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# Phospholipid-modified poly(lactide-*co*-glycolide) microparticles for tuning the interaction with alveolar macrophages: *In vitro* and *in vivo* assessment

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ABSTRACT

Controlled drug delivery to the lungs is promising with plentiful advantages over current rapid release products. However, alveolar macrophage clearance has severely hindered the application of inhaled controlled release preparations. The objective of our study was to explore the feasibility to decorate poly(lactide-*co*-glycolide) (PLGA) microparticles with endogenous phospholipids found in the deep lungs, thus, to regulate the interplay with alveolar macrophages. The influence of the phospholipid amount and type on macrophage uptake of PLGA microparticles was investigated systemically under both *in vitro* (RAW264.7 and NR8383) and *in vivo* conditions. The uptake rate ($k$) by macrophages, *in vivo* elimination rate from the bronchoalveolar lavage fluid ($k'$) and elimination rate from the whole lung ($k''$) were used as parameters for evaluation. Our data showed that a modification with dipalmitoyl phosphatidylcholine (DPPC) enhanced the macrophage phagocytosis significantly over the unmodified counterparts. Thereafter, using the same modification ratio, remarkable enhancement of macrophage uptake was found in the presence of different types of other phospholipids, especially with distearoyl phosphatidylethanolamine (DSPE). When replaced by a poly(ethylene glycol)-conjugated version of DSPE the uptake of the modified PLGA microparticles was reduced by ~200%. Meanwhile, the drug content in the lung tissue was improved by 3-fold (area under the curve value). Finally, it was possible to establish a correlation between *in vitro* phagocytosis and *in vivo* lung elimination rate for the investigated formulations. Overall, our study demonstrated that phospholipids play an important role in modulating the clearance of microparticle-based drug delivery vehicles, which gives a meaningful insight into the development of prolonged drug release system for inhalation.

## 1. Introduction

In recent years, inhalation has gained great attention as a potential administration route for the local treatment of respiratory diseases, delivering an efficacious drug amount to the targeted area with minimized systemic exposure. In order to prolong drug residence in the lungs, to achieve an optimal therapeutic effect and to reduce dosing frequency, controlled release formulations are highly desirable [1]. However, the efficient clearance mechanisms found in the respiratory tract made the development of efficient controlled release preparations laborious [2]. As an example, alveolar macrophages represent the main defense barrier to deposited micron-scale foreign particles in the deep lungs. Certainly, phagocytosis/clearance of inhaled particles interferes with the concept of continuous drug release [3]. Thus, escaping macrophage phagocytosis was identified as a fundamental strategy in the design of sustained release preparations intended for lung delivery, with success achieved by designing large porous or swellable microparticles [4,5].

Once the inhaled particles are deposited in the deep lungs, they encounter a physiological environment mainly composed of phospholipids [6]. It is widely reported that the constituted “corona” (inhaled particles coated by lung lipid and protein) will determine the fate and action of the inhaled particles [7]. Study found that lipid modification enhanced the lung lipid adsorption of nanoparticles while PEGylated nanoparticles decreased the lung lipid adsorption and protein corona [8]. In this respect, we assume that the surface of polymer-based drug

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delivery vehicles once in contact with the lung lining layer is modified with endogenous phospholipids, which then could lead to an altered extent of macrophage uptake [9,10]. The inclusion of dipalmitoyl phosphatidylcholine (DPPC) in poly(lactide-*co*-glycolide) (PLGA) microspheres has been proposed to escape uptake by rat alveolar macrophages (NR8338 culture) [11,12]. In contrast to the results obtained from microparticles, an increased cellular uptake of phospholipid-coated nanoparticles (~200 nm in diameter) has been announced in murine alveolar macrophage culture (MH-S cell line) [13,14]. Accordingly, the effect of phospholipids on the interplay of polymer particles with alveolar macrophage under both *in vitro* and *in vivo* conditions seems to be largely unsettled.

The objective of our present study was to investigate the impact of phospholipid modifications on the interaction of polymer microparticles with macrophages. PLGA microparticles modified with various amounts of DPPC were prepared by a premix membrane emulsification/solvent evaporation method. Two other endogenous phospholipids (namely dipalmitoyl phosphatidylglycerol (DPPG) and distearoyl phosphatidylethanolamine (DSPE)) and a PEGylated version of DSPE were selected to elucidate the influence of the type of utilized phospholipid. The impact of the phospholipid amount and type on the physicochemical properties, *in vitro* drug release, *in vitro* macrophage uptake, *in vivo* lung retention and absorption of the PLGA microparticles was systemically investigated. Furthermore, a correlation between macrophage uptake *in vitro* and lung retention behavior *in vivo* was established for the diverse microparticle formulations.

## 2. Materials and methods

### 2.1. Materials

PLGA (Resomer® RG 503H; lactide:glycolide of 50:50; molecular weight of 28 kDa) was purchased from Boehringer Ingelheim (Germany). DPPC, DPPG, DSPE, and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-2000] ($PEG_{2000}$-DSPE) were obtained from A.V.T. Pharmaceutical Co., Ltd (Shanghai, China). Micronized budesonide (model drug) was acquired from Hubei Gedian Humanwell Pharmaceutical (China). Coumarin 6 (fluorescent dye) was purchased from Dalian Meilun Biotechnology Co., Ltd (Dalian, China). Lactohale® 200 was a gift from DFE Pharma (China). Poly(vinyl alcohol) (PVA 205) was donated by Kuraray Trading Co., Ltd. (Japan). Phosphate buffer solution (PBS) was purchased from Dalian Meilun Biotechnology Co., Ltd (Dalian, China). High performance liquid chromatography (HPLC)-grade methanol, dichloromethane and dimethyl sulfoxide were supplied by Shandong Yuwang Co., Ltd. (China). All other reagents, unless otherwise specified, were of analytical grade and used as received.

### 2.2. Preparation of phospholipid-modified PLGA microparticles

Phospholipid-modified PLGA microparticles were prepared by premix membrane emulsification/solvent evaporation [15]. Briefly, 66.7 mg budesonide or 6.0 mg coumarin 6 were dissolved in 3 ml of dichloromethane together with 600.0 mg of PLGA. A certain amount of phospholipid (DPPC:PLGA in 0.5:1, 1:1 and 2:1 of molar ratios; DPPG/DSPE/$PEG_{2000}$-DSPE:PLGA in 1:1 of molar ratio) was added to the mixture and the resulting oil phase was injected into 50 ml of aqueous phase containing 0.1% (w/v) of PVA under high speed homogenization (Ultra-Turrax® T25, IKA, Germany) for 40 s. This pre-emulsion was passed three times through a SPG membrane (SPG Technology Co, Ltd, Japan) with pore size of 5 μm. The final emulsion was subsequently added to 250 ml of aqueous phase containing 0.1% (w/v) PVA and stirred to evaporate the organic solvent (25 °C for 4 h). The obtained microparticles were collected by centrifugation at 2000 rpm for 5 min, washed with distilled water for three times and eventually freeze-dried (FDU-1100, Tokyo Rikakikai Co., Ltd., Japan).

### 2.3. Characterization of phospholipid-modified PLGA microparticles

#### 2.3.1. Particle size measurement

The geometric particle size (i.e. volumetric median diameter [$D_{v,50}$]) of the microparticles was measured by laser diffraction (Sympatec, HELOS/KP, Germany). The span value was calculated according to the following formula:

$$\text{Span} = \frac{D_{v,90} - D_{v,10}}{D_{v,50}}$$

#### 2.3.2. Theoretical mass median aerodynamic diameter ($MMAD_t$)

The theoretical mass median aerodynamic diameter ($MMAD_t$) of the formulations was determined according to the following equation:

$$MMAD_t = d(\rho/\rho_0\chi)^{1/2}$$

where $d$ is the geometric diameter which was obtained from the particle size measurement, ρ is equal to the tapped density (final volume of a sample of known weight after 500 times of tapping), $\rho_0$ is unit density and $\chi$ is the shape factor, which equals 1 for spheres [16].

#### 2.3.3. Encapsulation efficiency

The encapsulation efficiency of budesonide or coumarin 6 in the formulations was determined by the following procedure: 10.0 mg of sample were precisely weighed and dissolved in 1 ml of dichloromethane, which was then diluted to 50 ml with methanol. The mixture was centrifuged at 10,000 rpm for 10 min. For determination of budesonide concentration, the supernatant was subsequently subjected to HPLC analysis on a $C_{18}$-H column (150 mm × 4.6 mm, particle size: 5 μm) equipped with a pre-column at 40 °C. The detection wavelength was 248 nm and drug retention time was in the range of 5.1–5.2 min (mobile phase: water/methanol (28/72, v/v); flow rate: 1 ml/min). For quantification of coumarin 6, the fluorescence intensity of the supernatant was measured in a microplate reader (Model 680, Bio-Rad, USA) ($\lambda_{ex}$ = 466 nm, $\lambda_{em}$ = 504 nm). The encapsulation efficiency (EE) is defined as follows:

$$EE\% = (M_{\text{actual drug loaded}}/M_{\text{theoretical drug loaded}}) \times 100\%$$

### 2.4. In vitro cumulative release of budesonide and coumarin 6

To evaluate the *in vitro* release behavior of budesonide from the formulations, ~10.0 mg of budesonide-loaded microparticles were weighed and dispersed in 5 ml of phosphate buffer (10 mM, pH 7.3) containing 0.15% (w/v) of sodium dodecyl sulfate. The dispersion was constantly incubated in air-bath oscillator at 37 °C with a speed of 80 rpm. At predetermined time points, the release medium was analyzed for the drug content by HPLC. The difference in release profiles of the tested formulations was evaluated using a similarity factor ($f_2$), which was calculated according to the equation[17] found below:

$$f_2 = 50 \times \log([1 + (1/n)\sum_{t=1}^{n}(R_t - R'_t)^2]^{-0.5} \times 100)$$

where $n$ is the number of time points, $R_t$ and $R'_t$ are cumulative release amounts of two contrastive preparations at time $t$, respectively. A significant release distinction would be declared if $f_2$ is smaller than 50.

The procedure of *in vitro* release assay for coumarin 6 was similar to that of budesonide, except that the release medium was changed to Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% of fetal bovine serum (cell culture fluid) without added phenol red and the content was quantified by fluorescence spectroscopy as outlined above.

### 2.5. Cell viability assay

Viability of RAW264.7 cells treated with the phospholipid-modified

microparticles was evaluated using MTT assay as per manufacturer's instructions. RAW264.7 cells were seeded into a 96-well plate ($5 \times 10^3$ cells/well) and incubated with the tested formulations at predetermined concentrations for 48 h. MTT stock solution (5 mg/ml) was then added to each well and incubated for another 4 h. Thereafter, the media was replaced with 100 µl of DMSO to dissolve the formazan crystals and the absorbance was measured at 570 nm using a microplate reader. The cell viability was calculated according to the formula below:

$$\text{Cell viability\%} = (OD_{experiment} - OD_{blank})/(OD_{control} - OD_{blank}) \times 100\%$$

### 2.6. Macrophage uptake/binding studies

RAW264.7 macrophages (ATCC TIB-71, USA) or NR8383 macrophages (Shanghai Yubo Biological Technology Co., Ltd., China) were seeded in 6-well plates at a density of $5 \times 10^5$ cells/ml and incubated in DMEM supplemented with 10% of fetal bovine serum at 37 °C, 5% $CO_2$ for 24 h. Thereafter, the cells were washed with DMEM for three times and re-suspended with 2 ml of cell culture fluid. The prepared coumarin 6-loaded formulations were uniformly dispersed into the cell medium (~4 µg of coumarin 6 in total per formulation) and incubated with the macrophages at 37 °C or 4 °C. At specific time points, the medium was removed, the incubated cells were washed for three times with phosphate buffer and then collected. After centrifugation, the cells were redispersed into phosphate buffer saline supplemented with 1% of fetal bovine serum and 0.1% sodium azide. The cellular uptake or binding of particles to cells were evaluated by a Quanta SC flow cytometer (Beckman Coulter, USA), excluding non-phagocytosed particles by gating regions and quantifying fluorescence intensity within $Q_1$ and $Q_2$ regions. The uptake fluorescence intensity was plotted against the incubation time and the phagocytic kinetics was fitted to the first-order kinetics equation [18]:

$$\ln\left(1 - \frac{N(t)}{N(terminal)}\right) = -kt$$

where *N(t)* is the measured uptake fluorescent intensity at time *t*, *N (terminal)* is the maximum fluorescent value (after 48 h incubation) and *k* is the first-order velocity constant.

Images of cells were captured on an inverted microscope (ZEISS, Germany) after 24 h of incubation. The area of particles swallowed by RAW264.7 cells was calculated for each group by Image Pro Plus 6.0 software and then the mean area of intracellular particles was calculated by formula:

$$\text{Mean area of intracellular particles} = \text{Intracellular dark area/Cell count}$$

### 2.7. Animals

Male Sprague Dawley rats (~200 g) were purchased from the Laboratory Animal Center of Shenyang Pharmaceutical University. Rats were housed under the condition of temperature at 23 ± 3 °C with a relative humidity of 55 ± 10% and a 12 h light-dark cycle. All the procedures were approved by the ethics committee and Shenyang Pharmaceutical University for the care and use of laboratory animals.

### 2.8. In vivo lung retention and absorption studies

The residence behavior and tissue absorption of budesonide-loaded microparticles were analyzed after intra-tracheal powder insufflation to the airspace of rats as previously described [4]. Briefly, the formulations were administered utilizing a DP-4 (Dry Powder Insufflator™, Penn-Century, USA) after an intraperitoneally anesthetization procedure (4% (w/v) chloral hydrate solution at a dose of 7 ml/kg). At predetermined time points (immediately, 4, 12, 24 and 48 h), the animals were sacrificed by a cervical dislocation maneuver and the bronchoalveolar lavage fluid (BALF) was collected using 2 ml of dimethyl sulfoxide repeated for 3 times. The lungs were resected and homogenized in 1 ml PBS using a tissue grinder (IKA, Germany). 1 ml of the pooled BALF or tissue homogenate was mixed with 2 ml of methanol to precipitate proteins and extract the drug. All the mixtures were then centrifuged at 10,000 rpm for 10 min and the supernatants were analyzed by HPLC. The elimination rate constant of the formulations from the lungs was calculated according to the first-order dynamics equation of single-compartment model shown below [19]:

$$\ln A_t = -kt + \ln A_0$$

where $A_t$ is the measured drug content in the BALF or whole lung at time *t*, $A_0$ is the initial drug content in the BALF or whole lung at time 0 and *k* is the first-order elimination velocity constant. The pharmacokinetic parameters of the diverse formulations were obtained using the DAS2.0 software.

### 2.9. Statistical analysis

*In vitro* data are presented as mean ± standard deviation (SD) from at least three independent measurements. *In vivo* data are expressed as mean ± standard error of means (SEM) from at least four independent measurements. Statistical analysis was performed using Prism 5 (GraphPad Software, USA) and one-way ANOVA followed by Dunnett's test of mean values. A probability value of $p < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Characterization of phospholipid-modified PLGA microparticles

Budesonide (model drug) or coumarin 6 (fluorescent dye) was embedded into PLGA microparticles. As outlined in Table 1/Tab. S1, the phospholipid (DPPC as an example) modification ratio and phospholipid type (DPPC, DPPG, DSPE and $PEG_{2000}$-DSPE) had no significant effect on the particle size (~3 µm) and drug/fluorescent dye loading

**Table 1**
Physicochemical properties of budesonide-loaded phospholipid-modified PLGA microparticles prepared by premix membrane emulsification (PME)/solvent evaporation.

| Formulation | Homogenization speed [rpm] | Pore size for PME [µm] | Phospholipid:PLGA [mol/mol] | Particle size [µm] (span value) | EE [%] | Tap density [g/ml] | $MMAD_t$ [µm] |
|---|---|---|---|---|---|---|---|
| PLGA | 8000 | 5 | – | 2.9 ± 0.0 (2.0) | 85 ± 3 | 1.25 ± 0.04 | 3.2 ± 0.0 |
| DPPC-PLGA 0.5:1 | 8000 | 5 | 0.5:1 | 3.0 ± 0.1 (2.0) | 83 ± 3 | 1.15 ± 0.03 | 3.2 ± 0.1 |
| DPPC-PLGA 1:1 | 8000 | 5 | 1:1 | 2.6 ± 0.0 (1.9) | 84 ± 3 | 1.10 ± 0.06 | 2.8 ± 0.1 |
| DPPC-PLGA 2:1 | 8000 | 5 | 2:1 | 2.6 ± 0.1 (1.7) | 81 ± 3 | 1.07 ± 0.03 | 2.7 ± 0.1 |
| DPPG-PLGA 1:1 | 8000 | 5 | 1:1 | 2.8 ± 0.1 (2.2) | 82 ± 3 | 1.12 ± 0.05 | 3.0 ± 0.1 |
| DSPE-PLGA 1:1 | 8000 | 5 | 1:1 | 2.8 ± 0.1 (2.0) | 85 ± 3 | 1.11 ± 0.05 | 2.9 ± 0.1 |
| $PEG_{2000}$-DSPE-PLGA 1:1 | 8000 | 5 | 1:1 | 3.5 ± 0.1 (1.6) | 89 ± 4 | 1.22 ± 0.04 | 3.8 ± 0.0 |

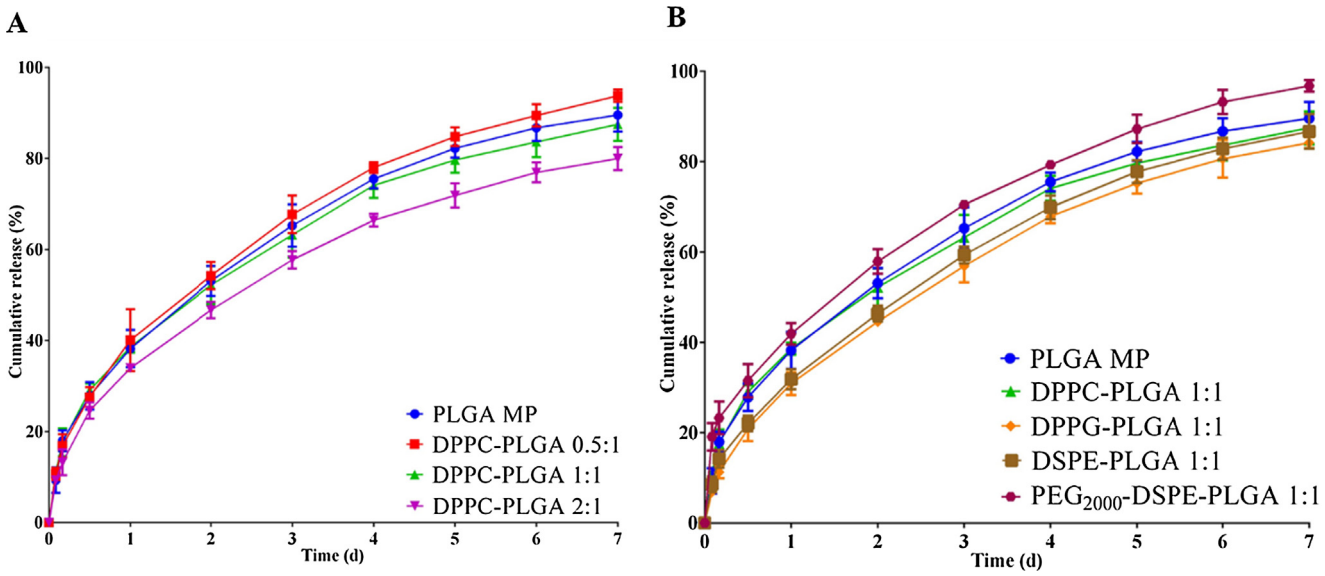


**Fig. 1.** *In vitro* drug release behavior of budesonide loaded microparticles modified with different amounts of DPPC (A) or different types of phospholipids (DPPC, DPPG, DSPE or PEG-DSPE) (B). Values are presented as the mean ± SD ($n = 3$).

(EE of > 80%). All formulations exhibited rational aerodynamic diameters (i.e. 2–5 μm) for deep lung deposition (Table 1).

### 3.2. In vitro release study

The *in vitro* budesonide release profiles from the diverse microparticle formulations are illustrated in Fig. 1. The release pattern was diffusion-dominated (Higuchi equation, $R^2 > 0.99$) [20] and lasted for up to 7 d throughout this study. For the influence of phospholipid modification ratio (Fig. 1A), the varying DPPC amounts did not alter the release profiles ($f_2 > 50$). For the influence of phospholipid type (Fig. 1B), the presence of DPPG and DSPE on the particle surface led to a comparable release kinetics with the DPPC modified system ($R^2 > 0.99$). No statistical difference in their release rates was found ($f_2 > 50$). Besides, the inclusion of PEG into the microparticles did not affect the drug release pattern ($R^2 > 0.99$), but its release rate was accelerated compared with the DSPE-modified formulation ($f_2 < 50$) (Fig. 1B) [21].

For coumarin 6 loaded microparticles, no relevant *in vitro* leakage of the fluorescent dye (coumarin 6) was detected from all the microparticle formulations (< 1.5% within 48 h) when tested in cell culture medium (Fig. S1). Thus, phospholipid modification ratio and phospholipid type (DPPC, DPPG, DSPE and $PEG_{2000}$-DSPE) had limited effect on drug release, and the cellular uptake of released/free coumarin 6 can be neglected.

### 3.3. Macrophage uptake study

Before the macrophage uptake study, *in vitro* cytotoxicity of the prepared formulations was determined using RAW264.7 cells. As shown in Fig. S2, all the microparticles displayed limited cytotoxicity to RAW264.7 at the concentration used in *in vitro* uptake assays (~0.2 mg/ml).

The macrophage uptake study was performed in both RAW264.7 cells (murine macrophage cell line) and NR8383 cells (cell line from rats). After incubation with the tested formulations, macrophages were collected at indicated time points and analyzed by flow cytometry (Fig. S3).

For RAW264.7 cells, the influence of DPPC ratio on macrophage phagocytosis was depicted in Fig. 2A. The cellular uptake of all the formulations increased along with time and the uptake saturation was observed at 48 h. Compared with the non-modified PLGA microparticles, all DPPC modified formulations enhanced the phagocytosis significantly irrespective of the molar ratio of DPPC/PLGA. This trend was further visualized by optical microscopy (Fig. 2E, Table 2). There was no relevant difference in macrophage uptake among the tested DPPC/PLGA ratios. Next, the uptake rate ($k$) was calculated for RAW264.7 cells (Table 3). The highest uptake rate was observed at a DPPC/PLGA ratio of 2:1 ($k = 0.12\,h^{-1}$, $R^2 = 0.97$). For a DPPC/PLGA ratio of 0.5:1 $k$ equaled to $0.09\,h^{-1}$ ($R^2 = 0.96$).

The influence of phospholipid type (DPPC, DPPG, DSPE and $PEG_{2000}$-DSPE) on RAW264.7 phagocytosis was depicted in Fig. 2B. A phospholipid/PLGA ratio of 1:1 was selected to fabricate microparticles with DPPC, DPPG, DSPE and $PEG_{2000}$-DSPE. Compared with the PLGA microparticles, DPPC, DPPG and DSPE promoted microparticle phagocytosis by RAW264.7 ($p < 0.05$) and the DSPE modification resulted in the fastest uptake by macrophages ($R^2 = 0.99$), followed by the DPPG ($R^2 = 0.96$) and DPPC group ($R^2 = 0.96$) (Table 3). On the contrary, the presence of $PEG_{2000}$-DSPE reduced the macrophage uptake significantly ($k = 0.05\,h^{-1}$, $R^2 = 0.99$), compared with both PLGA microparticles (0.85-fold) and the DSPE-PLGA group (0.65-fold) (Fig. 2B and Table 3). These tendencies of phagocytosis could be confirmed by optical microscopy which underlined the results from flow cytometry (Fig. 2E, Table. 2).

For NR8383 cells, the influence of DPPC ratio on NR8383 phagocytosis was shown in Fig. 3A. Compared with the PLGA microparticle group, a significantly enhanced uptake was observed for the DPPC/PLGA 1:1 and 2:1 groups ($p < 0.05$) after 24 h. In addition, a statistically significant difference was observed between the DPPC-PLGA 2:1 and 1:1 group ($p < 0.05$), indicating a DPPC ratio-dependent uptake behavior for the NR8383 macrophages. The influence of phospholipid type (DPPC, DPPG, DSPE and $PEG_{2000}$-DSPE) on NR8383 phagocytosis was shown in Fig. 3B. Similarly, all microparticle based on pulmonary endogenous phospholipids modification performed an accelerated uptake behavior. And the evasion of macrophage uptake was also confirmed in NR8383 cell line for $PEG_{2000}$-DSPE modified microparticles.

### 3.4. Macrophage binding study

To investigate the phospholipids-mediated mechanism of enhanced macrophage phagocytosis, cellular binding assay was conducted using RAW264.7 macrophages at 4 °C to depress the energy-dependent internalization [22]. As shown in Fig. 2C and D, it was found that the mean fluorescence intensity for binding was only around 10% of that at

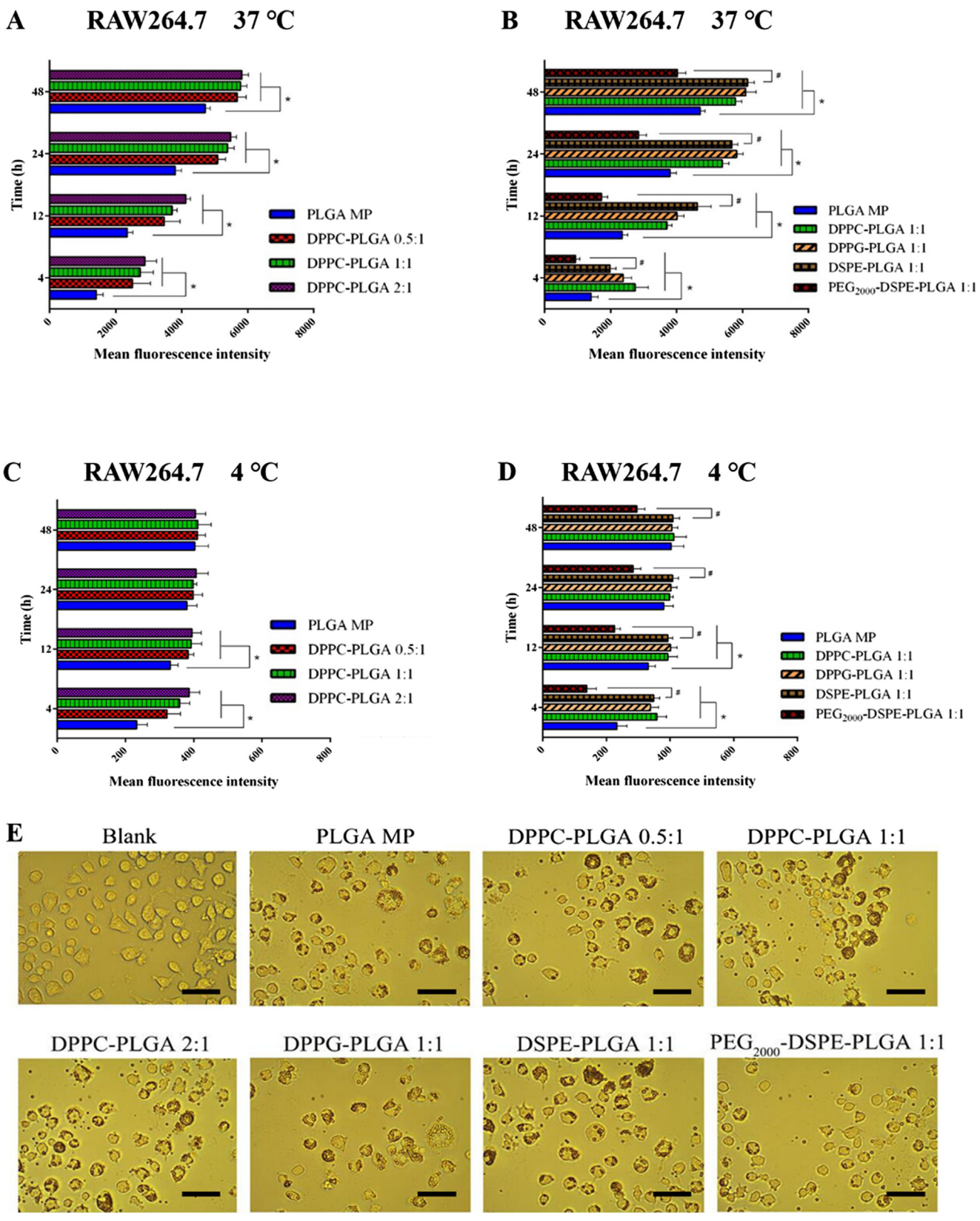


**Fig. 2.** Mean fluorescence intensity in RAW264.7 cell line as quantified by flow cytometry after 4, 12, 24 and 48 h of incubation at 37 °C (A and B) and 4 °C (C and D) for PLGA microparticles modified with different amounts and types of phospholipids. Data are shown as mean ± SD ($n = 3$). Comparison is made with the PLGA microparticle group (*$p < 0.05$) and DSPE-modified PLGA microparticle group (#$p < 0.05$). Images of RAW264.7 cells cultivated with coumarin 6-loaded formulations after 24 h at 37 °C (scale bar = 50 μm) are presented in (E) (with enhanced contrasting).

**Table 2**
The semi-quantitative statistics of intracellular particles in photomicrograph by Image Pro Plus 6.0.

| Formulation | Mean area of intracellular particles |
|---|---|
| PLGA | 6.61 ± 0.29 |
| DPPC-PLGA 0.5:1 | 7.59 ± 0.32* |
| DPPC-PLGA 1:1 | 8.40 ± 1.15* |
| DPPC-PLGA 2:1 | 8.21 ± 1.04* |
| DPPG-PLGA 1:1 | 9.59 ± 1.64* |
| DSPE-PLGA 1:1 | 8.11 ± 0.98* |
| $PEG_{2000}$-DSPE-PLGA 1:1 | 4.42 ± 0.23* |

Values are presented as the mean ± SD ($n = 3$). *$p < 0.05$ compared to the PLGA group.

**Table 3**
The uptake rate constant of the diverse formulations by RAW264.7 macrophages ($k$), *in vivo* elimination rate from the BALF ($k'$) and elimination rate from the whole lung ($k''$) of lung-delivered formulations.

| Formulation | $k/h^{-1}$ | $k'/h^{-1}$ | $K''/h^{-1}$ |
|---|---|---|---|
| PLGA | 0.07 | 0.04 | 0.04 |
| DPPC-PLGA 0.5:1 | 0.09 | – | – |
| DPPC-PLGA 1:1 | 0.11 | 0.10 | 0.10 |
| DPPC-PLGA 2:1 | 0.12 | 0.19 | 0.07 |
| DPPG-PLGA 1:1 | 0.13 | 0.21 | 0.07 |
| DSPE-PLGA 1:1 | 0.15 | 0.23 | 0.10 |
| $PEG_{2000}$-DSPE-PLGA 1:1 | 0.05 | 0.03 | 0.03 |

37 °C, implying the phagocytosis was energy dependent.

The influence of DPPC ratio on cellular binding was shown in Fig. 2C. Significantly enhanced macrophage binding was found for DPPC modified microparticles within 12 h compared with PLGA MP group ($p < 0.05$), and no statistical difference among the different groups was found at 24 and 48 h (Fig. 2C). In addition, no statistical difference in cellular binding capacities among the DPPC modified microparticles with varying ratios (from 0.5:1 to 2:1) was found ($p > 0.05$).

The influence of phospholipid type (DPPC, DPPG, DSPE and $PEG_{2000}$-DSPE) on cellular binding was shown in Fig. 2D. The binding of lung endogenous phospholipids (DPPC, DPPG, DSPE) modified particles to macrophages was significantly larger than that of non-modified microparticles within 12 h ($p < 0.05$), which was in line with those observed in the uptake study (Fig. 2B). However, no significant variation in binding was found between them ($p > 0.05$). Contrary to the behaviors of other groups in binding study, microparticles modified with $PEG_{2000}$-DSPE exhibited an obviously poor cellular binding until 48 h of incubation ($p < 0.05$) (Fig. 2D), which was a convincing explanation for reduced macrophage uptake.

### 3.5. *In vivo* lung retention and absorption study

The lung retention behavior of encapsulated budesonide was tested after administration of respective formulations to rats. The control, a physical mixture of budesonide and lactose showed a rapid clearance from the lungs, with no measurable drug content in the BALF after 4 h. By contrast, all formulations presented prolonged residence times ($p < 0.05$) (Figs. 4A and 5A) due to their sustained drug release properties.

Compared with the non-modified PLGA microparticles, the presence of DPPC accelerated macrophage clearance, indicated by the lower drug content in BALF (Fig. 4A). It was noticed that higher amount of DPPC (DPPC/PLGA 2:1) significantly reduced drug retention (area under the curve (AUC)) in the BALF ($p < 0.05$) (Fig. 4A and Table 4).

The drug content found in the lung tissue homogenate corresponds to the portion of drug being released/absorbed, which is associated with the permeation of deposited particles through lung surfactant layer as well as intra-macrophage capture. As shown in Fig. 4B, in the physical mixture (budesonide/lactose blend) group, the budesonide content reached its peak at 4 h and declined rapidly thereafter. Compared with the PLGA microparticles, the DPPC/PLGA (molar ratio of 1:1) group did not display a significant difference of drug content in lung tissues ($p > 0.05$). The calculated AUC values indicated that upon an elevation of the DPPC/PLGA ratio to 2:1, the drug content in the lung tissue increased remarkably (Table 4), which can be attributed to the enhanced phagocytosis by alveolar macrophages.

As presented in Fig. 5A and Table 4, the remaining drug levels for the DPPG- and DSPE-modified microparticles in the BALF were significantly lower than those of the non-modified PLGA microparticle group ($p < 0.05$). The phospholipid type had a great effect on the retention behaviors of modified microparticles, with lower retention observed for the DSPE group compared with that of the DPPG group ($p < 0.05$). Although the DPPC-modified microparticles displayed the most pronounced lung retention among the phospholipids investigated, they also showed the lowest tendency for accumulation in the lung tissue ($p < 0.05$). DPPG had the largest effect on the lung tissue absorption (with AUC 1.2 and 2.8 fold higher than that of DSPE, and DPPC based groups respectively).

Notably, the PEG modification of the PLGA microparticles improved their residence time in the respiratory lining layer compared with the non-modified and DSPE-modified PLGA microparticles ($p < 0.05$) (Fig. 5A and Table 4), with an increase of the AUC value by 135 and

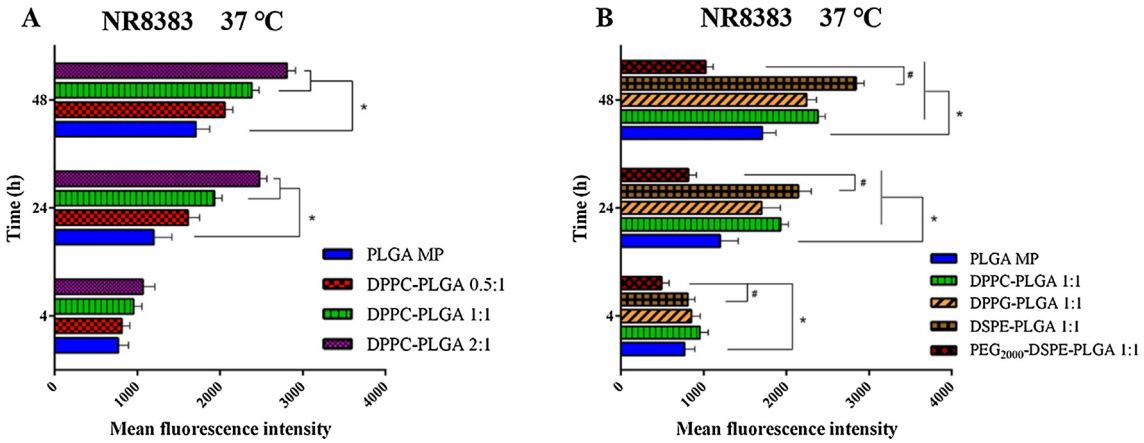


**Fig. 3.** Mean fluorescence intensity quantified by flow cytometry in NR8383 cell line after 4, 24 and 48 h of incubation at 37 °C with PLGA microparticles modified with different amounts of DPPC (A) or different types of phospholipids (DPPC, DPPG, DSPE or PEG-DSPE) (B). Data are shown as mean ± SD ($n = 3$). Comparison is made with the PLGA microparticle group (*$p < 0.05$) and DSPE-modified PLGA microparticle group (#$p < 0.05$).

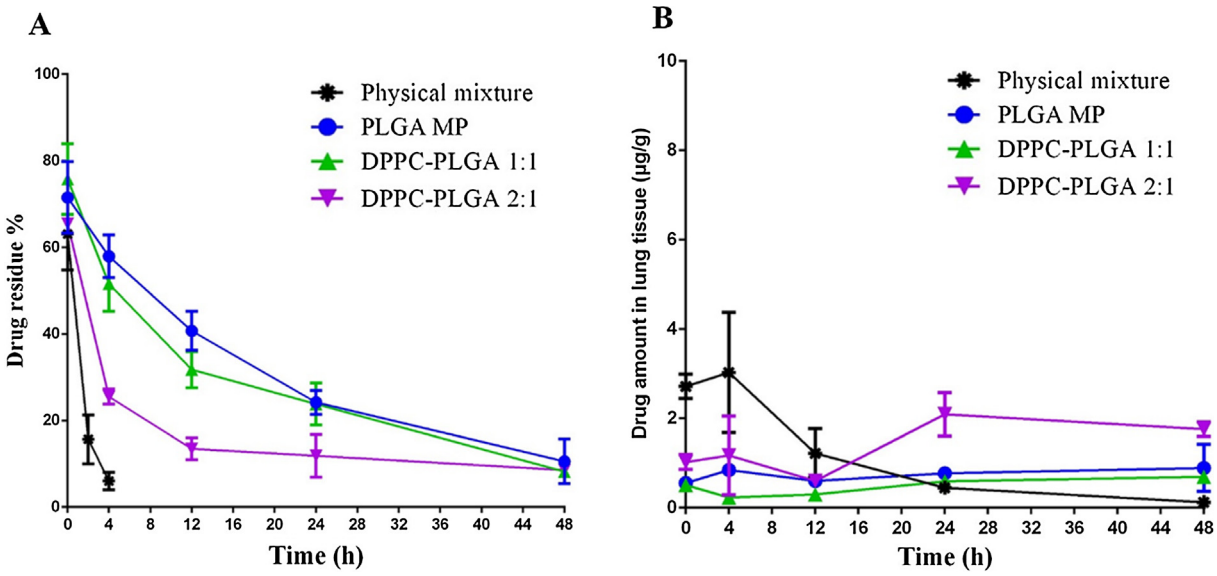


**Fig. 4.** Impact of the utilized DPPC amount on the *in vivo* drug content in the BALF (A) and absorbed drug amount found in the lung tissue (B) following lung delivery of budesonide-loaded PLGA microparticles. Values are presented as the mean ± SEM ($n = 4$).

**Table 4**
Retention (given as area under the curve (AUC)) of budesonide in the BALF and lung tissue as a function of the administered formulation.

| Formulation | AUC (0–48 h) [µg*h] in the BALF | AUC (0–48 h) [µg*h] in rat lung tissues |
|---|---|---|
| Physical mixture | 320 ± 12 | 45 ± 5 |
| PLGA | 1913 ± 39* | 37 ± 3 |
| DPPC-PLGA 1:1 | 1741 ± 28* | 24 ± 3* |
| DPPC-PLGA 2:1 | 1266 ± 42*#& | 74 ± 5*#& |
| DPPG-PLGA 1:1 | 1015 ± 20*#& | 69 ± 4*#& |
| DSPE-PLGA 1:1 | 854 ± 32*#& | 57 ± 4*#& |
| $PEG_{2000}$-DSPE-PLGA 1:1 | 2593 ± 32*#& | 167 ± 3*#& |

Values are presented as the mean ± SEM ($n = 4$). AUC comparison: *$p < 0.05$ compared to the physical mixture group, #$p < 0.05$ compared to PLGA MP group; &$p < 0.05$ compared to DPPC-PLGA 1:1 group.

209%, respectively. Moreover, in addition to the prolonged lung retention, $PEG_{2000}$-DSPE modification also enhanced drug absorption into the lung tissue, with a 4.5 and 3.0-fold increase of AUC over the unmodified and DSPE-modified group, respectively (Table 4). Accordingly, the effect of PEG moiety on the particle surface was displayed by the slowest macrophage uptake and lung elimination rate.

Based on the above findings, the macrophage phagocytosis *in vitro* was compared with the *in vivo* lung elimination rate ($k'$ in BALF and $k''$ in whole lungs). For DPPC based formulations, consistent with its faster phagocytosis, a higher elimination rate from the lung cavity was found for DPPC-PLGA 2:1 group, compared with that of DPPC-PLGA 1:1 ($k'$ 0.193 $h^{-1}$ versus 0.097 $h^{-1}$) (Table 3). However, with larger extent of absorption reflected by the higher AUC value in Table 4, the $k''$ of DPPC-PLGA 2:1 group was smaller than that of DPPC-PLGA 1:1, indicating that the absorption step could reduce drug elimination rate within the whole lung.

For phospholipid type, the microparticles showing the fastest macrophage uptake rate *in vitro* underwent the fastest lung elimination (i.e.,

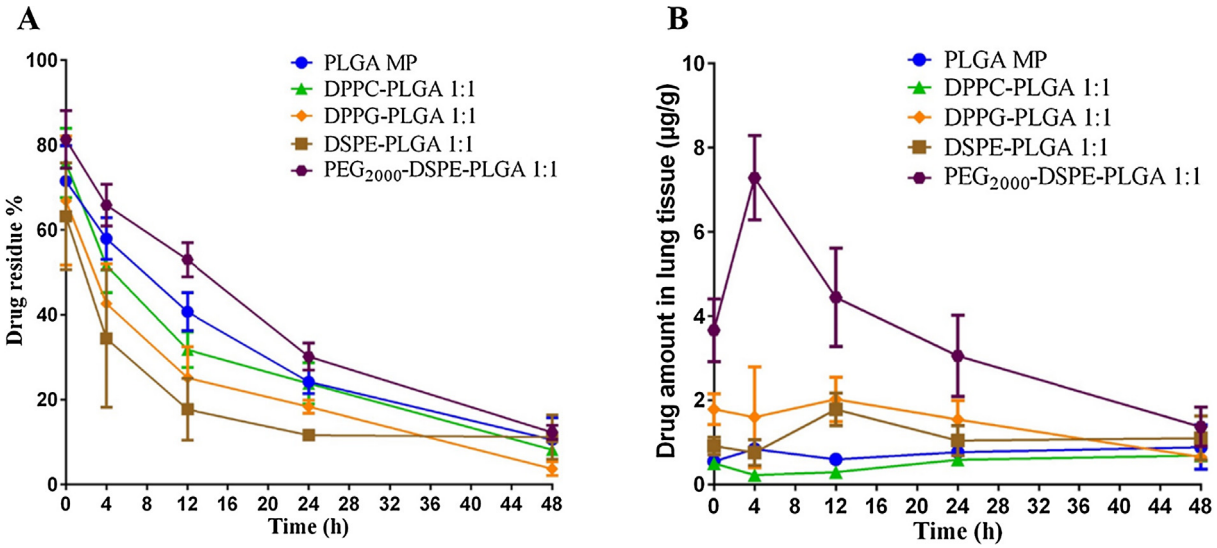


**Fig. 5.** Impact of the utilized phospholipid on the *in vivo* drug content in the BALF (A) and absorbed drug amount found in the lung tissue (B) following lung delivery of budesonide-loaded PLGA microparticles. Values are presented as the mean ± SEM ($n = 4$).

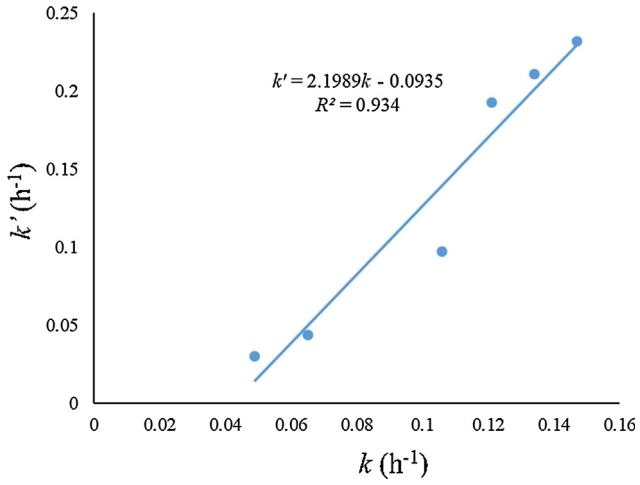


**Fig. 6.** Linear fitting between macrophage uptake rate ($k$) and elimination rate in BALF ($k'$).

DSPE modification). The variation of elimination constant in the whole lung ($k''$) was also a function of the phospholipids modification. A modification with DPPG had the highest AUC in the lung tissues (Table 4) and thus, exhibited the most steady elimination velocity from the whole lung among the tested phospholipids (Table 3).

Overall, good correlation between uptake rate ($k$) and elimination rate in the BALF ($k'$) was observed with excellent quantitative fitting (Fig. 6), which may potentially predict the effects of inhaled formulations on *in vivo* retention behaviors from *in vitro* uptake experiments.

## 4. Discussion

Due to the efficient physiological defense mechanisms of the lungs, e.g. alveolar macrophage clearance in the respiratory zone, inhaled formulations undergo a limited-time deposition at the targeted site, incapable of maintaining continuous drug release and providing desired therapeutic effects. In this study, the feasibility of using pharmaceutics to regulate microparticle-macrophage interaction was demonstrated both *in vitro* and *in vivo*, based on partially mimicking lung microenvironment (phospholipid in lung surfactant fluid). The results showed enhanced macrophage uptake by endogenous phospholipid modification in ratio-dependent and type-dependent manner, as well as reduced macrophage clearance by PEGylated phospholipid decoration. The potential mechanism under which the investigated particles interacted with macrophages was investigated to some extent, and a correlation between macrophage phagocytosis *in vitro* and retention behavior of these formulations in the lung of rats was proposed.

It has been demonstrated that physicochemical properties of particles, such as particle size and surface chemistry, can influence phagocytosis [23]. Studies have been conducted and shown that microspheres with diameters between 2 and 5 μm were phagocytosed more readily [24]. In order to eliminate the interference of particle size on cellular uptake and emphasize the role of phospholipids played in macrophage phagocytosis, all the prepared microparticles were controlled in size of ~3 μm with good size distribution by O/W emulsion-solvent evaporation combining with premix membrane emulsification (PME) technique.

As a hydrophobic fluorescent probe, coumarin 6 is usually encapsulated into particulate dosage form to study the cell uptake and transport mechanism of the drug delivery system [25]. Accordingly, fluorescent PLGA microparticles were fabricated with a desirable diameter and slow-release profile (< 1.5% cumulative release within 48 h making a negligible influence from passive diffusion of the fluorophore on cellular uptake), providing a good integrity to investigate macrophage phagocytosis. Flow cytometry analysis is generally developed to quantify the phagocytosis of fluorescent particles on a single cell at high speed. The tested cells can be distinguished from residual non-phagocytosed particulates (with small size and high fluorescence intensity) [26]. RAW264.7 is a commonly used macrophage model, and it has been reported that there are many similarities between RAW264.7 and NR8383 in terms of phagocytic behavior [27]. However, in order to be more rigorous, a supplementary uptake experiment using NR8383 is necessary. In accordance to what was found with RAW264.7 incubation, NR8383 macrophages exhibited an equivalent uptake trend on the tested preparations, which confirmed our findings.

In consideration that macrophage phagocytosis is a continuous process, the comparison of phagocytic fluorescence at a single time point is very limited and unreasonable. Thus, the phagocytic rate constant derived from phagocytic fluorescent signal versus time is proposed here to better describe the overall macrophage phagocytic behavior.

It is generally considered that the uptake mechanism of particles with diameter of > 1 μm is classified as phagocytosis, in which two distinctive steps, attachment and internalization, can be defined [28]. Particles must be attached to the cells before engulfed by the plasma membrane. To explore the uptake promotion of phospholipid, the two steps of phagocytosis were separately assessed. Internalization, which is energy-dependent due to the mobilization of actin cytoskeleton, was suppressed under a low incubation temperature (4 °C) so that the fluorescence intensity detected was mainly originated from the particles binding to incubated cells. An explanation of the accelerated macrophage uptake to phospholipid-modified particles is that the fusion effect between phospholipid and cell plasma-membrane promotes the binding course [29], while the uptake reduction found in $PEG_{2000}$-DSPE decoration is attributed to the steric hindrance of PEG [30]. Overall, the attachment step is a prerequisite to the subsequent internalization and determines the phagocytic outcomes.

It was reported previously that the incorporation of different amounts of DPPC in the powder could greatly affect its aerosol performance [31]. However, after deposition, the effect of DPPC content on the interplay of inhaled particles to endogenous lung fluid or cells has not been investigated. In this study, the different degrees of DPPC localization at the particle surface by varying DPPC/PLGA molar ratio was found to generate distinctive fusion capacities with cell membrane. With the increasing of DPPC modification, the membrane fusion effect was enhanced, resulting in (DPPC) amount-dependent uptake manner.

Relating to the microenvironment of respiratory zone, the effect of phospholipid category on macrophage uptake was explored using three kinds of endogenous phospholipids in lung surfactant components, including DPPC, DPPG and DSPE. Based on the calculated $k$ values, the types of phospholipid used have exerted an obvious influence on the macrophage uptake. However, this is not owing to the membrane fusion concerning that no distinction in binding result was observed. Considering the cell membrane constitution [32], the characteristics of phospholipid may be the cause of this phenomenon. It is widely reported that phospholipids in biological membranes are distributed unsymmetrically, with phosphatidylethanolamine (PE) and phosphatidylserine (PS) usually residing in the inner leaflet. Similar to the rolling-over effect of PS phospholipid on accelerating macrophage clearance [33], the presence of DSPE phospholipid on the surface of particles has expedited the uptake rate, resulting in the largest $k$ value among the three phospholipids.

Different from the outcomes of phospholipids, PEGylated phospholipid ($PEG_{2000}$-DSPE) has displayed a superior macrophage uptake escape on account of less attachment priority. The PEGylated molecules are prone to be attached with the hydrophobic PLGA chains through hydrophobic alkyl anchor, leaving PEG head groups protruding into surrounding medium [34]. By formation of hydration shell, the stereo-hindrance effect of PEG prevents the contact of modified particles with cells. In this manner, PEGylated phospholipid can protect PLGA microparticles from binding/engulfing by macrophages, which is a

potential strategy to be further developed for sustained lung delivery.

The effect of the modification by phospholipid with varying ratios or types was not only revealed in macrophage uptake, but also in the *in vivo* lung retention. The hypothesis that pulmonary retention *vs*. clearance of inhaled particles is controlled by drug dissolution as well as phagocytosis-dependent physiological mechanism has been proved recently [35], which is in accordance with our findings. In this study, with identical drug dissolution profiles, the residence property of prepared formulations in the lung is mainly associated with macrophage uptake. When modified with different amounts of DPPC, inhaled particles in a higher modification ratio (DPPC-PLGA 2:1) had less retention time in the lung, most likely due to the stronger macrophage clearance. With the three phospholipids decorated, DSPE or DPPG based microparticles showed lower lung retention than that of DPPC group, which was similarly induced by an excessive uptake by macrophages. Whereas for PEGylated phospholipid, an extended lung residence was achieved in relation to the macrophage phagocytic evasion effect. To further illustrate the underlying relevance, elimination rate in the BALF ($k'$) or the whole lung ($k''$) were compared with the uptake rate ($k$) among the formulations, and a correlation between cellular uptake and *in vivo* pulmonary retention was profoundly established. Overall, phospholipid amounts or species on the surface of inhaled particles has an influence on the retention behaviors in the lungs by a macrophage uptake-dependent mechanism. From this point of view, the endogenous phospholipid can act like a modulator to adjust the host clearance of inhaled preparations.

The interactions of inhaled particles with lung surfactant (LS) has been recognized as a practical tool in programming retention time and bioavailability of poorly soluble pharmaceutics [36]. LS is commonly known as a mixture of surface-active components and the primary barrier of contact between pulmonary zone and inhaled foreigners, playing a vital physicochemical role in the non-ciliated bronchial tree [37]. It has been acknowledged that solid microspheres with diameter smaller than 5 μm can penetrate to peripheral airways (LS-abundant alveolar region of respiratory system) via inhalation [38], which is the premise for our prepared formulations to interact with LS. Multiple surveys based on Langmuir trough experiments have demonstrated the respiratory function of endogenous phospholipid relating to pulmonary mass transfer from interfacial region to sub-phase [39]. Recent studies reported that LS could be the basis of ideal decorative materials to vehiculize hydrophobic drugs along the respiratory surface [40,41]. Therefore, surface-decoration of PLGA microparticles by LS component (phospholipid) may be beneficial for effective pulmonary entry after inhalation, appreciable particle immersion, and even thorough touch to the beneath cellular surface. This hypothesis has been demonstrated with a higher DPPC/PLGA molar ratio (2:1) possessing larger AUC (drug content in lung tissues versus time), where DPPC-PLGA 1:1 could not achieve the desired effect. Nevertheless, advantageous over DPPC-PLGA 1:1, DPPG and DSPE (1:1) was able to improve the bioavailability of drugs in lung tissues, indicating the impact of phospholipid type on the interaction of particles with LS.

Previous studies have shown that inhaled micelles composed of PEG can not only prolong pulmonary retention of incorporated drugs, but also efficiently translocate them across the air-blood barrier [42]. In accordance with this statement, the absorption of $PEG_{2000}$-DSPE modified formulation by lung tissues was larger than the other tested groups, implying a good biocompatibility with LS and alveolar epithelium. It is due to the evasive physiological clearance mechanism and trended LS penetration that PEGylated phospholipid modification has accomplished sustained lung retention. Consequently, prolonged retention in pulmonary cavity and increased absorption by lung tissues have altogether led to improved whole-lung conservation.

## 5. Conclusions

In this study, the feasibility of using phospholipid modification strategy to regulate the macrophage phagocytosis on lung-delivered microparticles has been systemically investigated under both *in vitro* and *in vivo* conditions. It was found that phospholipids do play an important role as modulators in the clearance of particles deposited in the deep lungs, which is both phospholipid ratio and type dependent. Among the phospholipids investigated (DPPC, DPPG and DSPE), when studied at the same molar ratio, DSPE is the most effective in enhancing macrophage uptake. After conjugation with PEG, $PEG_{2000}$-DSPE modified microparticles reduced the uptake of macrophages by 209% compared to DSPE modification with diminishing membrane fusional effect, and the drug content in lung tissues was also improved by 3-fold. It is proved that the behaviors of inhalable microspheres on promoting/escaping macrophage phagocytosis have actually influenced their *in vivo* retentions and absorptions, which should be given more consideration for further design and application of inhalable sustained-release formulations. Taken together, our study demonstrated the feasibility of using phospholipid modification to manipulate the microparticles interaction with macrophages, as well as resultant drug absorption in the lung tissue.

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejpb.2019.08.017.

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