Calcifediol-loaded liposomes for local treatment of pulmonary bacterial infections

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Supplementary material

Methods

Vitamin D3 liposome preparation

Liposome formulations containing vitamin D3 were prepared as described in the main manuscript for 25(OH)D liposomes, using the molar ratio as shown in Table S1.

Assessment of liposome stability

The storage stability of 25(OH)D-loaded liposomes was assessed by characterisation of physical (size, polydispersity index (PDI) and zeta potential - Zetasizer Nano, Malvern Instruments Ltd, Worcestershire, United Kingdom) and chemical (amount of incorporated 25(OH)D, see main manuscript) properties at various time points after storage at 4 °C.

Pilot study in infected mouse model

All animal experiments were approved by the "Landesamt für Soziales, Gesundheit und Verbraucherschutz" of the State of Saarland following the German guidelines for animal treatment. Ten week old C57BL/6N mice (Janvier France, Saint-Berthevin Cedex, France) were first anaesthetised by an intraperitoneal injection with ketamine and xylazine (7 mg/kg and 105 mg/kg respectively, Bayer, Germany, Leverkusen) in sodium chloride. Mice were then treated with 25(OH)D-containing liposomes (0.241 g 25(OH)D/kg) or empty liposomes (1.4 g DPPC/kg) via intubation in a volume of 50 µl, or with the same volume of air (as control), as described previously [1]. After 24 h the mice were again anaesthetised and infected intranasally with $5.2x10^6$ viable *P. aeruginosa* in 40 µl PBS.

At 24 h post-infection mice were euthanised by a lethal dose of ketamine and xylazine (35 mg/kg and 525 mg/kg) in sodium chloride by intraperitoneal injection. The lungs of mice were lavaged with 1 ml PBS. Bronchoalveolar lavage fluid (BALF) was centrifuged for 10 min at 300 x g and 4 °C. BALF cells were counted with a haemocytometer, and differentiated after staining on cytospins (Cellspin II, Tharmac, Waldsolms, Germany; Diff-Quick, Medion, Gräfelfing, Germany). Left lung lobes were finally homogenised in 1 ml of PBS and used diluted for colonisation analysis. Cytokines were measured in cell-free BALF and lung tissue homogenate by ELISA, according to the 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.

Figures and Table

Table S1. Physico-chemical characteristics of vitamin D3:DPPC liposomes (PD3). Size, polydispersity index (PDI) and zeta potential are shown. The concentration of vitamin D3 in the liposomal dispersion is also given. All data represent mean ± SEM (n=3).

	Molar ratio vitamin D3:DPPC	Size (nm)	PDI	Zeta potential (mV)	Concentration (ug/ml)
PD3	1.6:2	141.50±7.19	0.1±0.008	-37.30±0.35	19.06±0.32

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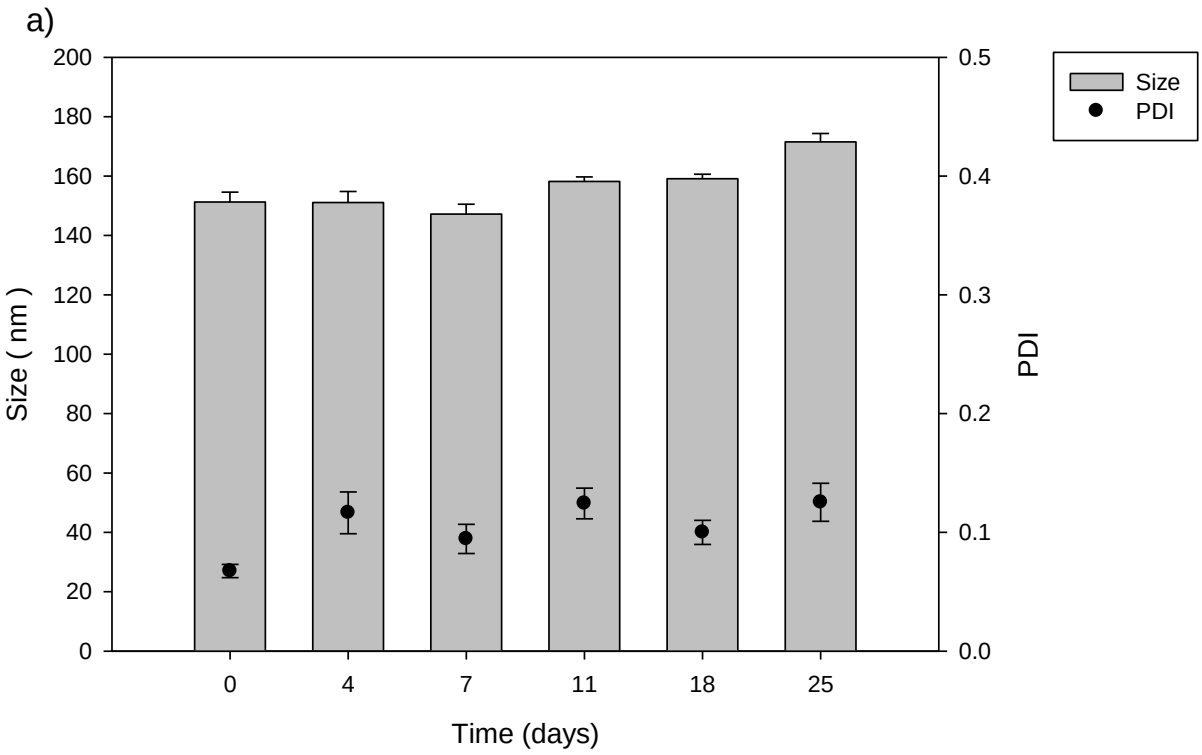
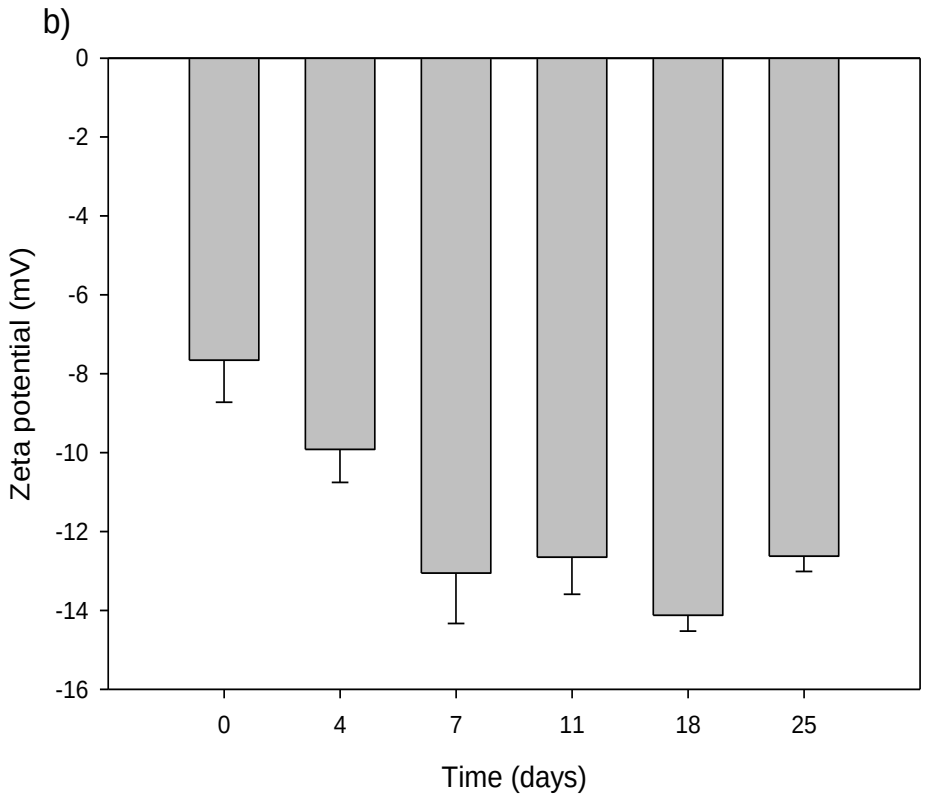

a)
Size (nm)
PDI
Time (days)
Size
PDI
0
20
40
60
80
100
120
140
160
180
200
0.0
0.1
0.2
0.3
0.4
0.5
0
4
7
11
18
25

59
60


b)
Zeta potential (mV)
Time (days)
0
-2
-4
-6
-8
-10
-12
-14
-16
0
4
7
11
18
25

61

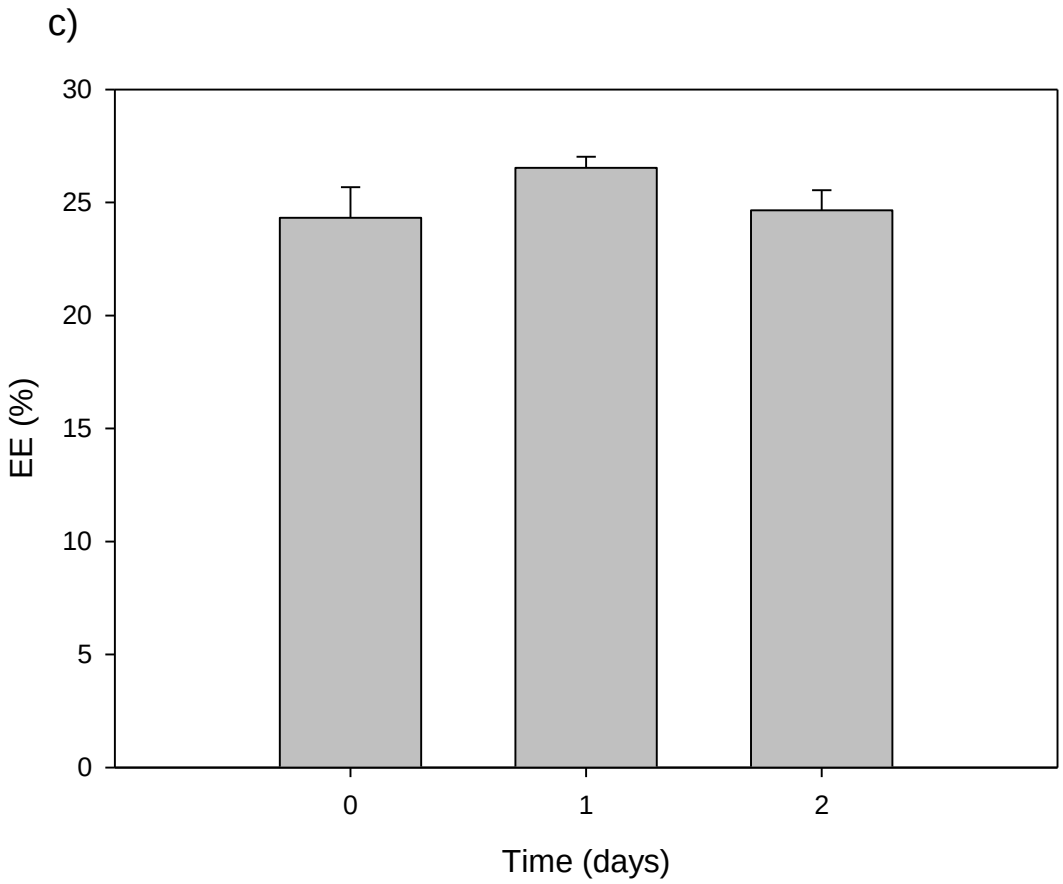


Figure S1. Physical and chemical stability of the 25(OH)D loaded liposomal formulation (PD) over time. (a) size and polydispersity index (PDI); (b) zeta potential; (c) the amount of 25(OH)D analysed via HPLC was used to calculate the encapsulation efficiency (EE%). Data represent mean ± SEM (n=3) for (a) and (b); for (c) the amount was calculated for one batch.

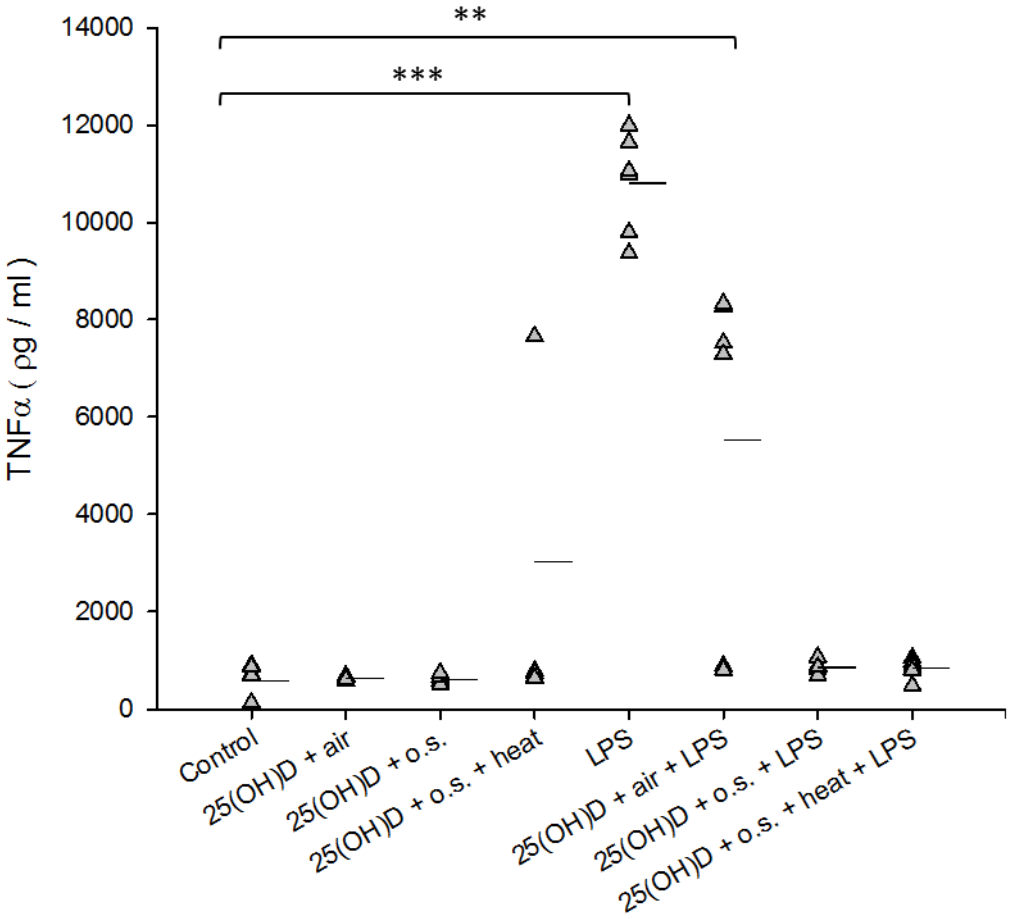


Figure S2. Activity testing of 25(OH)D following exposure to liposome preparation-relevant conditions. Continued activity was defined by a low elicited release of TNF-α from U937 cells (a human leukemic monocyte lymphoma cell line), following incubation with 25(OH)D which was previously exposed to various treatment conditions. TNF-α release was measured after incubation of U937 cells with 25(OH)D exposed for 1 h to air (25(OH)D + air), 25(OH)D treated with organic solvent (25(OH)D + o.s.), and 25(OH)D exposed to organic solvent plus heat ((25(OH)D + o.s + heat). The mixture of organic solvents and temperatures used were in accordance with the preparation protocol of liposomes. In all cases, U937 cells were stimulated with treated 25(OH)D preparations for 6 h. A second set of U937 cells was stimulated in an identical manner, however with a further 17 h of LPS treatment. The release of TNF-α from all U937 cells was then measured. Triangles represent individual TNF-α

measurement, while the black line represents the mean within a treatment group. ** = <0.01 and *** = <0.001, with respect to one-way ANOVA with post-hoc Bonferroni analysis.

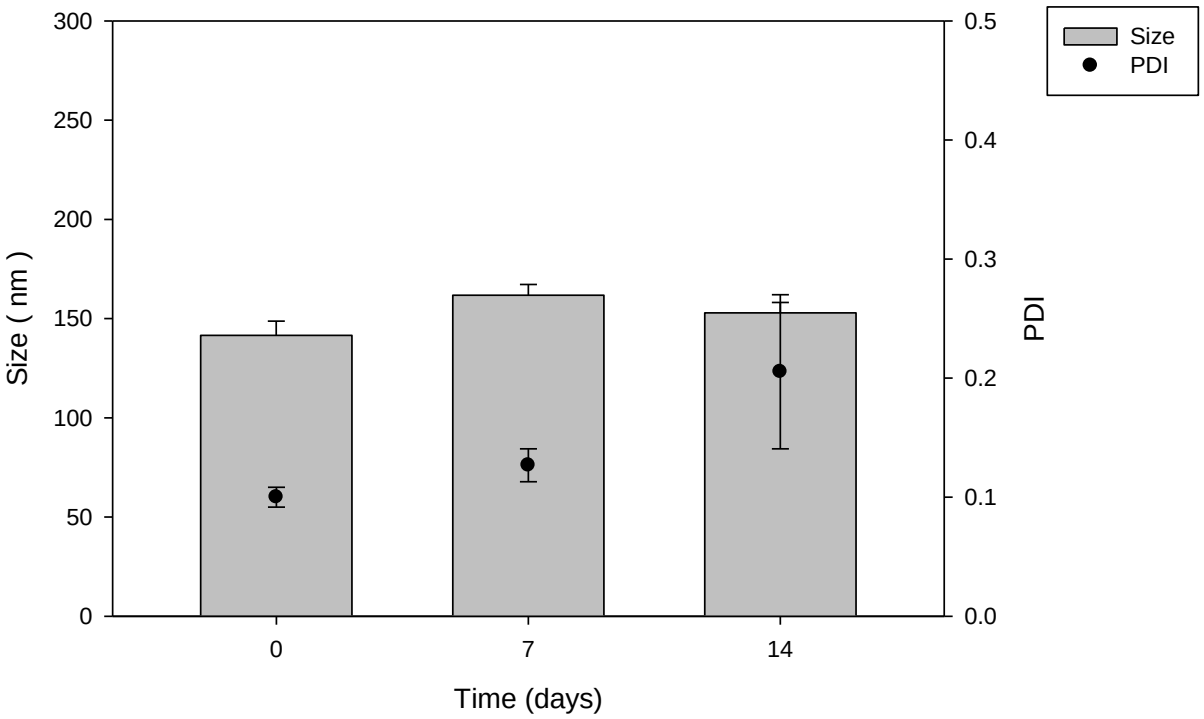
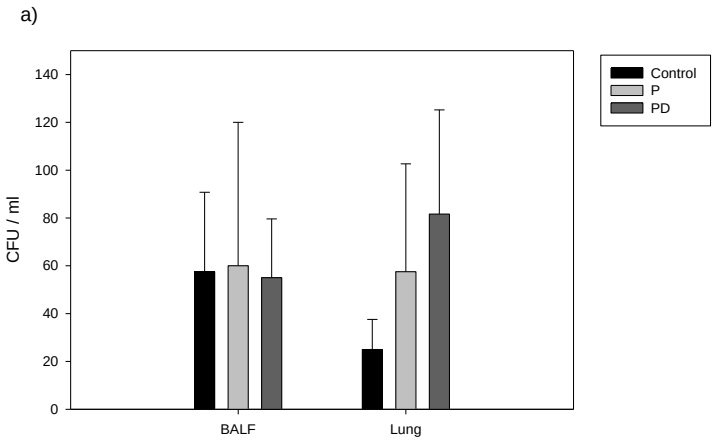
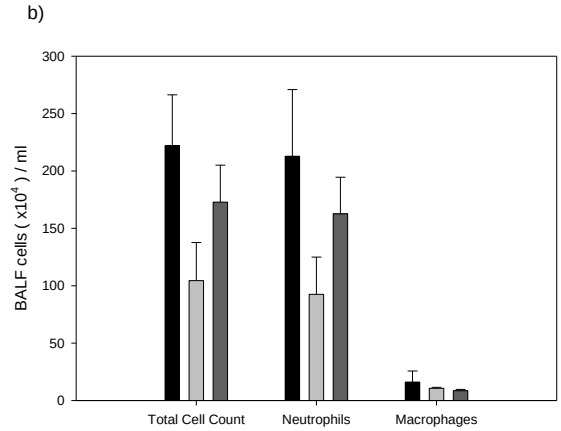


Figure S3. Physical stability of vitamin D3:DPPC liposomes (PD3) over time. Size and PDI of liposomes is shown. All data represent mean ± SEM (n=3).





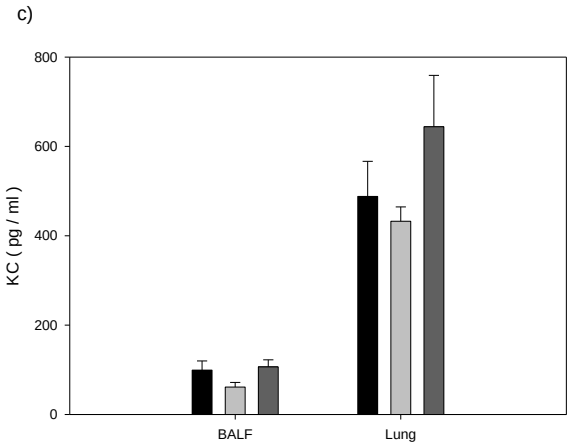


Figure S4. Mouse model infection pilot study. Ten week old C57BL/6N mice were intubated with air (control), empty, drug-free liposomes (P) or 25(OH)D-loaded liposomes (PD). After 24 h the mice were infected with *P. aeruginosa*; the number of viable bacteria in BALF and lung (a), the cell composition of the BALF (b) and the concentration of KC in BALF and lung (c) were analysed after a further 24 h. Data represent mean ± SEM (n = 4).

References

[1] Bivas-Benita, M., et al., Non-invasive pulmonary aerosol delivery in mice by the endotracheal route. Eur J Pharm Biopharm, (2005). 61(3): p. 214-8.