-bridged cochleates with and without the presence of sub-threshold concentration of Ca²⁺ and compared that with Ca²⁺-bridged cochleates. A synergistic model of cochleation was proposed when metallic and non-metallic bridging agents were used. Amikacin-bridged cochleates showed lower internal structural rigidity while conventional Ca²⁺-bridged cochleates showed higher rigidity due to stronger interaction of the DOPS head groups with Ca²⁺. Whereas, in the presence of sub-threshold Ca²⁺ concentrations, amikacin and Ca²⁺ synergistically bind to DOPS headgroups leading to form a tightly packed lipid phase resulting in the observed rigidity. As a consequence, cochleates with higher AE, AL and long-term stability were obtained. The in-vitro amikacin release also reflected the tight packing of the internal structures due to increased Ca²⁺ concentrations. The drug release kinetics studied by Higuchi model revealed that the rate constants showed linear correlation with the square root of Ca²⁺ concentrations used to prepare these cochleates. This implies to the

possibility of achieving tuneable drug release by simply varying the Ca^{2+} concentrations in sub-threshold range. Further, the long-term stability measured by half-lives of the cochleates showed positive effect of using Ca^{2+} in sub-threshold concentrations. Thus, the insights provided by this study on the formation of cochleates by non-conventional bridging agents helps in enabling outcome driven designing of cochleate formulations for future pharmaceutical products.

CHAPTER – 3

PREPARATION OF ARTEMISININ-LOADED COCHLEATES AND THEIR ALGINATE COATING FOR EFFECTIVE ORAL ADMINISTRATION

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3.1 Synopsis

Poor aqueous solubility is a major problem faced in the formulation of active pharmaceutical ingredients. Although several formulation strategies have been proposed to solve this problem, modest success has been achieved in meeting the requirements of commercially viable drug delivery systems. Therefore, development of new drug delivery systems for effective delivery of poorly water-soluble drugs is necessary. In the current chapter, we report the development of cochleate-based formulation for the oral administration of poorly water-soluble drug, artemisinin (ART). Cochleates offer great potential as vehicles for the oral delivery of ART due to their high drug encapsulation and controlled-release capabilities. The optimized ART-loaded cochleates showed encapsulation efficiency of $61.8 \pm 9.1\%$ and drug loading of 71.5 ± 4.9 mg ART/g. We developed for the first time, a facile method of coating cochleates by treatment with sodium alginate (ALG). The coated ALG-cochleates gave better control over in-vitro ART release under simulated gastro-intestinal conditions than uncoated SEL-cochleates. ALG-cochleates showed a cumulative

ART release of ~12%, while SEL-cochleates showed double ~22% release in simulated gastric fluid (SGF) over the first 1 h. In simulated intestinal fluid (SIF), the SEL-cochleates and ALG-cochleates showed a cumulative release of almost 100% and ~85%, respectively after 12 h. The controlled release is attributed to crosslinking of the alginate with surface Ca^+ on the cochleates and protonation of the carboxylate groups of the alginate. Freeze-dried powder of ALG-cochleates showed high storage stability over 12 weeks. This study highlights the advantages of loading ART in cochleates and demonstrates the benefits of coating these cochleates with alginate for enhanced oral delivery of ART and similar drugs.

3.2 Materials and methods

3.2.1 Chemicals and materials

Lipids, 1,2-dioleoyl-sn-glycero-3-phospho-L-serine [sodium salt] (DOPS) and 1,2-dimyristoyl-sn-glycero-3-phospho-L-serine [sodium salt] (DMPS) powders were purchased from Avanti Polar Lipids Inc. Artemisinin, Calcium chloride, sodium chloride, TRIZMA[®] base [2-amino-2-(hydroxymethyl)-1,3-propanediol] (Tris), sodium alginate (low viscosity), ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma-Aldrich. Polyethylene glycol (PEG-8000) was purchased from Thermo Fisher Scientific and dextran-500,000 was purchased from Acros Organics. All other solvents, chemicals and reagents used in the study were of analytical grade.

3.2.2 Preparation of ART-loaded cochleates by trapping film method

To prepare cochleates by trapping film method, small unilamellar vesicles (SUVs) of the lipids were obtained first by thin film hydration method before treatment with CaCl_2 . Briefly, 20 mg of lipid (DOPS or DOPS&DMPS) was dissolved along with pre-determined amount of ART (based on lipid:ART molar ratio) in 2 mL chloroform:methanol (3:1 v/v) in a round bottom flask. The solution was evaporated under vacuum (using Buchi R-210 Rotavap) to give a thin film. Traces of solvents were removed by placing the flask under vacuum for 1 h. The dried lipid film was then hydrated with 4 mL Tris buffer (25 mM Tris + 150 mM NaCl; pH 7.4) to final lipid concentration of 5 mg/mL (~6.2 mM). The resulting suspension was subjected to sonication for 5 min followed by extrusion through polycarbonate membrane of 100 nm pore size using a mini extruder (Avanti Polar Lipids Inc.) to produce uniform dispersion of SUVs. To the suspension of SUVs (4 mL), 250 μL of CaCl_2 stock solution (100 mM CaCl_2 in 25 mM Tris + 150 mM NaCl; pH 7.4) was added slowly at a rate of 10 $\mu\text{L}/\text{min}$ using a syringe pump under vigorous stirring, until a final CaCl_2 concentration of 6.2 mM was reached. Stirring was continued for 6 h. All processes including hydration, sonication and extrusion were carried out above the chain melting temperature (T_m) of the lipids. The final suspension containing ART-loaded cochleates was stored at 4 °C for immediate use, and for long-term use, they were stored as a freeze-dried powder at 4 °C.

3.2.3 Preparation of ART-loaded cochleates by hydrogel isolation method

A 1 mL suspension of SUVs (lipid concentration of 20 mg/mL) containing predetermined amount of ART (ART-SUVs) was obtained as mentioned earlier.

The SUVs suspension was mixed with 1 mL of 40% (w/v) dextran-500,000 to get a ratio of 1:1 (v/v). This mixture was then injected using a syringe into equal volume (2 mL) of 15% (w/v) PEG-8000 under constant stirring. At this stage, an aqueous–aqueous emulsion (4 mL total volume) of ART-SUVs/dextran droplets dispersed in a PEG continuous phase was obtained with a final lipid concentration of 5 mg/mL (~6.2 mM). A stock solution of 100 mM CaCl₂ (in 25 mM Tris + 150 mM NaCl; pH 7.4) was added to the emulsion slowly at a rate of 10 µL/min using a syringe pump to a final CaCl₂ concentration of 6.2 mM, under vigorous stirring. Stirring was continued for 6 h to allow the slow formation of small-sized cochleates. The polymers were washed by the addition of 8 mL of washing buffer (25 mM Tris + 150 mM NaCl; pH 7.4) containing 1 mM CaCl₂ by repeated centrifugation at 6000×g for 30 min. All processes, except the final washing, were carried out above the chain melting temperature (T_m) of the lipids. After removal of the polymers, the obtained ART-loaded cochleates were suspended in 4 mL of washing buffer and stored at 4 °C for immediate use, and for long-term use, they were stored as freeze-dried powder at 4 °C.

3.2.4 Characterization of ART-loaded cochleates

Structure and morphology of the cochleates were analysed using SEM as described previously in Section 2.2.3. Particle size and zeta potential of the SUVs and cochleates were measured as described in Section 2.2.4. IR spectroscopy and SAXS analyses of cochleates were performed as described in Section 2.2.8 and Section 2.2.9, respectively.

3.2.5 Determination of encapsulation efficiency and drug loading

Freshly prepared ART-loaded cochleates suspension was centrifuged at 6000×g for 10 min to separate cochleates from the supernatant. Pellets containing cochleates were resuspended in de-ionized water and freeze-dried under vacuum to obtain cochleate powder (buffer free). To accurately weighed freeze-dried cochleates (about 2 mg), 100 µL of EDTA (100 mM) prepared in 0.1 N NaOH (pH > 10) was added to disrupt the cochleate structure. The resulting suspension of large unilamellar vesicles was then solubilized by adding methanol to release the encapsulated ART. The amount of ART encapsulated in the cochleates was determined by using UV–Vis spectrometry (Thermo Evolution™ 300 UV-Vis Spectrophotometer) by measuring the absorbance at 291 nm using pure ART as the external standard reference according to the reported procedure.¹³⁹ The encapsulation efficiency (EE) in percentage (Eq. 3.1), drug loading (DL) in mg ART/g of cochleates (Eq. 3.2) and overall product yield in percentage (Eq. 3.3) were calculated as follows,

$$EE = \frac{\text{Total ART encapsulated in cochleates (mg)}}{\text{Total ART taken to prepare cochleates (mg)}} \times 100\% \dots (\text{Eq. 3.1})$$

$$DL = \frac{\text{Amount of ART encapsulated in cochleates (mg)}}{\text{Total amount of cochleates taken (mg)}} \times 10^{-3} \dots (\text{Eq. 3.2})$$

$$\text{Yield} = \frac{\text{Amount of recovered cochleates (mg)}}{\text{Total theoretical amount of lipids+ART taken (mg)}} \times 100\% \dots (\text{Eq. 3.3})$$

3.2.6 Alginate coating of the ART-loaded cochleates

Aqueous solution of sodium alginate (2.5% w/v) was prepared in Tris buffer (25 mM Tris + 150 mM NaCl; pH 7.4). The ART-loaded cochleate suspension was prepared in Tris buffer to get an equivalent concentration of 5 mg/mL in the final

reaction mixture and an appropriate volume of ALG stock solution was added to achieve the desired concentration. The total volume of the reaction mixture was maintained constant (400 μ L) by adjusting the volume of Tris buffer. The suspension was then kept under constant stirring for 3 h. Further, the coated ART-loaded cochleates were separated by centrifugation at 6000 $\times$ g for 20 min and the obtained pellet was washed three times by resuspending in ALG-free Tris buffer (25 mM Tris + 150 mM NaCl; pH 7.4) to remove excess alginate. The obtained alginate-coated ART-loaded cochleates (referred to as ALG-cochleates) were stored at 4 °C for immediate use and for long-term use, they were stored as a freeze-dried powder at 4 °C.

3.2.7 In-vitro artemisinin release from ART-cochleates

The in-vitro release profile of ART from ART-loaded cochleates was studied using dialysis bag diffusion technique in phosphate buffered saline (PBS, physiological of pH 7.4) at physiological temperature of 37 °C.^{53,54} The ART-loaded cochleate suspension encapsulating 1 mg equivalent of ART was introduced in a dialysis bag (cellulose membrane, molecular weight cut-off 14,000 Da) and immersed in 50 mL of release medium maintained at 37.0 $\pm$ 0.5 °C with continuous stirring at 100 rpm. The ART release was monitored for 24 h by withdrawing samples at pre-determined time points and the volume of samples withdrawn was replaced by fresh buffered saline to maintain sink conditions. The samples were analysed for ART using UV-Vis spectrophotometer. In addition, ART release was conducted in simulated gastric fluid (SGF, pH 1.2) and simulated intestinal fluid (SIF, pH 6.8).³⁸ SGF and SIF were prepared according to US Pharmacopeia (USP) standards. Release studies in SGF and SIF were also performed using dialysis bag diffusion technique as

described above. Samples of SGF were neutralized as soon as withdrawal by adding 0.1 N NaOH to facilitate the analysis of ART by UV-Vis spectrophotometer (Section 3.2.5).

$$\% \text{ ART release} = \frac{\text{Cumulative amount of ART in a release sample (mg)}}{\text{Total amount of ART taken as cochleates (mg)}} \times 100 \quad \dots$$

(Eq. 3.4)

3.2.8 Long-term stability studies of the ART-cochleates

To study long-term storage stability, ART-cochleate samples were stored in the form of suspensions (in 25 mM Tris + 150 mM NaCl + 6.17 mM CaCl₂; pH 7.4; alginate coated cochleates suspensions were stored in CaCl₂-free buffer) and freeze-dried powders at 4 °C for 12 weeks. Parameters such as particle size (diameter), zeta potential and ART-loading were analysed to study the storage stability of these formulations.

3.3 Results and discussion

3.3.1 Preparation and characterization of ART-DOPS-cochleates

We initially prepared the ART-DOPS-cochleates by the trapping film and the hydrogel isolation methods using DOPS:ART molar ratio of 10:1 to examine the suitability of the two methods. The cochleates were formed by treating the ART-loaded DOPS SUVs with Ca²⁺ (molar ratio is 1:1) followed by purification using dialysis bag to remove residual buffers and salts used during their formation. SEM images of the prepared cochleates using both methods showed typical rolled-up and elongated cylindrical structure of cochleates (Figure 3.1). The ART-DOPS-cochleates from both methods showed similar average diameter of 218.2±43.1 nm and 205.4±12.7 nm, respectively (Figure 3.1). Both also showed

similar EE of ~50%. Since there were no significant differences in the encapsulation and average particle size and since the trapping film method was simple to perform, gave reproducible and consistent results, it was used throughout this work.

Next, using the trapping film method, we prepared cochleates with variable molar ratios of ART w.r.t, DOPS to examine the optimum ratio at which high encapsulation efficiency (EE) and drug loading (DL) of ART can be achieved. At DOPS:ART molar ratios of 10:10 (equimolar), 10:2 and 10:1, the cochleates showed EE of 6.2 ± 2.8 , 34.8 ± 5.4 and 52.8 ± 4.7 %, respectively, and a corresponding DL of 21.4 ± 0.6 , 20.3 ± 1.1 and 18.8 ± 0.9 mg ART/g of cochleates, respectively. Based on these results, the EE increased as the ratio of ART decreases, while DL is marginally influenced and even slightly decrease as EE increases. However, for successful development of drug delivery systems, high EE and DL are required. Hence, a combination of cholesterol (Cho) with DOPS and another phosphatidylserine (PS) (as they are known to readily form cochleates), DMPS was used with DOPS to prepare the cochleates in anticipation to increase the EE and DL.

3.3.2. Preparation of the ART-DOPS&Cho-cochleates and comparison to ART-DOPS-cochleates

Trapping film method was used with various DOPS:Cho:ART ratios of 9:1:2 and 9.5:0.5:2 (molar) to prepare ART-DOPS&Cho-cochleates. A slightly higher EE of 42.4 ± 3.3 % and DL of 27.1 ± 0.9 mg ART/g of cochleate were observed for DOPS:Cho:ART ratios of 9:1:2 compared to ART-DOPS-cochleates alone (EE of 34.8 ± 5.4 %, and a corresponding DL of 20.3 ± 1.1 mg ART/g of cochleates).

Further, the EE and DL for cochleates prepared with DOPS:Cho:ART ratios of 9.5:0.5:2 was comparable to that of ART-DOPS-cochleates. But structural analysis of these samples revealed aggregates and sheets with only fewer well-formed cochleate structures (Figure 3.2). Cholesterol is known to stabilize lipid membranes and aid in improving the drug encapsulation.¹⁴⁰ However, DOPS:Cho ratio was previously reported to have a significant effect on the formation of cochleates.³⁸ Pham et al., reported that the formation of cylindrical cochleate particles did not occur when cholesterol proportion was 20% or higher while, DOPS:Cho ratio of 9:1 resulted in the formation of cochleates due to lower cholesterol proportion.³⁸ Nevertheless, we did not observe well-formed cochleate structures with DOPS&Cho lipid system even with much lower cholesterol concentration.

Figure 3.1 SEM images of ART-DOPS-cochleates (DOPS:ART, 10:1) prepared by (A) trapping film method and (B) hydrogel isolation method

Figure 3.2 Cochleates prepared using DOPS&Cho showing aggregates and large sheets (dotted-line arrow heads) with fewer well-formed cochleates (solid line arrow heads) at DOPS:Cho:ART molar ratios of (A, B) 9:1:2 and (C, D) 9.5:0.5:2

3.3.3 Preparation of the ART-DMPS-cochleates and ART-DOPS&DMPS-cochleates, and comparison to ART-DOPS-cochleates

Using the trapping film method, we prepared the ART-DMPS-cochleates (DMPS:ART, 10:4 molar) and ART-DOPS&DMPS-cochleates using DOPS&DMPS lipid combination at various DOPS:DMPS:ART ratios of 9:1:4, 8:2:4 and 6:4:4 (molar) (Table 3.1). For comparison, we also prepared the ART-DOPS-cochleates where the DOPS:ART is 10:4 (molar) (Table 3.1). All of the prepared cochleates using pure DMPS, various combinations of DOPS&DMPS and pure DOPS showed well-formed cochleate structures (Figure 3.3) and were

obtained in over 96% yield with a diameter between 218 nm and 399 nm (Table 3.1). The rolling of bilayer sheets to produce the cylindrical morphology of the cochleates is clearly seen (Figure 3.3). We noted that the diameter of the ART-DOPS&DMPS-cochleates is roughly double that of ART-DMPS-cochleates or ART-DOPS-cochleates (Table 3.1). This is attributed to the integration of DMPS with DOPS forming homogeneous lipid bilayer and causing the enlargement due to the difference in their acyl chain length after they are rolled-up by Ca^{2+} . Again, the dispersity of the ART-DOPS&DMPS-cochleates is also much higher than that of ART-DMPS-cochleates or ART-DOPS-cochleates (Table 3.1). Empty DOPS&DMPS-cochleates (without using ART) were also prepared (Figure 3.3 F). These empty DOPS&DMPS-cochleates (DOPS:DMPS 9:1) showed a similar structure or morphology to ART-DOPS&DMPS-cochleates. The well-formed structure of ART-DOPS&DMPS-cochleates is the result of similarity in the head-groups of DOPS and DMPS which exert comparable structure and membrane fluctuation properties allowing for the formation of the integrated lipid-bilayers and consequent rolling-up to form the cochleates upon addition of Ca^{2+} .¹⁴¹ In contrast, combination of PS with neutral lipids such as phosphatidylcholine (PC) resulted in PS clustering and formation of long bilayer sheets due to restricted membrane dehydration capacity of PC upon the addition of cations (e.g. Ca^{2+}) which may inhibit the rolling-up process to form cochleates.^{142,143}

Figure 3.3 ART-loaded cochleates made from different lipid compositions,
(A) DOPS:ART, 10:4; (B) DMPS:ART, 10:4; (C) DOPS:DMPS:ART,
9:1:4; (D) DOPS:DMPS:ART 8:2:4; (E) DOPS:DMPS:ART 6:4:4 and (F)
Empty DOPS&DMPS-cochleates (without ART; DOPS:DMPS 9:1)

Table 3.1 Effect of the lipid on the characteristics of ART-loaded cochleates

Sl. No.	Composition (Lipid:ART, 10:4)	Product yield (%)	Diameter (nm)	Dispersity	EE (%)	DL (mg ART/g of cochleate)
1	DOPS	98.4±0.8	218.2±43.1	0.18	18.6±1.2	22.0±0.6
2	DMPS	96.7±1.3	185.8±20.3	0.15	12.2±0.9	15.6±0.5
3	DOPS&DMPS (9:1)	98.9±0.9	399.3±147.4	0.37	61.8±9.1	71.5±4.9
4	DOPS&DMPS (8:2)	98.2±0.6	389.9±159.7	0.41	49.5±6.7	64.6±7.1
5	DOPS&DMPS (6:4)	97.5±1.1	298.2±97.5	0.55	49.5±9.5	60.5±2.9

3.3.4 FTIR analysis of the cochleates

We measured the IR spectra of ART, empty DOPS&DMPS-cochleates (prepared without using ART) and ART-DOPS&DMPS-cochleates (DOPS:DMPS:ART, 9:1:4) to provide evidence for successful ART encapsulation. The FTIR spectrum of pure ART showed a stretching vibration at 1737 cm^{-1} corresponding to the lactone —C=O and another vibration at 883 cm^{-1} corresponding to the endo-peroxide bridge (Figure 3.4 A).^{72,144} Spectral analysis of the empty DOPS&DMPS-cochleates showed characteristic peaks of the lipids at $\sim 1737\text{ cm}^{-1}$ attributed to the ester —C=O , at 2852 cm^{-1} and 2923 cm^{-1} attributed to the CH_2 symmetric and asymmetric stretching, respectively, and at 1063 cm^{-1} and 1236 cm^{-1} attributed to the —PO_2 symmetric and asymmetric stretching, respectively (Figure 3.4 A). The —PO_2 stretching values confirm the dehydration of phosphate groups due to the formation of a bidentate complex between Ca^{2+} and the phosphate ester of the lipid head groups; an important requirement for the formation of cochleate structures.^{44,145} The ART-DOPS&DMPS-cochleates showed the characteristic stretching of the lactone and ester —C=O , —CH_2 and —PO_2 at similar values as shown by ART and empty DOPS&DMPS-cochleates (Figure 3.4 A). Additionally, the characteristic endo-peroxide bridge vibrational band of ART was clearly observed at 883 cm^{-1} confirming the encapsulation of ART in the ART-DOPS&DMPS-cochleates (Figure 3.4 B).

Figure 3.4 (A) FTIR spectra of (a) pure ART, (b) Empty DOPS&DMPS-cochleates and (c) ART- DOPS&DMPS-cochleates; (B) expansion of the FTIR region corresponding to the endo-peroxide bridge stretching

3.3.5 SAXS analysis of the cochleates

SAXS analysis of the prepared ART-DOPS-cochleates, ART-DMPS-cochleates and ART-DOPS&DMPS-cochleates showed diffraction patterns with an intense Bragg's reflection along with higher order weak reflections, typical of cochleate systems corresponding to the presence of uniform multilamellar structure (Figure 3.5). The first reflection peak in the diffraction pattern corresponds to the characteristic domain spacing in the cochleates (or the interlamellar repeat

distance) (Figure 3.5). ART-DOPS-cochleates showed an interlamellar repeat distance of 51.3 Å with sharp, equidistant Bragg's reflections at 0.12 Å and 0.25 Å which agreed with the results reported elsewhere.³⁰ Further, ART-DMPS-cochleates showed sharp reflections at 0.14 Å and 0.29 Å with an interlamellar repeat distance of 43.4 Å as reported for DMPS-Ca²⁺ complexes.¹⁴⁶ However, SAXS patterns of ART-DOPS&DMPS-cochleates (DOPS:DMPS:ART was 9:1:4 and 6:4:4) showed interlamellar repeat distances of 50.7 Å and 48.6 Å, respectively and reflections at 0.13 Å and 0.25 Å, and 0.13 Å and 0.26 Å, respectively. The bilayer repeat distance values of ART-DOPS&DMPS-cochleates (both, DOPS:DMPS:ART = 9:1:4 and 6:4:4) was found to be in between the range of repeat distance values for ART-DOPS-cochleates and ART-DMPS-cochleates. These intermediate values may be attributed to the homogenous mixing of DOPS and DMPS lipids throughout the cochleate bilayer structures. Further experiments were not conducted to study the homogeneity or lipids clustering since well-formed cochleate structures were observed.

3.3.6 Encapsulation efficiency and ART loading in the cochleates

All of the cochleates prepared using a mixture of DOPS&DMPS gave better EE and DL than cochleates made from either pure DOPS or pure DMPS (Table 3.1). Initially, for cochleates prepared with DOPS:DMPS:ART ratio of 9:1:2 we obtained an EE of 45.9±7.6% and a corresponding DL of 30.5±2.5 ART/g of cochleates. Further, ART-DOPS&DMPS-cochleates with 9:1:4 mole ratios gave the highest EE of 61.8±9.1% and highest DL of 71.5±4.9 mg ART/g of cochleates (Table 3.1). ART-DOPS-cochleates (10:4) and ART-DMPS-cochleates (10:4) showed similar EE below 18% and DL below 22 mg ART/g of cochleates. The EE and DL of ART-DOPS&DMPS-cochleates (9:1:4, Table

3.1, entry 3) are higher or at par with recent reports on lipid formulations containing ART and its derivatives. For example, ART-loaded PEGylated long-circulating liposomes showed an EE of ~70.0% while liposomal formulations of dihydroartemisinin and artemether showed EE of 69% and 66%, respectively.^{85,89,91} Artemether-loaded lipid nanoparticles showed a loading capacity of 77.4 mg/g of lipid nanoparticles while chitosan-coated, artesunate-loaded lipid nano capsules showed drug loading capacity of 17 mg/g of particles.^{75,93}

Figure 3.5 SAXS patterns showing typical Bragg's peaks characteristic of multilamellar cochleate systems, (a) DOPS:ART, 10:4; (b) DMPS, 10:4; (c) DOPS:DMPS:ART, 9:1:4; and (d) DOPS:DMPS:ART 6:4:4

3.3.7 Coating of cochleates with alginate

We selected ART-DOPS&DMPS-cochleates (9:1:4) (herein referred to as SEL-cochleate) with the best EE and DL (Table 3.1, entry 3) and treated it with alginate in anticipation of achieving controlled-release of ART and enhancing its stability. Achieving controlled drug release from nanoparticles with high surface area such as cochleates is desirable and challenging at the same time.⁹⁹

Since a major limitation in the oral delivery of ART is its rapid gastro-intestinal transit time, coating with mucoadhesive agents should delay the gastric emptying and increase the residence time.¹⁰⁰ Although cochleates are known to be stable due to their tightly packed multilamellar structure, some studies have shown that cochleates dissociate under the low pH gastric conditions resulting in the drug release.³⁸ Alginate (ALG) is a natural biopolymer which tend to shrink at acidic pH, swell at neutral pH, and most importantly, show controlled-release characteristics especially in gastro-intestinal milieu making it suitable for oral administration; and therefore, it was selected in this study to coat cochleates (Figure 3.6).^{103,104}

Figure 3.6 Schematic for coating ART-loaded cochleates with alginate

SEL-cochleates was coated by treatment with varying concentrations of ALG (0, 0.5, 1.0, 1.5 and 2.0% w/v). Under these conditions, partial coating of the cochleates is expected. The coated cochleates retained a DL of 64–68 mg ART/g of cochleates and showed a high overall product yield (92–95%) (Table 3.2). Further, the coated cochleates were characterized using SEM, SAXS, FTIR and their zeta potential values measured. In all cases, intact cochleate structures were obtained, however, with some differences in the size and extent of aggregation. SEM images of the cochleates prepared using ALG concentration of 0.5 and 1%

(W/V) showed cochleate structures with similar extent of aggregation as SEL-cochleates. However, the cochleates prepared using 1.5% and 2.0% of ALG concentrations showed smaller and better-dispersed aggregates of cochleates (Figure 3.7).

Figure 3.7 SEM images of SEL-cochleates after treatment with different concentrations of ALG (%W/V), (A) 0% ALG (SEL-cochleates); (B) 0.5% ALG; (C) 1.0% ALG; (D) 1.5% ALG and (E) 2.0% ALG

3.3.8 SAXS analysis of the ALG-cochleates and comparison to SEL-cochleates

In support of the results from SEM images (Figure 3.7), SAXS analysis of the ALG-cochleates confirmed the intact cochleate structure after ALG coating. The SAXS patterns of the SEL-cochleates and the ALG-cochleates (SEL-cochleates coated using 2.0% w/v ALG) were similar with sharp reflections at 0.13 Å and 0.25 Å and with bilayer repeat distance of 50.7 Å (Figure 3.8). Further, the presence of a monotonic increase in the SAXS signal towards small angle region in ALG-cochleates may be due to the diffused scattering caused by smaller, well-dispersed cochleate particles, further, supporting the observation made from SEM images (Figure 3.7 E).⁴¹

Figure 3.8 (A) SAXS patterns showing typical Bragg's peaks characteristic of multilamellar cochleate systems in (a) SEL- cochleates; (b) ALG-cochleates (2.0% w/v ALG), and (B) FTIR spectra of (a) sodium alginate; (b) SEL-cochleates; (c) ALG-cochleates (1.5% w/v ALG)

3.3.9 FTIR analysis of ALG-cochleates and comparison to SEL-cochleates

FTIR spectral analysis confirmed the crosslinking of ALG with SEL-cochleates. The FTIR spectrum of sodium alginate shows two characteristics bands at 1596

cm^{-1} and 1404 cm^{-1} , corresponding to the asymmetric and symmetric vibrational stretching of the carboxylate group, respectively (Figure 3.8 B).¹⁰⁹ Additionally, it also shows strong broad bands at $\sim 3350\text{ cm}^{-1}$ characteristic of the stretching vibration of the O—H groups of ALG. The spectrum of the ALG-cochleates also shows the above characteristics peaks but with a small shift of the asymmetric and symmetric vibrational stretching of the carboxylate group to 1590 cm^{-1} and 1414 cm^{-1} , respectively, proving successful crosslinking of the alginate carboxylate group with Ca^{2+} (Figure 3.8 B).¹⁴⁷ It is important to note that during formation of cochleates, Ca^{2+} interacts with PS head groups located both in the plane of only one bilayer and the two apposed bilayer membranes leading to the formation of cis- and trans-complexes, respectively, and subsequently promote rolling of bilayer sheets.²⁵ Thus, resulting in the formation of cochleate cylinders when PS to Ca^{2+} mole ratio of 2 to 1 is used (note that the ratio used in our study PS: Ca^{2+} was 1:1).²⁹ During this process, the PS headgroups on the exposed cochleate-surface are left with valent Ca^{2+} which was recently shown by Bozo et al., as the main cause of aggregation in cochleates.⁴¹ Further, the interaction of sodium alginate with Ca^{2+} was described by the egg-box model where the electronegative cavities on alginate are involved in both unidentate and bidentate coordination modes with Ca^{2+} leading to crosslinking.^{107–109} Hence, the FTIR data confirms the association and crosslinking of ALG with SEL-cochleates. Thus, the coordination between exposed Ca^{2+} on the surface of the cochleates and the carboxylate groups of ALG facilitated the interaction through the crosslinking and association effects rather than uniform layer-by-layer coating on the cochleate particles.¹⁴⁸

3.3.10 Zeta potential of ALG-cochleates

In order to understand the change in zeta potential values over the different stages of preparation of ALG-cochleates, we measured the zeta potential of ART-loaded DOPS&DMPS SUVs, SEL-cochleates and ALG-cochleates (Table 3.2). There was an increase in the value of zeta potential from -66.4 ± 2.7 mV for ART-loaded DOPS&DMPS SUVs to -41.6 ± 1.4 mV for SEL-cochleates and then a general trend of decrease in the value as the concentration of ALG to make the ALG-cochleates increases (Table 3.2). The increase in zeta potential is attributed to the complexation of the negatively charged phosphatidylserine with Ca^{2+} and consequently, partial charge neutralization. Further, the decrease in the zeta potential upon treatment of SEL-cochleates with increasing concentrations of ALG (0–2% w/v) is due to crosslinking and association of negatively charges ALG with surface Ca^{2+} on SEL-cochleates (Table 3.2). These results agree with the conclusions obtained from FTIR (Figure 3.8 B).

We observed a significant increase in zeta potential of ALG-cochleates only for ALG concentrations $>0.5\%$ (w/v) used to treat SEL-cochleates (data is not shown for ALG concentration $<0.5\%$ w/v). In addition, at much higher ALG concentration of 1.5 and 2.0% (w/v) this effect was more prominent indicating higher degree of association of negatively charged ALG on surface Ca^{2+} of SEL-cochleates (Table 3.2). This increase in zeta potential values may also come from the negative carboxylate groups of ALG after association with SEL-cochleates. At concentrations $<0.5\%$ (w/v), repulsive interaction between ALG and SEL-cochleates may restrain ALG from gaining access to surface Ca^{2+} and this repulsive force is overpowered by ALG for concentrations $>0.5\%$ (w/v) leading to their association. Further, coating of ALG with SEL-cochleates may have

caused the ALG-cochleate to show better dispersion as seen from Figure 3.7 D, E, and Figure 3.8 A.

3.3.11 In-vitro ART release studies

The in-vitro release profiles of ART from SEL-cochleates and ALG-cochleates (1.5% w/v ALG) in phosphate buffered saline (PBS, pH 7.4), simulated gastric fluid (SGF, pH 1.2) and simulated intestinal fluid (SIF, pH 6.8) are shown in (Figure 3.9). In PBS, both SEL-cochleates and ALG-cochleates showed a similar biphasic drug release profile and ALG-cochleates showed better controlled-release of ART than SEL-cochleates (Figure 3.9 A). A rapid burst release was seen up to 2 h, within which ~45% and ~35% of ART was released from the SEL-cochleates and ALG-cochleates, respectively (Figure 3.9 A). Another relatively slow and continuous release phase lasted for almost 24 h and showed a cumulative release of ~90% and ~84% from SEL-cochleates and ALG-cochleates, respectively (Figure 3.9 A). This biphasic ART release is attributed to the strong interaction of hydrophobic ART with the lipidic- bilayer structure of cochleates. Similar biphasic drug release profiles from cochleates have been reported for different drugs including amphotericin B, miltefosine, recombinant factor VIII, fisetin, doxorubicin, etc.^{38,48,52–54} Overall, ALG-cochleates showed better controlled release of ART than SEL-cochleates (Figure 3.9 A). The ALG associated with the surface of ALG-cochleates may be the reason for better controlled release since ART molecules have to diffuse through an additional barrier layer of ALG.

In SGF, SEL-cochleates and ALG-cochleates showed a cumulative ART release of ~22% and ~12%, respectively, over 1 h (Figure 3.9 B). These results agree

with the observation of Pham et al.,³⁸ and indicates unrolling (de-cochleation) of the cochleate bilayer sheets, and contrasts with the observation of Zarif who found cochleates to be stable with tightly-packed lipid bilayer sheets under the gastrointestinal conditions.¹⁵ Nevertheless, it was evident from the release results that even under these conditions, cochleates showed controlled release of ART. Upon changing the release medium to SIF, the SEL-cochleates and ALG-cochleates showed a cumulative release of ~62% and ~43%, respectively after 2 h. Further, all of the ART encapsulated in the SEL-cochleates was released into the SIF by 12 h, whereas the ALG-cochleates showed a cumulative release of ~85%. The lower rate of ART release from the ALG-cochleates in both SGF and SIF is attributed to the ALG coat. Under gastric conditions (low pH values, SGF), protonation of the carboxylate groups of the alginate occurs and the unionized alginic acid formed renders them less soluble in water. Further, the decreased water uptake shrinks the alginate to form tightly packed alginate-cochleate coat which in turn protects the cochleates and results in the observed reduced ART release.^{147,149} Similar results of drug release from core materials coated with alginate under simulated gastro-intestinal conditions have been reported.^{104,150} On the other hand, alginate swells at neutral pH (SIF, pH 6.8) and reduce the association between alginate and cochleates leading to increased rate of ART release in the intestinal tract which is a necessary requirement for oral administration of ART. However, ART has rapid gastro-intestinal transit time of about 3 h.⁹⁸ Therefore, under gastro-intestinal conditions, coating with ALG is advantageous in providing higher controlled-release characteristics and given the fact it also has desirable mucoadhesive properties which might help in

avoiding gastric emptying of the cochleates thus increasing their residence time which is essential for oral absorption of ART.^{104,105}

Figure 3.9 ART release profiles from SEL-cochleates and ALG-cochleates in (A) PBS and (B) SGF-SIF

3.3.12 Long-term stability studies

We stored SEL-cochleates and ALG-cochleates (1.5% w/v ALG) in suspension and freeze-dried powder forms at 4 °C to study their stability since few extensive long-term stability studies of drug-loaded cochleates have been reported. Samples of these cochleates were assessed by measuring changes in their particle size (diameter), zeta potential values and drug loading over 12 weeks (Figure 3.10). All the cochleates showed an overall reduction in the particle size. However, the reduction was lower for freeze-dried cochleates compared to cochleates in suspensions suggesting better stability (Figure 3.10 A). ALG-cochleates stored as powder showed the least change in particle size. This is in contradiction to previous studies which reported no observable trend and/or change in the particle size of freeze-dried cochleates.^{38,48} The bilayer sheets of cochleates are known to open-up into large vesicles under Ca^{2+} deficient

conditions.¹⁶ The reduction in size could be attributed to the dissociation of outer bilayer sheets of cochleates due to the loss of Ca^{2+} on prolonged storage, especially for samples stored as suspension we believe this effect to be significant. Both the freeze-dried and cochleates in suspension forms showed an overall increase in the zeta potential values. Especially, both suspension and freeze-dried SEL-cochleates showed a drastic increase in the zeta potential from -35 mV to -55 mV and from -33 mV to -50 mV, respectively (Figure 3.10 B). This trend agreed with the report by Asprea et al., but contradicted with the observation by Pham et al., who reported no change in the zeta potential values under these conditions.^{38,48} This observation may also be attributed to the loss of Ca^{2+} on cochleate-surface or to the dissociation of outermost bilayer sheets forming free PS vesicles which present higher negative zeta-potential compared to cochleates (Table 3.2). Overall, there was a reduction in ART-loading over time from all cochleate samples (Figure 3.10 C). In general, freeze-dried samples retained higher ART-loading over the period of storage compared to cochleates in suspension form. Freeze-dried ALG-cochleates showed the highest retention of ART-loading ($\sim 85\%$ corresponding to 51.7 mg ART/g cochleates) compared to SEL-cochleate samples ($\sim 75\%$ corresponding to 48.8 mg ART/g cochleates). The stability of these cochleates and their ability to retain ART is superior to several liposomal and other lipid-based formulations of ART. For example, ART loading in a conventional liposome and PEGylated liposome formulations after only 25 days was found to be under 80% .⁸⁹ Artesunate-loaded-chitosan-coated lipid nano capsules showed a reduction of over 25% in the drug loading after storage for 30 days.⁹³ Overall, ALG-cochleates in their freeze-dried powder form were the most stable during the storage period of 12 weeks.

Figure 3.10 Effect of storage on (A) particle size; (B) zeta potential and (C) drug loading of SEL-cochleates (SEL) and ALG-cochleates (ALG) where Pd. – powder and Sus. – suspension

Table 3.2 Effect of ALG treatment on SEL-cochleates as a function of increasing ALG concentrations

Entry	ALG conc. (% w/v)	Zeta Potential (mV)	DL (mg ART/g cochleates)	Product yield (%)
0 (ART-SUVs)				
1	DOPS&DMPS	-66.4 ± 2.7	—	—
2	0 (SEL-cochleates)	-41.6 ± 1.2	67.3 ± 4.1	93.1 ± 1.2
3	0.5	-46.4 ± 1.5	65.2 ± 2.3	92.2 ± 2.8
4	1.0	-50.8 ± 1.0	64.5 ± 5.2	92.9 ± 4.5
5	1.5	-53.2 ± 1.4	66.2 ± 3.1	93.7 ± 2.4
6	2.0	-56.4 ± 0.5	67.8 ± 3.7	95.4 ± 1.8

3.4 Conclusions

Cochleates as lipid-based supramolecular systems offer great opportunity for effective delivery of drugs having poor bioavailability. ART, a drug recommended by WHO for the treatment of malaria, shows therapeutic limitations due to its poor solubility and rapid metabolism. To overcome these limitations, we formulated ART in stable alginate-coated cochleate system intended for effective oral administration. The ART-loaded alginate-coated cochleates showed higher ART encapsulation and loading capacities along with better controlled-release and increased stability. We optimized this system and found that DOPS&DMPS (9:1) lipids combination offered highest EE of

61.8±9.1% and DL of 71.5±4.9 mg ART/g. The facile alginate-coating technique that we developed achieved controlled-release of ART under conditions mimicking gastro-intestinal tract. The release of ART from this system was controlled by the crosslinking of alginate with surface Ca⁺ on the cochleates and by the carboxylate groups of the alginate in addition to the multilamellar lipid structures of the cochleates. The freeze-dried powder of alginate-coated ART-loaded cochleates (ALG-cochleates) showed high stability when stored at 4 °C for 12 weeks. We believe that the coating of cochleates with alginate could be an effective method to enhance oral administrability of cochleates for the delivery of drugs with low bioavailability like artemisinin.

CHAPTER – 4

CONTINUOUS PRODUCTION OF DRUG-LOADED COCHLEATES USING OFF-THE-SHELF FLOW FOCUSING DEVICE

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4.1 Synopsis

Lipid cochleates have gained increasing interest as drug-carriers. However, their preparation strategies rely on conventional batch processes that are complex, time consuming and lack batch-to-batch reproducibility, presenting a bottleneck for clinical translation. We report an efficient continuous preparation process for artemisinin-loaded cochleates (ART-cochleates) using inexpensive off-the-shelf flow focusing device. By carefully controlling the flow focusing parameters, we showed along with the mechanism that, ART-cochleates of uniform and tuneable size (~180 nm in width and ~1030 nm in length) were obtained with low dispersity (0.18 in width and 0.27 in length), narrow size distribution and high reproducibility compared to the batch process. The device achieved high throughput of 11.5 g/day with ART encapsulation of $64.24 \pm 2.5\%$ and loading of 83.37 ± 3.68 mg ART/g of cochleates. Art-cochleates were non-toxic and showed sustained in-vitro release of ART with effective transepithelial permeability across intestinal Caco-2 monolayer (~60% and ~25% transport for pure ART and ART-cochleates, respectively) resulting in better in-vitro bioavailability.

The off-the-shelf device is envisioned to be highly promising platform for continuous and high-throughput manufacturing of drug-loaded cochleates in a controlled and reproducible manner. It has potential to enable clinical translation of drug-loaded cochleates with predictable drug release, absorption, and bioavailability.

4.2 Materials and methods

4.2.1 Chemicals and materials

Lipids, 1,2-dioleoyl-sn-glycero-3-phospho-L-serine [sodium salt] (DOPS) and 1,2-dimyristoyl-sn-glycero-3-phospho-L-serine [sodium salt] (DMPS) powders were purchased from Avanti Polar Lipids Inc. Artemisinin, Calcium chloride, sodium chloride, TRIZMA® base [2-amino-2-(hydroxymethyl)-1,3-propanediol] (Tris) and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma-Aldrich. All other solvents, chemicals and reagents used in the study were of analytical grade.

The device was assembled using standard parts available in the market purchased from IDEX Health & Science, USA. It was constructed from an off-the-shelf chip P-729 - Cross Assembly PEEK 0.020, F-300; characterized by crossflow geometry with three inlets and one outlet to give two-dimensional (2D) flow focusing (Figure 4.1). The cross assembly was made from inert and chemically resistant polyether ether ketone (PEEK) and was designed with a 500 μm thru hole that delivers a low swept volume of 0.72 μL . The inlets of the chip were connected to the syringes using Teflon (PTFE) tubes with an inner diameter (i.d.) of 500 μm and 1/16" outer diameter (o.d.) via F-300 fittings provided with the cross assembly. Outlet was also a PTFE tube of 500 μm i.d., 1/16" o.d. and length

(l) 1000mm connected to chip via F-300 fittings. SPLab02 syringe pumps purchased from Baoding Shencheng Precision Pump Co. Ltd, China, were used to control the flow rate of the liquids pumped through the device.

4.2.2 Preparation of ART-cochleates by trapping film method

ART-cochleates were prepared by trapping film method as described in Section 3.2.2. Herein, ART-cochleates prepared by the modified trapping film method will be referred to as “ACB – Artemisinin Cochleates from Batch”. In addition, cochleates without ART were prepared similarly as control samples for the in-vitro assays using the same lipid composition (DOPS:DMPS; 9:1) and are referred to as “EC - Empty Cochleates”.

4.2.3 In-flow preparation of ART-cochleates using off-the-shelf device

The ART-cochleates were prepared by injecting a solution of ART-lipid mixture (DOPS:DMPS:ART in 9:1:4, mole ratio) in methanol from the central channel of the cross-assembly, while injecting aqueous Tris- Ca^{2+} buffer (5 mM CaCl_2 in 25 mM Tris + 75 mM NaCl; pH 7.4) into the two side channels to intersect with the solution from the central channel inside the chip (Figure 4.1). The methanol stream with the ART-lipids mixture was introduced into the central channel using a gastight glass syringe and the aqueous Tris- Ca^{2+} buffer was introduced into the side channels using plastic syringes with the help of syringe pumps (Figure 4.1). All fluids were filtered prior to injection using 0.2 μm pore size filters to prevent contamination and clogging of the device by particulate matter.

Figure 4.1 Schematic representation of continuous ART-cochleate production in off-the-shelf cross-assembly chip using 2D flow focusing with highlighted possible mechanism of cochleate formation inside the PTFE microchannel

The ART-cochleates were synthesized using the off-the-shelf device under varying flow focusing conditions of flow rate ratios (FRR) and total volumetric flow rates (Q_t) (or average flow velocities, v_f). The FRR is defined as the ratio of the buffer volumetric flow rate (Q_b) to the methanol volumetric flow rate (Q_m) given by Eq. (4.1), and the total volumetric flow rate (Q_t) is given by Eq. (4.2).

$$FRR = \frac{2Q_b}{Q_m} \dots (\text{Eq. 4.1})$$

$$Q_t = 2Q_b + Q_m \dots (\text{Eq. 4.2})$$

The ART-cochleates were collected as a suspension from the outlet of the mixing channel (PTFE microchannel connected to the chip with length, $l=1000$ mm) in centrifugation tubes, stored at 4 °C for immediate use, and for long-term use, they were stored as a freeze-dried powder at 4 °C. Herein, ART-cochleates

prepared in-flow using off-the-shelf flow focusing device were named as “ACF – Artemisinin Cochleates from Flow”.

4.2.4 Studying the effect of flow conditions using CFD simulations

It is known that amphiphilic phospholipids are soluble in nonpolar solvents but spontaneously self-assemble into bilayer structures as the polarity of the surrounding fluidic environment increases. Since we used buffer containing calcium ions as bridging agent to spontaneously induce the formation of the cochleates from the bilayer structures, it is essential to gain better understanding of the mixing process. Computational fluid dynamics (CFD) simulations were performed to understand the effects of different flow focusing conditions on cochleate formation by studying the mixing and concentration distribution profiles of the central methanol stream w.r.t two adjacent aqueous Tris-Ca²⁺ buffer streams using COMSOL Multiphysics 5.3 software.

A two-dimensional model of the plan view of the cross-assembly along with the microchannel was considered to best represent the off-the-shelf device for a qualitative correlation of the microfluidic mixing conditions and cochleate formation. Microchannels leading to the flow focusing area were truncated. Microchannel downstream of the cross assembly was limited to a length of 10 mm along with a cross assembly as the point of interest. Laminar flow models for stationary steady state governed by incompressible Navier-Stokes and continuity equations were followed assuming incompressible Newtonian fluids as below,

$$\rho \left[\frac{\delta v_f}{\delta t} + (v_f \cdot \nabla) v_f \right] = -\nabla p + \mu \nabla^2 + k \dots \text{(Eq. 4.3)}$$

$$\rho[\nabla \cdot (v_f)] = 0 \dots (\text{Eq. 4.4})$$

Where, ρ is the fluid density, v_f is the flow velocity, μ is the dynamic viscosity of the fluid, p is the pressure ($p = 0$ Pa for outlet boundary function of pressure) and k is other forces. For dilute systems with dissolved solute, the mass transport of species is given by a convection-diffusion model given below,

$$N = -D\nabla c + cv_f \dots (\text{Eq. 4.5})$$

$$\frac{\delta c}{\delta t} + (\nabla \cdot N) = R \dots (\text{Eq. 4.6})$$

where, N is the molar flux ($\text{m}^{-2}\text{s}^{-1}$), c is the species concentration (mol.m^{-3}), D is the diffusion coefficient of the species (m^2s^{-1}) and R is a net volumetric source for the species concentration ($R = 0$ as no chemical reaction leading to formation or depletion of species). Numerical simulations were performed to solve the set of equations (Eq. 4.3, 4.4, 4.5 and 4.6) above iteratively until steady state was reached. A no-slip boundary condition for the microchannel walls was applied which imposes zero velocity at the channel walls implies,

$$v_f(x_w) = 0 \dots (\text{Eq. 4.7})$$

where, x_w denotes the location of the microchannel wall. Further, tetrahedral elements were used to mesh the 2D flow-focusing geometry of off-the-shelf chip.

4.2.5 Characterisation of the prepared cochleates

Structure and morphology of the cochleates were analysed using SEM as described previously in Section 2.2.3. Particle size and zeta potential of the SUVs and cochleates were measured as described in Section 2.2.4. IR

spectroscopy and SAXS analyses of cochleates were performed as described in Section 2.2.8 and Section 2.2.9, respectively. Further, the encapsulation efficiency (EE) in percentage, drug loading (DL) in mg ART/g of cochleates and overall product yield in percentage were determined as described earlier in Section 3.2.5.

4.2.6 In-vitro artemisinin release

The in-vitro release profiles of ART from both ACB and ACF were studied separately using dialysis bag diffusion technique in phosphate buffered saline (PBS, physiological of pH 7.4), simulated gastric fluid (SGF, pH 1.2) and simulated intestinal fluid (SIF, pH 6.8) as release media at physiological temperature of 37 °C (Section 3.2.7). The released ART in the samples were analysed by UV–Vis spectrophotometry as described earlier in Section 3.2.5.

4.2.7 In-vitro cytotoxicity using MTT assay

Caco-2 cells of human colon carcinoma were cultured in 75 cm² culture flasks using Dulbecco's Modified Eagle's Medium (DMEM), high glucose (4500 mg/L), L-glutamine (without pyruvate), 16% heat-inactivated foetal bovine serum (FBS), 1% MEM nonessential amino acids and 1% penicillin-streptomycin (100 U/mL stock each) solution, incubated at 37 °C and 5% CO₂. The cells were washed with PBS, trypsinized, pelleted at 1500 rpm for 5 min and re-suspended in DMEM, seeded in 96 well plates at 1×10⁵ cells/well and incubated overnight. The medium was then replaced with samples of ART (25, 75 and 150 µg/mL), EC, ACB and ACF each at 0.3, 1.0, and 2.0 mg/mL and incubated for 24 h. The wells were then rinsed with PBS to remove excess particulates before replacing with MTT solution. After incubation with MTT for

3 h, the cell-viability was measured following the addition of DMSO, by measuring the absorbance at 570 nm (Tecan Infinite M200 microplate reader). Untreated cells were used as controls, corresponding to 100% cell-viability against which cell-viabilities of other samples were reported. All samples were tested in replicates (n=6).

4.2.8 In-vitro intestinal absorption of ART from ACB and ACF

Caco-2 cells were cultured and maintained as mentioned in the Section 4.2.7 above. The cells were seeded onto 12-well Transwell® cell-culture plates at a seeding density of 3×10^5 cells/well. The cells were then maintained for a period of 21–24 days before the transport test with a fresh medium replacement interval of 2–3 days. The integrity of the monolayer culture was monitored by measuring its transepithelial electrical resistance (TEER) using an EndOhm tissue resistance measurement chamber equipped with ‘chopsticks’ electrodes. The transport experiments across the cell monolayers were performed from the apical to the basolateral direction in Hanks' Balanced Salt solution (HBSS) at 37 °C using orbital shaker (100 rpm). Before the start of the experiment, the cell-culture medium was removed, the Transwell® filters and receiver chambers were rinsed with HBSS (with 1% methanol, v/v), preincubated in HBSS (1% methanol, v/v) for 15 min at 37 °C. After that, 0.5 mL of pure ART (150 µg/mL), suspensions of ACB, and ACF (2.0 mg/mL each) with ART concentration of ~150 µg/mL were pipetted into the apical side of the chambers. Samples were drawn from the permeated basolateral side of the chambers at different time-intervals (up to 240 min) and an equal volume of fresh prewarmed HBSS (1% methanol, v/v) was added. At the end, contents of both apical (donor) and basolateral (receiver) chambers were collected. The permeated ART

concentrations were measured using UV–Vis spectrophotometry and apparent permeability coefficients (P_{app}) were calculated using the Eq. 4.8 below. All transport experiments were conducted in triplicates (n=6).

$$P_{app} = \frac{\Delta Q}{\Delta t A C_0} (\text{cm.s}^{-1}) \dots (\text{Eq. 4.8})$$

where, $\Delta Q/\Delta t$ is the permeability rate ($\mu\text{g}/\text{min}$), C_0 is the initial concentration ($\mu\text{g}/\text{mL}$) and A is the surface area of the monolayer (1.1 cm^2).

4.3 Results and discussion

4.3.1 Preparation of ACB under batch process conditions

Initially, we prepared three independent batches of ART-cochleates under batch process (ACB) in order to compare their morphology, particle size and particle size distribution with ART-cochleates prepared under flow process (ACF). The three ACB were prepared according to our previous process using the trapping film method with DOPS:DMPS:ART ratio of 9:1:4 (molar). All three ACB showed typical well-formed and rolled-up cylindrical structure as verified from SEM images represented by Figure 4.2 A.

A significant difference was observed in the particle size (both width and length) of these cochleates from batches 1–3 ($p < 0.05$) as shown in Figure 4.2 B and C. Additionally, the three batches showed wide particle size distribution range as represented in Figure 4.2 D (width of the cochleates) and Figure 4.2 E (length of the cochleates) from batch 3. The wide differences in the size and particle size distributions of the ACB is mainly due to the continuous and uncontrolled aggregation of lipid sheets during their formation.

Figure 4.2 (A) Representative SEM of ACB from batch 3; (B) and (C) Particle size of ACB from different independent batches in width and length of the cochleates, respectively; (C) and (D) Representative particle size distribution of ACB from batch 3 in width and length of the cochleates, respectively (n=number of randomly selected particles measured for size individually)

4.3.2 Preparation of ACF: Influence of flow rate ratio (FRR) and mixing characteristics

To overcome the challenges associated with batch processes, we examined a continuous in-flow preparation of ART-cochleates (ACF) using off-the-shelf HFF device. HFF is envisioned to give precise control over the microfluidic mixing in the off-the-shelf device used to prepare ART-cochleates. In HFF, a central stream of ART and lipids in methanol is sheathed between two adjacent streams of buffered Tris and Ca^{2+} at pH 7.4 and hydrodynamically focused, which narrows the central stream to force rapid diffusive mixing across its width. The fluids would flow as parallel layered streams after they converge at the hydrodynamic focusing region (cross-assembly) due to the micrometre-scale laminar flow regime. In addition, the use of ‘off-the-shelf’ device configuration instead of the commonly used PDMS, COC or glass 2D chips not only helps to reduce the fabrication cost of microfluidic chips but also, their readily availability in the market as standard HPLC components and simple device assembly makes this device an appealing alternative.^{13,116}

Mixing in the microchannels of off-the-shelf device is an important parameter as it controls the diffusion of molecules from the side streams (aqueous Tris- Ca^{2+}) across the width of the focused stream (methanolic lipids+ART) leading to the self-assembly of cochleates. Firstly, we calculated the Reynold's number (Re) which represents the ratio of the inertial forces to the viscous forces, to ascertain the fluid flow regime inside the device. Re is an important dimensionless parameter for transport phenomena in fluid flows and is expressed as follows,

$$Re = \frac{\rho v_f d_h}{\mu} \dots \text{(Eq. 4.9)}$$

where, ρ is the fluid density, v_f is the average flow velocity, d_h is the hydraulic diameter and μ is the dynamic viscosity of the fluid. The Re values for our system were found to be lower than 1800 (i.e., laminar flow). Hence, it showed that higher flow velocities could be used maintaining the laminar fluid flow regime within the device which also aids in achieving higher throughputs from our device. The Re values for our system ranged from 35.4 to 565.9 which is typical of microfluidic devices.^{117,152} The Re values lower than 1800 under all our operation conditions indicated that even at higher flow velocities the laminar flow regime was maintained.

Under microfluidic laminar flow regime, the mixing time (τ_{mix} - time required for the complete diffusive mixing of parallel methanol and aqueous streams) mainly depends on the FRR of aqueous to methanol streams. It is an important parameter for the preparation of the ART-cochleates in the HFF device. It gives an estimate of the time required for the complete diffusive mixing of the methanol (ART and lipids) and aqueous (Tris and Ca^{2+}) streams to occur under laminar flow regime. Alternatively, the mixing time can be interpreted as the minimum residence time required to attain complete mixing for the formation of the cochleates through the length of the microchannel. However, the residence time (t_{res}) which is inversely proportional to the average flow velocity, mainly governs the length of the microchannel. Assuming that the fluid flow profile was not significantly altered, as the difference between viscosities of alcohol and aqueous streams is small and considering most of the mixing happens due to the diffusion of molecules after the three fluid streams have converged downstream

of the cross assembly used for focusing, the mixing time (τ_{mix}) for cochleate self-assembly can be approximated according to Eq. (4.10) using a simple two-dimensional model as follows:

$$\tau_{mix} \approx \frac{w_f^2}{4D} \approx \frac{w^2}{9D} \frac{1}{(1+FRR)^2} \dots \text{(Eq. 4.10)}$$

where, w_f is the width of the focused stream, w is the channel width (diameter in our case), FRR is the flow rate ratio and D is the diffusivity of methanol. Hence, the effect of FRR or device mixing characteristics on cochleate formation was studied by preparing ART-cochleates under several different FRRs with constant average flow velocity (v_f) (Figure 4.3).

The τ_{mix} under different conditions of FRR tested ranged from 0.3 to 3.4 s which were significantly less than the residence time ($t_{res} = 5.9$ s at $Q_t = 2.0$ mL/min or $v_f = 170$ mm/s) ensuring complete mixing of the fluid streams within the microchannel. Also considering that self-assembly of cochleates is a fast process (millisecond scale),¹⁹ the difference between τ_{mix} and t_{res} was insignificant for their formation. We observed well-formed cochleates under all tested conditions of FRR. However, at higher FRR of 5 (with lowest τ_{mix} , 0.3 s), fewer cochleates were formed with higher number of aggregated intermediate lipid structures compared to those prepared with smaller FRRs (Figure 4.3). The optimum FRR was 2.5 where the ACF were well formed with minimum intermediate lipid structures (Figure 4.3 C). The influence of FRR or τ_{mix} on formation of the ACF particle size was also analysed. We observed smaller cochleates size at lower FRRs (1.0, 2.0 and 2.5) and larger cochleates size at higher FRRs (3.0, 4.0 and 5.0) across both their width and length (Figure 4.4). The lowest dispersity was

obtained with FRR 2.5 and a gradual increase in dispersity was seen, as the FRRs increased from 2.5 to 5.0 for both the width and length of the cochleates.

Figure 4.3 SEM showing the effect of FRR (or τ_{mix}) on the ACF prepared under a constant v_f (or Q_t) with different FRR (A) 1.0, (B) 2.0, (C) 2.5, (D) 3.0, (E) 4.0, and (F) 5.0

Figure 4.4 Particle size and dispersity in (A) width and (B) length of ACF prepared at different FRRs with constant Q_t of 2.0 mL/min. n = number of randomly selected particles measured individually; * $p < 0.05$ comparing with FRR 1.0; ** $p < 0.05$ comparing with both FRR 2.0 and 2.5

4.3.3 Preparation of ACF: Effect of the total volumetric flow rate or average flow velocity

The influence of the total volumetric flow rate (Q_t) or average flow velocity (v_f) on the preparation of ACF was investigated. Parameters of well-formed cochleate particles, particle size and dispersity were studied at different Q_t under

several constant FRRs. In addition to directly affecting the throughput in continuous flow, Q_t plays an important role in the formation of cochleates. It was shown that slower flow rates resulted in the formation of densely aggregated lipid particles which was due to arrest of cochleate formation at the intermediate stages, whereas higher flow rates resulted in well-formed cochleates.³⁷ While only a few well-formed cochleates among dense lipidic aggregates were seen at low v_f (42 and 85 mm/s), more and more cochleate structures formed along with disappearance of the dense aggregates with the increase in the v_f (170, 340 and 680 mm/s) (Figure 4.5). However, increasing the v_f further beyond 680 mm/s resulted in higher aggregates and fewer cochleates (Figure 4.5 F), proving that Q_t plays a critical role and must be carefully tuned to obtain well-formed cochleates with a desirable throughput.

To study the effect of Q_t or v_f on the particle size and dispersity, we prepared ART-loaded cochleates at a constant FRR of 2.0 using Q_t of 2.0, 4.0 and 8.0 mL/min, with which well-formed cochleates were obtained (Figure 4.5). At lower FRR of 2.0, Q_t showed no significant effect on the size of the cochleates in both width and length ($p < 0.05$) (Figure 4.6). However, formation of cochleates could depend on v_f within a certain focusing regimes at a specific magnitude of FRRs.¹¹² Hence, we chose slightly higher FRRs such as 2.5 and 3.0 to prepare ACF using the previously chosen Q_t . At FRR of 2.5, we observed a significant difference in the size of particles at faster 8.0 mL/min compared to slower Q_t (2.0 and 4.0 mL/min) (Figure 4.6). However, at FRR of 3.0, slightly larger particles both by width and length were obtained with slower 2.0 mL/min Q_t . Further, upon increasing the Q_t to 4.0 and 8.0 mL/min, a trend of decreasing particle size was seen with significant differences in size for each value of Q_t

($p < 0.05$) (Figure 4.6). Like FRR of 2.0, higher FRRs of 2.5 and 3.0 also showed higher dispersity of the cochleate size prepared at Q_t of 2.0 mL/min. However, it is important to note that the dispersity of the ACF prepared with higher Q_t of 8.0 mL/min and FRR of 2.5 was significantly lower both in width and length ($p < 0.05$) (Figure 4.6). Hence, it is implied that the ART-cochleates of highly uniform size with narrow size distribution can be continuously prepared with higher control on the particle properties using our off-the-shelf device (Figure 4.6 C and D).

4.3.4 Formation of ACF in the off-the-shelf flow focusing device

The formation of cochleates is a nucleation-dependent continuous self-assembly process which occurs through several intermediate steps.^{17–19} It is known that amphiphilic phospholipids are soluble in nonpolar solvents but spontaneously self-assemble into bilayer structures as the polarity of the surrounding fluidic environment increases. Since we used buffer containing calcium ions as bridging agent to spontaneously induce the formation of the cochleates from the bilayer structures, it is essential to gain better understanding of the mixing process since it affects the formation of ACF, their size and dispersity. In flow focusing, most of the mixing occurs downstream of the cross assembly used for focusing of streams and the width of the central stream can be controlled by controlling the flow rates of both central and side streams.^{112,113} The mixing of methanol with the aqueous buffer in the microchannel environment was verified by performing CFD simulations. By following the present literature with consideration to the effects of microfluidic conditions such as mixing and flow velocity (with the help of CFD simulations), we tried to qualitatively correlate the formation and growth of the cochleates via the intermediate structures (Figure 4.1).

Figure 4.5 SEM of ART-cochleates prepared in-flow (ACF) using off-the-shelf device at different total volumetric flow rates (Q_t) (A) 0.5, (B) 1.0, (C) 2.0, (D) 4.0, (E) 8.0 and (F) 10.0 mL/min with corresponding v_f of 42, 85, 170, 340, 680 and 850 mm/s, respectively

Figure 4.6 Effect of Q_t (or v_f) on the particle size and dispersity in (A) width and (B) length. Representative particle size distribution of ACF (FRR = 3.0 and Q_t = 8.0 mL/min) in (C) width and (D) length. n = number of randomly selected particles measured individually; * $p < 0.05$ comparing with both Q_t 2.0 and 4.0 mL/min; ** $p < 0.05$ comparing with both Q_t 2.0 and 4.0 mL/min; * $p < 0.05$ comparing with Q_t 4.0 mL/min**

Different values of FRRs and Q_t (at slower 1.0 ml/min and faster 8 ml/min) that correspond to experimental conditions were simulated using COMSOL Multiphysics to obtain methanol concentration profile in the modelled microchannel design (Figure 4.7). In our simulations, the mixing of methanol with water was assumed to be homogeneous at the microscale, thereby neglecting microheterogeneities at the molecular scale. Our simulations follow a similar approach to that described previously in several reports in which a two-phase fluid flow system is expressed as a single-phase system with concentration-dependent diffusion.^{112,113,153} However, for simplicity we start by explaining the effects of mixing (or FRR), followed by flow velocity (or Q_t) and in turn the flow regimes on cochleate formation inside our off-the-shelf device.

At low FRRs (such as 1.0, 2.0 and 2.5) the methanol (lipid+ART) stream remains wider with a relatively low surface/volume ratio between methanol and aqueous (Tris- Ca^{2+}) streams (Figure 4.7 A, B and C). This leads to lower concentration gradient across the focused stream causing its depletion by diffusion to be slower. Thus, methanol concentration remains high (>50% v/v) in the interior of the focused stream downstream of the cross-assembly resulting in a large fraction of the lipids to remain solubilized for a longer duration. Hence, only a small fraction of lipid molecules gain access to the transition region between methanol/aqueous interface (Figure 4.7 A, B and C). Under microfluidic environments, the alcohol-phase concentration critically affects the onset of nucleation process at the methanol/aqueous interface.³⁷ Although phospholipid bilayer structures are only known to form at alcohol concentrations below 50% (v/v),¹⁵⁴ formation of cochleates was shown to occur even at higher alcohol concentrations (up to 90% v/v).³⁷ At higher alcohol concentrations, phospholipid

head groups interact with alcohol by means of hydrogen bonding while increasing alkyl chain disorder. This in turn decreases the bending energy of phospholipid bilayers.^{154,155} Under such conditions, exposure to Ca^{2+} leads to the nucleation followed by progressive aggregation of lipid bilayers resulting in the generation of hydrophobic surfaces on lipid stacks which would be stabilized by methanol. This stabilizing effect coupled with lowering of bending energy explains the smaller dimensions of the cochleates formed at lower FRRs (Figure 4.4).

Figure 4.7 Methanol concentration profiles inside the off-the-shelf device used to produce ACF as obtained from CFD simulations performed at flow focusing conditions with FRRs of (A) 1.0, (B) 2.0, (C) 2.5, (D) 3.0, (E) 4.0 and (F) 5.0 at relatively slower and faster Q_t of 1.0 mL/min (left panel) and 8.0 mL/min (right panel), respectively

At high focusing (FRR 3.0 and 4.0), the width of the central stream becomes narrowed with a shorter diffusion length resulting in relatively higher

surface/volume ratio of methanol to aqueous streams (Figure 4.7 D and E). This gives rise to a higher concentration gradient leading to rapid depletion of the focused stream by two-dimensional convective-diffusive transport which dominates the mixing. Consequently, lipid molecules initially dissolved in the focused methanol stream become exposed to an increasingly polar fluidic environment with decreasing lipid solubility (Figure 4.7 D and E).^{112,115} This leads to rapid initiation of most of the nucleation (exposing majority of the lipids to Ca^{2+} at the interface) to take place within the focusing region forming small oblate micelles as soon as the critical polarity is reached (Figure 4.1).^{19,112,156} Under the increased aqueous environment, lipid aggregation continues due to the bridging effect of Ca^{2+} forming bilayer stacks. This Ca^{2+} chelation has a dehydrating effect on the bilayer stacks which results in highly condensed alkyl chains with high degree of order. The resulting stacks aggregate rapidly due to their hydrophobic surfaces leading to the self-assembly of larger planar sheets. These sheets further undergo curling to reduce their surface area and form cochleate cylinders of relatively larger size than at low focusing conditions (Figure 4.4).^{30,37} However, under large FRRs (5.0), the abrupt depletion of the focused stream with rapid diffusive mixing could arrest the cochleate self-assembly at intermediate stages leading to the presence of increased number of aggregated lipid structures (Figure 4.3 F). Similar aggregated structures were formed in batch process when the bulk of the Ca^{2+} was rapidly mixed with vesicle suspension.

Further, due to the nucleation process at focusing region, the diffusion coefficient of lipid sheets tends to decrease while increasing its tendency to advent along the streamlines in downstream microchannel. At this stage, shear

forces acting at the methanol/aqueous interface influences the formation of cochleates. When FRR is kept constant, the width of the focused stream (methanol with lipids+ART) remains constant (assuming the liquids are incompressible) in turn maintaining a constant dilution or mixing rate (i.e., mixing depends only on FRR but not on the magnitude of Q_t) (Figure 4.7). Thus, increasing Q_t (or v_f) at constant FRR increases shear forces at the interface of two fluids. These increasing shear forces affect aggregation of lipids leading to the formation of smaller stacks and sheets which in turn results in the growth of smaller cochleates. This explains the reduction in size of ACF formed at FRR of 2.5 and 3.0 when Q_t was increased from 2.0 to 8.0 mL/min (Figure 4.6 A and B). However, at FRR 2.0 varying Q_t (or v_f) showed no such effect (Figure 4.6 A and B). Hence, it is important to recall that the formation of cochleates depends on Q_t (or v_f) within a certain focusing regimes only (i.e., at specific FRRs). This was true for several studies on liposome formation in microfluidic HFF where shear forces had a minor to no role in providing control over size of liposomes.^{112,117,152} In contrast, microfluidic formation of micro-spherical cochleate complexes showed a trend between particle size and Q_t which agrees with our results.³⁷ Similar effects of shear stress on microfluidic formation of emulsions and polymeric nanoparticles was also seen where the size of dispersed droplets or particle nucleates decreased with increasing flow rates.^{112,114,124,127}

Hence, for all practical applications with similar off-the-shelf flow focusing devices to produce well-formed cochleates with higher reproducibility, we identify optimizing the flow focusing conditions to be crucial. In our case, focusing regimes at FRR of 3.0 and Q_t of 4.0 – 8.0 mL/min (or v_f 340 – 680 mm/s) was found to be favourable whereas, unfavourable or extreme focusing

(FRR 5.0) and flow rate ($Q_t < 2.0$ and > 8.0 mL/min) conditions arrest the cochleate formation at the intermediate stages to give dense lipid aggregates (Figures 4.3 and 4.5). While our simulations simplify the complex mixing process between methanol and water, our aim is to qualitatively establish a correlation between different microfluidic mixing conditions and the formation of the cochleates. We shall note that, to validate the numerical simulations it is important to compare of CFD simulation results with experimental results. For this purpose, in general the width of the focused stream (w_f) needs to be determined experimentally by visualizing the mixing inside chip and compared with simulated data. However, in our case the experimental results are limited by the nature of the device. It is impossible to visualize the mixing inside the off-the-shelf chip due to the opaque nature of the material, one of the disadvantages of off-the-shelf devices. Nevertheless, the results of CFD simulations and the resulting ART-cochleates under those mixing conditions (or process parameters) largely show qualitative agreement w.r.t, the particle characteristics like size and morphology. However, integration of molecular and fluid dynamics simulations including interactions between lipids and intermediate structures, as well as the varying kinetic effects of diffusion and advection on these structures is necessary to fully understand complex interplay of molecular and hydrodynamic phenomena involved in the microfluidic formation of cochleates.

4.3.5 Production rate of ACF using the off-the-shelf device

The production rate or throughput of cochleates in a continuous flow device depends on the concentration of lipids, dimensions of the device and process conditions such as Q_t (or v_f).^{113,117,157} A lipid concentration of 5 mg/mL (~6.25 mM) is usually considered optimum to produce cochleates in batch process as

increased concentrations result in higher degree of aggregation and incomplete cochleation.^{33,35} Recently, a much lower lipid concentration of 0.1 mg/mL was deemed necessary to continuously produce uniform micro-spherical cochleate complexes.³⁷ Nevertheless, we used a lipid concentration of 2 mg/ mL (~2.5 mM) to ensure complete solubility of the lipids and ART in the methanol phase (~5.0 mM for lipids) and avoid formation of aggregates prior to cochleation. Further, we calculated the ACF production rate using Q_t of 4.0 mL/min and FRR of 3.0 to be 8 mg/min i.e., 480 mg/h, equating to 11.5 g/day (with a corresponding space-time-yield of 120 g/L.h). This productivity is over 7-fold higher than the current production.³⁷ Additionally, cochleate production rate is affected by the average flow velocity. While there is an upper limit to the flow velocity based on the flow regimes that could be employed during flow focusing (i.e., transition from laminar to turbulent flow), a parameter that practically restrains from using higher velocity is the pressure limitations of the device itself. The build-up of high inlet pressures due to increasing flow velocity can cause a substantial deformation at the channel walls of devices fabricated with materials such as PDMS and such deformations could lead to leakage or perturbations in flow-focusing.^{113,117} However, the PEEK device components and PTFE microchannels used in our study are fabricated with a purpose to withstand high-pressure operations and hence are suitable for use with higher flow velocities to achieve higher throughputs. Furthermore, the modular nature, inexpensive and readily availability of the components used in the off-the-shelf device further promotes paralleling this device on a single microfluidic platform to achieve enhanced production rates. Hence, this work shows that the off-the-shelf device could be a highly promising platform to enhance the ART-cochleate

production rate and achieve high throughputs with an excellent control over the particle properties.

4.3.6 FT-IR analysis of ART-cochleates

The IR spectra of ART, DOPS+DMPS lipids (hydrated form), EC, ACB and ACF were analysed. Firstly, lipid phase features such as phosphate head group hydration, ester carbonyl group hydration, and acyl chain order of pure lipids (DOPS+DMPS) in hydrated form and different cochleates formulations were studied to confirm complete cochleate formation (Figure 4.8 A, B and C). IR spectra of pure lipids showed rather featureless bands for symmetric and asymmetric phosphate stretching at 1065 cm^{-1} and 1219 cm^{-1} , respectively (Figure 4.8 A). In all the cochleate samples, symmetric phosphate stretching band showed four distinct peaks at 1062 cm^{-1} , 1075 cm^{-1} , 1102 cm^{-1} and 1112 cm^{-1} , and the asymmetric phosphate stretching band shifted to 1238 cm^{-1} , indicating bidentate complexation of phosphate with Ca^{2+} and dehydration of the phosphate head group.⁴⁴ Similar observations were made for ester carbonyl groups. Cochleates showed four distinct narrow stretching bands at 1703 cm^{-1} , 1720 cm^{-1} , 1732 cm^{-1} and 1747 cm^{-1} which appeared due to Ca^{2+} complexation with polar head group. Whereas, in pure lipids this was characterized by a broader stretching band centred at 1730 cm^{-1} (Figure 4.8 B).⁴⁴ In addition, the symmetric and antisymmetric methylene stretching vibrations of the acyl sidechain dominated at 2853 cm^{-1} and 2924 cm^{-1} , respectively, indicating a tighter lipid packing in cochleate phase (Figure 4.8 C).^{41,44}

In pure artemisinin spectrum, the lactone —C=O showed a peak at 1737 cm^{-1} (Figure 4.8 B) and the endo-peroxide bridge showed the characteristic peak at

883 cm^{-1} (Figure 4.8 D).⁷² The presence of the additional endo-peroxide vibrational band at 883 cm^{-1} in ART-cochleates and its absence in pure lipids and EC spectra, confirmed encapsulation of ART in both ACB and ACF alike (Figure 4.8 D).

Figure 4.8 IR spectra of (a) pure ART, (b) pure lipid (DOPS+DMPS in hydrated form), (c) EC, (d) ACB and (e) ACF showing (A) phosphate, (B) ester carbonyl, (C) acyl chain C—H and (D) ART specific endoperoxide bridge stretching regions

4.3.7 SAXS analysis of ART-cochleates

Small angle X-ray scattering (SAXS) was used to confirm the multilamellar ultrastructure of the ACB and ACF (Figure 4.9). The SAXS diffraction patterns

showed sharp equidistant Bragg's reflections at 0.124, 0.249 and 0.373 $\text{\AA}^{-1}$. Such a pattern was commonly observed for cochleate systems due to their highly ordered and periodic lamellar internal structure.³⁰ Since cochleates are uniform multilamellar structures, the interlamellar repeat distance or the characteristic domain spacing of each lipid bilayer membrane can be given by the first reflection peak in the diffraction pattern. We obtained an interlamellar repeat distance of 50.7 $\text{\AA}$ for both the ART-cochleates as in agreement with our previous results. This confirmed that well-formed cochleates were obtained from both batch process and in-flow using off-the-shelf device alike.

Figure 4.9 SAXS patterns of (a) EC, (b) ACB and (c) ACF showing Bragg's reflections typical of multilamellar cochleate ultra-structures

4.3.8 Zeta-potential of ACF

Zeta-potentials of ACB and ACF were measured to study the effect of different conditions used to prepare these cochleates. The hydrated lipid vesicles (i.e., the DOPS+DMPS SUVs) used to prepare ACB showed a zeta-potential of -66.4 ± 2.7 mV, whereas the cochleates themselves showed an increase in zeta potential of -41.6 ± 1.4 mV. This increase in zeta potential was attributed to the complexation of negatively charged phosphatidylserine with Ca^{2+} during

cochleation thus resulting in partial charge neutralization. Further, ACF also showed similar zeta-potential value of ~ -41 mV. In addition, the process conditions used in the off-the-shelf device such as FRR and Q_t for the preparation of ACF did not show significant difference in the zeta-potentials ($p < 0.05$).

4.3.9 Encapsulation efficiency and ART loading

We previously prepared the ACB and found that the DOPS:DMPS:ART molar ratio of 9:1:4 gives an optimum EE of $61.8 \pm 9.1\%$, DL of 71.5 ± 4.9 mg ART/g of cochleates and overall product yield of $98.9 \pm 0.9\%$ (Section 3.3.6). Therefore, we used the same DOPS:DMPS:ART ratio of 9:1:4 to prepare ACF to compare the EE, DL and yield. Water miscible organic solvents such as ethanol, methanol and isopropanol are commonly used to produce lipid-based nanoparticles by HFF as reported in literature.^{37,112,154} In this study, methanol was used as the solvent to dissolve the lipids and ART since the solubility of ART is lower in methanol compared to ethanol and isopropanol.¹⁵⁸ This assisted the partitioning of more and more ART in the lipid cochleate phase when exposed to buffer under HFF conditions resulting in higher EE and DL compared to other solvents (data not shown). The effect of HFF conditions in the off-the-shelf device on encapsulation and loading of ART into cochleates was studied under different FRRs as preliminary studies showed no significant effect of Q_t on EE or DL under constant FRR ($p < 0.05$). We observed significantly higher encapsulation and loading at intermediate FRRs 2.5 and 3.0 ($p < 0.05$, $n=3$) (Figure 4.10 A). Lower FRR resulted in considerably lower encapsulation due to higher concentration of methanol (up to 50% v/v at FRR 1.0) which restricted the partitioning of ART in lipids leaving the unencapsulated ART in supernatant. However, at high FRR (4.0 and 5.0) the encapsulation of ART is apparently

lower due to the presence of lesser well-formed cochleates. As discussed earlier, at high FRR the dilution of the central stream occurs rapidly due to smaller mixing time, causing aggregation of lipids. This rapid dilution of methanol stream also results in precipitation of ART which was washed off during the subsequent steps. The EE of $64.24 \pm 2.5\%$ and DL 83.37 ± 3.68 mg ART/g of cochleates of the ACF prepared at FRR 2.5 (Figure 4.10 A) was comparable to several ART delivery systems reported in the literature recently.^{91,94,159,160}

4.3.10 In-vitro ART release under simulated gastro-intestinal conditions

Release profiles of ART from the ACB and ACF were studied by invitro dialysis method using phosphate buffered saline (PBS, pH 7.4), simulated gastric fluid (SGF, pH 1.2) and simulated intestinal fluid (SIF, pH 6.8) as release media. In PBS, a similar biphasic ART release profile was seen for both ACB and ACF. It was characterized by a rapid burst release with up to 40% of ART released in the first 2 h followed by a relatively slow and continuous release phase with a cumulative release close to 80% of ART over the next 10 h. Both ACB and ACF sustained the release of ART over 12 h in PBS release medium (Figure 4.10 B). Similar drug release profiles from cochleates were reported previously especially with hydrophobic drugs like ART due to its strong interaction with the lipid bilayer structure of cochleates.⁷³ To study the release profile of ART under simulated gastro-intestinal conditions, both ACB and ACF were subjected to in-vitro release in SGF medium for 1.5 h followed by replacing SGF with SIF as release medium. Both the samples of cochleates showed a cumulative release of up to 20% of the ART encapsulated over the first 1.5 h in SGF (Figure 4.10 B). Cochleates are generally regarded as stable under gastric conditions due to their tightly packed lipid bilayer structure. However, in SGF some de-

cochleation of the bilayer lipid sheets occurs leading to release of ART in a controlled manner as reported in literature.^{15,38} When the release medium was changed to SIF, a cumulative ART release of up to 90% occurred over 6 h and all of the ART encapsulated in the cochleates was released into the SIF within 12 h (Figure 4.10 B). We concluded that both ACB and ACF show similar ART release profile where process of preparation does not impact the release characteristics of ART from the cochleates.

4.3.11 In-vitro cytotoxicity study of ART-cochleates using MTT assay

The biocompatibility of the formulated ART-cochleates was studied by assessing the cytotoxicity of pure ART, EC, ACB and ACF samples on Caco-2 cells using MTT assay. For this, the survival rate of Caco-2 cells after 24 h of exposure to the ART at concentrations of 25, 75 and 150 µg/mL and cochleate concentrations (EC, ACB and ACF) of 0.3, 1.0, and 2.0 mg/mL was determined. In cytotoxicity tests, cell viabilities above 50% are necessary to consider a particular sample concentration as safe.⁴⁹ Hence, under the tested concentrations as shown in Figure 4.10 C, ART, EC, ACB and ACF showed neither apparent cytotoxicity nor dose-dependent cytotoxicity on Caco-2 cells. Similar results using ART and EC were reported previously.^{49,76} Thus, even at higher concentrations, ART-cochleates can be considered safe due to their weak cytotoxicity and little to no interference in cell integrity.

4.3.12 In-vitro permeability of ART through Caco-2 cell

The transepithelial permeability of ART (pure, ACB and ACF) was directly measured by studying its transport across Caco-2 cells monolayer.^{49,76,161} Pure ART, ACB or ACF were dispersed in the transport medium and the cumulative

amounts of ART permeated through Caco-2 cell monolayers were calculated at different intervals over 4 h to obtain the transportation curves (Figure 4.10 D). A safer ART concentration of 150 $\mu\text{g/mL}$ (and corresponding cochleate concentrations $\sim 2.0 \text{ mg/mL}$) was chosen based on the results of cytotoxicity studies. The TEER readings were taken before the beginning and at different intervals throughout the transport studies. Apparent permeability (P_{app}) was also calculated for all the samples using the linear part of the transportation curve, where no significant change in TEER readings at the end of the experiment indicated that the membrane integrity was maintained throughout the transport interval (Table 4.1). Pure ART showed higher permeability coefficients where $\sim 60\%$ ART was transported across the Caco-2 monolayer. This higher permeation rate is attributed to the hydrophobic nature of ART and its higher solubility in lipids.^{76,161} However, both ACB and ACF showed lower ART transport rate ($\sim 25\%$ in 4 h; $p < 0.05$, $n = 6$) resulting in lower P_{app} compared to pure ART (Figure 4.10 D and Table 4.1). The lower P_{app} values was attributed to the slow release of ART from both ACB and ACF due to multiple bilayer structure and lack of internal aqueous phase. A major bottleneck for oral administration of ART is its poor aqueous solubility and hence erratic/poor oral bioavailability.⁷³ This combined with its rapid gastro-intestinal transit time results in high recrudescence, despite the use of higher dosages for treatment.⁷³ Due to the sustained release characteristics of ART-cochleates, combined with effective intestinal absorption of ART, it should be possible to achieve a sufficiently high ART concentration over a sufficiently long period of time to achieve parasitocidal effect.^{73,76} However, extensive in-vivo studies need to be conducted to draw any conclusion on the efficacy of ART-cochleates following

oral administration as it is the result of an interplay between many factors including drug solubility, dissolution rate, membrane permeability, regional pH differences and first-pass metabolism.

Figure 4.10 (A) Effect of focusing conditions on EE and DL and product yield of ACF, (B) In-vitro ART release profiles from ART-cochleates studied under different release media including simulated gastro-intestinal conditions, (C) In-vitro cytotoxicity of ART and ART-cochleates on Caco-2 cell line of intestinal mucosa and (D) Intestinal drug absorption of ART-cochleates studied in-vitro using Caco-2 cells

Table 4.1 TEER values of Caco-2 cell-monolayers before and after the ART transport study conducted to assess in-vitro oral-bioavailability of ART-cochleates along with their apparent permeability (P_{app})

Sample	P_{app} ($\times 10^{-6} \text{cm.s}^{-1}$)	TEER Before ($\Omega \text{ cm}^2$)	TEER After ($\Omega \text{ cm}^2$)
ART	37.6 $\pm$ 1.5	651 $\pm$ 34	633 $\pm$ 27
ACB	15.8 $\pm$ 6.5	670 $\pm$ 29	631 $\pm$ 35
ACF	18.2 $\pm$ 5.4	641 $\pm$ 68	613 $\pm$ 51

4.4 Conclusions

We developed an off-the-shelf flow focusing device for the continuous production of ART-loaded cochleates to overcome the inherent problems associated with conventional batch processes. The device produced ART-cochleates with low dispersity, narrow size distribution, high reproducibility, and high throughput as well as with high ART encapsulation and loading. Under simulated gastro-intestinal conditions, the ART-cochleates showed high stability and more sustained release of ART. They also showed high ART absorption with neither apparent nor dose-dependent cytotoxicity. The device is a promising platform for the continuous production of drug-loaded cochleates with excellent reproducibility and control over the cochleate properties. The modular nature, inexpensive and readily availability of the device components aids in multiplexing this device in turn to achieve higher throughputs.

CHAPTER – 5

CONCLUSIONS AND FUTURE PROSPECTS

5.1 Overview of the dissertation

Chapter 1 of this thesis highlighted the importance and motivation for the current need to develop cochleate system for applications in drug delivery. Along with the state-of-the-art literature in the field, highlights of the current research status relevant to cochleate drug delivery systems were critically discussed. Further, the significance of using amikacin as a model drug and the need for encapsulating artemisinin in cochleates was discussed in detail. Also discussed was the need for the development of a continuous preparation method for drug-loaded cochleates. Finally, the specific objectives of this dissertation were given.

In Chapter 2, the consequences of the design of cochleate drug delivery system on their functional properties to achieve a desired outcome was demonstrated. An account on the use of important class III aminoglycoside drug, amikacin and Ca^{2+} in sub-threshold concentration range to prepare cochleates and its impact on the resulting properties such as morphology, internal ultrastructure, amikacin encapsulation and loading, in-vitro release kinetics, and their stability were given. Additionally, the use of techniques such as scanning electron microscopy, Fourier transformed infrared spectroscopy, and small angle X-ray scattering for the characterization of cochleates were described.

Chapter 3 reported the development of a cochleate-based formulation for the effective oral administration of artemisinin, a drug recommended by WHO for the treatment of malaria. The importance of optimising lipid composition to prepare artemisinin-loaded cochleates with desired encapsulation efficiency and drug loading characteristics was presented. We also showed the influence of lipid composition on the structural features of the cochleates. Further, a facile method of coating the cochleates with alginate to achieve controlled release of artemisinin, especially in gastro-intestinal milieu, was described along with the mechanism.

Chapter 4 dealt with the development of an inexpensive, off-the-shelf flow focusing device for the continuous production of artemisinin-loaded cochleates. The optimization of flow-focusing conditions such as flow rate ratio and total flow rate to obtain well-formed cochleates with high reproducibility was described in detail. The mechanism of formation of cochleates under the flow focusing conditions was discussed with the help of computational fluid dynamics simulations. The characteristics of resulting cochleates such as artemisinin encapsulation and loading, their cytotoxicity, in-vitro release, and the intestinal transport of artemisinin were also discussed in detail.

This chapter contains highlights from the previous chapters and a compilation of the conclusions drawn from them. It also provides an insight into the prospects for cochleates as advanced drug delivery system and the future scope for parallel studies on cochleates and cochleate-like materials for applications beyond drug delivery.

5.2 Research outcome and conclusions

Cochleates are lipid-based supramolecular systems that offer great opportunity as a vehicle for drug delivery. This dissertation sheds light on the aspects of cochleates that are seldom covered in the literature that help to achieve better design of this system backed by an efficient and reliable preparation technique. Studies on the mechanism of formation of cochleates bridged by non-metallic cation, amikacin with and without the presence of Ca^{2+} in sub-threshold concentrations compared with conventional Ca^{2+} -bridged cochleates showed a significant impact on the resulting functional properties. We proposed a synergistic model of cochleation when metallic and non-metallic bridging agents were used together. While amikacin-bridged cochleates showed lower internal structural rigidity, the rigidity increased when Ca^{2+} was used for bridging, due to stronger interaction of the DOPS head groups with Ca^{2+} . Whereas, when amikacin was used with Ca^{2+} in sub-threshold concentrations, they synergistically bind to DOPS headgroups, resulting in the tightly packed lipid phase of the cochleates. The drug release kinetics studied using diffusion-based Higuchi model showed potential for achieving tuneable drug release by simply varying the concentration of Ca^{2+} in the sub-threshold range. The insights from this work aid in the functional designing of cochleates to achieve a specific functional outcome which is crucial in drug delivery. Further, similar non-metallic bridging agents other than amikacin can be used to formulate cochleate-based systems for the encapsulation and delivery of drugs to meet specific needs such as desired rate of drug release, higher stability of the formulation, etc.

Having studied in detail the functional interdependence of cochleates on the mechanism of their formation, we then developed a cochleates-based system for the oral administration of artemisinin, a drug having poor bioavailability. Artemisinin is a WHO recommended drug for the treatment of malaria and shows therapeutic limitations due to its poor bioavailability caused by poor solubility and rapid metabolism. We optimized the lipid composition of cochleate system to attain high encapsulation and loading of artemisinin and found that a combination of DOPS and DMPS lipids to be effective. A facile technique of coating these cochleates developed in this study resulted in a better controlled-release of artemisinin under conditions mimicking gastro-intestinal environment. This was due to the crosslinking of alginate with surface Ca^+ on the cochleates. Overall, these artemisinin-loaded alginate-coated cochleates showed higher encapsulation and loading capacities along with better controlled-release and increased stability. While the functional coating of cochleate surface with polymers like alginate is considered as an effective method to enhance the oral administrability of cochleates, it also shows potential for active targeting prospects using cochleates.

Apart from the functional design of cochleate-based drug delivery system, the development of a facile device for the continuous preparation of cochleates is envisioned as a step forward, towards their translation to industry with several practical advantages. Hence, to overcome the inherent problems associated with existing conventional batch methods used for their preparation, we developed an off-the-shelf flow focusing device for the continuous production of drug-loaded cochleates. This device produced artemisinin-loaded cochleates having low dispersity and narrow size distribution with enhanced reproducibility. These

cochleates were non-toxic with neither apparent nor dose-dependent cytotoxicity. They also showed effective transepithelial permeability for the absorption of artemisinin across intestinal Caco-2 monolayer indicating better in-vitro bioavailability. This device is a promising platform for the continuous production of drug-loaded cochleates with excellent reproducibility and control over the cochleate properties. Moreover, the modular nature, low cost, and the ease of availability of the device components facilitate multiplexing in this device to achieve even higher throughputs. The production of drug-loaded cochleates under continuous aseptic process is also believed to support their smooth clinical translation.

Therefore, this dissertation promotes further advancement of cochleate system with better understanding of the mechanism of their formation and surface modification capabilities which may help in the development of tailor-made formulations to meet expected therapeutic outcomes as required by the patients. Furthermore, the continuous flow-based method using simple, inexpensive, off-the-shelf device is believed to meet the demands of robust and reproducible production of these formulations to support industrial applicability of the cochleate drug delivery system.

5.3 Future prospects

Cochleates are a promising platform for applications in drug encapsulation and delivery. They have gained tremendous interest in the recent past both in academia and industries alike. One of the several companies that invested in research on cochleate-based drug formulations, Matinas BioPharma, has several candidates in various clinical trial phases. For example, phase-2 clinical trials

have been completed for an oral cochleates formulation of amphotericin B with fast track, qualified infectious disease product (QIDP) & orphan drug designations granted by FDA and currently waiting approvals for phase-3 trials. Also, an oral Amikacin cochleate-based formulation is currently in phase-1 clinical trials, which has shown less toxicity and high efficacy against non-tuberculosis mycobacterial lung disease. Hence, the general focus for cochleates has been in the development of oral formulations with improved efficacy for some of the orally ineffective therapeutic drugs. Considering the rapid advances, one can at least expect drugs such as amphotericin B, amikacin, to be available in cochleate-based formulations over the coming years. Further, cochleates may have potential applications in the field of veterinary science for use in vaccine formulations. Agriculture, food, and nutraceutical industries might also benefit from cochleates as a vehicle for the encapsulation of essential micronutrients with low stability, like vitamins and minerals, and other valuable compounds like flavours, preservatives, etc. In addition, the availability of inexpensive, low purity lipid raw materials and development methods to prepare cochleates using these lipids could further facilitate the industrial scale production of cochleate-based formulations.

However, certain specific research problems that we believe would be worthwhile to address are mentioned in this section. The sustained release of encapsulated drugs is a highly desired property for micro- and nano-scale drug delivery systems. However, the initial rapid (or burst) release of drugs remains a major challenge in developing these systems. This burst release can cause release-associated drug toxicity as well as the reduction in the effective lifetime of the drug delivery system itself. Various approaches like surface coating of

these carriers can be used to minimize the extent of burst release, as described in this thesis. However, there is further scope for development of these techniques specifically for cochleate drug delivery systems. In this view, the integration of cochleate surface modification methods like alginate coating with the established continuous flow process for cochleate preparation would be an interesting value addition. This microfluidic approach could be a convenient way to prepare cochleate embedded alginate microcapsules having uniform size and structure to allow tuning of the release properties of incorporated drug(s) in a well-controlled manner. Another important aspect of cochleate production that remains a challenge is the purification of final product or removal of unwanted salts, contaminants, and non-entrapped drug molecules. The existing separation techniques such as dialysis, centrifugation, and chromatography are time-intensive and might result in a diluted final product or a reduced product yield. Furthermore, the feasibility of large-scale operations with these techniques is limited. This issue of final product purification may be addressed by using an in-line purification unit based on a tangential flow-filtration with a modular, easily exchangeable membrane, in tandem with our established off-the-shelf flow focusing device. Such flow-based system could facilitate the design of a robust process with easily scalable operating prospects for the purification of final cochleate-based drug formulation.

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