

Formulation development and characterization of nanocochleates for the improvement of permeability of drug

Akash Yadav*¹,
Shaily Chaudhary^{2,3}

¹IPS Academy, College of
Pharmacy, Indore (M.P.)

²Faculty of Pharmacy, Pacific
University, Udaipur (Rajasthan)

³Acropolis Institute of
Pharmaceutical Education and
Research, Indore (M.P.)

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ABSTRACT

Rifampicin is the first choice drug for treatment of tuberculosis (TB), but it shows variable absorption in presence of other anti-TB drugs, so high dose is required. To overcome this problem nanocochleates of rifampicin were prepared through the interaction of calcium ions with the negatively charged phospholipids. It was done by two step process; first the liposomes of the drug were prepared by film hydration method using rotary evaporator, then dropwise calcium ions were added to the liposomes under constant stirring to form nanocochleates. Liposomes were evaluated for parameters of vesicle size and percentage entrapment, and the best formulations were selected for preparing nanocochleates. Cochleates thus prepared were subjected under particle size analysis, size distribution and SEM, which shows the elongated rod shaped sub micron size particles. Permeability studies were performed using the ex-vivo model, everted rat intestine was used to determine apparent permeability of rifampicin and comparing it to the apparent permeability shown by the rifampicin loaded nanocochleates. A significant increase in the apparent permeability of rifampicin (3.56×10^{-6} cm/sec) was shown when given as nanocochleates of rifampicin (7.78×10^{-6} cm/sec), thus this strategy can be used to improve the absorption of rifampicin through small intestine.

Keywords: Rifampicin, nanocochleates, cochleates, apparent permeability, liposomes.

INTRODUCTION

Cochleates are elongated rolled microstructures that consist of a series of lipid bilayers formed as a result of condensation of small unilamellar negatively charged liposomes. These structures were first reported by Papahadjopoulos and Wilschut (Papahadjopoulos and Wilschut, 1979). These are solid particulates which are made of continuous lipid bi layer sheets rolled up into a spiral structure by interaction with multi cationic metal ion such as Ca^{2+} and have no internal aqueous space. Cochleate particles are stable phospholipid-cation precipitates composed of simple, naturally occurring phospholipids, such as dioleoylphosphatidylserine (Zarif, 2002) and/or a mixture of lipids containing atleast phosphatidylserine, phosphatidylinositol, phosphatic acid, phosphatidyl glycerol upto 75% by weight with phosphatidylcholine,

phosphatidylethanolamine and other less abundant phospholipids (Sankar and Reddy, 2010). Because of this unique structure, cochleates have the ability to encapsulate hydrophilic as well as hydrophobic moieties of any shape and size thus making them the most versatile carrier for delivery of a wide range of drug molecules, proteins and peptides. They also protect the entrapped molecule from harsh conditions of pH, temperature and lipase degradation.

Rifampicin is a semisynthetic antibiotic (bactericidal) produced from *Streptomyces mediterranei*. It is a broad antibacterial spectrum, having activity against several forms of Mycobacterium. It is the first choice drug in the treatment of tuberculosis but requires a high dose for a period of 4-6 months in Multi Drug Therapy (MDT) along with isoniazid, ethambutol and pyrazinamide. The BCS classification of rifampicin puts it under BCS class II (low solubility, high permeability) having an apparent permeability of about $5.79 \pm 0.053 \times 10^{-6}$ cm/s which is above the critical limit of 2×10^{-6} cm/s (Biganzoli et al., 2009), and it is expected to have a bioavailability of over 90% (Artursson and Karlsson, 1991). But low rifampicin

Address for correspondence

Dr. Akash Yadav
IPS Academy, College of Pharmacy, Indore (M.P.) India
E-mail: akash.ipsa@gmail.com

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concentrations have been observed in tuberculosis (TB) patients. Also the apparent permeability coefficient of rifampicin is variable and changes throughout the intestinal mucosa. Concentrations of antimycobacterial drugs below than normally expected during tuberculosis (TB) therapy may cause appearance of resistant strains of *Mycobacterium tuberculosis* (Peloquin, 2002). Low antimycobacterial drug concentrations can be due to inadequate dosing and irregular drug intake. However, other factors that are reported to compromise the bioavailability of anti-TB drugs are malabsorption due to intestinal function impairment under treatment, and when they are consumed with food or antacids.

The determining factor for any drug absorption is the absorptive capacity of the intestinal mucosa, which may be altered in several clinical situations; eg- HIV infected TB patients have low serum concentrations of rifampicin and ethambutol. Nanocochleates loaded with rifampicin can be a good alternative solution for overcoming its variable absorption through intestinal mucosa. Reported strategies to improve drug absorption through cross membrane diffusion include drug delivery using a lipid based system. Nanocochleates can entrap the hydrophobic drug molecule effectively and the entrapped moiety remains unaffected by its surrounding environment thus providing a reasonable solution for the problem. Formulating rifampicin as nanocochleates helps in increasing its permeability through intestinal mucosa, lowering the dose of rifampicin and reduction of gastrointestinal tract side-effects such as abdominal cramps and diarrhoea.

2. MATERIALS AND METHODS

2.1 Materials

Rifampicin was obtained as a gift sample from Modern Laboratories, Indore, India. Soya lecithin was obtained as generous gift from Lipoids, Germany and used as vesicle forming phospholipid, cholesterol (Sigma-Aldrich) provides flexibility to the formed liposomal vesicles thus maintaining its shape. Chloroform (Loba

Chem, India) was used as the organic solvent used to dissolve both soya lecithin and cholesterol. Bovine serum albumin (Sigma-Aldrich) was used as an aggregation inhibitor while formation of nanocochleates to stop them from aggregating and forming clumps. Calcium chloride (Loba Chem, India) was used as the cochleation agent as the Ca^{2+} cations are required to roll up the lipid bilayer sheets into cochleate structures. All solvents used were of analytical grade.

2.2 Preparation of small unilamellar vesicles by lipid film hydration method

Negatively charged liposomes were prepared by lipid film hydration method. Constitutive lipids (Soya Lecithin: Cholesterol) were taken in different molar ratios (5:5, 6:4, 7:3, 8:2, 9:1) and were dissolved in minimum volume of chloroform. Known amount of drug rifampicin (100 mg) dissolved in organic phase (chloroform) was added to the lipid solution. Organic solvent was slowly removed using a rotary evaporator (Lab India) at temperature of about $37 \pm 2^\circ\text{C}$, so that a thin film of dry lipid was deposited on the inner wall of the flask. This lipid film was left overnight to allow any remaining traces of organic solvent to evaporate. The thin lipid film was dispersed in phosphate buffer saline (PBS, pH 7.4) to give a final phospholipid concentration of 20 mg/ml by mechanical agitation by wrist shaking for 3 hours. Liposomes thus obtained were subjected to ultra sonication for 2 cycles of 10 min each to obtain small unilamellar vesicles.

2.3 Vesicle size and percent drug entrapment of small unilamellar vesicles

The small unilamellar vesicles thus formed were studied for vesicle size, percent entrapment and external morphology by photo microscopy. Nanotracc-150, USA was used to determine vesicle size. Nanotracc uses a newer, advanced power spectrum analysis of Doppler shifts to produce a full spectrum of vesicle sizes provided as a vesicle size distribution of volume (mass) percent and intensity. The liposomal

suspension is directly taken in a cell. The combined light passes through a fiber-optic cable to a single detector, and advanced electronics and software analyze the signals to calculate the Doppler shifts corresponding to particle size.

Percent drug entrapment was determined and expressed as the ratio of experimentally measured amount of drug in dispersion and initial amount used for entrapment. Vesicles were lysed by adding 1.0ml of 0.1% (v/v) Triton X-100, and were subjected to centrifugation at about 13000 rpm for 15 min at 4°C, and supernatant was taken for analysis of rifampicin using UV spectrophotometric method at a maximum wavelength of 334 nm.

2.3 Preparation of small cochleate cylinders.

Bovine serum albumin was added to the liposomal preparation as an aggregation inhibitor such that the final concentration of it remains to about 1% of the cochleate composition. Cochleate cylinders were then formed from liposomal preparations by trapping method, following addition of stock solution of calcium chloride in liposomal preparation while under constant stirring. The final Ca²⁺ concentration was 10 mM. The size of cochleate cylinders was controlled by subjecting the preparation to 2 cycles of ultrasonication of about 10 min each. After the evaporation of water solid form of cochleates were extracted.

2.4 Size measurement of cochleate particles

The vesicle size and size distribution of nanocochleates were measured by Malvern Zetasizer 2000 (Malvern, UK). Vesicular suspensions were mixed with the appropriate medium (PBS, pH 7.4) and the measurements were taken in a multimodal mode. All measurements were performed at 25°C after 5 min of thermal equilibration. The Malvern Zetasizer 2000 (Malvern, UK) detects backscattering at an angle of 173° for an improved sizing of especially larger particles at higher concentrations. Viscosity of the medium of 0.89 mPas and refractory index of 1.33 were assumed.

2.5 Scanning electron microscopy studies

Surface morphology of the specimens will be determined by using a scanning electron microscope (SEM), Model JSM 840A, JEOL, Japan. The samples are dried thoroughly in vacuum desiccator before mounting on brass specimen studies. Gold-palladium alloy of 1200A was coated on the sample using sputter coating unit (Model E5 100 Polaron U.K.) in Argon at ambient of 8-10 Pascal with plasma voltage about 20MA. The sputtering was done for nearly 5 minutes to obtain uniform coating on the sample to enable good quality SEM images. The SEM was operated at low accelerating voltage of about 10KV with load current of about 80MA. The objective lens aperture has a diameter of 240 microns and the working distance (WD) of 39mm.

2.6 Permeability studies (ex-vivo evert gut sac model)

Male Albino Wistar rats (150-200 g) were used for the present study. The rat was sacrificed humanely by cervical dislocation, the abdomen was opened by a midline incision, and the entire small intestine was then washed out with a solution of 0.9% (w/v) NaCl containing 0.3% (w/v) glucose. The whole of the small intestine was then removed by cutting across the upper end of the duodenum and the lower end of the ileum and manually stripping the mesentery from the intestine.

To evert the gut, a glass rod (150 mm long, 1.5 mm diameter) was used to push the ileal end of the gut into the gut lumen, and the eversion was completed by rolling the proximal half of the intestine on the rod. The everted intestine was then slipped off the glass rod and placed in glucose saline at room temperature in a petri dish. 5 cm of everted segment was mounted to permeability apparatus. Now, the apparatus containing intestinal segment was placed in a 250 ml beaker containing 1000 µg/ml drug (rifampicin) solutions. Another similar assembly was placed in similar beaker containing nanocochleates equivalent to 1000 µg/ml rifampicin. The mucosal side of the intestine was perfused with kreb's solution. This

assembly (beaker and apparatus with tissue) was placed on a magnetic stirrer and a magnetic bead was allowed to rotate at 25 rpm in beaker and the temperature was maintained at 37°C with adequate aeration. The samples were collected at every 5 min for 1 hour and analyzed by HPLC for estimation of permeability.

The apparent permeability (P_{app}) was calculated by the equation.

$$P_{app} = \frac{dQ}{dt} * \frac{1}{A * C_0} = v * \frac{dC}{dt} * \frac{1}{A * C_0}$$

(Le Ferrec et al.)

Where,

- P_{app} : Apparent permeability co-efficient
- dQ/dt : Cumulative amount of drug (Q) appearing in the acceptor compartment as a function of time, and was obtained from the slope of the linear portion of the amount transported-versus-time plot
- A: Surface area of the intestine (cm^2), taking 0.18 cm as radius (Shirasaka et al., 2008)
- C_0 : Initial concentration of drug in the donor compartment ($\mu g/ml$)
- V: Volume of sample (ml)

3. RESULTS AND DISCUSSION

3.1 Characterization of vesicles formed

The vesicles formed were spherical in shape and unilamellar in nature. However, the vesicle size of the formed vesicle tends to vary as the molar ratio of the

structural lipids is changed. The vesicles also show variable entrapment efficiency with the maximum percent entrapment of $39.86 \pm 0.38 \%$. The results are compiled under table 1.

Table 1: Characterization of small unilamellar vesicles

S. No.	Composition (molar ratio)	Vesicle size (nm)	Entrapment (%)
1.	Soya lecithin: Cholesterol (5:5)	401	27.63 ± 0.15
2.	Soya lecithin: Cholesterol (6:4)	435	34.73 ± 0.52
3.	Soya lecithin: Cholesterol (7:3)	431	39.86 ± 0.38
4.	Soya lecithin: Cholesterol (8:2)	451	26.95 ± 0.19
5.	Soya lecithin: Cholesterol (9:1)	506	25.29 ± 0.44

3.2 Size and size distribution of nanocochleates formed

The nanocochleates were subjected to particle size analysis and particle size distribution and average diameter (Z- Average) was found out to be 1005 nm with peak obtained at 522.6 nm, following results were obtained:

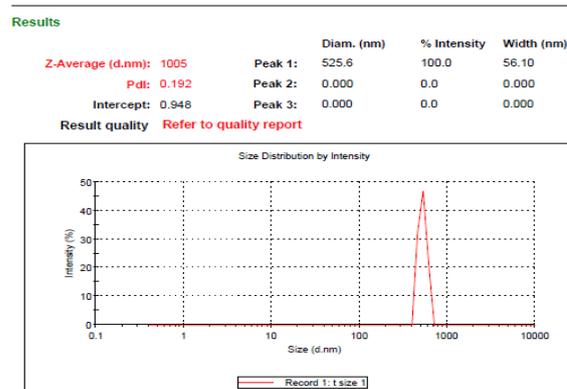


Figure 1: Particle size of cochleates by Malvern zetasizer

Measurement Date and Time: Wednesday, May 16, 2012 1:12:48 PM

Z-Average (nm): 1005.249 Derived Count Rate (kcps): 15927.14512...
 Standard Deviation: 0 Standard Deviation: 0
 %Std Deviation: 0 %Std Deviation: 0
 Variance: 0 Variance: 0

Size d.nm	Mean Intensity %	Std Dev Intensity %	Size d.nm	Mean Intensity %	Std Dev Intensity %	Size d.nm	Mean Intensity %	Std Dev Intensity %	Size d.nm	Mean Intensity %	Std Dev Intensity %
0.4000	0.0		5.615	0.0		78.82	0.0		1106	0.0	
0.4632	0.0		6.503	0.0		91.28	0.0		1281	0.0	
0.5365	0.0		7.531	0.0		105.7	0.0		1484	0.0	
0.6213	0.0		8.721	0.0		122.4	0.0		1718	0.0	
0.7195	0.0		10.10	0.0		141.8	0.0		1990	0.0	
0.8332	0.0		11.70	0.0		164.2	0.0		2305	0.0	
0.9649	0.0		13.54	0.0		190.1	0.0		2669	0.0	
1.117	0.0		15.69	0.0		220.2	0.0		3091	0.0	
1.294	0.0		18.17	0.0		255.0	0.0		3580	0.0	
1.499	0.0		21.04	0.0		295.3	0.0		4145	0.0	
1.736	0.0		24.36	0.0		342.0	0.0		4801	0.0	
2.010	0.0		28.21	0.0		396.1	0.0		5560	0.0	
2.326	0.0		32.67	0.0		458.7	32.1		6439	0.0	
2.696	0.0		37.84	0.0		531.2	46.7		7456	0.0	
3.122	0.0		43.82	0.0		615.1	21.1		8635	0.0	
3.615	0.0		50.75	0.0		712.4	0.0		1.000e4	0.0	
4.187	0.0		58.77	0.0		825.0	0.0				
4.849	0.0		68.06	0.0		955.4	0.0				

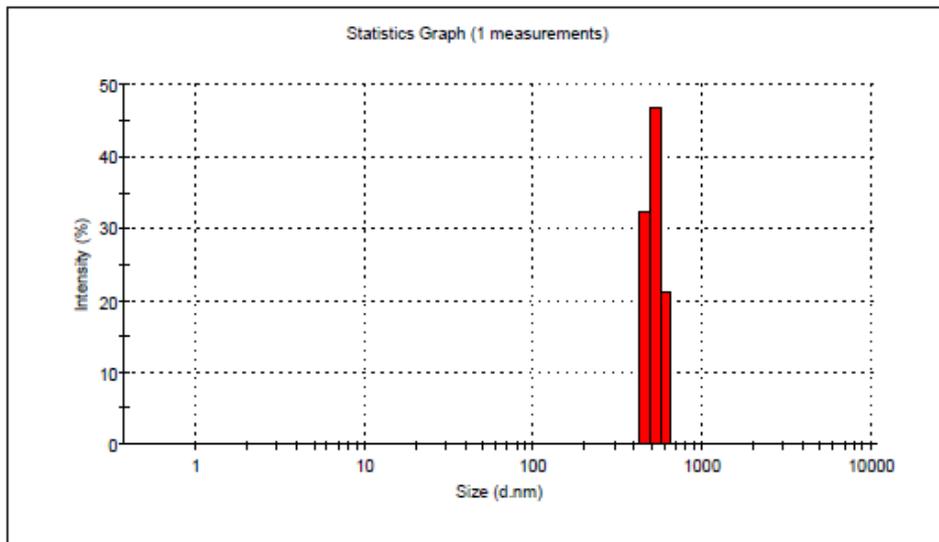


Figure 2: Size Distribution of Cochleates by Malvern Zetasizer

3.3 Surface morphology of cochleate particles (SEM studies).

The images are shown in Figure 3. The micro size rods and clusters of such elongated rod shaped cochleates were observed.

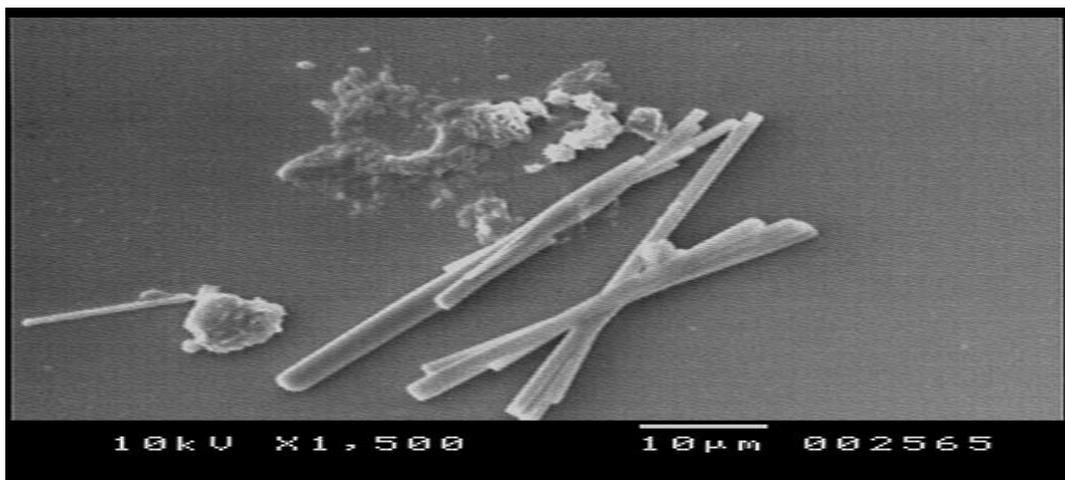


Figure 3: SEM images of cochleate particles

3.4 Permeability estimation

The absorption rate as the cumulative concentration obtained at various time intervals across the intestinal segment is depicted in Fig. 4 for rifampicin and rifampicin loaded nanocochleates. The absorption of both was linear after initial lag phase and then had a plateau. The slope of the linear phase was used to

determine the dQ/dt and then the apparent permeability co-efficient. The P_{app} values were found to be 3.56×10^{-6} and 7.78×10^{-6} cm/sec respectively for rifampicin and rifampicin loaded nanocochleates respectively.

Table 2: Absorption kinetics of rifampicin and rifampicin loaded nanocochleates by everted rat intestine method

S. No.	Time (sec)	Cumulative Concentration (μg)	
		Rifampicin	Rifampicin loaded nanocochleates
1.	300	36.075 \pm 4.5	96.135 \pm 7.5
2.	600	51.870 \pm 5.2	238.485 \pm 17.6
3.	900	70.590 \pm 8.3	292.110 \pm 21.3
4.	1200	109.394 \pm 9.6	365.235 \pm 23.3
5.	1500	187.590 \pm 12.3	437.580 \pm 24.6
6.	1800	243.490 \pm 23.4	524.745 \pm 26.7
7.	2100	300.300 \pm 22.1	587.925 \pm 21.2
8.	2400	302.835 \pm 21.4	737.100 \pm 20.2
9.	2700	327.210 \pm 23.7	771.615 \pm 27.8
10.	3000	369.525 \pm 23.4	807.495 \pm 21.9
11.	3300	376.155 \pm 24.4	828.750 \pm 23.3
12.	3600	381.030 \pm 21.2	842.945 \pm 25.6

Absorption kinetics of rifampicin and rifampicin loaded nanocochleates using everted intestine apparatus.

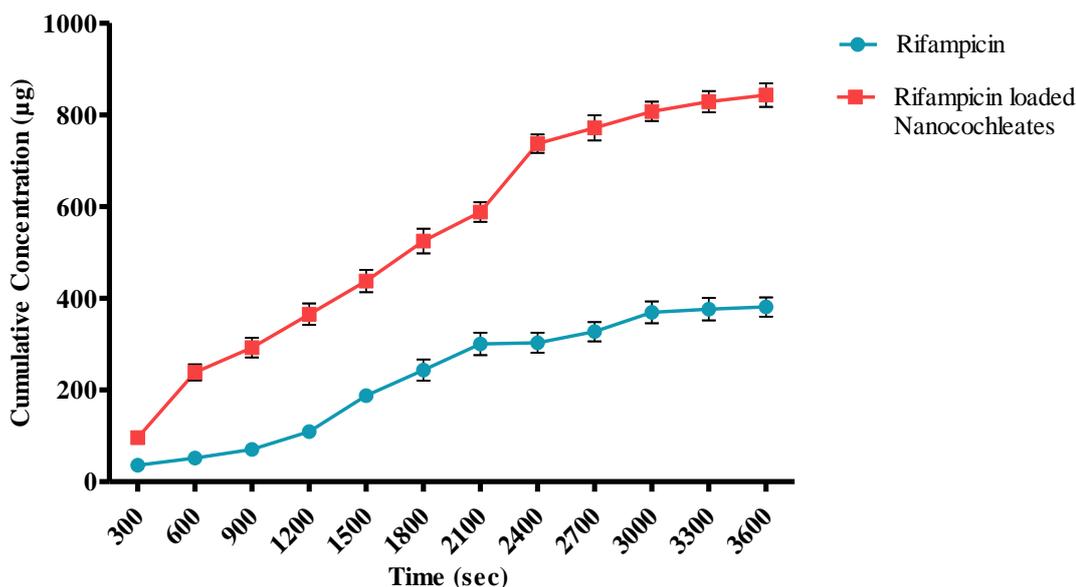


Figure 4: Absorption kinetics of rifampicin and rifampicin loaded nanocochleates using everted intestine apparatus

4. CONCLUSION

Formulation of nanocochleates of rifampicin is a promising and good strategy for improving its absorption through intestinal mucosa. It was observed that the cochleate formulation of rifampicin shows significant increase in apparent permeability.

Since the cochleate structure are formed from the interaction of divalent Ca^{2+} ion with small unilamellar vesicles of rifampicin, the formulation was started by selecting a suitable lipid, cholesterol composition for the preparation of SUV. Soya lecithin was selected as the structural phospholipid as it is easily available and

consists of three types of phospholipids; phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI) which can be used in formulation of negatively charged vesicles used for formulation of cochleates.

Initial experiments were conducted to optimize the liposomes formed by the process of film hydration method. The parameters studied included vesicle size and percent entrapment. It was observed that the vesicles formed by taking soya lecithin and cholesterol in the molar ratio of 7:3 shows maximum percent drug entrapment at about 39.86 ± 0.38 %. This can be attributed to the lipophilic nature of the drug since the entrapment is dependent upon lipid: aqueous phase ratio. While the vesicle size of various formulations varied marginally between 400-500 nm. The vesicle size of liposomes increases with the increase in the phospholipid content in the formulation. Furthermore, morphologically the vesicles were spherical in shape and unilamellar in nature.

Liposomal formulation showing the best percent drug entrapment of rifampicin was taken for formulation of cochleates. Initial experiments were carried out using variable concentration of Ca^{2+} ion, which suggested that for the better cochleation of liposomal bilayer into rod shaped structure and formation of small cochleate structures concentration of Ca^{2+} ion should be increased. Ca^{2+} concentration of 10 mM was maintained in the final formulation. On microscopic study of the formulation it was observed that the cochleate cylinders tend to aggregate to form large clumps which can affect the absorption kinetics, therefore bovine serum albumin (1% w/v of the total cochleate formulation) was used as an anti-aggregation agent, it was incorporated in the liposomal solution prior to the addition of Ca^{2+} ions.

Characterization of nanocochleates on various parameters such as particle size and size distribution, scanning electron microscopy were performed. Average diameter (Z- Average) of cochleates was found out to be 1005 nm with peak obtained at 522.6 nm, this difference in the average diameter and peak

can be attributed to the elongated shaped structure of cochleate particle. Size distribution report of the formulation also shows maximum particle intensity in the range of 450-620 nm.

To further confirm the morphological structure of the cochleates, the particles were subjected to scanning electron microscopy (SEM) and elongated rod shaped structures of cochleates were observed. Permeability studies of the cochleate formulation of rifampicin shows more than two fold increase in the apparent permeability over normal rifampicin. The apparent permeability of rifampicin was found to be 3.56×10^{-6} cm/s. Formulation of drug as nanocochleates increased its absorption through the instestinal mucosa. This resulted in a significant increase in the apparent permeability to 7.78×10^{-6} cm/s. Hence the presented work proves a marked increase in permeability of rifampicin through small instestine by its formulation as nanocochleates, which is a good strategy to overcome the problem of malabsorption of rifampicin in presence of other anti-TB drugs as well as food and antacid, thus lowering the dose and side effects of the drug and requires further clinical studies to confirmation.

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