Fabrication and application of dimyristoyl phosphatidylcholine biomaterial-based nanocochleates dry powder inhaler for controlled release resveratrol delivery

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Abstract

Background: Resveratrol, a bioactive phytoconstituent, is used to treat chronic respiratory diseases. However, its clinical application was hampered due to its poor bioavailability. In the present study, controlled release of resveratrol loaded nanocochleate-based dry powder inhaler was investigated to improve its biopharmaceutical properties for pulmonary drug delivery. The in vivo toxicity study was performed in the healthy male albino Wistar rats by intracheal administration.

Results: Resveratrol loaded nanocochleate-based dry powder inhaler was prepared by lyophilizing the resveratrol loaded dimyristoylphosphatidylcholine sodium and calcium ion-based nanocochleates using mannitol as cryoprotectant. Resveratrol loaded nanocochleates showed a particle size and encapsulation efficiency of 329.18 ± 9.43 nm and 76.35 ± 3.65%, respectively. Resveratrol loaded nanocochleate-based dry powder exhibited a particle size of 102.21 ± 9.83 μm and satisfactory flowability with initial burst release followed by extended release up to 96 h. The in vitro drug deposition pattern using multistage cascade impactor showed 1.28-fold improvement in fine particle dose, and the in vivo toxicity potential by histopathological study in albino rats revealed safety of formulation.

Conclusions: Resveratrol loaded nanocochleate-based dry powder inhaler could serve as an efficient delivery system for the treatment of chronic respiratory diseases.

Keywords: Resveratrol, Biomaterial, Nanocochleates, Dry powder inhaler, Controlled release, Drug delivery, Cascade impactor

Background

Chronic respiratory diseases (CRDs) are one of the leading causes of death worldwide, characterized by airflow restriction, bronchoconstriction, emphysema, chronic inflammation, and mucus hypersecretion. The tobacco smoke, inhalation of noxious particles, air pollution, genetics, and occupational chemicals are the leading factors which prominently contribute to the development of CRDs. At present, some of the most widespread CRDs are asthma, chronic obstructive pulmonary disease (COPD), and occupational lung diseases [1, 2]. Recently, as per the World Health Organization (WHO), there are more than 100 million people of all age groups suffering from CRDs across the globe [2]. Considering the impact of CRDs on a universal level, it is clear that this disease presents a major public health challenge which further impact on the quality of individual life. The present line of treatment for CRDs is symptomatic which does not control or cure the progression of the disease by...
restoring usual lung function. Further, for CRDs, a very narrow treatment alternatives are available at present conditions such as inhaled corticosteroids (e.g., budesonide, fluticasone), anticholinergics (e.g., tiotropium, ipratropium), and β2-agonists (e.g., salmeterol, indacaterol, formoterol). In some cases, CRDs become refractory to inhaled corticosteroids and the treatment fails. More recently, few scientific reports have confirmed the postulation of oxidative injury in CRDs and a direct injury to epithelial cells by reactive oxygen species (ROS) and reactive nitrogen species (RNS). These situations are worsened in smokers, a few studies in the literature have shown that cigarette smoke and oxidative stress increase histone acetylation which leads to increased expression of inflammatory genes. Hence, oxidative stress can be a key pharmaceutical target for the treatment of CRDs and antioxidant molecules have the potential to reinstate the response to inhaled corticosteroids [1, 3, 4].

Recently published literature has demonstrated that polyphenolic phytoconstituents can regulate the production of ROS and RNS to control oxidative stress chronic inflammatory diseases [1, 5]. Resveratrol (RES) is a polyphenolic phytoalexin. Chemically, it is 3,5,4′-trihydroxytrans-stilbene and isolated from the roots of *Veratum grandiflorum* O. Loes (Melanthiaceae). It shows antioxidant potential by inhibiting ROS by activating AMPK (5′-adenosine monophosphate-activated protein kinase). It displayed better anti-radical activity as compared to other polyphenols such as kaempferol, catechin, naringenin, myricetin, and α-tocopherol which justifies its useful effects in oxidative stress-mediated disease conditions. RES is also well known for treatment of various intrapulmonary and extrapulmonary diseases, such as cystic fibrosis, COPD, pulmonary hypertension, and lung cancer. In spite of potent antioxidant activity, light-sensitive nature, short biological half-life (<15 min), extensive first-pass metabolism, poor water solubility (0.0688 mg/mL), and high lipophilicity (log P of 3.06) are the major challenges for successful delivery RES in clinically [6–9].

Drug delivery techniques currently available for CRDs treatment are nebulizers, soft mist inhalers (SMIs), pressurized metered-dose inhalers (pMDIs), and dry powder inhalers (DPIs). Administration of drugs through nebulization is a helpful treatment but only for stationary use. The pMDIs are more useful but need good coordination between dose actuation and inhalation. The DPIs were developed to overcome the weaknesses of nebulizers and pMDIs. DPIs present better physicochemical stability, do not need cold-chain storage or reconstitution, and no coordination problem [10–12]. Furthermore, the powder is dispersed into fine particles by the patient’s inspiratory flow, and generally, the device is inexpensive, thus suitable for the treatment of CRDs. A variety of molecules such as phytoconstituents, anticancer agents, proteins, and peptides can be delivered to the pulmonary region using DPIs for treatment of various intrapulmonary and extrapulmonary diseases such as COPD, bronchiectasis, tuberculosis, lung cancer, and pulmonary hypertension.

Nanocochleates (NC) are lipid-based supramolecular assemblies [13, 14]. They are mainly made up of three constituents: the negatively charged phospholipid bilayers (liposomes), the multivalent metal ions, and the therapeutic agent to be delivered; on varying one or more of these elements, various permutation and combinations are feasible. NC has an elongated shape, cigar-like spiral rolls, or a carpet roll-like morphology. They vary from liposomes in having a water-free inner core and rigid stable structure. These unique morphological properties make nanocochleates a great platform for the delivery of drugs [15]. NC structure is resistant to diffusion of oxygen and as a result less susceptible to oxidation of encapsulated agent. It also provides protection from degradation for an encapsulated agent in biological fluids. Since the whole NC structure is a series of lipid layers, components within the core of the NC structure remain intact, although its outer lipid layers may be exposed to harsh environmental surroundings. NC has demonstrated their capability by improving physicochemical, biopharmaceutical properties, and therapeutic efficacy of a variety of molecules such as phytoconstituents like fisetin, anticancer agents (paclitaxel, doxorubicin), vitamins (retinol), and also macromolecules. Therefore, it is of interest to improve the RES activity of antioxidant by developing NC drug delivery system [15–18].

The aim of the present study was to formulate RES loaded NC dry powder inhaler (RES-NC- DPI) to improve its physicochemical and biopharmaceutical properties. Using the trapping method, RES-loaded dimyristoylphosphatidylcholine sodium vesicles were converted into NC by addition of calcium ions. Besides, RES-NC was lyophilized using mannitol which acted as cryoprotectant as well as DPI carrier. Developed RES-NC were examined for particle size, zeta potential, encapsulation efficiency, and surface morphology. Furthermore, the lyophilized RES-NC-DPI powder was characterized using powder X-ray diffraction (PXRD), differential scanning calorimetry (DSC), scanning electron microscopy (SEM), and powder flow properties. Additionally, RES-NC-DPI was studied for various physicochemical properties, in vitro drug release in phosphate buffer saline (pH 7.4), in vitro drug deposition pattern using multistage cascade impactor, and in vivo toxicity potential in albino rats.

**Methods**

**Materials**

*Trans*-resveratrol (99 %) and dimyristoylphosphatidylcholine sodium (DMPC Na+) were obtained as a gift...
sample from Mega resveratrol, USA, and Lipoid GmbH, Germany, respectively. Cholesterol was purchased from Fine Chem Industries Ltd., India. Dialysis membrane bag (molecular weight cutoff 12,000 Da) was purchased from Sigma-Aldrich Pvt. Ltd., India. Inhalation grade lactose (Respitose® ML001) was obtained from DFE Pharma, Germany. All other solvents and chemicals used were of analytical grade.

Preparation of RES-loaded vesicle
RES-loaded vesicles (RES-V) were prepared according to the previously published method with slight modification [17, 19]. As shown in Table 1, specific amount of DMPC sodium, cholesterol was dissolved in 3 mL of chloroform whereas RES was dissolved in 3 mL of ethanol and mixed together with heating to the phase transition temperature of DMPC sodium. The resulting mixture was rapidly injected into 10 mL deionized water under continuous stirring at 500 rpm under vacuum until complete evaporation of chloroform and ethanol to obtained vesicles. The final volume was adjusted with deionized water. Lastly, prepared RES vesicles were purified by filtering through a 0.45-μm membrane filter. The filtered RES-loaded vesicles were used to prepare the NC (Fig. 1).

Preparation of RES-loaded nanocochleates (RES-NC)
RES-NC was prepared by a previously reported method [15, 17]. A 10 μl of 0.1 M calcium chloride (CaCl2) solution was added drop-wise into the RES-V under vortex. RES-V phase instantaneously turned opaque because of NC formation. Precipitated NC was refrigerated at 2-8 °C.

Characterization of RES-NC
The mean particle size and zeta potential were estimated by laser diffraction technique (Malvern 2000 SM; Malvern Instruments, Malvern, UK). Powder X-ray diffraction (PXRD) studies were performed using X-ray diffractometer 2 (PW 1729, Philips, Netherlands). Thermal properties were studied using differential scanning calorimetry (DSC) coupled with an intercooler and STAR® software (821e; Mettler-Toledo, Switzerland). Surface morphology was studied using scanning electron microscopy (SEM; Cambridge Stereoscan S120, Cambridge, UK) at ×2000 magnification. Besides, flow properties of prepared powders were characterized in terms of bulk density, tap density, angle of repose (θ), Carr’s index (CI), and percent porosity by previously reported method [23–25]. Further, percent porosity (ε) is used to determine compressibility of powder which is the degree of volume reduction due to an applied pressure is measurement of porosity changes during compaction and is calculated using following formula

\[
E = 1 - \left( \frac{P_b}{P_t} \right) \times 100
\]

where Pb and Pt are bulk and tapped density of DPI.

| Formulation | RES (mg) | DMPC (mg) | Cholesterol (mg) | EE (%) | Particle size (μm) | Zeta potential (μm) |
|-------------|----------|-----------|------------------|--------|-------------------|---------------------|
| RES-V1      | 1        | 60        | 20               | 61.42 ± 2.82 | 193.65 ± 13.62 | −31.44 ± 1.80       |
| RES-V2      | 3        | 60        | 20               | 72.26 ± 3.34 | 206.12 ± 14.12 | −38.12 ± 2.71       |
| RES-V3      | 6        | 60        | 20               | 59.61 ± 3.12 | 277.77 ± 11.32 | −35.89 ± 3.34       |
| RES-NC      | 3        | 60        | 20               | 76.35 ± 3.65 | 329.18 ± 9.43  | −35.40 ± 3.52       |

All the determinations performed in triplicate and values are expressed as mean ± SD (n = 3)
In vitro release study

The in vitro release of RES from RES-DPI and RES-NC-DPI was investigated at physiological pH of 7.4 and temperature of 37 ± 0.5°C. The dialysis bag having molecular weight cutoff 12,000 Da was used to carry out diffusion study. Briefly, formulation equivalent to 400 mcg of RES was loaded into a dialysis membrane bag, hermetically closed, and immersed into 100 mL of phosphate buffer saline with a pH of 7.4. The whole system was kept at 37 ± 0.5°C with constant stirring at 100 rpm/min. Aliquots of 1 mL were withdrawn at predetermined time intervals and replaced with the same volume of fresh medium to retain constant volume. The withdrawn aliquots were filtered through Whatman filter paper (0.45 μm), and the amount of RES in the aliquots was quantified using a UV-VIS absorption spectrophotometer (Shimadzu UV-1601, Japan) at 306 nm after suitable dilution. Cumulative RES release amount at pre-determined time intervals was mapped as the percentage released versus the time. The RES release mechanism from RES-NC-DPI and RES-DPI was analyzed by different kinetics models such as zero-order release \( \frac{M_t}{M_\infty} = k_0t \), first-order release \( \frac{M_t}{M_\infty} = 1 - \exp(-k_1t) \), and Higuchi \( \frac{M_t}{M_\infty} = k_Ht^{1/2} \) equation. The data analysis was performed using PCP Disso software (V3; Poona College of Pharmacy, Pune, India). The results of triplicate measurements and their means were reported.

In vitro deposition study

In vitro drug deposition pattern of RES from RES-DPI and RES-NC-DPI was determined using an eight-stage, non-viable Andersen cascade impactor (ACI, Westech Instruments, UK). The ACI was equipped with USP induction port (IP) and pre-separator (PS), which was coupled to a vacuum pump and a critical flow controller (Westech Instruments, UK). Prior to analysis, the pre-separator was placed with 15 mL of methanol for sample recovery and the ACI plates were coated with glycerol containing silicone oil (1 %, w/v) to ensure efficient sample collection. Accurately weighed 25 mg of each formulation (equivalent to 400 mcg of RES) was filled into size 3 hard gelatin capsules under controlled environmental conditions. For each trial, the formulation was actuated into the ACI at a constant flow rate of 60 L/min via a Rotahaler® inhaler device (\( n = 3 \)). Following aerosolization testing, the ACI was dismounted and each plate was washed with a known volume of methanol. The amount of powder deposited at different stages was measured using a UV-VIS absorption spectrophotometer (Shimadzu UV-1601, Japan) at 306 nm after suitable dilution. The aerosolization efficiency was characterized in terms of emitted dose (ED), fine particle size (FPD), mass median aerodynamic diameter (MMAD), and geometric standard deviation (GSD). Additionally, the effective inhalation index (EI) and
dispersibility was measured using the following equation

\[ E_I = EM + FPF \] (2)

where \( EM \) is the percent ratio of the ED to RD and FPF is fine particle fraction

\[ E_I = \sqrt{FPF^{ED}} \times 100 \] (3)

**In vivo toxicity assessment in rats**

Healthy male Wistar albino rats (200–250 g) were purchased from the National Toxicology Centre, Pune. Animals were housed under a controlled environmental condition (25 ± 2°C, 12-h dark-light cycle) with free access to water before experiments. During this adaptation phase, the health status of the rats was supervised daily. Animals were fasted overnight but offered free access to water before the initiation of the experiment. In vivo toxicity studies were performed using a previously published method. Briefly, healthy male Wistar albino rats were randomly divided into three groups \((n = 6)\).

The animals were observed continuously for the first 2 h and 24 h to identify the behavioral changes. However, there are no noticeable behavioral changes and mortality occurred. The acute toxicity assessment was performed to check the acceptability of the NC for the route of inhalation. Group I (control group) did not receive any treatment. Group II received a single intratracheal dose of RES-NC-DPI (equivalent to 400 mcg of RES) and group III received a single intratracheal dose of RES-DPI (equivalent to 400 mcg of RES). The suturing was carried out after dosing at the incision area. To prevent any infection, the povidone gel was applied. All groups are treated for 10 successive days. Twenty-four hours after the last treatment the animals were sacrificed by deep ether anesthesia, and the lung and trachea sections (5 μm thick) were collected and fixed in 10% formalin solution for histological examination. The obtained sections were stained with hematoxylin-eosin dye and examined under a light microscope (Olympus CH02). Further, the performed study did not contain any hamane end-points.

**Statistical methods**

All the experiments were performed in triplicates. Data were recorded as mean ± standard deviation. The statistical analysis was carried out using online Quick Calcs Graph Pad software. Data analyzed in following manner; 

\( ^*p \leq 0.1 \) (not statistical significant), \( ^{**}p \leq 0.01 \) (statistical significant), \( ^{***}p \leq 0.001 \) (very statistical significant), and \( ^{****}p \leq 0.0001 \) (extremely statistical significant).

**Results**

**Particle size distribution**

The particle size distribution of RES and RES-NC-DPI samples are shown in Table 2. The RES showed a mean particle size of 3.34 ± 1.88 μm. Formulated RES-NC-DPI mean particle size of 102.21 ± 9.83 μm \((p \leq 0.01)\), while Respitose® ML001 and conventional RES-DPI showed a mean particle size of 126.88 ± 11.43 and 132.33 ± 10.78 μm, respectively. The span value for all analyzed samples was ≤ 1.0 representing uniform particle size distribution. Such uniform particle size distribution is useful in terms of efficacy of the DPI because high polydispersity of PSD might lead to high variations in drug deposition pattern.

**Powder X-ray diffraction studies**

PXRD analysis was performed to conclude the physical nature of RES within the final formulation. PXRD analysis results were shown in Fig. 2. The diffraction patterns of RES showed characteristic high-intensity diffraction peaks at 2θ values of 16.4°, 19.3°, 20.4°, 22.5°, 23.6°, 23.8°, 25.3°, and 28.5° signifying crystalline nature, while formulated RES-NC-DPI did not show any characteristic high-intensity diffraction peaks representing the amorphous nature of RES-NC-DPI [20, 26].

**Differential scanning calorimetry**

In addition to PXRD analysis, DSC study was performed to verify the physical nature of RES within the final formulation. The DSC thermograms of RES and RES-NC-DPI are showed in Fig. 3. The DSC thermogram of RES displayed a single sharp endothermic peak at 267.7°C with the heat of fusion 46.8 ml/mg attributed to its melting point and confirming its crystalline nature, whereas no remarkable peak was observed in the DSC thermograms of RES-NC-DPI confirming its amorphous nature. DSC analysis shows that the crystalline RES was converted into the amorphous state.

**Scanning electron microscopy**

The scanning electron microscopy (SEM) of RES and RES-NC-DPI are shown in Fig. 4. The surface morphology of RES-NC-DPI appeared absolutely different from RES. The SEM exhibited morphology of RES-NC-DPI varied from irregular plate to smooth rod. The pure RES exhibited the existence of irregular rough plate-shaped particles with several asperities, whereas RES- NC-DPI showed uniform rod-shaped particles with a smooth surface. In addition, SEM analysis of the particle size is in good conformity with the laser sizing particle size analysis (Table 2).
Powder flow properties
Powder flow properties were determined using static (CI) and a dynamic method (angle of repose). The considerable difference was observed in the powder flow properties RES-NC-DPI and RES-DPI. The values of CI, angle of repose, Hausner ratio, and porosity for RES-NC-DPI and RES-DPI are given in Table 2. The flow properties of RES-NC-DPI and RES-DPI was within the acceptable limits [25, 27, 28].

In vitro release study
In vitro release of the RES from RES-DPI and RES-NC-DPI was studied by diffusion bag technique. The in vitro release study was carried out in phosphate buffer saline (pH 7.4) instead of simulated lung fluid (SLF) as its pH varies considerably with time. The in vitro release pattern of RES-DPI and RES-NC-DPI was showed in Fig. 5. RES-DPI displayed around 30% RES release within the first 30 min, whereas RES release from RES-NC-DPI displayed a biphasic release pattern with initial burst release (27%) within the first 2 h followed by controlled release up to 96 h (75.07%). Further, the release kinetic model results are depicted in Table 3. As per the release kinetics, RES-DPI and RES-NC-DPI best fitted ($R^2$ values are 0.916 and 0.997) to the Higuchi kinetic model that indicated diffusion controlled release mechanism from the NCs.

Table 2 Particle size and flowability properties of RES-NC-DPI and RES-DPI (mean ± SD, n = 3)

| Formulation    | Particle size (µm) | Bulk density | Tapped density | Angle of repose (θ) | Carr’s index | Hausner ratio | Percent porosity |
|----------------|--------------------|--------------|----------------|--------------------|--------------|---------------|-----------------|
| RES-NC-DPI     | 102.21 ± 9.83**    | 0.083 ± 0.01 | 0.085 ± 0.02   | 31.72 ± 2.12*      | 21.17 ± 4.11* | 0.97 ± 0.01   | 42 ± 2.14       |
| RES-DPI        | 132.33 ± 10.78     | 0.14 ± 0.04  |                |                    |              |               |                 |
| 0.24 ± 0.12    | 39.33 ± 3.44       | 16.24 ± 3.16 | 0.58 ± 0.21    | 3 ± 1.11           |              |               |                 |
| RES alone      | 3.34 ± 1.88        | –            | –              | –                  |              |               |                 |
| Respitose® (ML001) | 126.88 ± 11.43 | –            | –              | –                  |              |               |                 |

All the determinations are performed in triplicate, and values are expressed as the mean data are mean ± SD (n = 3); *p ≤ 0.1 (not statistical significant), **p ≤ 0.01 (statistical significant) when compared to the control group RES-DPI.
In vitro deposition study

The in vitro aerodynamic performance of RES-DPI and RES-NC-DPI was analyzed using ACI assembly equipped with USP IP and commercially available capsule-based inhaler device, i.e., Rotahaler® (Cipla, India) at a constant flow rate of 60 L/min. Stage-wise deposition pattern in the ACI assembly for formulated RES-DPI and RES-NC-DPI are showed in Fig. 6 and Table 4 display deposition profiles for RES actuated through the Rotahaler® device. RES-DPI showed FPD and MB of 780.08 ± 90.22 μg and 89.94 ± 7.00 %, respectively, whereas RES-NC-DPI displayed FPD and MB of 986.31 ± 82.43 μg and 91.60 ± 8.33 %, respectively. RES-NC-DPI showed 1.26-fold improvement in FPD as compared to RES-DPI (p ≤ 0.01). Besides, RES-DPI and RES-NC-DPI showed similar MMAD of 2.30 ± 0.99 and 2.15 ± 1.09 μm, respectively. Lower MMAD values exhibited suitable RES deposition pattern using the Rotahaler® inhaler device. However, amounts of RES deposited within the non-sizing components (device, capsule, mouthpiece, IP and PS) were varied remarkably. Additionally, formulated RES-NC-DPI showed 1.21 and 1.22 fold higher percent recovery and emitted dose as compared to RES-DPI. These results indicated that RES-NC-DPI had effective aerodynamic performance most likely because of the particles smooth surface which control the cohesive-adhesive forces between the particles, therefore ease the aerosolization of DPI. Formulated RES-NC-DPI exhibited higher FPF of 54.87 ± 9.32 % as compared to previously reported spray dried resveratrol-PCL-microparticles (46.48 %), co-spray
dried resveratrol and budesonide microparticles (42.50\% \cite{3, 28}), and spray dried resveratrol microparticles (39.90\%) \cite{3}.

**In vivo toxicity study**
RES is a well-known polyphenolic antioxidant. It plays an important role in chronic pulmonary diseases by controlling oxidative stress. But its clinical application can be limited by the risk of irreversible and perhaps life-threatening lung tissue injury. Thus, RES-DPI and RES-NC-DPI were tested for lung tissue toxicity in the Wistar rats by performing the macroscopic examination on lung and trachea. As shown in Fig. 7, RES-NC-DPI did not cause any significant tissue toxicity on the lung and trachea as compared to conventional RES-DPI. However, mild congestion was observed in the treated group which may be because of the installation procedure.

**Fig. 5** In vitro release profile of RES alone and RES-NC-DPI in phosphate buffer saline (pH 7.4)

**Fig. 6** Mass of RES deposited within the ACI assembly using the Rotahaler® inhaler device at a flow rate of 60 L/min
Discussion

The RES-NC-DPI was developed to achieve the enhanced lung deposition and anticancer activity of RES by using the trapping method. Additionally, the lyophilized RES-NC-DPI was characterized for the physicochemical and biopharmaceutical properties along with the in vitro lung deposition by using the ACI.

Different acidic phospholipids are utilized to develop uniform sized and shaped vesicles [17, 29, 30]. In the present study, we preferred DMPC Na⁺ as a lipid for vesicles formation because it is a vital constituent of the mammalian cell membrane and also shows superior compatibility for human use. With the aim of loading the maximum amount of RES into the lipid vesicles, vesicles were formulated to hold an increasing amount of RES (1, 3, and 6 mg) and analyzed for entrapment efficiency, particle size, and zeta potential. RES is a lipophilic bioactive, gets easily solubilized into the lipid phase of vesicles. At higher concentration of RES, though, the mean vesicle size increased but with lower entrapment efficiency because of exclusion of components from the lipid vesicles. The formulation RES-V2 represented satisfactory entrapment efficiency; therefore, the maximum amount that can be loaded into the vesicles was fixed at 3 mg.

RES-V2 lipid vesicles prepared by ethanol injection were transformed into NC using divalent cation. Various divalent cations (Ca²⁺, Mg²⁺, Ba²⁺, and Zn²⁺) are reported for preparing NC. It has been reported that Ca²⁺ forms a more greatly ordered, firmly packed and less hydrated arrangement than does Mg²⁺ with phospholipids. In addition, it is needed in much lower concentration than Mg²⁺. The addition of calcium ions to lipid vesicles stimulate fusion of lipid membrane and the development of planar sheets, which ultimately roll around a primary point of folding to form rod shape cylindrical NC. Furthermore, it is well reported that Ca²⁺ plays an essential role in natural membrane fusion phenomena whereas other cations noted above are ineffective in the majority of such systems. Therefore, it is most compatible with the body. Hence, Ca²⁺ is the most appropriate divalent cation for preparing NC and thus used in the present study [15].

The enhancement in the mean particle size was because of free RES entrapment and showed better entrapment efficiency as compared to RES-V2 vesicles. The reduction in the zeta potential than RES-V2 could be because of the interaction between Ca²⁺ and lipid resulting fractional neutralization heading to poor availability of anionic lipid on the surface. RES-NC were successfully lyophilized using 1–5% mannitol as a cryoprotectant as well as the carrier (RES-NC-DPI). Mannitol is one of the most commonly used cryoprotectant and/or bulking agents as compared to other sugars. It helps to provide
adequate structure to powder. The nature of lyophilized powder also depends on the ratio of drug and cryoprotectant [29, 31]. PXRD of RES-NC-DPI confirms that the RES is fully integrated within the NCs [20, 26]. The trapping method holds the lipid vesicles tightly and preserved the amorphous state of RES. The variation in the surface morphology and size of particles might change the aerodynamic deposition of the inhaled formulations.

As per the release data, the initial burst release may be because of the dissolution of free RES absorbed on the surface of the NCs, whereas the controlled release might be due to slow diffusion of RES from NC. The drug encapsulated into the spiral structure release slowly and needed a longer duration of time to release. The in vitro aerodynamic drug deposition behavior of RES-NC-DPI and RES-DPI was conducted using Anderson cascade impactor (ACI) assembly equipped with commercially available inhaler device Rotahaler (low resistance device).

In both cases, the amount of RES deposited on the non-sizing parts of the assembly, i.e., induction port (IP) and pre-separator (PS) varied immensely with the RES-DPI displaying a much better deposition in non-sizing part, whereas the RES-NC-DPI showed a better deep lung deposition characteristic with more than 50 % deposition on the latter stages of the assembly. To sum up, the deposition profile displayed by the RES-NC-DPI was far more superior than the RES-DPI with better deep lung deposition. The uniform rod-shaped particles with a smooth surface with tapering end may be the major contributing factor which results in the enhanced lung deposition (Fig. 8). This characteristic shape of the NCs was useful to increase not only the trajectory but also the in vitro aerodynamic efficacy to reach the deeper region of the lungs. Further, there was observed marked changes in the zeta potential of the vesicles and NCs. Initially, the higher end negative charges were observed on the vesicles in the preparation of NCs. But in the process of neutralization by using the divalent cation, there was a marked decrease in the zeta potential of the RES-NC-DPI which helpful in the spiraling of the NCs reflecting its cylindrical structure. Further, RES-NC-DPI has shown negative surface charge $-35.40 \pm 3.52 \text{ mV}$. The negative surface charge on the respiratory tract and RES-NC-DPI was more prominently responsible for repulsive forces as per the charge theory which may increasing the time of flight and consequently lung deposition of the RES.

Further, the main reason behind the low toxicity for RES-NC-DPI could be accredited to the slow release of the RES from the NCs over a prolonged period of time which is released in 96-h duration as confirmed in in vitro release study. Briefly, results showed no signs of tissue toxicity in RES-NC-DPI-treated group representing its non-toxic nature. The results accomplished that developed novel formulation was safe for the pulmonary administration.

**Conclusion**

In the present study, RES-NC was successfully prepared by trapping method and transformed in the inhalable microparticles. In the case of RES-NC, a mixture of physiological lipid and cholesterol was used to form smooth surface rod-shaped NC and the lyophilized powders showed particle size, shape, and flow properties suitable for the deep lung deposition of RES. The RES-NC-DPI showed satisfactory aerodynamic performance with fine particle dose of 986.31 $\mu g$ vs 780.08 $\mu g$ for RES-DPI. Further, formulated RES-NC-DPI displayed controlled release up to 96 h in phosphate buffer saline (pH 7.4). A

### Table 4 Aerodynamic assessment of RES-NC-DPI and RES-DPI at a flow rate of 60 L/min (mean ± SD, n = 3)

| Aerodynamic parameters | RES-DPI       | RES-NC-DPI   |
|------------------------|---------------|--------------|
| RD (μg)                | 1470.67 ± 110.22 | 1797.50 ± 109.65** |
| ED (μg)                | 1281.08 ± 132.44 | 1557.08 ± 120.22 |
| FPD (μg)               | 780.08 ± 90.22  | 986.31 ± 82.43** |
| FPF (%)                | 53.04 ± 9.08    | 54.87 ± 9.32  |
| MB (%)                 | 89.74 ± 7.00    | 91.60 ± 8.33  |
| MMAD (μm)              | 2.15 ± 0.99     | 2.30 ± 1.09   |
| GSD (μm)               | 3.60 ± 1.22     | 3.16 ± 1.21   |
| D (%)                  | 60.89 ± 8.89    | 63.34 ± 9.77  |
| EI                     | 11.84 ± 1.09    | 11.90 ± 1.03  |

**p ≤ 0.01 (statistically significant) when compared to the control group RES-DPI**

RD: recovered dose, ED: emitted dose, FPD: fine particle dose, FPF: fine particle fraction, MB: mass balance, D: dispersibility, MMAD: mass median aerodynamic diameter, GSD: geometric standard deviation, and EI: effective inhalation index.

**Fig. 8** Transmission electron photomicrographs of RES-NC.
preliminary in vivo toxicity study showed no significant tissue toxicity in the lung and trachea. It still remains to be investigated whether the formulations effectively give in vivo deep lung deposition. Briefly, in depth in vivo study will be require so as to evaluate the deep lung deposition, pharmacokinetics performance, and antioxidant potential of these formulations.

Abbreviations
CRDs: Chronic respiratory diseases; COPD: Chronic obstructive pulmonary disease; WHO: World Health Organization; ROS: Reactive oxygen species; RNS: Reactive nitrogen species; RES: Resveratrol; AMPK: 5’-Adenosine monophosphate-activated protein kinase; SMs: Soft mist inhalers; PMDs: Pressurized metered-dose inhalers; DPIs: Dry powder inhalers; NC: Nanocochleates; RES-NC-DPI: RES loaded nanocochleate dry powder inhaler; PXRD: Powder X-ray diffraction; DSC: Differential scanning calorimetry; SEM: Scanning electron microscopy; DMPC Na+: Dimyristoylphosphatidylcholine sodium; ACT: Andersen cascade impactor; ED: Emitted dose; FPD: Fine particle size; MMAD: Mass median aerodynamic diameter; GSD: Geometric standard deviation.

CPCSEA: Committee for the purpose of control and supervision of the experiment of animals; IAEHC: Institutional Animal Ethics Committee; SLF: Simulated lung fluid.

Acknowledgements
The authors would like to express their heartfelt thankfulness to Om sai Pharmaceuticals Ltd, Uttarakhand, India for Resveratrol gift sample. The authors would like to acknowledge the support from Bharati Vidyapeeth Deemed to be University, Poona College of Pharmacy, Pune, Maharashtra, India.

Authors’ contributions
We declare that this study was conducted by the authors named in this article. A J Mali: Data drafting and interpretation of the work and designing and presentation of the data. P A Joshi: The execution of the work and collection of the data. C Bothiraja: The data analysis and presentation. A P Pawar: The idea generation, analysis, and execution. The authors read and approved the final manuscript.

Funding
There was no funding.

Availability of data and materials
The all data is available for the publication of research work upon request.

Ethics approval and consent to participate
A research proposal was prepared according to the guidelines of the Institutional Animal Ethics Committee (IAEC) of Poona College of Pharmacy, Pune (CPCSEA/15/2015).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests regarding the present work.

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