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# Cochleates bridged by drug molecules

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ABSTRACT

A new type of cochleate, able to microencapsulate water-soluble cationic drugs or peptides into its inter-lipid bi-layer space, was formed through interaction between negatively charged lipids and drugs or peptides acting as the inter-bi-layer bridges instead of multi-cationic metal ions. This new type of cochleate opened up to form large liposomes when treated with EDTA, suggesting that cationic organic molecules can be extracted from these cochleates in a way similar to multivalent metal ions from metal ion-bridged cochleates. Cochleates can be produced in sub-micron size using a method known as "hydrogel isolated cochleation" or simply by increasing the ratio of multivalent cationic peptides over negatively charged liposomes. When nanometer-sized cochleates and liposomes containing the same fluorescent labeled lipid component were incubated with human fibroblasts cells under identical conditions, cells exposed to cochleates showed bright fluorescent cell surfaces, whereas those incubated with liposomes did not. This result suggests that cochleates' edges made them fuse with the cell surfaces as compared to edge free liposomes. This mechanism of cochleates' fusion with cell membrane was supported by a bactericidal activity assay using tobramycin cochleates, which act by inhibiting intracellular ribosomes. Tobramycin bridged cochleates in nanometer size showed improved antibacterial activity than the drug's solution.


## 1. Introduction

According to Egan and Lauri (2002), most of the drugs are absorbed across the tissue membrane by passive diffusion. While particulate therapeutics may be taken up through endocytosis of lymphatic epithelial cells such as Peyer's patches, its efficiency is low because of the limited population of these cells (Camenish et al., 1996; Egan and Lauri, 2002). Hydrophilic molecules can be absorbed through the water filled tight junctions (Salama, 2006) but only a small portion of drug molecules can have access to the tight junctions along the entire intestinal wall. Although some of the hydrophilic drugs such as nucleosides, anti-folates, and some small peptides are absorbed cross the membrane by attaching to membrane transporters, these transporters are specific to certain therapeutics (Sai and Tsuji, 2004; Huang and Sadee, 2006). Moreover, structural modifications of drug molecules are often required to facilitate receptor-mediated drug molecule absorption, which may alter the pharmacological activity of the drug molecule (Morishita and Peppas, 2006). Therefore, there is a need to develop delivery system, which could facilitate diffusion of the drugs across the cell membrane.

Reported strategies to improve drug absorption through cross membrane diffusion included pro-drug analogue design, application of absorption enhancers and enzyme inhibitors, and delivery by using lipid-based systems (Panchagnula and Sood, 2001; Kaparissides et al., 2006). Lipid-based delivery systems including liposomes attracted enormous research efforts as a cross membrane drug delivery vehicle because of their structural resemblance with cell membrane (Helens and Bentz, 1984; Lu and Huang, 1989; Torchillin et al., 1993; Gabizon and Papahadjopoulos, 1998; Drummonds and Zignanai, 2000; Leroux et al., 2001; Turk et al., 2002; Feng et al., 2004; Fahr et al., 2005). Utilization of liposomes to improve oral absorption of hydrophilic drugs remains unsuccessful mainly due to their poor mechanical stability, low-drug loading capacity (Lu and Huang, 1989; Morishita and Peppas, 2006), and probably the lack of mechanism to facilitate cross membrane diffusion in intestine. Cochleates are solid particulates made of large continuous, lipid bi-layer sheets rolled up in a spiral structure with no internal aqueous phase (Zarif, 2002). These unique structures were first observed and reported by Papahadjopoulos and Wilschut (1979) and later by others (Goldstein and Lukaynov, 1997; Lee and Lukaynov, 1998; Lee and Carlson, 1999; Price and Patchan, 1991). Utilization of cochleates to deliver drugs such as anti-fungal agents,

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DNA, and subunit vaccines were reported by Mannino et al. (1998), and Zarif and Mannino (2000).

Cochleates were initially prepared in micrometer sizes, either by direct addition of multivalent ion solution to liposome's solution or by the dialysis method (Vekleig et al., 1974; Papahadjopoulos, 1978; Papahadjopoulos et al., 1978; Wilschut and Papahadjopoulos, 1979). However the particle size could not be reduced to nanometer range. Jin et al. (2000) and Zarif et al. (2000) reported a method named "hydrogel isolated cochleation" to prepare nanometer-sized cochleates. By this method, a very small amount of liposome is isolated in each dextran droplet dispersed in a polyethylene glycol (PEG) continuous phase resulting in the formation of nanometer-sized cochleates (nanocochleates) upon interaction with multivalent ions. These nanocochleates when loaded with amphotericin B showed significantly improved oral absorption and anti-fungal activity as compared to drug solution and micrometer-sized cochleates (Jin et al., 2000; Zarif et al., 2000). This enhanced activity may be attributed to the membrane fusion capability of cochleates and an increased cell-cochleate contact by increasing number of particles due to their size reduction. The high tension at the bi-layer edges of cochleates (Helfrich, 1986; Mannino et al., 1998) is suggested to be the driving force of cochleate's interaction with the tissue membrane.

Though the membrane fusion capability of cochleates is more important for the delivery of hydrophilic drugs across the membrane, cochleates and nanocochleates have not demonstrated an effective microencapsulation of hydrophilic drugs. However, there are some reports suggesting that hydrophilic biomolecules such as proteins or DNA could be encapsulated in cochleates during calcium ion induced cochleation (Zarif et al., 2000). The possibility that these charged molecules were co-precipitated during the interaction of multivalent cations with liposomes rather than their own microencapsulation in the lipid assemblies cannot be ruled out. Since cochleates are formed by ionic interaction between negatively charged liposomes and bivalent cations, microencapsulation of cationic drugs using themselves as the bridging agents of cochleation can be rationalized. In order to test this hypothesis, two water soluble and multivalent cationic molecules, i.e., tobramycin and polylysine (oligo-peptide) were used to form cochleates with negatively charged liposomes.

## 2. Materials and methods

### 2.1. Materials

1-Oleoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino] hexanoyl]-*sn*-glycero-3-phosphocholine (fluorescent lipid) and dioleoylphosphatidylserine (sodium salt, molecular weight 810.3) were purchased from Avanti Polar (AL, USA). Tobramycin, poly L-lysine hydrobromide (molecular weight 500–2000), dextran (molecular weight 500,000), and PEG (molecular weight 18,000) were obtained from Sigma Chemicals (MO, USA). Ethylenediamine-tetraacetic acid (EDTA) was supplied by Fluka (WI, USA). LB Broth Base and Select Agar were purchased from Gibco-BRL Life Technologies (UK). *Escherichia coli* strain was obtained from Neal Copeland (USA) whereas yeast culture was purchased from Invitrogen (CA, USA). Primary human foreskin fibroblast (HFF) cells were prepared from skin tissue samples and grown in Dulbecco's modified Eagle medium plus 10% fetal calf serum at 37 °C in a humidified atmosphere of 3% $CO_2$, 97% air (Wang et al., 2005). They were regularly split to 1:3 dilutions by using conventional procedures with 0.05% trypsin plus 0.02% EDTA in HEPES buffered saline.

### 2.2. Methods

#### 2.2.1. Preparation of small unilammelar vesicles

A 500 μl of dioleoylphosphatidylserine (DOPS) solution in chloroform (10 mg/ml) was added into a 5 ml clear vacutainer, followed by solvent evaporation using nitrogen stream. Then, the dried lipid was hydrated with 1 ml of distilled water in nitrogen seal, and sonicated for few minutes until the milky liquid become transparent, indicating formation of nanometer-sized liposomes. Similarly, fluorescent liposomes were prepared by following the same procedure except addition of 2% of fluorescent lipid to the DOPS.

#### 2.2.2. Cochleation without multivalent metal ions

A 100 μl of polylysine (10 mg/ml, pH 2) or tobramycin (1 mg/ml, pH 2) solution was added drop-wise to 100 μl of liposomes (5 mg/ml) under vortex. A precipitation was formed and the samples were refrigerated at 2–8 °C.

#### 2.2.3. Nanometer-sized cochleation with tobramycin as bridging agent

A 500 μl of liposomes (5 mg/ml) prepared as above was added to 2 ml dextran solution (40%, w/w) and then dispersed with 20 ml of PEG solution (15 %, w/w, pH 2). The sample was stirred with a magnetic bar for 2 h. A 500 μl of tobramycin solution (1 mg/ml, pH 2) was added drop-wise to the stirred dextran/PEG emulsion, followed by continuous stirring at 4 °C for 6 h. Nanometer-sized tobramycin cochleates were then collected by rinsing dextran and PEG with 200 ml of water, followed by centrifugation. This method is referred to as hydrogel isolated cochleation (HIC) (Jin et al., 2000).

#### 2.2.4. Nanometer-sized cochleation with polylysine as bridging agent

A 100 μl of liposomes (5 mg/ml) prepared as above was added to 400 μl of polylysine (10 mg/ml, pH 3.0) under vortex. A precipitation was formed and the sample was refrigerated at 2–8 °C.

#### 2.2.5. Cochleation using calcium chloride

Calcium bridged cochleates were prepared by adding 200 μl calcium chloride solution (20 mM) drop-wise to 200 μl of liposome (5 mg/ml) under vortex. A precipitation was formed and the sample was refrigerated at 2–8 °C.

#### 2.2.6. De-cochleation with EDTA

A drop of cochleate sample (either bridged by tobramycin, polylysine, or calcium) was added on a glass slide, covered with a glass cover slip, and loaded to a microscope equipped with oil immersion lens of 100× magnification. A drop of EDTA (200 mM, pH 8) was placed at the edge of the glass cover slip, allowing EDTA to diffuse in the cochleate carrying medium and to react with the cochleate. Microscopic images of the sample were taken before and after the EDTA treatment through a built-in computerized data acquisition system. The momentary changes during the transformation of cochleates into large liposomes were observed under microscope.

#### 2.2.7. Electron microscopic images

A drop of each cochleate sample (either bridged by tobramycin, polylysine, or calcium) was placed on the glass slide already mounted to the sample holder, air dried under the hood, sputter coated, and loaded into Amray 1830I scanning electron microscope for images recording.

#### 2.2.8. Cochleates fusion with cell membrane

To examine cochleates fusion with the cell membrane, 2% of 1-oleoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-

*sn*-glycero-3-phosphocholine was mixed with DOPS prior to liposomes preparation. The fluorophore of the fluorescent lipid is the ring structure at the terminal end of the *sn*-2 fatty acid of the molecule. Two sample plates (six well counts) already cultured with fibroblast cells were incubated with liposome and cochleate samples. The cell count was $3 \times 10^5$ cells/well. Fluorescent labeled nanocochleates were incubated at 25 °C with yeast and human fibroblasts cells for 2 h and then rinsed to remove cochleates' suspension. Yeast cells were rinsed with water whereas HFF cells were rinsed with extra growth medium. Fluorescent liposomes were also incubated with yeast and HFF cells under identical conditions to compare with cochleates' cellular fusion. The HFF cell culture was observed under Olympus 1×50 inverted Fluorescent microscope using 40× magnification lens. Fluorescent microscopic images of the sample were taken through a built-in computerized data acquisition system. Yeast cells were observed under Olympus B×61 fluorescent microscope using 40× magnification lenses. Fluorescent microscopic images of the yeast cells were taken through a built-in computerized data acquisition system.

#### 2.2.9. Anti-microbial activity test

A drop of *E. coli* culture (DH5α) was added to 2 ml of LB broth solution in tube, mixed well, incubated at 37 °C, and shaken at 200 rpm for 24 h. DH5α (1:10) culture solution was formed. Base dishes were inoculated with this culture solution. At the same time tobramycin solution, tobramycin loaded large size cochleate suspension, and tobramycin loaded nanocochleate suspension samples were prepared with a final concentration of 0.5 μg/ml, 1.0 μg/ml, 2.5 μg/ml and 5.0 μg/ml. A 50 μl of each test sample was added to the base dishes, incubated at 37 °C, and shaken at 200 rpm for 24 h, followed by counting the surviving colonies.

## 3. Results and discussion

### 3.1. Cochleation by using organic cations as bridging agents

Adding tobramycin (an antibiotic drug with five amino groups) or polylysine solution (at pH < 3 to ensure full ionization of the amino groups of the cationic drugs) to a nanometer-sized liposome suspension of DOPS resulted in precipitation. Fig. 1A1, B1 and C1 shows the microscopic images of the precipitates formed by adding polylysine, tobramycin, and calcium chloride to liposomes, respectively. The precipitates formed with polylysine were fibrous in shape whereas as those formed with tobramycin possessed round shape particles. However none of them showed a typical cylindrical shape of the metal ion-bridged cochleates. This result suggests that cochleate's morphology is strongly dependent upon the structure of the bridging agent. Interestingly enough, all of these precipitated particles of different morphology opened up to form large liposomes when treated with EDTA (Fig. 1A2 and B2), indicating that organic cations can be extracted from solid–lipid bi-layer assembly in a way similar to multivalent metal ions from metal ion-bridged cochleates (Fig. 1C2). The similarity of tobramycin, polylysine and calcium ions in terms of precipitating anionic liposomes as well as being extracted from the lipid precipitates by EDTA suggest that tobramycin and polylysine functioned in the same way as calcium cations in cochleation, i.e., ionic interaction of lipid bi-layers (composed of the array of negative charges) to form a solid multi-layer assembly. To further confirm the morphological structures of the lipid precipitates, the particles formed with tobramycin, polylysine and calcium ions were subjected to scanning electron microscope (SEM) and their images are shown in Fig. 2. The branched fibrous shape structure of polylysine bridged cochleates may be comparable to those formed by DNA-cationic liposome's interaction of positively charged liposomes with negatively charged DNA which resulted either in spherical clusters or micro size rods depending upon the molar ratio of two components (Gershon et al., 1993; Pouton, 1998).

**Table 1**
Particle size data of liposome sample and the nanocochleates produced by mixing polylysine and liposomes in 4:1 and 3:1 ratio

| | Particle size data | | |
|---|---|---|---|
| | Liposome | Sample 1 | Sample 2 |
| Description | Liposome solution (10 mg/ml) | Cochleates | Cochleates |
| Charge ratio | n/a | 4:1 | 3:1 |
| pH | – | 3 | 3 |
| Mean | 58.7 nm | 111.3 nm | 160.1 nm |
| S.D. | 29.169 nm | 57.813 nm | 88.857 nm |
| Coefficient of variation | 0.498 | 0.529 | 0.532 |

### 3.2. Organic cationic bridged cochleates of reduced sizes

Tobramycin bridged cochleates were prepared in sub-micron sizes using the hydrogel isolated cochleation method (Jin et al., 2000). Polylysine bridged cochleates were produced in sub-micron sizes by increasing the ratio of positively charged polylysine over negatively charged liposomes. The cochleates formed were analyzed for particle size using Nicomp nanometer particle sizer. Fig. 3 shows the particle size distribution of the tobramycin cochleates prepared by HIC method. The data shows particle size distribution in a dynamic diameter range of 60–1000 nm with a mean of 280 nm. For polylysine cochleates, the desired particle size can be achieved by selecting the ratio of cationic polymer and negatively charged liposomes. In this study polylysine bridged cochleates were prepared by direct addition of polylysine to liposomes at the charge ratio of 2:1, 3:1 and 4:1. When mixed in 3:1 and 4:1 peptide to liposome charge ratio, particles were formed in nanometer size, whereas mixing in 2:1 ratio resulted in the formation of micrometer size particles. Increasing polylysine to liposome ratio (charge ratio) from 3:1 to 4:1 resulted in particle size reduction. However, the change was not significant. The particle size data for polylysine nanocochleates was collected by Nicomp Sub-micron Particle Sizer, Model 370. Particle size data of the polylysine cochleates formed at the charge ratio of 3:1 and 4:1 is listed in Table 1. The mean particle size was reduced from 160 nm to 111 nm when the charge ratio was increased from 3:1 to 4:1. The cochleates formed at 2:1 ratio were out of the detection limit of the sub-micron particle sizer, suggesting that particles were in micrometer size range. Fig. 4 shows the particle size distribution functions of the polylysine cochleates formed at charge ratio of 3:1 and 4:1, as well as liposome's particle size prior to cochleation. It is hypothesized that it is probably the electric repulsion generated by extra positive charge that caused the formation of cochleates in nanometer sizes. The charge-ratio dependent particle size control has been reported (Berre and Duffin, 1998; Pouton, 1998; Zhu and Li, 2003) for polymeric complexes (polyplexes and lipoplexes). Polyplexes and lipoplexes are formed due to the ionic condensation between DNA-cationic polymers and DNA-cationic liposomes, respectively, in the gene delivery studies. Such a charge-ratio dependent particle size control is consistent with the observations of this study.

This has been previously reported that cochleates (formed with $Ca^{2+}$ or $Zn^{2+}$ ions) in nanometer sizes showed considerable improvement in the absorption of amphotericin B, an anti-fungal drug normally given by intravenous injection (Jin et al., 2000). Particle size of amphotericin B played a crucial role in improving oral absorption of the drug (Zarif et al., 2000). Amphotericin cochleates with dynamic diameters below 1 μm showed considerable absorp-

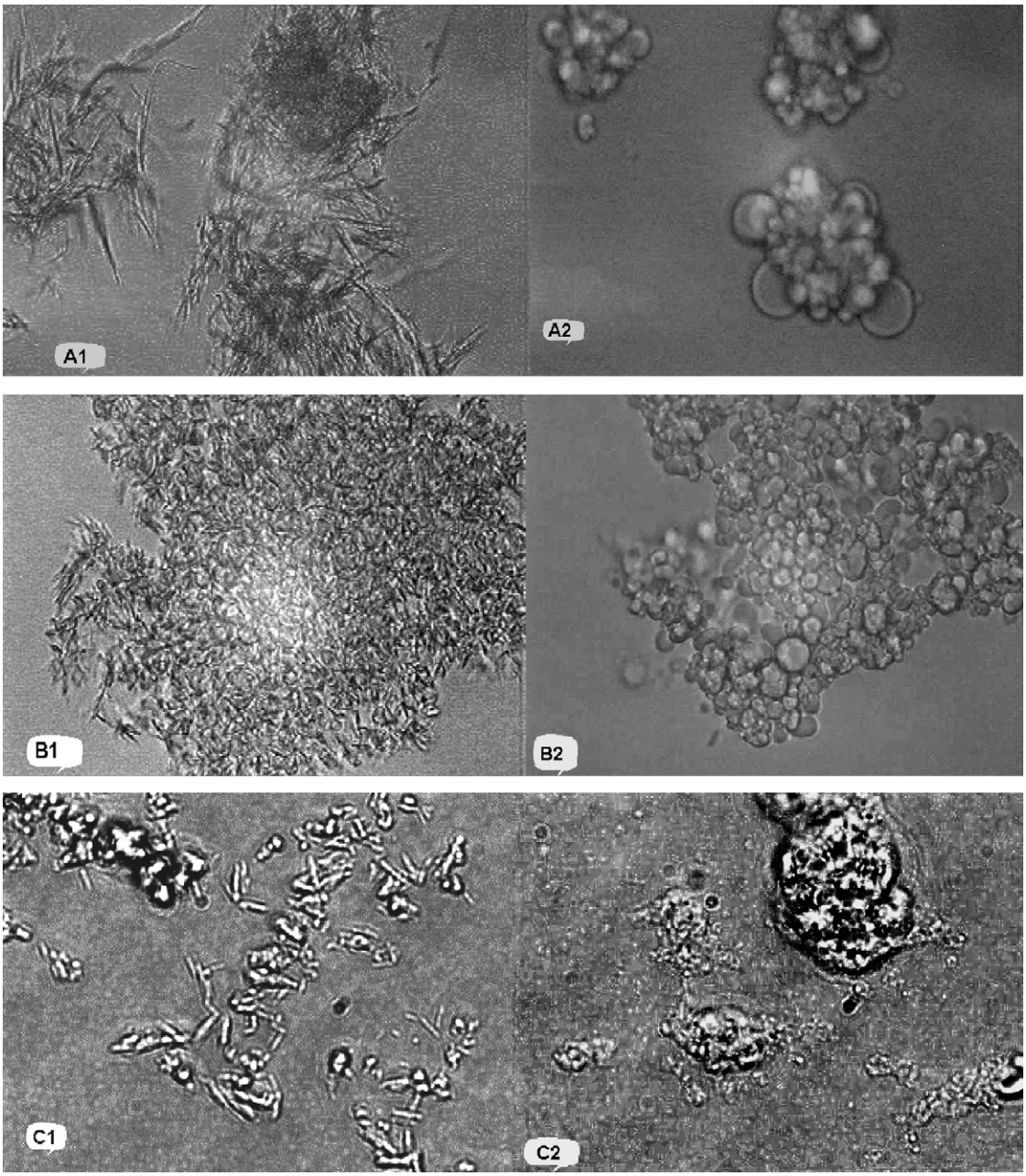


**Fig. 1.** Optical images: polylysine (A1 and A2), tobramycin (B1 and B2), and calcium (C1 and C2) cochleates. Left side images depict cochleates and right side images show formation of large liposomes on the application of EDTA to the respective cochleates.

tion, whereas those with dynamic diameters of several microns failed to show any absorption. We hypothesize that reducing particle size of the cochleates in nanometer range facilitates the cross membrane permeability of cochleates.

### 3.3. Cochleates–cell interaction

To examine the interaction of cochleates with cell membrane, 2% fluorescent lipid was mixed with DOPS to form fluorescent liposomes (Sampaio et al., 2005; Villa et al., 2005). When cochleates interact with cell membrane involving a fluorescent lipid transfer, cell surfaces become fluorescent under fluorescent microscopes. Fluorescent nanocochleates were prepared by adding polylysine or tobramycin to the fluorescent labeled liposomes through a charge-controlled cochleation or HIC method respectively. The fluorescent nanocochleates and fluorescent liposomes were incubated with yeast and HFF cells for 2 h. Then the cells were rinsed with fluorescent-free medium (or water) and observed under a fluorescent microscope. The cells treated with nanocochleates showed a bright fluorescent contour, whereas those treated with liposomes did not (Figs. 5–7). Fig. 5A and B shows the fluorescent microscopic images of the yeast cells incubated with fluorescent polylysine nanocochleates and fluorescent liposomes respectively, while Fig. 6A and B shows the fluorescent microscopic images of the yeast cells treated with fluorescent tobramycin nanocochleates and fluorescent liposomes respectively. Fig. 7 shows the photo images of the mammalian cells (HFF) exposed to fluorescent nanocochleates of polylysine (Fig. 7A) and fluorescent liposomes (Fig. 7B). Figs. 5A and 6A show fluorescent dots on the surface of the yeast cells, suggesting that nanocochleates were adsorbed

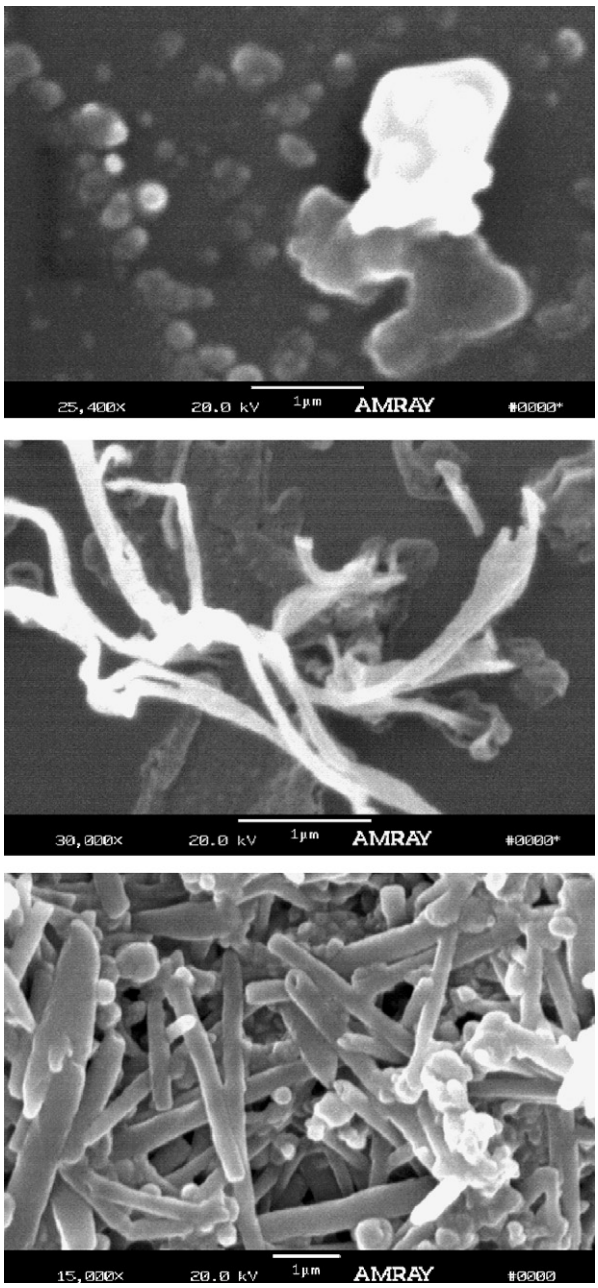


**Fig. 2.** Scanning electron microscope images of cochleates formed with tobramycin (A), polylysine (B) and calcium ions (C).

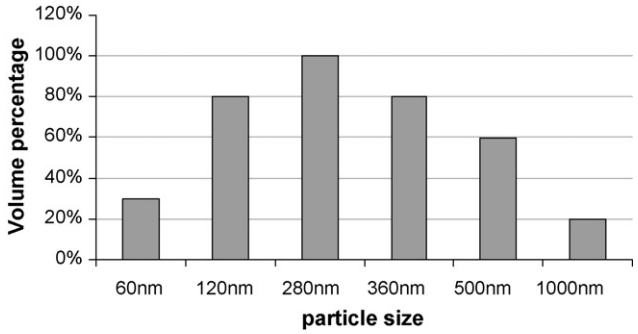


**Fig. 3.** Particle size distribution of tobramycin loaded nanocochleates, using Nicomp sub-micron particle sizer model 370.

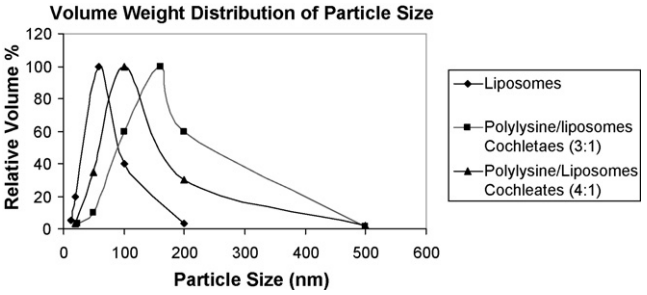


**Fig. 4.** Particle size distribution data of cochleates samples and liposome.

to the yeast cell walls. Fluorescent image of the HFF cells treated with fluorescent nanocochleates (Fig. 7A) shows distinctly bright and uniform cell line contours. The fluorescent cell line contours of HFF cells as compared to fluorescent dots on yeast cells, suggest that nanocochleates fused with HFF cell membranes rather than adsorbed to the cell surfaces. If the interaction between nanocochleates and HFF cells would have been mainly due to adsorption, it should have resulted in the presence of fluorescent dots at the HFF cell surfaces similar to once at the yeast cell walls rather than smooth and bright cell contours. In terms of cochleate–cell membrane fusion, the high tension of the lipid bilayer edges (line tension of the membrane edges) of cochleate as

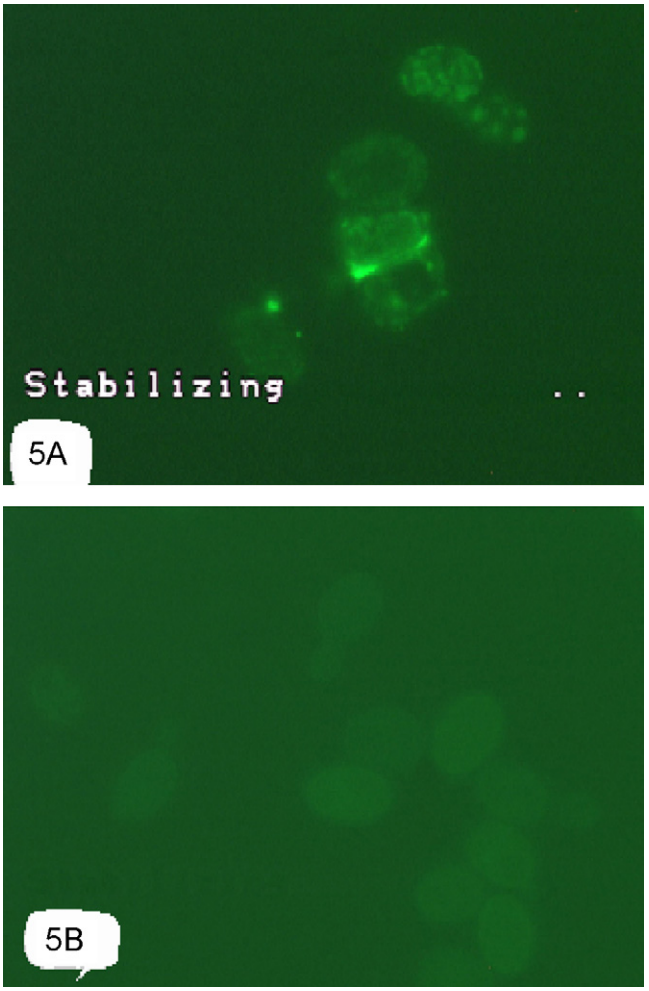


**Fig. 5.** Images of yeast cells exposed to fluorescent polylysine nanocochleates (A) and fluorescent liposomes (B).

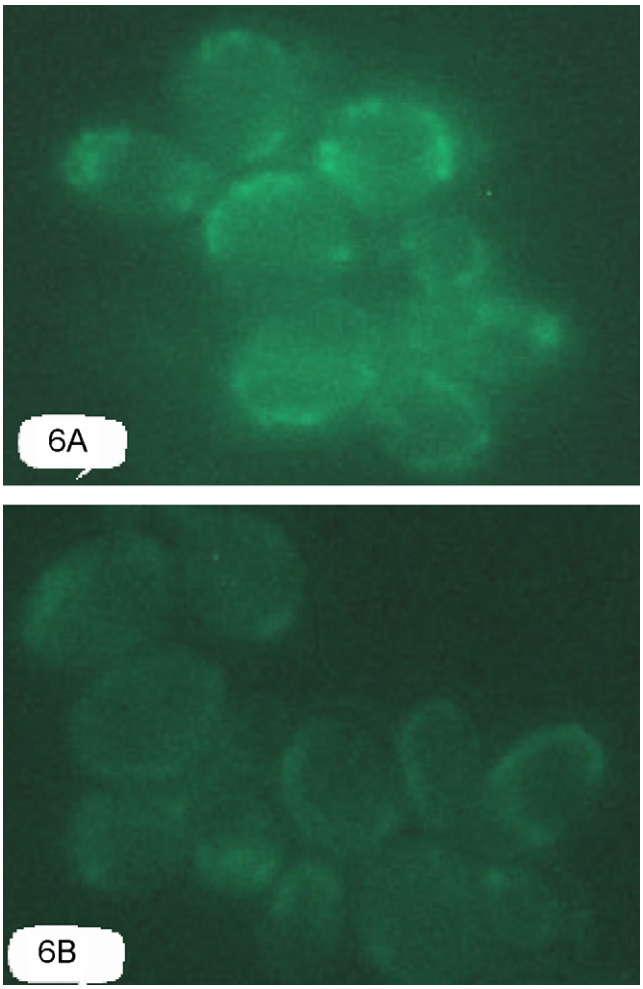


**Fig. 6.** Fluorescent images: yeast cells exposed to fluorescent nanocochleates of tobramycin (A) v/s fluorescent liposomes (B).

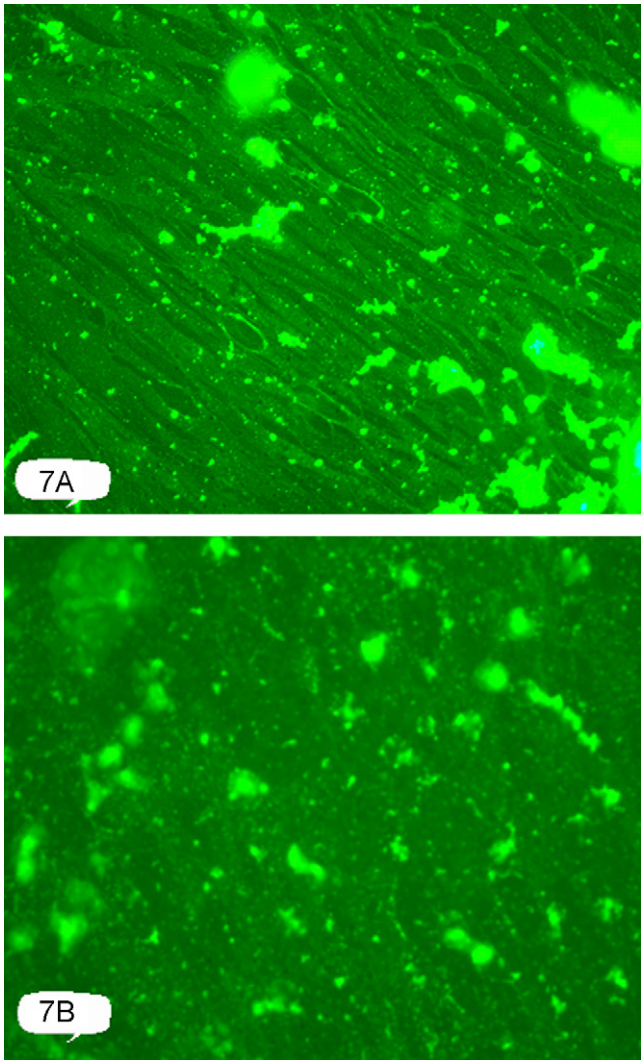


**Fig. 7.** Image of the mammalian skin cells (HFF) exposed to the polylysine nanocochleates (A) and fluorescent liposome (B).

compared to edge free spherical liposome is suggested to be the driving force (Lundbaek and Maer, 2000; May, 2000).

To examine cross membrane delivery of the inter-bi-layer contents of the cochleates, tobramycin cochleates (of both nanometer and micrometer sizes) were incubated with *E. coli* culture. Tobramycin was selected as the model drug for this study, as the acting site of this drug is not receptors at cell surfaces but the ribosomes inside the bacterial cell. Fig. 8 shows the results of antibacterial activity of tobramycin nanocochleates in comparison with tobramycin solution and tobramycin loaded cochleates in micrometer sizes. At low dose of tobramycin (0.5 μg/ml), both tobramycin solution and tobramycin nanocochleates showed significant antibacterial activity, while the numbers of *E. coli* colonies reduced by the tobramycin solution were higher than those reduced by nanocochleates formulation. However by increasing the dose to 1 μg/ml, the numbers of surviving *E. coli* colonies after incubation with nanocochleates were significantly lesser than those treated by tobramycin solution. Antibacterial activity of tobramycin loaded in nanocochleates was lower than the tobramycin solution at 0.5 μg/ml but higher at 1.0 μg/ml. While free molecular tobramycin itself showed considerable anti *E. coli* activity, the dose dependency between tobramycin loaded in nanocochleates and free tobramycin solution suggests that nanocochleates delivered their drug loading across the cell through a different pathway than free molecules. The lower anti *E. coli* activity of the nanocochleates formulations at low dose (0.5 μg/ml) may be attributed to insufficient number of cochleate particles accessible to *E. coli* cells. For example, micrometer size cochleates, which have remarkably reduced number of particles for an equivalent dose, when treated with *E. coli* showed significantly reduced antibacterial activity (Fig. 8).

The proposed mechanism of the cross membrane delivery of lipophobic drugs loaded in the inter-bi-layer spaces of cochleates

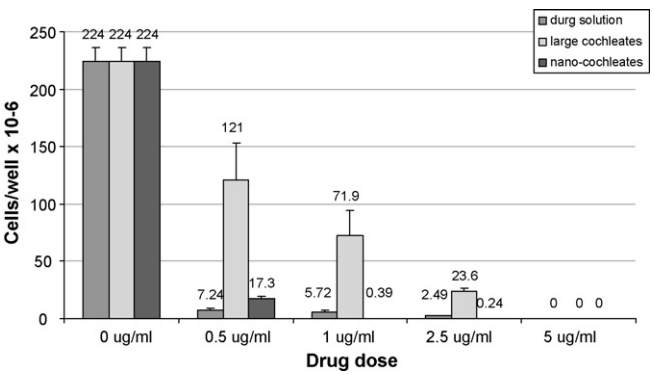


**Fig. 8.** Anti-microbial activity of tobramycin cochleates, nanocochleates, and drug solution against *E. coli*.

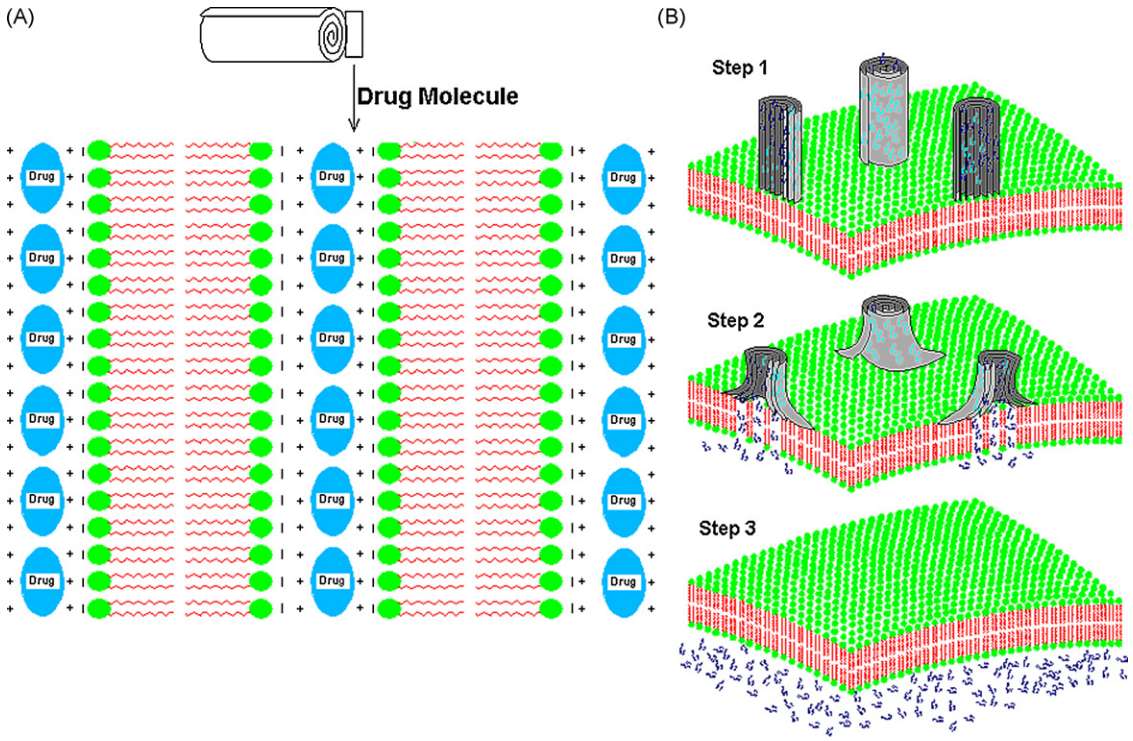


**Fig. 9.** New cochleation schematics (A) and cochleates' fusion with tissue membrane (B).

is shown in Fig. 9. We hypothesize that inter-bi-layer contents of cochleates are delivered into cells, when lipid bi-layer structure of nanocochleates fuses with the cell membrane.

## 4. Conclusion

Hydrophilic and multi-cationic drugs and peptides can be encapsulated into cochleates and nanocochleate particles as cochleates' bridging agents. This new type of cochleate is inherent in the physico-chemical properties of metal ion-bridged cochleates in terms of their conversion back to liposomes upon the extraction of cation bridging agent and their ability to fuse with the cell membrane. These unique properties enable cochleates and nanocochleates to deliver charged hydrophilic drugs across the tissue membrane.

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