

1                   **Calcifediol-loaded liposomes for local treatment of**  
2                   **pulmonary bacterial infections**

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## 28 **Abstract**

29 The influence of vitamin D3 and its metabolites calcifediol (25(OH)D) and calcitriol on  
30 immune regulation and inflammation is well described, and raises the question of potential  
31 benefit against bacterial infections. In the current study, 25(OH)D was encapsulated in  
32 liposomes to enable aerosolisation, and tested for the ability to prevent pulmonary infection  
33 by *Pseudomonas aeruginosa*. Prepared 25(OH)D-loaded liposomes were nanosized and  
34 monodisperse, with a negative surface charge and a 25(OH)D entrapment efficiency of  
35 approximately 23%. Jet nebulisation of liposomes was seen to yield an aerosol suitable for  
36 tracheo-bronchial deposition. Interestingly, 25(OH)D in either liposomes or ethanolic solution  
37 had no effect on the release of the proinflammatory cytokine KC from *Pseudomonas*-infected  
38 murine epithelial cells (LA-4); treatment of infected, human bronchial 16-HBE cells with  
39 25(OH)D liposomes however resulted in a significant reduction in bacterial survival. Together  
40 with the importance of selecting an application-appropriate *in vitro* model, the current study  
41 illustrates the feasibility and practicality of employing liposomes as a means to achieve  
42 25(OH)D lung deposition. 25(OH)D-loaded liposomes further demonstrated promising effects  
43 regarding prevention of *Pseudomonas* infection in human bronchial epithelial cells.

44

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

47

## 48 **Introduction**

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

62

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

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

79

## 80 **Materials and Methods**

### 81 **Material**

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

92

### 93 **Liposome preparation and characterisation**

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

98 solvent mixture was then evaporated under reduced pressure and with a rotation speed of 145  
99 rpm at 70 °C using a rotavapor (Büchi, Essen, Germany). The resulting homogenous thin lipid  
100 film was then re-hydrated by the addition of 5 ml of deionised water, followed by further  
101 rotation at 60 °C for 1 h. The formed liposomal dispersion was sonicated in a sonication bath  
102 (Bandelin Sonorex, Berlin, Germany) for 10 min and then extruded (LiposoFast extruder,  
103 Avestin, Mannheim, Germany) repeatedly through 200 nm pore size membranes (AMD  
104 Manufacturing Inc., Ontario, Canada) to achieve size reduction and uniformity. Liposomes  
105 were then diluted 1:10 with deionised water and stored at 4 °C under nitrogen until further  
106 use. Physical characterisation of diluted liposomal formulations was performed by dynamic  
107 light scattering (size and size distribution) and electrophoretic mobility (zeta potential) at 25  
108 °C using a Zetasizer Nano (Malvern Instruments Ltd, Worcestershire, United Kingdom).

109

#### 110 **Determination of liposomal DPPC and 25(OH)D content**

111 The amount of 25(OH)D incorporated within liposomes was determined via HPLC,  
112 performed on a Dionex HPLC system (Thermo Scientific, Bremen, Germany) composed of a  
113 P680 pump, an Elite degassing System, an Asta-medica AG 80 column oven and a UV  
114 detector. A LiChrospher<sup>®</sup> RP-18 (5 µm, 125 x 4 mm) column (Merck KGaA, Darmstadt,  
115 Germany) was employed. A mobile phase of methanol/acetonitrile (30:70 v/v) was used, with  
116 an injection volume of 100 µl, a flow rate of 2 ml/min and a temperature of 30 °C. For sample  
117 analysis, liposomes were first dissolved in a mixture of 50% ethyl acetate/methanol (4:1) and  
118 50% acetonitrile. The 25(OH)D content of dissolved liposome samples was determined using  
119 UV detection at a wavelength of 265 nm, and calculated in reference to standard solutions of  
120 25(OH)D. The determined amount of 25(OH)D was then used to calculate the encapsulation  
121 efficiency (EE) of liposomes, defined as the measured amount of 25(OH)D as percentage of  
122 the initially added amount [20].

123

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

138

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

142

### 143 **Aerosolisation of liposomes**

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

149

150 For investigating the aerodynamic properties of nebulised liposomes, a next-generation  
151 impactor (NGI, Copley Scientific, Nottingham, UK) was used. Deposition experiments were  
152 conducted according to the procedure specified in the European Pharmacopoeia [23] and as  
153 detailed further in the supplementary material. The amount of deposited liposomes in each  
154 NGI stage was determined by measuring the fluorescence of Rh-DPPE using a plate reader  
155 (Genios Pro Tecan, Männedorf, Switzerland, excitation wavelength = 560 nm, emission  
156 wavelength = 662 nm). To predict pulmonary deposition *in vivo*, parameters of Mass Median  
157 Aerodynamic Diameter (MMAD), Geometric Standard Deviation (GSD) and Fine Particle  
158 Fraction (FPF) were calculated. For determination of the MMAD and GSD, probit analysis  
159 [24] was employed. FPF was defined as the mass of aerosolised material with an aerodynamic  
160 diameter of less than 5 µm.

161

## 162 **Bacteria cultivation**

163 To determine the influence of 25(OH)D on the immune response to infection, heat inactivated  
164 or viable *P. aeruginosa* PAO1 cultured as described previously [25] were used. The viable  
165 bacterial suspension was diluted 1:10 in phosphate-buffered saline (PBS, without Ca<sup>2+</sup> and  
166 Mg<sup>2+</sup>, pH 7.4, Life Technologies, Darmstadt, Germany) prior to application. For heat  
167 inactivation the undiluted bacterial suspension was incubated for 10 min at 95 °C, and  
168 subsequently stored in aliquots corresponding to 3x10<sup>7</sup> colony-forming units (CFU)/ml at -20  
169 °C. To determine bacterial concentrations prior to use, serial dilutions were plated on LB-agar  
170 and cultured overnight.

171

## 172 **Cell culture**

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

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

178

179 The human bronchial epithelial cell line 16-HBE was cultured in DMEM-HamF12 (1:1) with  
180 10% FBS, penicillin (100 U/ml), and streptomycin (0.1 mg/ml) at 37 °C with 5% CO<sub>2</sub>, and  
181 split at regular intervals. For infection experiments, cells were seeded under standard  
182 conditions on 12-well Transwell<sup>®</sup> plates (Corning Inc., Acton, MA, USA). After reaching  
183 confluency the medium was removed from the upper compartment, in order to achieve an air  
184 liquid interface setup for subsequent stimulation [26], and the medium of the basolateral  
185 compartment was changed to DMEM-HamF12 (1:1) containing only 2% Ultrosor-G.

186

#### 187 **25(OH)D pre-treatment and bacterial challenge**

188 In all cases, cells were first pre-treated with 25(OH)D either within liposomes or dissolved in  
189 0.5% ethanol (40 ng of 25(OH)D/well), or appropriate controls (empty liposomes at 376.7 ng  
190 of DPPC/well, or 0.5% ethanol alone) for 24 h. For all experiments, the dose of empty DPPC  
191 liposomes administered was standardised on the amount of lipid calculated to be contained  
192 within a dose of 25(OH)D liposomes. Following pre-treatment, murine LA-4 cells were  
193 stimulated with heat inactivated *P. aeruginosa*, while 16-HBE cells were infected with  $1 \times 10^3$   
194 CFU/well live bacteria, diluted in PBS to a final volume of 100  $\mu$ l. In the case of LA-4 cells,  
195 following a 6 h incubation period, apical release of the murine IL-8 homologue KC was  
196 determined by enzyme-linked immunosorbent assay (ELISA) as described below. Survival of  
197 bacteria in the apical compartment of 16-HBE cultures was quantified by plating on LG-agar  
198 and CFU counting after overnight incubation. The final CFU in pre-treated samples was  
199 expressed as a percentage of the CFU of infected samples without pre-treatment.

200

201



## 202 **Measurement of cytokines**

203 The concentration of KC in cell culture supernatants was determined by ELISA according to  
204 the kit manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). A TECAN Ultra  
205 384 ELISA reader together with Magellan software (Mainz, Germany) was employed for  
206 quantification.

207

## 208 **Statistical analysis**

209 Data are expressed as mean  $\pm$  standard error of the mean (SEM). The data was analysed using  
210 SigmaPlot Version 11 (Systat Software Inc., San Jose, CA, USA). Comparisons between  
211 groups were performed using Student's t test (two-sided), or ANOVA with post-hoc  
212 Bonferroni adjustment for experiments with more than two subgroups. Results were  
213 considered statistically significant at p values  $<0.05$ .

214

## 215 **Results**

### 216 **Liposome preparation and characterisation**

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

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

231

### 232 **Aerosol deposition studies**

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

240

### 241 **Effect on *P. aeruginosa* infection**

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

248

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

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

## 254 **Discussion**

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

261

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

275

276 Due to its lipophilicity, 25(OH)D is expected to be more concentrated within vesicular lipid  
277 bilayers rather than in the bilayer spaces or aqueous core of liposomes [27]. The steroid-like  
278 structure of 25(OH)D in combination with this intra-bilayer location is also known to have the  
279 advantage of condensing and stabilising liposomal bilayers without the need for other  
280 membrane-stabilising components, such as cholesterol [27]. Accordingly, incorporation of  
281 25(OH)D into liposomes in the current work appeared to reduce liposome size and PDI (Table  
282 1). The gained stability data, which showed a high and constant level of 25(OH)D entrapment  
283 and consistent colloidal characteristics over time (Figure S1), further confirmed the stabilising  
284 effect of 25(OH)D on liposomes.

285

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

301

302 Somewhat surprisingly, neither application of PD liposomes nor of an ethanolic solution of  
303 25(OH)D to infected, mouse-derived LA-4 cells was noted to have an anti-inflammatory  
304 effect (Figure 3). This observation was also supported by an *in vivo* pilot study in mice  
305 (Figure S4). The lack of effect of 25(OH)D either as ethanolic solution or in liposomes in  
306 such mouse-based models points to significant species differences, with the consequence that  
307 murine models may not be suitable for investigation of 25(OH)D-mediated  
308 immunomodulation in the context of a potential clinical application to patients. This view is  
309 also supported by recent reports on inter-species difference in the immunomodulatory effects  
310 of vitamin D3 and its metabolites between mice and primates [29-31]. The predictive value of  
311 murine models, either *in vitro* or *in vivo*, for the translation of this therapeutic approach into  
312 the clinic, may therefore be limited.

313

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

319

## 320 **Conclusion**

321 A liposomal formulation of 25(OH)D with favourable and robust physico-chemical properties  
322 for local delivery to the lung could be successfully prepared. A beneficial effect of 25(OH)D  
323 with respect to *P. aeruginosa* infection was clearly observed in a human cell line. In this case,  
324 a significant improvement in antibacterial action was observed as a result of incorporation of  
325 25(OH)D into liposomes as compared with administration in ethanolic solution. 25(OH)D-  
326 loaded liposomes therefore appear as a promising anti-infective therapy for CF-related lung

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

330 **Declaration of interest**

331 The authors declare that they have no competing interests.

332

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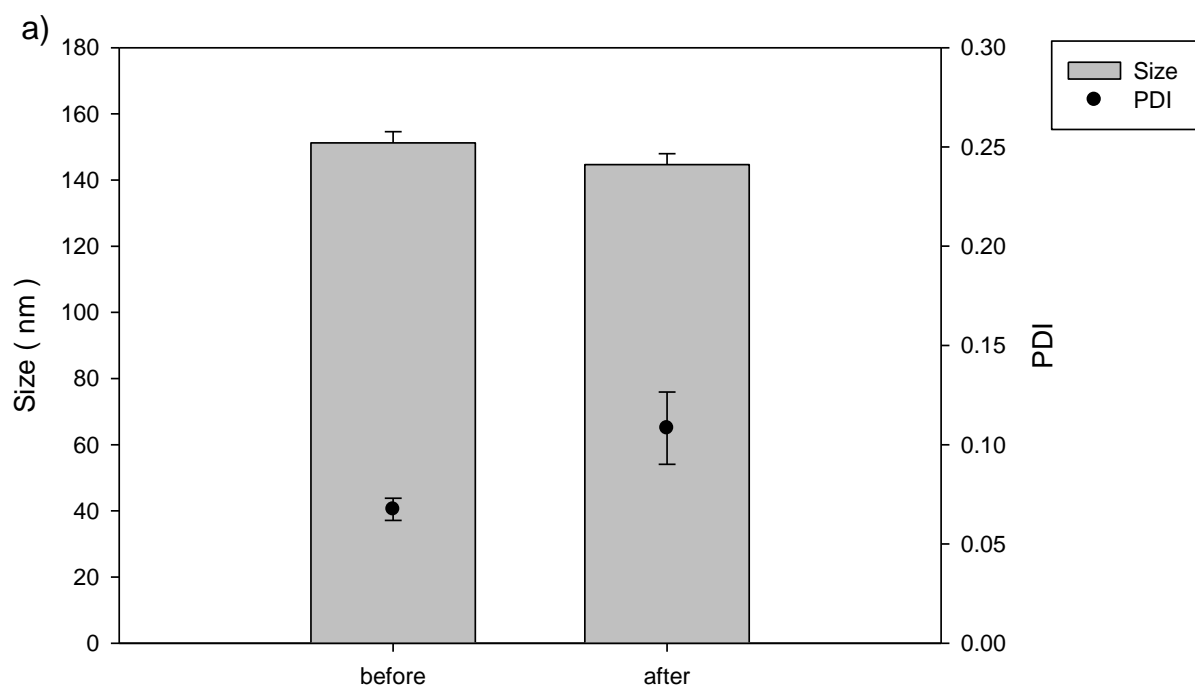
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410 **Figures and Tables**

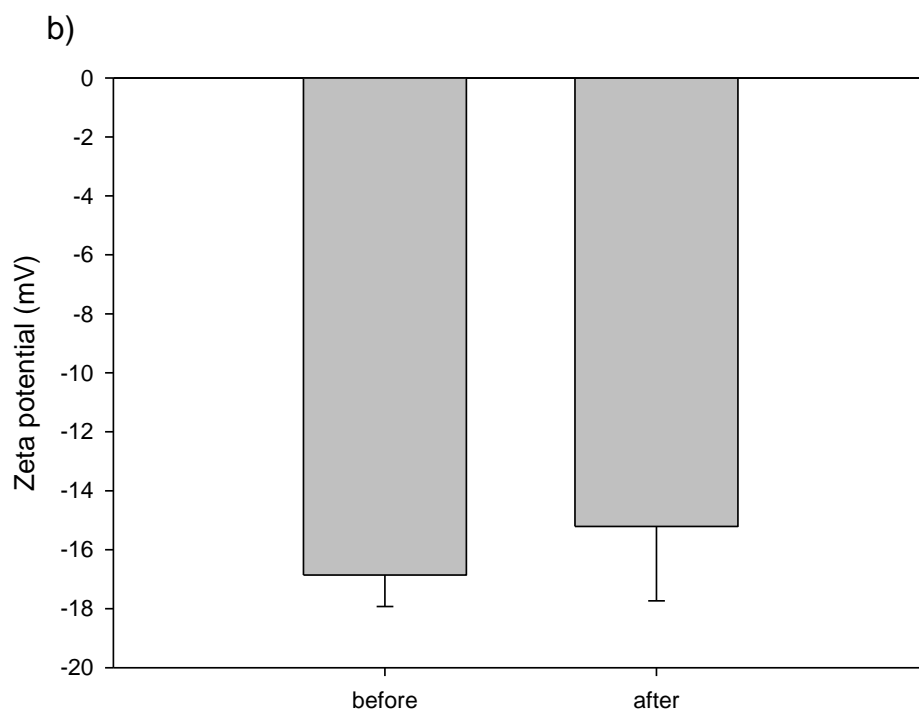
411 Table 1. Physico-chemical characteristics of 25(OH)D:DPPC liposomes (PD) and DPPC liposomes  
412 (P). Size, polydispersity index (PDI) and surface charge (zeta potential) of PD and P are shown. The  
413 encapsulation efficiency (EE) and the loading capacity (LC) of PD are also given. All data represent  
414 mean  $\pm$  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 $\pm$ 3.3	0.067 $\pm$ 0.005	-7.6 $\pm$ 1.1(***)	23.4 $\pm$ 7.9	46.3 $\pm$ 4.6
P	0:1	180.3 $\pm$ 1.7	0.121 $\pm$ 0.006	-25.6 $\pm$ 0.1	--	--

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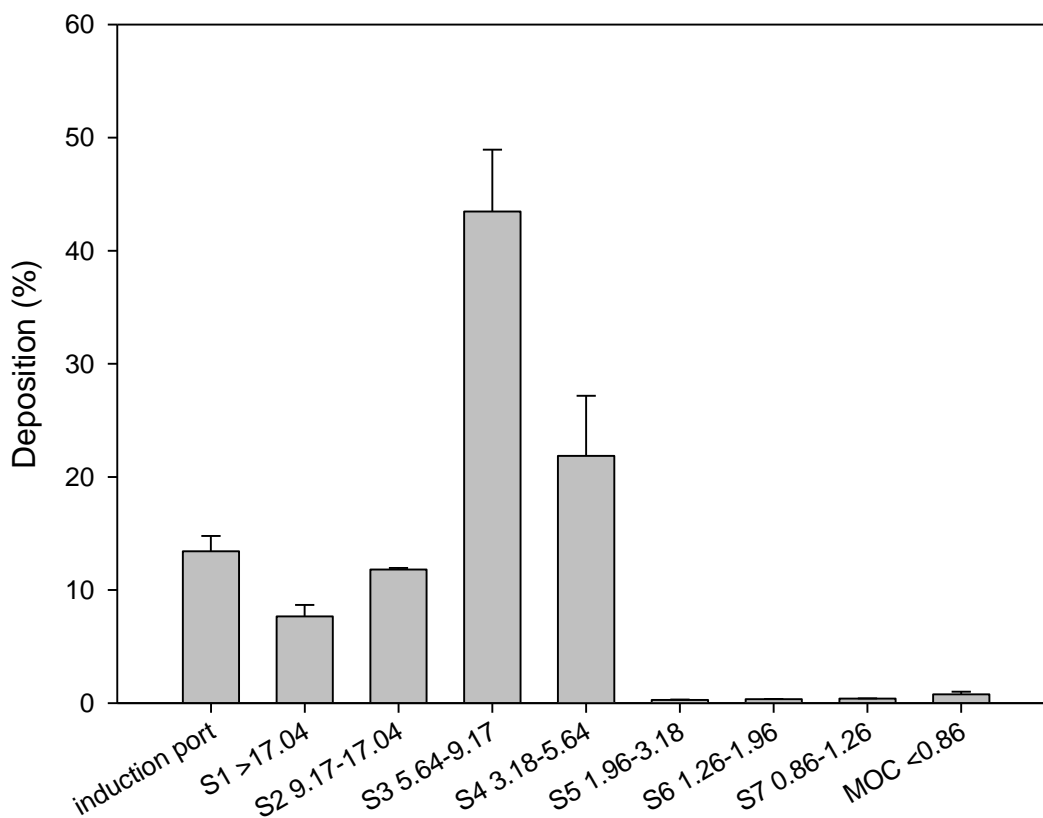
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419 Figure 1. Physical stability of 25(OH)D-loaded liposomes (PD) subjected to nebulisation.

420 Physical characteristics of PD before and after the nebulisation process are shown. (a) size

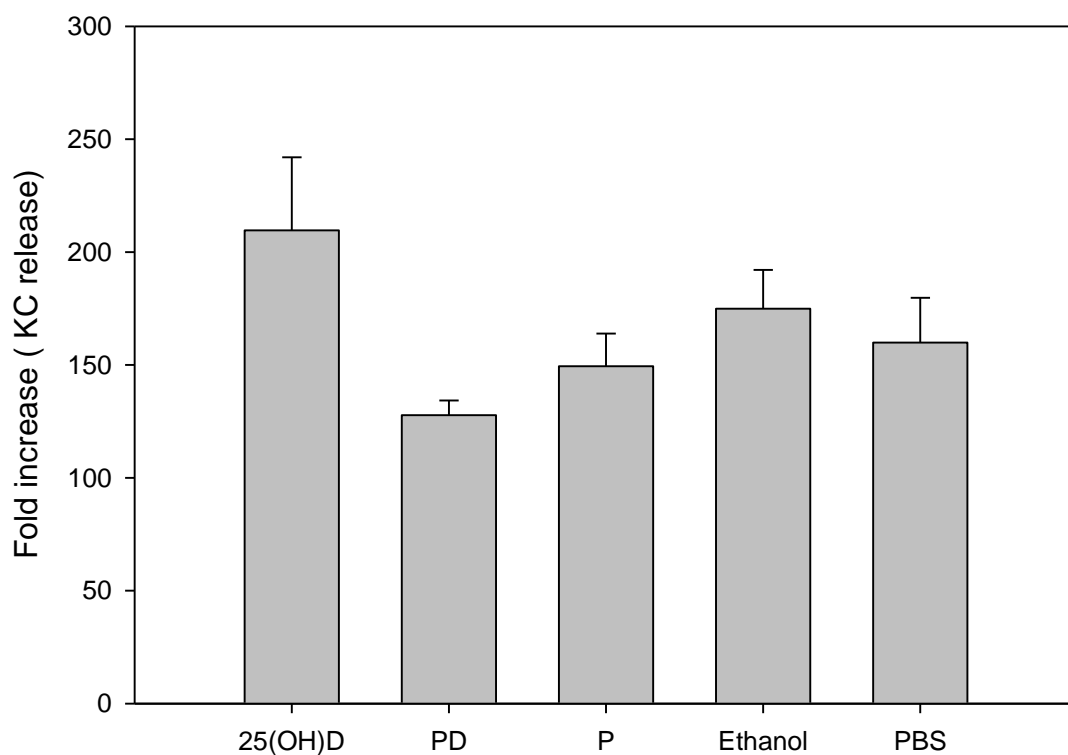
421 and polydispersity index (PDI); (b) zeta potential. Data represent mean  $\pm$  SEM (n=3).

422



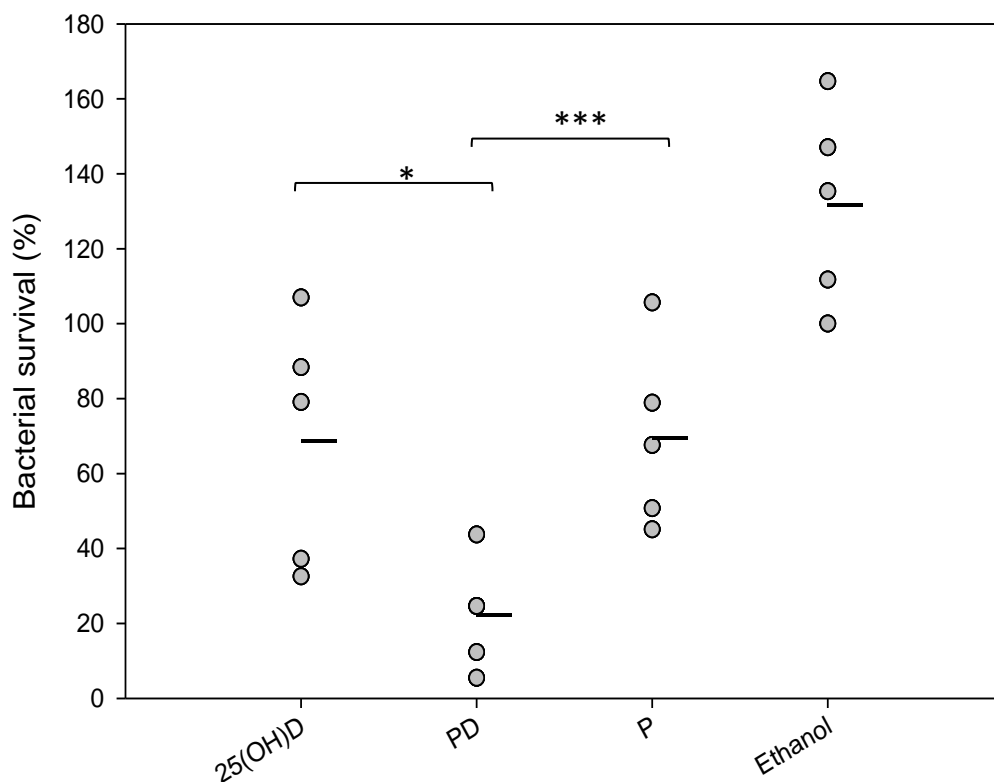
423  
 424 Figure 2. NGI aerosol stage deposition profiles of 25(OH)D-loaded liposomes (PD). Drug  
 425 deposition from the induction port to stage 8 (micro-orifice collector, MOC) of the NGI  
 426 specifically is shown. Data represent mean  $\pm$  SEM (n = 3).

427  
 428



429

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



436

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  $\pm$  SEM, n = 5. \* = p<0.05,  
 444 \*\*\* = p<0.001.

445