European Journal of Pharmaceutics and Biopharmaceutics 117 (2017) 270–275

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

Dispersion and stabilization of cochleate nanoparticles

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ARTICLE INFO

Article history:
Received 4 January 2017
Revised 28 March 2017
Accepted in revised form 27 April 2017
Available online 29 April 2017

Keywords:
Cochleate
Citrate
Aggregation
Stabilization
Calcium
AFM
SAXS
FTIR

ABSTRACT

Cochleates, calcium-stabilized membrane rolls of nanoscale diameter, promise a unique and efficient way of delivering lipid-soluble drugs, proteins or nucleic acids into biological systems because they protect the encapsulated material against enzymatic or chemical degradation. Self-aggregation, which typically arises during production and storage is a major obstacle that has so far precluded the development of an efficient cochleate-based drug-delivery system. Here we show that citric acid, added transiently in a narrow concentration range, effectively disperses cochleate aggregates, stabilizes the disperse state for long-term storage and preserves the canonical ultrastructure and topological characteristics of cochleate nanoparticles.

1. Introduction

Cochleates are biocompatible, prospective drug-delivery vehicles. They are formed of spirally rolled bilayer sheets of negatively charged phospholipids stabilized by Ca^{2+} ions [1] so that the lipid-to-calcium binding ratio is thought to be 2:1 [1–3]. Several studies have shown their applicability as vaccine adjuvants [4–8] and carriers of antibiotics [9–12], systemic antimycotic agents [13–16] or factor VII for replacement in hemophilia A [17,18]. However, their widespread use is severely hindered by a major technological obstacle: Ca^{2+} ions, which are essential in the formation and maintenance of cochleate structure, consistently provoke aggregation of the particles. Aggregation is a serious drawback as it prevents the efficient separation from byproducts, the assessment and monitoring of cochleate formation, the modification and functionalization of cochleate surface and the manufacturing of a disperse, long-shelf-life pharmaceutical delivery system. A recent patent claimed to remedy this problem by adding half and half, milk, casein, albumin or methylcellulose [19]. However, the use of protein excipients may lead to aggravated problems of reduced stability and safety, and altered pharmacokinetics. Furthermore, methylcellulose caused only a partial disruption of aggregates. A long list of other polymeric materials (e.g., celluloses, gums, starches, etc.) has also been suggested as potential aggregation inhibitors, but feasibility is uncertain [19]. A very recent comparison between a novel, microfluidics-based strategy and conventional cochleate production methods has underlined that aggregation prevails as a major, unsolved problem [20]. In the present work we developed an efficient method of preparing stable, disperse cochleates. Dioleoyl phosphatidylserine liposomes, which are the precursors of cochleates, exhibit outstanding colloidal stability. However, upon the addition of Ca^{2+} they quickly form aggregates. Aggregation is caused by the surface-charge-modifying effect of Ca^{2+} [21]. Therefore, by removing calcium the aggregates are expected to disassemble into their component nanoparticles. Removal of calcium, however, may lead to unfavorable consequences, because it is the Ca^{2+} ions themselves that stabilize the inner structure of cochleates. Indeed, it has been known since the discovery of cochleates that upon the addition of EDTA, a strong chelator of calcium, cochleate particles loose their rolled-up structure and fuse into giant vesicles [1]. We hypothesized that a molecule with an affinity to Ca^{2+} lower than that of EDTA may be effective to disperse aggregates by removing Ca^{2+} ions from the outer surface of

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http://dx.doi.org/10.1016/j.ejpb.2017.04.030
0939-6411/

Table 1
Association constants of some carboxylic acids to Ca^{2+} and H^+.

Acid	pK_a to Ca^{2+}	Ref.	pK_a[c] to H^+	Ref.
Acetic acid	0.53[a]	[24]	4.75	[25]
Tartaric acid	1.8[b]	[26]	3.04; 4.37	[25]
Citric acid	3.17[a]	[24]	3.13; 4.76; 6.40	[25]
EDTA	10.75[a]	[27]	0.0; 1.5; 2.0; 2.68	[28]

[a] At 25 °C and 0.15 M ionic strength.
[b] At 25 °C and cca. 0.2 M ionic strength.
[c] Refers only to carboxyl groups at 25 °C and 0 ionic strength.

cochleates but leaving their inner structure intact. To test this hypothesis, we systematically examined sodium salts of mono-, bi- and tricarboxylic acids: acetate, tartrate and citrate. All of these acids are in their fully deprotonated form at pH 7.4, and their association constant to Ca^{2+} increases with the number of their carboxyl groups (Table 1).

We show that the addition of sodium citrate in a narrow concentration regime can fully disperse aggregated cochleates, and after citrate removal a colloidally stable system is obtained in which the topology and ultrastructure of the nanoparticles are well preserved.

2. Materials and methods

2.1. Materials

Dioleoyl phosphatidylserine (1,2-Dioleoyl-sn-Glycero-3-[Phospho-L-Serine], DOPS) was purchased from Avanti Polar Lipids, Inc. (Alabaster, Alabama, USA). All other chemicals were from Sigma Aldrich Kft. (Budapest, Hungary).

2.2. Preparation of lipid vesicles and cochleates

Thin DOPS lipid film was formed on the wall of a clean glass tube by evaporating the organic solvent from 500 μl 20 mg/ml DOPS/chloroform stock solution under a stream of N_2. Organic solvent traces were removed by placing the tube in vacuum (<20 Hgmm) for 30 min. The lipid film was then hydrated with 100 μl aliquots of 100 mM TRIS (pH 7.4) to 1 ml final volume while vigorously vortexing. This multilamellar vesicle (MLV) suspension was extruded 41 times through a polycarbonate membrane filter with 100 nm pore diameter in an AvantiPolar Mini Extruder (Avanti Polar Lipids, Inc., Alabaster, AL, USA). The resultant small unilamellar vesicle (SUV) suspension of 12.4 mM DOPS concentration was diluted 1 to 1 with 100 mM TRIS (pH 7.4), and 400 μl of this solution was dialyzed for 24 h against 100 ml 6.2 mM $CaCl_2$, 100 mM TRIS (pH 7.4). The obtained cochleate suspension had a nominal 6.2 mM concentration for both DOPS and Ca^{2+}. The sample was stored at room temperature until use. The preparatory steps were done at room temperature.

2.3. Dispersion of cochleates

Aqueous solutions of the tested potential dispersing agents (sodium acetate trihydrate, sodium tartrate dihydrate, trisodium citrate dihydrate, disodium edetate) were prepared in a buffer containing 100 mM TRIS (pH 7.4). We added 10 μl of the solution to 10 μl cochleate sample and mixed intensively by drawing through a narrow pipette tip 50 times. Samples were examined within 10 min after being mixed with dispersing agents. The actual concentrations of dispersing agents are given in figure captions. Control experiments were carried out with buffer only.

To obtain a stabilized colloidal system, 20 μl of cochleate sample freshly dispersed with citrate was diluted with 80 μl of 100 mM TRIS (pH 7.4), then clarified by centrifugation (Heraeus Biofuge Pico, Life Technologies Magyarország Kft., Budapest, Hungary, 12800*g*, at 25 ± 1 °C, 5 min). The supernatant was gently removed and the pellet was resuspended in 90 μl of 100 mM TRIS (pH 7.4). The procedure was repeated 3 times. To test the effect of Ca^{2+} on resuspended cochleates, the pellets were resuspended with 100 mM TRIS (pH 7.4) containing 6.2 mM $CaCl_2$.

2.4. Phase contrast microscopy

Micrographs were recorded with a Nikon Eclipse Ti-U inverted microscope (Auro-Science Kft., Budapest, Hungary) equipped with a uEye UI 1220 LE digital camera (IDS Imaging Development Systems GmbH, Obersulm, Germany) using either a 20x or a 40x Nikon S Planfluor phase contrast objective.

2.5. Atomic force microscopy

For AFM imaging 10 μl of the control or dispersed cochleate sample was diluted with 50 μl 100 mM TRIS (pH 7.4) then applied to the substrate and, after 1 min incubation, washed gently with MilliQ water and dried in a stream of N_2 gas. For control cochleates freshly cleaved mica sheets were used as substrate. In the case of dispersed cochleates we used mica sheets pre-coated with 100 μl 0.01% poly-L-lysine for 20 min. Cochleates were imaged with a Cypher instrument (Asylum Research, Santa Barbara, CA) with 0.5–2.5 Hz line-scanning rate in air at 29 ± 1 °C. A silicon cantilever (OMCL AC-160TS, Olympus, Japan) was used in non-contact mode, oscillated at its resonance frequency (300–320 kHz, typically). Images with 512 × 512 pixel dimensions were collected and analyzed with the built-in algorithms of the AFM driver software.

2.6. Small-angle X-ray scattering

Small-angle X-ray scattering (SAXS) measurements on the cochleate samples were carried out with a CREDO instrument integrated with a GeniX^{3D} Cu ULD microfocus system (Xenocs SA, Sassenage, France) for producing X-rays and a Pilatus-300 k CMOS hybrid pixel position sensitive detector (Dectris Ltd, Baden, Switzerland) [22,23]. The recorded 2D scattering patterns were subjected to the standard on-line data reduction procedure of the instrument control software. Corrections were made for instrumental background, detector flatness, and sample self-absorption. The abscissa was calibrated with mesoporous silica into scattering variable units $q = (4 \cdot \pi \cdot \sin\theta) \cdot \lambda^{-1}$, where 2θ is the scattering angle and $\lambda = 0.154$ nm is the X-ray wavelength of Cu Kα radiation. Intensity was transformed from count rates to differential scattering cross-section ($cm^{-1} \cdot sr^{-1}$) units using a pre-calibrated glassy carbon specimen.

The MLV sample was used without further processing. Cochleate samples were clarified by centrifugation (see above). All the samples were filled into borosilicate capillaries of cca. 1 mm outer diameter and 0.01 mm wall thickness and centrifuged gently (Eppendorf Minispin microcentrifuge, Eppendorf AG, Hamburg, Germany, 67*g*; at 25 ± 1 °C, 5 min) to prevent sedimentation during experiment. The total exposure time was at least 2 h in each case, carried out in several 5-min exposures in order to monitor sample stability.

2.7. Infrared spectroscopy

ATR-FTIR spectra were collected with a Varian 2000 FTIR Scimitar Series (Varian Inc., Paolo Alto, CA) spectrometer equipped with a 'GoldenGate' (Specac Ltd., London, UK) single reflection diamond ATR accessory. The measurements were performed at room temperature: 5 μl sample was mounted on the top of the diamond