Calcifediol-loaded liposomes for local treatment of pulmonary bacterial infections

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Abstract

The influence of vitamin D3 and its metabolites calcifediol (25(OH)D) and calcitriol on immune regulation and inflammation is well described, and raises the question of potential benefit against bacterial infections. In the current study, 25(OH)D was encapsulated in liposomes to enable aerosolisation, and tested for the ability to prevent pulmonary infection by *Pseudomonas aeruginosa*. Prepared 25(OH)D-loaded liposomes were nanosized and monodisperse, with a negative surface charge and a 25(OH)D entrapment efficiency of approximately 23%. Jet nebulisation of liposomes was seen to yield an aerosol suitable for tracheo-bronchial deposition. Interestingly, 25(OH)D in either liposomes or ethanolic solution had no effect on the release of the proinflammatory cytokine KC from *Pseudomonas*-infected murine epithelial cells (LA-4); treatment of infected, human bronchial 16-HBE cells with 25(OH)D liposomes however resulted in a significant reduction in bacterial survival. Together with the importance of selecting an application-appropriate *in vitro* model, the current study illustrates the feasibility and practicality of employing liposomes as a means to achieve 25(OH)D lung deposition. 25(OH)D-loaded liposomes further demonstrated promising effects regarding prevention of *Pseudomonas* infection in human bronchial epithelial cells.

Keywords: Liposome aerosol, calcifediol, cystic fibrosis, poorly soluble drugs, *Pseudomonas aeruginosa*, pulmonary drug delivery

Introduction

Cystic fibrosis (CF) is an autosomal recessive genetic disease, characterised by persistent and recurring infection of the lungs [1, 2]. A chronic inflammation in response to the presence of pathogens also develops in CF patients, which is mainly characterised by the accumulation of neutrophils [3-6]. Continuing infection and inflammation lead to a progressive destruction of the lung tissue, with subsequent respiratory failure being the ultimate outcome [7]. The causative agents of infection in CF are limited to a relatively narrow spectrum of pathogens, with *Staphylococcus aureus, Haemophilus influenzae* and *Pseudomonas aeruginosa* being the most prevalent organisms [1, 6, 8]. Despite progress in the development of antibiotic therapy, pulmonary infections still dictate the fate of most CF patients. Effective antimicrobial treatment of CF-associated infection is presently limited by several factors, including development of bacterial resistance against the antibiotics commonly in use (exacerbated by the common need for regular or prophylactic antibiotic therapy), as well as a lack of novel anti-infectives currently in the pharmaceutical pipeline [9-11].

The role of vitamin D3 in the regulation of immune and host defence reactions is well described, as is its influence on the release of inflammatory mediators from neutrophils and macrophages [12-15]. In recent years a connection between vitamin D3 and pulmonary diseases such as asthma and chronic obstructive lung disease has been suggested, and a clear link between vitamin D3 deficiency and respiratory tract infections in patients has been postulated [16, 17]. Interestingly, low serum levels of vitamin D3 have been found specifically in CF patients, probably as a result of malabsorption [18]. Therefore, it may be hypothesised that the administration of vitamin D3 or its metabolites directly to the lung of CF patients could lead to an improved clinical outcome. Unfortunately however, the poor water solubility of these compounds necessitates dissolution in organic solvents such as

ethanol, which limits administration *in vivo*. Therefore, to enable pulmonary delivery of vitamin D3 and to study its potential effects on CF-relevant infections, aerosolisable liposomes of the vitamin D3 metabolite calcifediol (25(OH)D) were developed and characterised in the current work. The potential of 25(OH)D liposomes to act as a local delivery system to prevent *P. aeruginosa* infection was then tested *in vitro* in two different cell models.

Materials and Methods

Material

Dipalmitoylphosphatidylcholine (DPPC) was obtained as a kind gift from Lipoid GmbH (Ludwigshafen, Germany). Calcifediol (25(OH)D, Ph.Eur/USP) was provided by Dishman Netherlands (Veenendaal, The Netherlands). 1,2 dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) ammonium salt (Rh-DPPE) was purchased from Avanti Polar Lipids (Alabaster AL, USA). Distilled de-ionised water having a conductivity of less than 18.2 MΩ/cm at 25°C was used throughout the study. All the other solvents and chemicals used were of at least analytical grade. For cell cultivation, Ham's F12 medium containing 15% or DMEM-HamF12 (1:1) with foetal bovine serum (FBS) (all from Life Technologies, Darmstadt, Germany), penicillin and streptomycin (both Life Technologies, Darmstadt, Germany) and Ultroser-G (Pall, Fribourg, Switzerland) were used.

Liposome preparation and characterisation

Liposome formulations were prepared based on a modified version of the lipid film hydration method [19]. Briefly, DPPC and 25(OH)D or DPPC alone (total weight 75 mg) were dissolved in 5 ml of ethyl acetate/methanol (4:1 v/v) in a round-bottomed flask. Following dissolution, 0.1 ml of a 0.5 mg/ml solution of Rh-DPPE was added and mixed. The organic

solvent mixture was then evaporated under reduced pressure and with a rotation speed of 145 rpm at 70 °C using a rotavapor (Büchi, Essen, Germany). The resulting homogenous thin lipid film was then re-hydrated by the addition of 5 ml of deionised water, followed by further rotation at 60 °C for 1 h. The formed liposomal dispersion was sonicated in a sonication bath (Bandelin Sonorex, Berlin, Germany) for 10 min and then extruded (LiposoFast extruder, Avestin, Mannheim, Germany) repeatedly through 200 nm pore size membranes (AMD Manufacturing Inc., Ontario, Canada) to achieve size reduction and uniformity. Liposomes were then diluted 1:10 with deionised water and stored at 4 °C under nitrogen until further use. Physical characterisation of diluted liposomal formulations was performed by dynamic light scattering (size and size distribution) and electrophoretic mobility (zeta potential) at 25 °C using a Zetasizer Nano (Malvern Instruments Ltd, Worcestershire, United Kingdom).

Determination of liposomal DPPC and 25(OH)D content

The amount of 25(OH)D incorporated within liposomes was determined via HPLC, performed on a Dionex HPLC system (Thermo Scientific, Bremen, Germany) composed of a P680 pump, an Elite degassing System, an Asta-medica AG 80 column oven and a UV detector. A LiChrospher® RP-18 (5 µm, 125 x 4 mm) column (Merck KGaA, Darmstadt, Germany) was employed. A mobile phase of methanol/acetonitrile (30:70 v/v) was used, with an injection volume of 100 ul, a flow rate of 2 ml/min and a temperature of 30 °C. For sample analysis, liposomes were first dissolved in a mixture of 50% ethyl acetate/methanol (4:1) and 50% acetonitrile. The 25(OH)D content of dissolved liposome samples was determined using UV detection at a wavelength of 265 nm, and calculated in reference to standard solutions of 25(OH)D. The determined amount of 25(OH)D was then used to calculate the encapsulation efficiency (EE) of liposomes, defined as the measured amount of 25(OH)D as percentage of the initially added amount [20].

The amount of DPPC present in liposome formulations was assessed according to the Bartlett assay [21]. Briefly, a calibration curve was constructed from a stock solution of 0.05 mg/ml potassium phosphate (Sigma-Aldrich, St. Louis, Missouri, USA) diluted as required with deionised water to produce standards of known concentration. Both liposome samples and standards were dried completely in a sand bath at 180 °C prior to any analysis. A 450 µl volume of 70% perchloric acid (AppliChem, Darmstadt, Germany) was then added to both samples and standards, followed by incubation at 250-260 °C for 30 min. After cooling, 3.5 ml of deionised water, 500 µl of 2.5% w/v ammonium molybdate solution and 500 µl of 10% w/v ascorbic acid solution (both from VWR BDH Prolabo, Darmstadt, Germany) were added to vials of sample and standards, to initiate the colorimetric reaction. The final mixtures were vortexed and incubated in a water bath at 100 °C for 7 min. The reaction was then stopped by placing the vials in an ice bath. Subsequently, the UV absorbance of standard solutions and samples was measured at 820 nm (Lambda 35 UV/VIS Spectrophotometer, Perkin Elmer, Waltham, USA).

With the determined amounts of DPPC and 25(OH)D a loading efficiency (LE) was calculated, expressed as the quantified drug/lipid molar concentration ratio as percentage of the initial drug/lipid molar concentration ratio [22].

Aerosolisation of liposomes

Prior to deposition studies, the effect of the nebulisation process on the colloidal stability of liposomes was assessed. Liposomes were dispersed in water and nebulised using an electronic vibrating membrane inhaler (eFlow, PARI Medical Holding GmbH Starnberg, Germany). For stability, nebulised liposome samples were collected and the diluted liposomal aerosol was measured for size and zeta potential, as mentioned in the previous section.

For investigating the aerodynamic properties of nebulised liposomes, a next-generation impactor (NGI, Copley Scientific, Nottingham, UK) was used. Deposition experiments were conducted according to the procedure specified in the European Pharmacopoeia [23] and as detailed further in the supplementary material. The amount of deposited liposomes in each NGI stage was determined by measuring the fluorescence of Rh-DPPE using a plate reader (Genios Pro Tecan, Männedorf, Switzerland, excitation wavelength = 560 nm, emission wavelength = 662 nm). To predict pulmonary deposition *in vivo*, parameters of Mass Median Aerodynamic Diameter (MMAD), Geometric Standard Deviation (GSD) and Fine Particle Fraction (FPF) were calculated. For determination of the MMAD and GSD, probit analysis [24] was employed. FPF was defined as the mass of aerosolised material with an aerodynamic diameter of less than 5 μm.

Bacteria cultivation

To determine the influence of 25(OH)D on the immune response to infection, heat inactivated or viable *P. aeruginosa* PAO1 cultured as described previously [25] were used. The viable bacterial suspension was diluted 1:10 in phosphate-buffered saline (PBS, without Ca^{2+} and Mg^{2+}, pH 7.4, Life Technologies, Darmstadt, Germany) prior to application. For heat inactivation the undiluted bacterial suspension was incubated for 10 min at 95 °C, and subsequently stored in aliquots corresponding to $3x10^7$ colony-forming units (CFU) /ml at -20 °C. To determine bacterial concentrations prior to use, serial dilutions were plated on LB-agar and cultured overnight.

Cell culture

Cells of the murine epithelial cell line LA-4 were cultured at 37 °C with 5% CO_2, and split at regular intervals. For measuring inflammatory responses, cells were seeded in a 12-well plate (Greiner Bio-One GmbH, Frickenhausen, Germany; $0.26x10^4$ cells/cm^2) and cultured in

Ham's F12 medium containing 15% FBS and 1% penicilin-streptomycin. Cells were treated under submerged conditions.

The human bronchial epithelial cell line 16-HBE was cultured in DMEM-HamF12 (1:1) with 10% FBS, penicillin (100 U/ml), and streptomycin (0.1 mg/ml) at 37 °C with 5% CO_2, and split at regular intervals. For infection experiments, cells were seeded under standard conditions on 12-well Transwell® plates (Corning Inc., Acton, MA, USA). After reaching confluency the medium was removed from the upper compartment, in order to achieve an air liquid interface setup for subsequent stimulation [26], and the medium of the basolateral compartment was changed to DMEM-HamF12 (1:1) containing only 2% Ultroser-G.

25(OH)D pre-treatment and bacterial challenge

In all cases, cells were first pre-treated with 25(OH)D either within liposomes or dissolved in 0.5% ethanol (40 ng of 25(OH)D/well), or appropriate controls (empty liposomes at 376.7 ng of DPPC/well, or 0.5% ethanol alone) for 24 h. For all experiments, the dose of empty DPPC liposomes administered was standardised on the amount of lipid calculated to be contained within a dose of 25(OH)D liposomes. Following pre-treatment, murine LA-4 cells were stimulated with heat inactivated *P. aeruginosa*, while 16-HBE cells were infected with $1x10^3$ CFU/well live bacteria, diluted in PBS to a final volume of 100 µl. In the case of LA-4 cells, following a 6 h incubation period, apical release of the murine IL-8 homologue KC was determined by enzyme-linked immunosorbent assay (ELISA) as described below. Survival of bacteria in the apical compartment of 16-HBE cultures was quantified by plating on LG-agar and CFU counting after overnight incubation. The final CFU in pre-treated samples was expressed as a percentage of the CFU of infected samples without pre-treatment.

Measurement of cytokines

The concentration of KC in cell culture supernatants was determined by ELISA according to the kit manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). A TECAN Ultra 384 ELISA reader together with Magellan software (Mainz, Germany) was employed for quantification.

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). The data was analysed using SigmaPlot Version 11 (Systat Software Inc., San Jose, CA, USA). Comparisons between groups were performed using Student´s t test (two-sided), or ANOVA with post-hoc Bonferroni adjustment for experiments with more than two subgroups. Results were considered statistically significant at p values <0.05.

Results

Liposome preparation and characterisation

Liposomal formulations consisting of DPPC and 25(OH)D (PD), or DPPC alone as a control (P) were prepared, and characterised firstly in terms of colloidal properties. Both formulations had a mean initial size below 200 nm, a polydispersity index (PDI) below 0.1 and a negative zeta potential around -8 mV. With respect to chemical properties, an encapsulation efficiency and loading capacity of approximately 23% and 46% respectively was found for the PD preparation (Table 1). PD liposomes were also seen to exhibit constant colloidal properties upon storage for a period of at least 25 days (Figure S1a, S1b), and to retain the entire incorporated amount of 25(OH)D for a period of at least 3 days post-preparation (Figure S1c). Liposomal 25(OH)D was further determined to be active, confirming that the process of liposome preparation itself (involving for example the use of organic solvents and elevated

temperatures) had no adverse effect on 25(OH)D stability (Figure S2). The preparation process and employed conditions were moreover proven to be well translatable, as demonstrated by the ability to form liposomes utilising vitamin D3 itself as cargo (Table S1, Figure S3).

Aerosol deposition studies

The PD formulation was physically stable upon nebulisation, with no appreciable difference in liposome size and zeta potential noted before and after the nebulisation procedure (Figure 1). The deposition profile of PD liposomes is shown in Figure 2. A high percentage of liposomes was recovered in the initial stages of the NGI, in particular between stages 1 and 4, showing that the aerodynamic diameter of the majority of PD liposomes is higher than 3.18 µm. An MMAD of approximately 5.9 µm, a GSD of approximately 2.1 and an FPF of 41% were calculated from obtained NGI data.

Effect on *P. aeruginosa* infection

The efficacy of the 25(OH)D-loaded liposome formulation PD against *P. aeruginosa* infection was first investigated in cells of the murine epithelial cell line LA-4. Surprisingly, treatment of cells with 25(OH)D liposomes or even with 25(OH)D in ethanolic solution prior to bacterial stimulation was not seen to lead to a significant reduction in KC release, relative to cells which were treated with PBS alone (Figure 3). This lack of significant 25(OH)D effect was also noted in a pilot study in an *in vivo* mouse model (Figure S4).

In contrast, PD liposomes were seen to impact on *P. aeruginosa* infection in human 16-HBE cells. Loaded liposomes were compared with 25(OH)D dissolved in ethanol, and while both formulations demonstrated an anti-microbial effect, a significantly lower bacterial survival

was found following treatment with the PD liposomes compared to 25(OH)D dissolved in ethanol (Figure 4).

Discussion

In order to develop a novel anti-inflammatory and anti-infective approach for treatment of CF lung disease, and to overcome difficulties in administration resulting from the poor aqueous solubility of 25(OH)D, a stable liposomal formulation was successfully designed and prepared. While an anti-inflammatory and anti-infective activity of this formulation was not notable in murine models, studies in human-derived cell cultures showed a protective effect of liposomal 25(OH)D against *P. aeruginosa* infection.

Although the employed liposomal formulation was also seen to be compatible with vitamin D3 itself (see supplementary material), the vitamin D3 metabolite 25(OH)D was rather selected as the specific liposomal cargo in the current work, due to the absence of the vitamin D3-activating enzyme 25-hydroxylase in pulmonary epithelial and immune cells [17]. In addition to increasing its effective solubility, incorporation of 25(OH)D into liposomes was hypothesised to counteract the well-known instability of this compound. However, the effect of liposome preparation conditions on the stability and continued activity of 25(OH)D was unknown. The compound was therefore first dissolved in ethanol and subjected to different stresses (heat, organic solvent and air exposure) as encountered in the liposome preparation procedure. Encouragingly, only air exposure combined with lipopolysaccharide (LPS) administration was found to decrease 25(OH)D activity (supplementary material Figure S2). Therefore, in order to minimize air exposure and maintain 25(OH)D stability, liposomes were prepared under nitrogen.

Due to its lipophilicity, 25(OH)D is expected to be more concentrated within vesicular lipid bilayers rather than in the bilayer spaces or aqueous core of liposomes [27]. The steroid-like structure of 25(OH)D in combination with this intra-bilayer location is also known to have the advantage of condensing and stabilising liposomal bilayers without the need for other membrane-stabilising components, such as cholesterol [27]. Accordingly, incorporation of 25(OH)D into liposomes in the current work appeared to reduce liposome size and PDI (Table 1). The gained stability data, which showed a high and constant level of 25(OH)D entrapment and consistent colloidal characteristics over time (Figure S1), further confirmed the stabilising effect of 25(OH)D on liposomes.

As a further step in the formulation characterisation process, information about the lung deposition of PD liposomes was gained by nebulisation of liposomes into an NGI. A lack of appreciable difference in colloidal parameters pre- and post-nebulisation (Figure 1) indicated that the vibration of the nebuliser membrane did not destroy or considerably alter the liposomal structure, confirming the feasibility of deposition studies themselves. NGI experiments were considered as essential to investigate the aerodynamic deposition of the prepared liposomes, and to assess their potential for local airway administration (Figure 2). The calculated MMAD is an encouraging result, as it has been shown that particles with a size of up to 5 µm can effectively reach the bronchiolar region of the respiratory tract, where infection and inflammatory responses are mostly localised in CF patients [28]. FPF was calculated as a measure of the portion of the inhaled mass of liposomes capable of reaching the lower airways (alveolar region). According to NGI experiments, only 41% of the liposomal dispersion is expected to reach the alveolar region, while the remaining deposited fraction will be localised in the bronchiolar region where the action of 25(OH)D is specifically required.

Somewhat surprisingly, neither application of PD liposomes nor of an ethanolic solution of 25(OH)D to infected, mouse-derived LA-4 cells was noted to have an anti-inflammatory effect (Figure 3). This observation was also supported by an *in vivo* pilot study in mice (Figure S4). The lack of effect of 25(OH)D either as ethanolic solution or in liposomes in such mouse-based models points to significant species differences, with the consequence that murine models may not be suitable for investigation of 25(OH)D-mediated immunomodulation in the context of a potential clinical application to patients. This view is also supported by recent reports on inter-species difference in the immunomodulatory effects of vitamin D3 and its metabolites between mice and primates [29-31]. The predictive value of murine models, either *in vitro* or *in vivo*, for the translation of this therapeutic approach into the clinic, may therefore be limited.

However, when applied to *Pseudomonas*-infected human 16-HBE cells, PD liposomes showed a significantly higher bacterial killing compared with both the empty liposome formulation (P), and 25(OH)D dissolved in ethanol (Figure 4). In this setting therefore, incorporation of 25(OH)D into liposomes appears to be more effective than 25(OH)D in solution.

Conclusion

A liposomal formulation of 25(OH)D with favourable and robust physico-chemical properties for local delivery to the lung could be successfully prepared. A beneficial effect of 25(OH)D with respect to *P. aeruginosa* infection was clearly observed in a human cell line. In this case, a significant improvement in antibacterial action was observed as a result of incorporation of 25(OH)D into liposomes as compared with administration in ethanolic solution. 25(OH)D-loaded liposomes therefore appear as a promising anti-infective therapy for CF-related lung

infection. As all materials required for preparing the formulations used in this study may be regarded as safe for pulmonary administration in humans, this concept should soon be further evaluated in clinical studies.

Declaration of interest

The authors declare that they have no competing interests.

Acknowledgments

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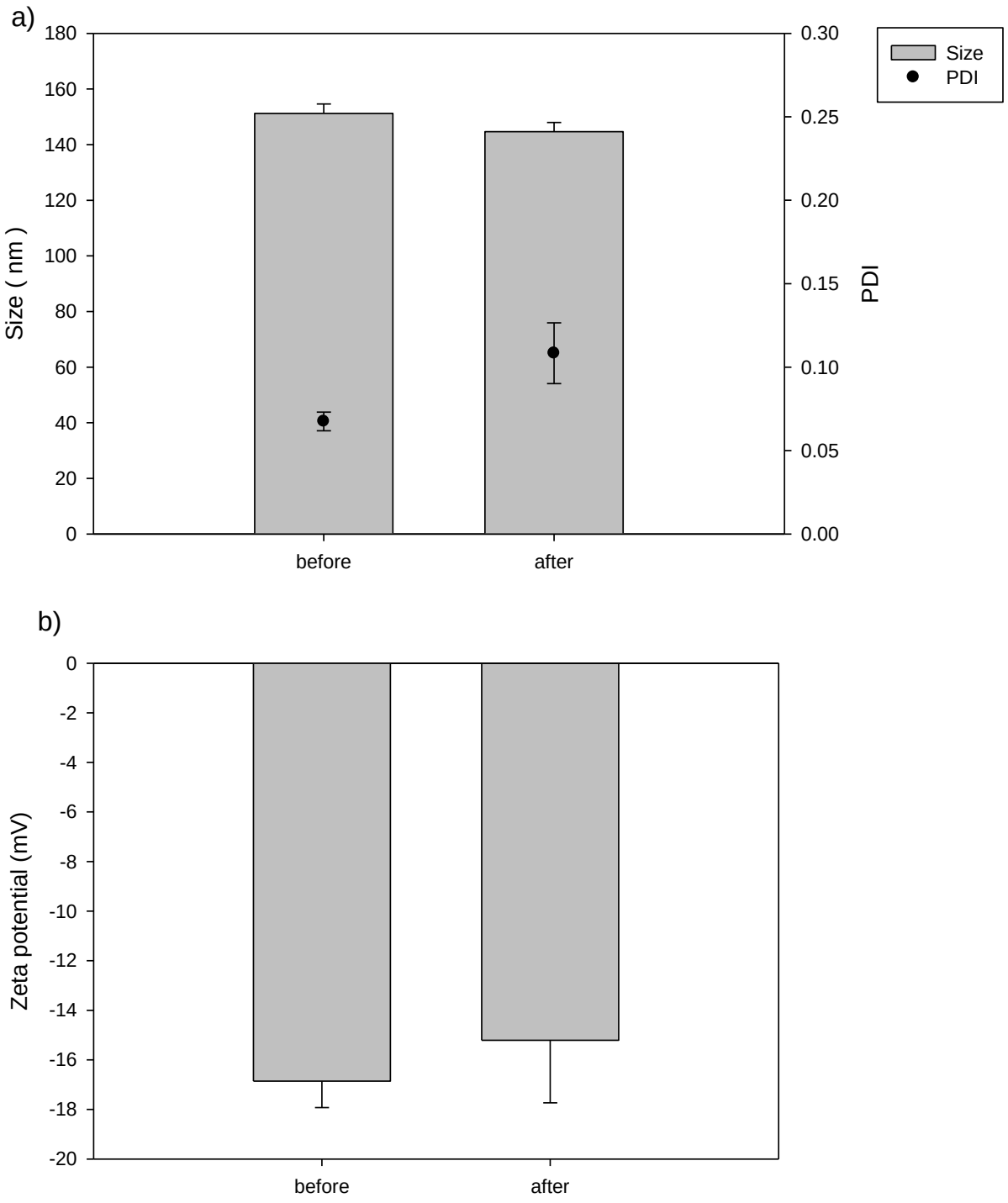
Figures and Tables

Table 1. Physico-chemical characteristics of 25(OH)D:DPPC liposomes (PD) and DPPC liposomes (P). Size, polydispersity index (PDI) and surface charge (zeta potential) of PD and P are shown. The encapsulation efficiency (EE) and the loading capacity (LC) of PD are also given. All data represent mean ± SEM (n=3) , *** = $p<0.001$.

	Molar ratio 25(OH)D:DPPC	Size (nm)	PDI	Zeta potential (mV)	EE (%)	LC (%)
PD	1.6:2	151.2±3.3	0.067±0.005	-7.6±1.1(***)	23.4±7.9	46.3±4.6
P	0:1	180.3 ±1.7	0.121±0.006	-25.6±0.1	--	--



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419 Figure 1. Physical stability of 25(OH)D-loaded liposomes (PD) subjected to nebulisation.
420 Physical characteristics of PD before and after the nebulisation process are shown. (a) size
421 and polydispersity index (PDI); (b) zeta potential. Data represent mean ± SEM (n=3).

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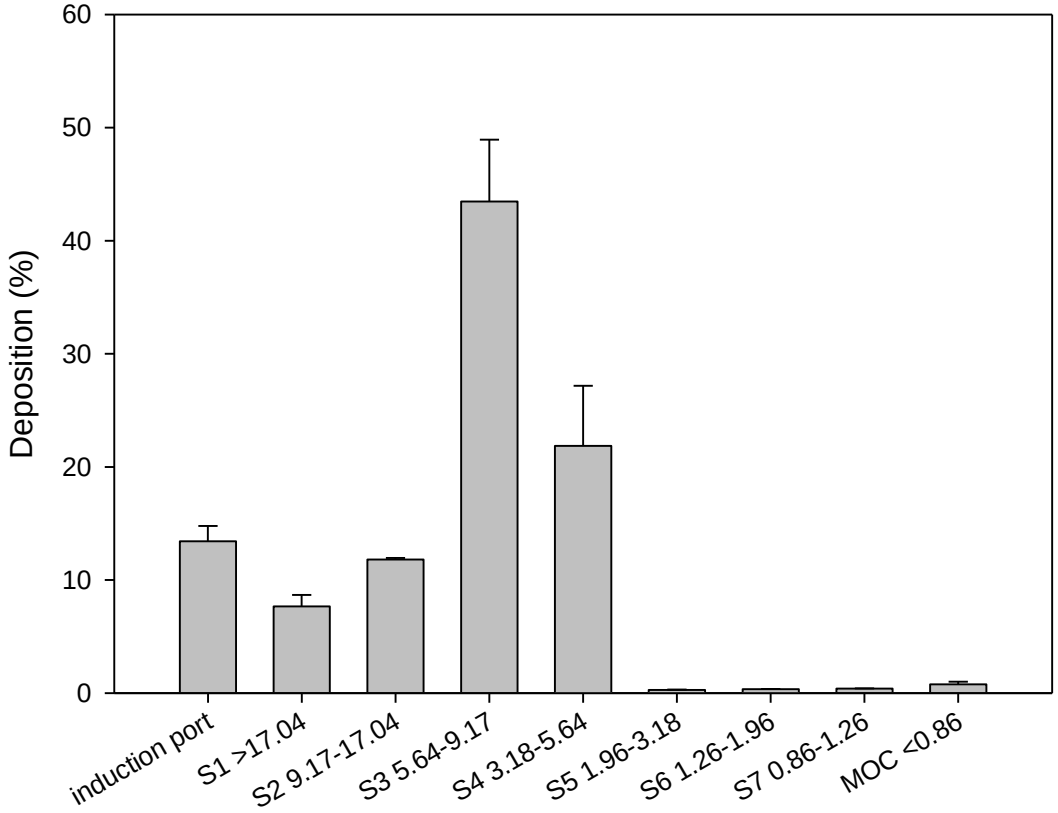

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424 Figure 2. NGI aerosol stage deposition profiles of 25(OH)D-loaded liposomes (PD). Drug
425 deposition from the induction port to stage 8 (micro-orifice collector, MOC) of the NGI
426 specifically is shown. Data represent mean ± SEM (n = 3).

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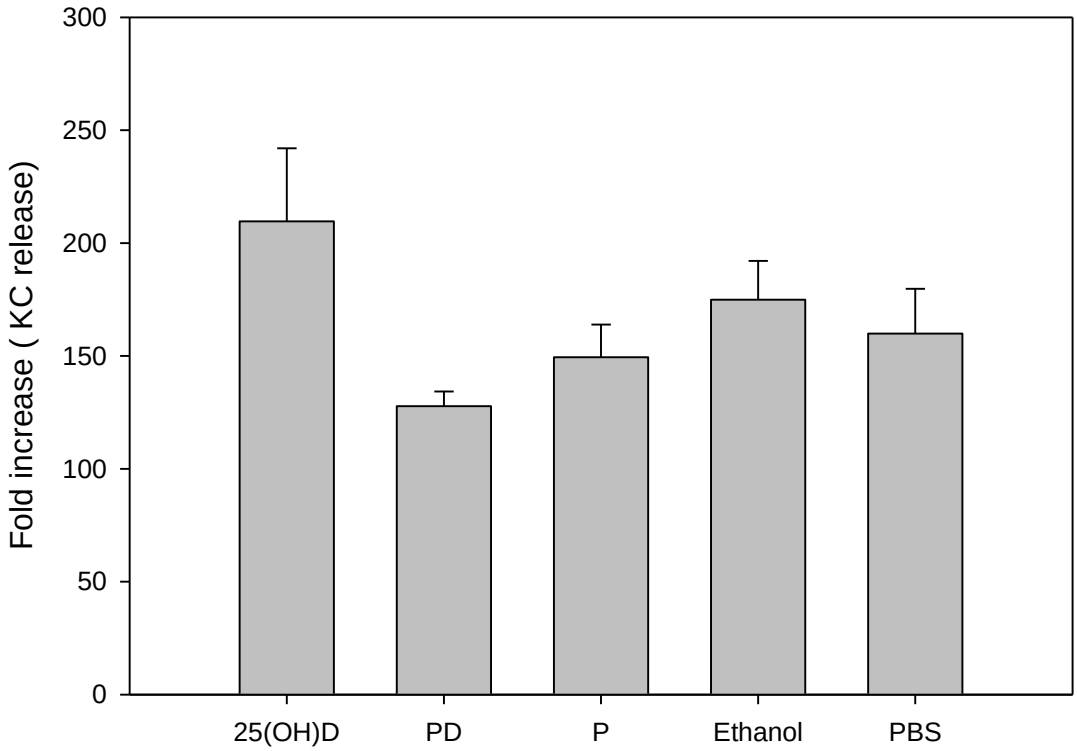
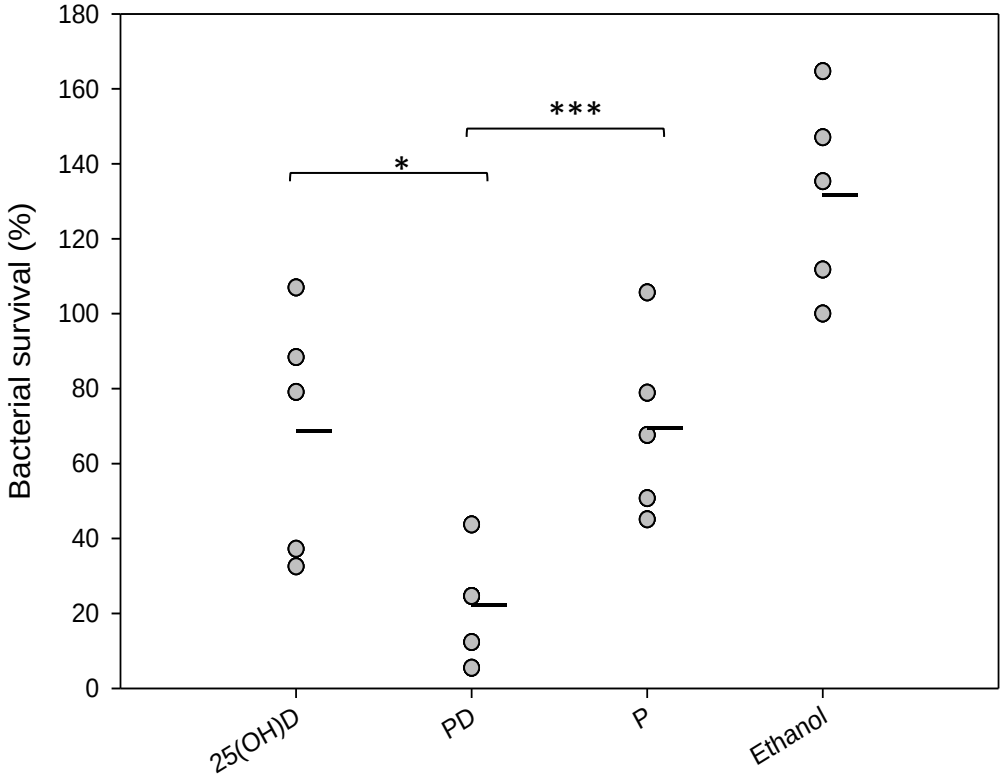


Figure 3. Release of KC from mouse-derived LA-4 cells. Cells were treated with 25(OH)D dissolved in ethanol (25(OH)D), 25(OH)D-loaded liposomes (PD), empty liposomes (P), ethanol, or PBS for 24 h. Cells were then stimulated with heat inactivated *P. aeruginosa* PAO1 for 6 h. Fold increase in KC release was calculated by diving the KC levels after bacterial stimulation by the respective baseline level before bacterial stimulation. Data represent mean ± SEM (n=6).



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437 Figure 4. Bacterial survival in response to treatment of human-derived 16-HBE cells. Cells
438 were pre-incubated with 25(OH)D either dissolved in 0.5% ethanol (25(OH)D) or within
439 liposomes (PD), or with drug free liposomes (P) or ethanol alone (Ethanol) as controls. Cells
440 were then stimulated with *P. aeruginosa*. Percentage of bacterial survival was calculated as
441 the number of living bacteria following treatment relative to the amount of living bacteria on
442 cells without treatment. Circles represent individual survival measurements, while lines
443 represent the mean within a treatment group. Data represent mean ± SEM, n = 5. * = $p<0.05$,
444 *** = $p<0.001$.

445