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Exploring polyvinylpyrrolidone in the engineering of large porous PLGA microparticles via single emulsion method with tunable sustained release in the lung: In vitro and in vivo characterization

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ABSTRACT

Sustained pulmonary drug delivery is regarded as an effective strategy for local treatment of chronic lung diseases. Despite of the progress made so far, there remains a need for respirable drug loaded porous microparticles, where porosity of the microparticles can be readily engineered during the preparation process, with tunable sustained drug release upon lung deposition. In this work, polyvinyl pyrrolidone (PVP) was used as a novel porogen to engineer PLGA-based large porous particles (LPPs) using single emulsion method, with fine tuning of the porosity, sustained drug release both in vitro and in vivo. Using cinaciguat as the model drug, influence of PVP content and PLGA type on the properties of the LPPs was characterized, including geometric particle size, drug encapsulation efficiency, tap density, theoretical and experimental aerodynamic particle size, specific surface area, morphology, and in vitro drug release. Solid state of cinaciguat in the LPPs was studied based on DSC and X-ray analysis. LPPs retention in the rat lung was carried out using bronchoalveolar lavage fluid method. Raw 264.7 macrophage cells were used for LPPs uptake study. Pharmacodynamic study was performed in mini-pigs in a well-established model of pulmonary arterial hypertension (thromboxane challenge). It was demonstrated that porosity of the LPPs is tunable via porogen content variation. Cinaciguat can be released from the LPP in a controlled manner for over 168 h. Significant reduction of macrophage phagocytosis was presented for LPPs. Furthermore, LPPs was found to have extended retention time (~36 h) in the rat lung and accordingly, sustained pharmacodynamics effect was achieved in mini-pig model. Taken together, our results demonstrated that this simple PLGA based LPPs engineering using single emulsion method with PVP as porogen may find extensive application for the pulmonary delivery of hydrophobic drugs to realize tunable sustained effect with good safety profile.

1. Introduction

In recent years respiratory drug delivery has drawn great attention. Characterized by large surface area, high vascularization and thin blood–alveolar barrier, drug delivery by pulmonary route has many outstanding features over oral or parenteral routes, including a targeted local lung action, very thin diffusion path to the blood stream and rich vasculature, rapid onset of therapeutic action, relatively low metabolic activity, circumvention of first-pass hepatic metabolism and fewer systemic side effects [1–4]. This route can be utilized for both local and systemic treatment, and it is extremely suitable for the treatment of local lung diseases such as asthma, cystic fibrosis, pulmonary arterial hypertension, chronic obstructive pulmonary disease, lung cancers and lung metastases. However, current available inhalation products in the market are mainly rapid release formulations. An obvious shortcoming concerning the present products is that drug concentration peaks immediately after application and declines promptly, which is the main cause for unpleasant adverse effects at the onset and inadequate therapy subsequently. In contrast, drug release rate modulation provides plentiful advantages over traditional preparations, such as less frequency of drug administration, improved patient compliance and releasing the drug in the physiological circumstance for longer time period with improved therapeutic efficacy [5,6]. However, mucociliary clearance and uptake by macrophages of the inhaled particles are the major challenges for developing controlled release formulations despite of their well-controlled in vitro release behavior [7,8].

So far, numerous efforts have been made to develop pulmonary controlled release delivery systems via particle engineering, including large

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porous microparticles (LPPs), swellable microparticles and nanoparticle aggregates [9–11]. Among them, the principle of LPPs is the most promising one. Characterized by large geometric diameter and low bulk density, LPPs decreased alveolar phagocytosis and tendency of particle aggregation, leading to higher aerosolization efficiency [9,12]. LPPs based on biocompatible and biodegradable poly (lactide-*co*-glycolide) acid (PLGA), have been suggested as potential sustained-release carriers for pulmonary drug delivery. Since PLGA particles prepared by the single emulsion solvent (o/w) method have been reported as being naturally less porous compared to those made by double emulsion (w/o/w) method [9], the LPPs were mainly produced via double emulsion methods and most of the therapeutic agents loaded into inhalable PLGA-LPPs were macromolecular drugs [9,13–16]. Different pore-forming agents (porogens) have been used to introduce desired porosity into the particles, including osmotic agents such as sodium chloride [17], albumins [18], cyclodextrin derivatives [13], effervescent agents such as ammonium bicarbonate [19], immiscible oils such as canola or silicon oil [20]. Porogen residue related safety issue, low drug loading and complicated processing are the main shortcomings for the above mentioned porogens. Until now, fewer reports on the manufacture of LPPs by a single emulsion method are available. In a single reference the preparation of PLGA-LPPs for pulmonary drug delivery using Pluronics® F68/F127 or their mixture was reported [21]. However, the particles were prepared in a first step and the active agents were then adsorbed/immobilized onto the particles in a second step, which is far different from the conventional single emulsion method.

Therefore, despite of the progress made so far, there remains a need for respirable drug loaded porous microparticles, where porosity of the microparticles can be readily adjusted and controlled during the preparation process, and being sustainable to release the pharmaceutically active agent during a predefined time period. In particular there are yet no satisfactory solutions for a single emulsion-solvent evaporation/extraction based method using extractable porogens, which do not have to be separated in a subsequent step and which do not leave potentially harmful residues in the resulting microparticles. Furthermore there are so far no satisfactory solutions for the preparation of LPPs for pulmonary drug delivery encapsulating hydrophobic small molecule drugs, especially if these cannot be formulated through the established double emulsion based protocols. Therefore, it is highly desirable to search for novel porogens applicable to LPPs system with good safety profile, fine tuning of porosity, sustained release and simple preparation process.

Thus, the objective of the present study is to develop a simple and efficient one-pot process for the preparation of porous microparticles with favorable aerodynamic properties, high drug encapsulation efficiency, flexibility in adjusting drug release rate via porosity tuning and enhanced sustained release in vivo. Here, cinaciguat (Cina), a hydrophobic small molecular drug, was selected as a drug model. It is a novel nitric oxide (NO)-independent activator of soluble guanylate, with poor water solubility, and can be used for the therapy of pulmonary arterial hypertension and chronic thromboembolic pulmonary hypertension [22,23]. In this paper, the feasibility of using PVP as porogen to prepare PLGA based LPPs by single emulsion method was studied, and properties of the LPPs were characterized systemically, including particle size, drug encapsulation, porous structure, solid state, in vitro release behavior, in vitro aerodynamic properties, in vivo residence time, macrophage phagocytosis, in vivo pharmacodynamics and histological analysis. To the best of our knowledge, this is the first report of PLGA-LPPs prepared using PVP as an extractable porogen combined with single emulsion method for sustained pulmonary drug delivery.

2. Materials and methods

2.1. Materials

Poly (D, L-lactide-*co*-glycolide) (PLGA) (Resomer® RG 502,502H, 503 and 503H; lactic acid:glycolic acid, 50:50; inherent viscosity 0.16–0.24 dL/g for 502 and 502H; 0.32–0.44 dL/g for 503 and 503H) were purchased from Boehringer Ingelheim (Germany). Cinaciguat (BAY 58-2667) was provided by Bayer Healthcare (Bayer Pharma AG, Wuppertal, Germany). Polyvinyl alcohol (PVA 205, 86.5–89 mol% hydrolyzed), polyvinyl pyrrolidone (PVP-k12) and Pluronic F127 were donated by Kuraray Trading Co., Ltd. (Japan), International Specialty Products Inc. (USA) and BASF in China respectively. Sodium Dodecyl Sulfate (SDS) was donated by BASF China. Dulbecco's modified eagle medium (DMEM, Gibco™) and RIPA lysis buffer were purchased from Thermo Fisher Scientific and Beyotime Biotechnology in China respectively. HPLC grade acetonitrile, dichloromethane (DCM) and 1-Methyl-2-pyrrolidon (NMP) were procured from Shandong Yuwang Co., Ltd. (China). All other reagents, unless otherwise specified, were of analytical grade.

31.5/600 = 0.05, 5% drug

2.2. Preparation of cinaciguat loaded PLGA-LPPs

Cinaciguat loaded PLGA large porous particles (Cina PLGA-LPPs) were prepared by a modified single emulsion (o/w) solvent evaporation method as described previously [24]. Briefly, 31.5 mg cinaciguat were dissolved in 3.0 mL of 1-Methyl-2-pyrrolidon (NMP)/Dichloromethane (DCM) (1:19, *v*/*v*) mixed solvent containing 600.0 mg of PLGA and porogens (PVP-k12 or Pluronic F127). The resulting organic phase was then injected into 300 mL aqueous phase containing 0.1% PVA as emulsifier under high speed homogenization (Ultra Turrax T25, IKA, Germany) at 8000–15000 rpm for 30 s. The final emulsion was subsequently stirred at 800 rpm to evaporate DCM under a fume hood at 25 °C for 5 h. The microparticles were collected by centrifugation and washed three times with distilled water, then lyophilized for 24 h (FDU-1100, Tokyo Rikakikai Co., Ltd., Japan).

2.3. Characterization of cinaciguat loaded PLGA-LPPs

2.3.1. Particle size measurement

The particle size and size distribution of the LPPs were measured using a laser diffraction particle size analyzer (Beckman-Coulter LS 230, USA). Approximately 20 mg of the dry LPPs were well dispersed in 0.1% PVA solution and the homogeneous suspension was used for measurement. The volume mean diameter (D_v) was used to evaluate the geometric diameter of the particles.

2.3.2. Encapsulation efficiency of cinaciguat in the microparticles

Encapsulation efficiency (EE%) of cinaciguat in the microparticles was measured according to the following procedures: 10.0 mg of drug loaded LPPs were weighed precisely and dissolved in 50.0 mL (±0.05 mL) 1-Methyl-2-pyrrolidon (NMP)/Acetonitrile mixed solvent (NMP/ACE, 1:19, *v*/*v*). 10.0 μL solution was then subjected to the RP-HPLC on a Phenomenex Gemini column with pre-column (150 mm × 4.6 mm, particle size: 5 μm) at 40.0 °C. Gradient elution method was used. The detection wavelength was 230 nm and drug retention time was in the range of 14.70–14.80 min. EE% was defined as the followed equation:

$$EE\% = \frac{M_{\text{actual drug loaded}}}{M_{\text{theoretical drug loaded}}} \times 100\% \quad (1)$$

2.3.3. Specific surface area measurement

The specific surface area of the LPPs was measured according to the Brunauer-Emmett-Teller (BET) and Barrett-Joyner-Halenda (BJH) isotherms methods [25] from the adsorption isotherms of N_2 (Beckman Coulter SA3100, USA). Briefly, approximately 100 mg of the particle samples were degassed for 24 h (25 °C) to remove any dissolved components that could interfere with the analysis. The particles were put in a sample cell placed within a liquid nitrogen filled cavity to maintain equilibrium temperature. Each sample was run for 24 h to obtain BET

and BJH isotherms. The average surface areas (m^2/g) were determined by both BET and BJH isotherms for all the formulations.

2.3.4. Scanning electron microscopy (SEM)

Morphology of the LPPs was observed using Hitachi S-3400N and SU-8010 scanning electron microscopes (SEM) (Hitachi Ltd., Japan). A small amount of powder was sprinkled onto double-sided adhesive tape attached to an aluminum stub and was sputter-coated with gold under vacuum. Photographs were taken at varied magnifications with an accelerating voltage of 4–5 kV to reveal surface characteristics of the porous particles.

2.3.5. Differential scanning calorimetry (DSC)

Thermodynamic analysis of cinaciguat, LPPs and the excipients were performed by differential scanning calorimetry (DSC-1, Mettler-Toledo, Switzerland). The samples (~3.0 mg) were weighed and placed in hermetically sealed aluminum pans. The samples were scanned in the range of 20 °C to 220 °C at the heating rate of 10 °C/min in nitrogen atmosphere. The melting temperature was determined from the endothermic peak of the DSC curve recorded.

2.3.6. X-ray powder diffraction

The drug crystallinity was further validated by X-ray powder diffraction. Samples were measured using an X-ray diffractometer (X'pert PRO, PANalytical B.V., The Netherlands) with Cu-K_α radiation generated at 40 mA and 35 kV. Samples were analyzed in a 2θ range of 4.5°to 40° with a step size of 0.033° and a counting time of 0.6 s per step.

2.4. In vitro release

To evaluate the in vitro release rate of cinaciguat from the PLGA LPPs, 10.0 mg of cinaciguat loaded LPPs were weighed precisely and dispersed in 5.0 mL phosphate buffer (10 mM PBS, pH = 7.40) containing 0.1% (*w*/*v*) SDS and 0.02% (*w*/*v*) NaN_3, then incubated at a speed of 80 rpm in an air bath (37.0 ± 0.5 °C). At predetermined time intervals, the supernatants were collected and withdrawn by centrifugation. And 10 μL of the removed supernatants was used for HPLC analysis. Then, LPPs were re-suspended in fresh media of equal volume and continued to be incubated.

2.5. In vitro aerosolization performance

2.5.1. The theoretical mass mean aerodynamic diameter ($MMAD_t$)

The theoretical mass mean diameter ($MMAD_t$) of the LPPs was calculated based on tapped density measurement according to previous report with modification [14]. Briefly, a known weight of LPPs (approximately 100 mg) was placed in a purpose-made 10 mL (±0.05 mL) graduated cylinder and the initial volume (mL) was recorded. The cylinder was then tapped 500 times until a consistent height (volume plateau) was achieved and the final volume (mL) was recorded. Tapped density was expressed as the ratio between sample weight (g) and the volume occupied after 500 times tapping (mL). $MMAD_t$ was calculated according to the following equation:

$$\mathrm{MMAD_t} = \mathrm{d} \times (\rho/\rho_0\chi)^{1/2} \ (\rho_0 = 1\ \mathrm{g/cm^3},\ \ \chi = 1) \tag{2}$$

where d is the geometric mean diameter, which was expressed as mean volume diameter in this study; ρ is the mass mean density of the particles, which was tapped density here; ρ_0 is a reference density (1 g/mL) and χ is the dynamic shape factor, which is 1 for a sphere [26].

2.5.2. The experimental aerodynamic properties

In accordance with United States Pharmacopeia (USP38_NF33) Chapter <601> on aerosols, the in vitro aerodynamic properties of LPPs and Cina-lactohale powder blend (micro-Cina/LH300/LH200, 5/20/75) were determined using Next Generation Impactor (NGI, MSP Corporation, USA) with a stainless steel induction port (i.e. USP throat) and pre-separator attachments. The impactor was equipped with a critical flow controller (Copley TPK 2000), a flow meter (Copley DFM 2000) and a vacuum pump (Copley HCP5) (Copley Scientific, UK).

Prior to measurement, the NGI plates need to be coated with a thin film to decrease the particle entrainment. Specifically, each plate was coated with a 10% Tween-20 in ethanol (*w*/*v*) solution and then ethanol was evaporated in the fume hood for 1 h. Vcaps®(No. 3 HPMC capsule) (Capsugel® from Suzhou, China) was used to load the powder samples with the inhaler device Cyclohaler® (Pharbita BV, The Netherlands) tightly connected to the NGI equipment. 20.0 mg powder was weighed precisely and loaded in one capsule. 10 capsules were used for one measurement. The standard flow rate for Cyclohaler® is 100 L/min and the actuation time lasted for 2.4 s for each capsule. After actuation, particles intercepted in each compartment were collected into separate volumetric flasks and dissolved, then analyzed by HPLC. The experimental mass median aerodynamic diameter ($MMAD_e$) was provided by the bundled software of NGI (the Copley Scientific Inc.) and the fine particle fraction (%) was calculated according to the following Eq. (3):

$$\text{Fine particle fraction (FPF)}\% = \frac{\mathrm{M}_{\text{stage 1 through 5}}}{\mathrm{M}_{\text{total}}(\text{Induction port} + \text{Pre}-\text{separator} + \text{all stages})} \tag{3}$$

2.6. Retention of the LPPs in the lung

In order to study the residence time of LPPs in the lung, cinaciguat content in the bronchoalveolar lavage fluid (BALF) was analyzed at different time points after pulmonary drug delivery to rats according to the procedures reported previously with modifications [25,27]. Adult male Sprague-Dawley (SD) rats were procured from Shenyang Pharmaceutical University Animal Center. All animal studies were performed in accordance with guidelines for the care and use of laboratory animals under a protocol approved by Shenyang Pharmaceutical University Animal Care and Use Committee.

Briefly, SD rats (6–8 week old, 180–230 g) were divided into 4 groups and administered (a) cinaciguat-lactohale powder blend (micro-cinaciguat/LH300/LH200 = 5/20/75), (b) LPPs (20% k12, 503H), (c) LPPs (40% k12, 503H) and (d) LPPs (20% k12, 502H). The entire administration dose was equivalent to 600 μg/kg (cinaciguat/rat). For each group, at least 5 rats were used for any time point. Firstly, the rats were anaesthetized using 7.5 mL/kg of 4.0% (*w*/*v*) chloral hydrate in normal saline. Then, the respective powder formulation was insufflated into the lungs of the anesthetic rats using a dry powder insufflator device (Dry Powder Insufflator™, DP-4-R) with a laryngoscope (Penn-Century, Philadelphia, PA) and a disposable 3.0 mL air syringe.

At different time points, rats were executed by cervical dislocation. The neck trachea of rat was subsequently isolated surgically and cut a small "T" type of incision on it. The chest was subsequently opened to expose the whole lung to facilitate the following process of BALF retrieval. A lavage needle with an air syringe was carefully inserted into trachea from the incision and down to the position of ~1 cm above the carina. Finally the incision was ligated. 2.0 mL NMP was injected slowly into the whole lung and left for 30 s, withdrawn and re-injected. BALF was finally collected after injection-withdrawal for 3 times. This procedure was repeated for 3 times with fresh NMP. All the collected BALF was combined for the drug content analysis. During the BALF collection process, the lung was gently squeezed with massage in order to improve the recovery of the lavage fluid. Thereafter, 3.0 mL acetonitrile was added to 1.0 mL BALF. The mixture was then under vortex for 15 min to precipitate proteins and impurities. Finally, the mixture was then centrifuged at 12,000 rpm for 10 min. The supernatant was withdrawn and acetonitrile removed by evaporation under nitrogen gas flow. The solution left was used for content analysis.

2.7. Macrophage uptake study

Raw 264.7 macrophage cells (ATCC TIB-71; USA) were seeded at a density of 5×10^5 cells/mL in 6-well plates and incubated for 24 h. Thereafter, the cells were washed 3 times with fresh DMEM. Then, 1.80 mg cinaciguat-lactohale powder blend (~80 μg Cina) and 2.1 mg LPPs (20% PVP, 503H) (~80 μg Cinaciguat) were precisely weighed and uniformly dispersed with 1.0 mL preheated DMEM medium. The mixture was then incubated with RAW264.7 cells for 2 h, 4 h and 8 h respectively. At determined time points, the medium was removed and the cells were washed 3 times with PBS, lysed with 500 μL RIPA lysis buffer for 30 min. Then, 300 μL of cell-lysed solution was taken out, mixed with NMP of equal volume and centrifuged (12,000 rpm, 10 min). The supernatant was used for drug content analysis. The rest of the sample was centrifuged (12,000 rpm, 10 min), and 10 μL was used for protein content determination by a BCA kit assay. The cell uptake was defined by the following equation:

$$\text{Cell uptake} = \frac{M_{\text{Cina}}}{M_{\text{protein}}} \quad (4)$$

2.8. Pharmacodynamic study in mini-pigs

Mini-pigs (6–8 week old, 2–6 kg BW, Göttinger Minipigs® Ellegaard, Dänemark) were used to establish the pathological model of pulmonary arterial hypertension. The animals were sedated by an intramuscular application of 25 mg/kg ketamin and 10 mg/kg azaperon. Anaesthesia was maintained by i.v. dosage of 30 mg/kg/h ketamin and 1–4 mg/kg/h midazolam as well as 150 μg/kg/h pancuronium bromid (z.B. Pancuronium-Actavis). Animals were intubated and ventilated (10–12 mL/kg, 35 breath/min; Avea®, Viasys Healthcare, USA, or Engström Carestation, GE Healthcare, Freiburg, Germany) to keep an end tidal CO_2-concentration of about 5%. Ventilation is performed with room air enriched with 40% oxygen. For hemodynamic assessment (e.g. pulmonary artery pressure (PAP), blood pressure (BP) and heart rate (HR)), catheters were placed in the A. carotis (BP) and a Swan-Ganz-Catheter is placed via the V. jugular into the pulmonary artery. Hemodynamics was recorded via pressure transducers (Combitransducer, B. Braun, Melsungen, Germany) and analyzed using Ponemah® aquisition software. To model pulmonary hypertension after instrumentation, a continuous infusion of a thromboxane A_2-analogon was started. And 0.3–0.75 μg/kg/min 9, 11-Dideoxy-9α, 11α-epoxymethanoprostaglandin $F_{2\alpha}$ (U-44069; Sigma, Kat.-Nr. D0400, or Cayman Chemical Company, Kat.-Nr. 16440) were dissolved in physiological NaCl and infused to reach a mean PAP of at least 25 mm Hg. The experiment was started when the steady state was reached (usually 30 min after the start of the infusion).

The respective powder formulations were then insufflated into the lungs of the mini-pigs using a specially customized dry powder insufflator device (Dry Powder Insufflator™, DP-4M) and an air pump (AP-1) assembly (Penn-Century, Philadelphia, PA) which was advanced via the tubus of the animals. A laryngoscope (Penn-Century, Philadelphia, PA) was used to visualize trachea and epiglottis to ensure a quicker and safer intubation and insertion of the dry powder insufflator.

For LPPs formulation and powder blend, the applied therapeutic dose was 18.75 μg Cina/kg. And a 3-fold higher dose (56.25 μg Cina/kg) was also investigated. The systemic arterial blood pressure and the pulmonary arterial blood pressure were monitored and recorded as evaluation parameters. Data were shown as mean ± SEM of several independent experiments per formulation.

At the end of the in-life part of the study, the animals were sacrificed and necropsy was performed. The lungs were removed from the thorax and one lobe was instilled with formalin via the bronchus. Samples of the other lobes were immersion-fixed in formalin. Additionally, lung associated lymph nodes, trachea, heart, liver, thymus, kidneys were immersion-fixed in formalin. The organs were trimmed, embedded in paraffin, cut at 4 μm and stained with hematoxylin and eosin (H&E). Subsequently, semi-quantitative histopathological evaluation was carried out.

2.9. Statistical analysis

Except for the pharmacodynamic study, all the other experimental results were depicted as the mean value ± standard deviation (SD) from at least three measurements (unless otherwise specified). Significance of difference was evaluated using one-way ANOVA at a probability level of 0.05.

3. Results

3.1. Effect of porogen concentration

Preliminary experiments demonstrated the feasibility of using PVP-k12 as a novel porogen to prepare cinaciguat loaded PLGA-LPPs using a single emulsion solvent evaporation method. Keeping drug loading 5% (*w*/*w*), PLGA 503H concentration 20% (*w*/*v*), PVA concentration 0.1% (*w*/*v*), organic phase/water phase ratio 3/300 (*v*/*v*), the influence of PVP-k12 concentration (0, 10, 20, 40%, *w*/*w* based on PLGA amount) on the properties of the LPPs was investigated. The final optimized D_v value overall, was in the range of 12–15 μm, except for LPPs prepared with 40% PVP (D_v, 18.85 ± 0.07 μm). The results are presented in Table 1. Compared with the non-porous PLGA microparticles, the inclusion of PVP-k12 caused decrease in drug encapsulation efficiency (EE%). When the PVP-k12 concentration was increased from 10% to 20%, no statistical decrease in EE value was found ($P > 0.05$).

Influence of PVP addition on the morphology of the LPPs was observed by SEM, as shown in Fig. 1(A–D). Non-porous PLGA microspheres with smooth surface were obtained when no PVP was added to the formulation (Fig. 1A). Along with the increase of PVP concentration from 10% to 40%, the number of pores and the corresponding diameter of the pores increased obviously based on surface observation (Fig. 1B, C and D). The internal porous structure was further disclosed from the cross-section image (Fig. 1H). Moreover, increasing PVP concentration on the porosity of the LPPs was evaluated quantitatively via specific surface area measurement. With the comparable particle size, the surface area of the microspheres increased from 1.03 m^2/g (non-porous) to 2.62 m^2/g (20% PVP LPP). And despite of the larger particle size, LPPs prepared with 40% PVP exhibited the highest porosity (3.48 m^2/g), demonstrating increased porosity in the microspheres along with the increase of PVP concentration. This is in good agreement with the SEM observation.

In vitro release of cinaciguat loaded PLGA-LPPs with different PVP-k12 concentrations was carried out under sink conditions and the release profiles are shown in Fig. 2A. The burst release (defined as drug release in the first 2 h, listed in Table 1) increased with the increase of PVP-k12 concentration and a remarkable increase was found when PVPk12 concentration was increased from 20% to 40%, with burst release from 35.8% to 87.1%. For the non-porous PLGA microspheres, it presented a plateau state after 10% burst release. In contrast, drug release from LPPs was biphasic, with a burst release followed by a sustained release behavior. More importantly, drug release rate from the LPPs was PVP concentration dependent, implying the release behavior of the LPPs formulation was tunable via adjusting the porogen content. However, since this in vitro release is investigated under sink condition, its correlation with in vivo retention and in vivo release need further investigation.

3.2. Effect of PLGA type

Keeping drug loading 5% (*w*/*w*), PLGA concentration 20% (*w*/*v*), PVA concentration 0.1% (*w*/*v*), organic phase/water phase ratio 3/300 (*v*/*v*),

Table 1
The properties of different cinaciguat loaded PLGA-LPPs.

0.05 x 0.68 = 0.036, 3.7% drug loading

Formulations	Porogen (PVP, %)	D_v (μm)	EE (%)	Specific surface area (m^2/g)	Burst release (%)
PLGA503H	0	15.09 ± 1.03	85.87 ± 1.59	1.03	10.46 ± 3.87
	10	12.82 ± 0.04	81.70 ± 2.83	1.38	22.81 ± 3.22
	20	12.39 ± 0.02	77.94 ± 2.43	2.62	35.75 ± 3.10
	40	18.85 ± 0.07	68.63 ± 8.31	3.48	87.14 ± 5.41
PLGA503	20	13.97 ± 0.08	83.42 ± 3.20	2.28	31.37 ± 4.62
PLGA502	20	12.51 ± 0.07	68.10 ± 2.60	2.74	50.30 ± 3.15
PLGA502H	20	13.27 ± 0.13	71.41 ± 2.39	2.37	59.54 ± 4.20

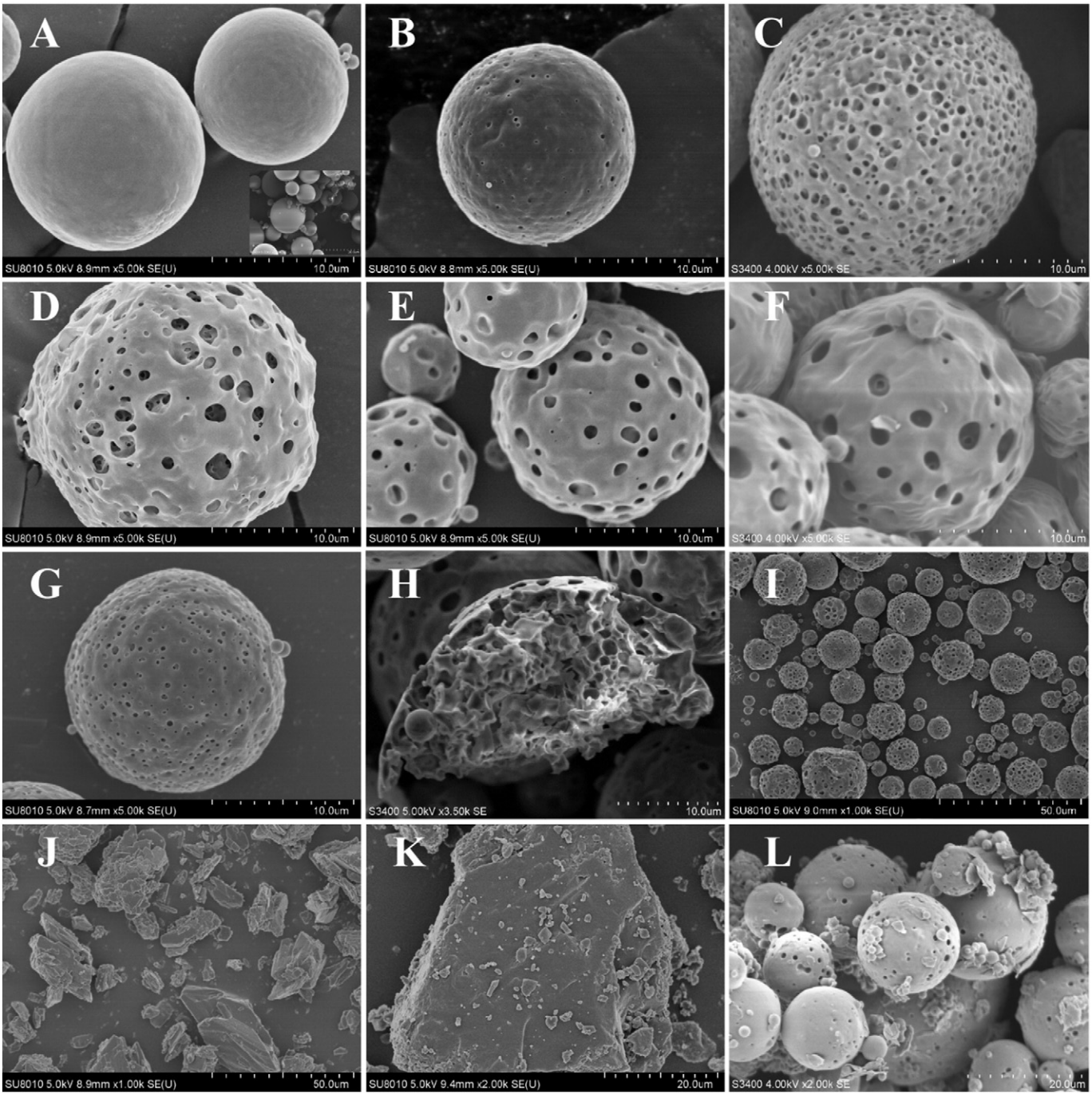

Fig. 1. Morphology of the particles observed by SEM: (A) non-porous Cina-PLGA microspheres (503H); (B) Cina-503H-LPPs (10% PVP); (C) Cina-503H-LPPs (20% PVP); (D) Cina-503H-LPPs (40% PVP); (E) Cina-503-LPPs (20% PVP) (F) Cina-502-LPPs (20% PVP) (G) Cina-502H-LPPs (20% PVP) (H) cross-section of Cina-503-LPPs (50% PVP); (I) overall field of vision of Cina-503-LPPs (20% PVP); (J) coarse-Cina (K) powder blend of micro-Cina and inhalable lactose and (L) Cina-502-LPPs (50% F127).

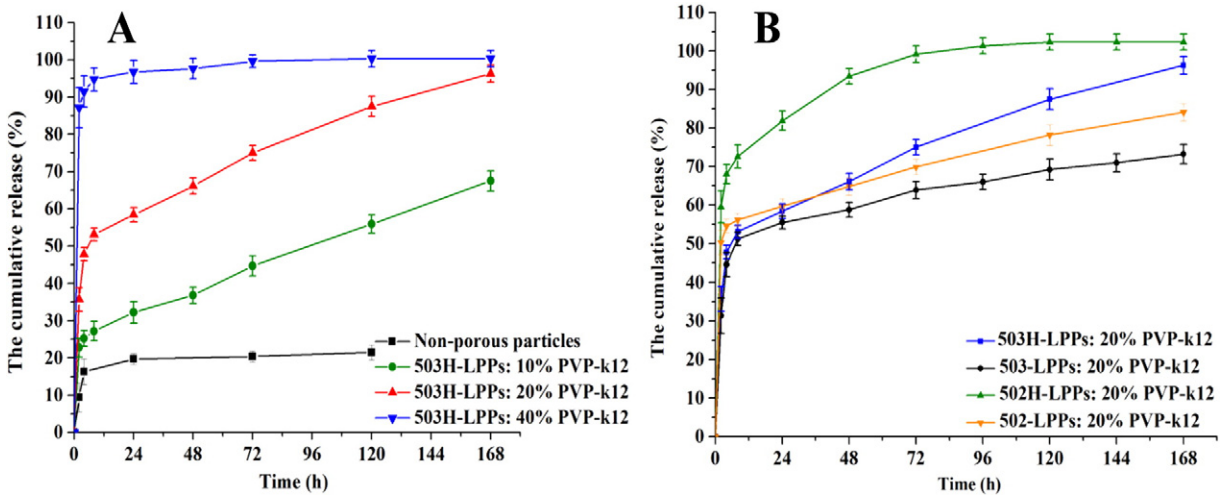

Fig. 2. Influence of PVP-k12 concentration and PLGA type on the in vitro release of cinaciguat from PLGA-LPPs.

PVP-k12 concentration 20% (*w*/*w*), all the particle size (D_v) was kept in the range of 12–14 μm, influence of PLGA type (503H, 503, 502H and 502) on the properties of LPPs was investigated and the results are presented in Table 1. The drug encapsulation efficiency was influenced by PLGA molecular weight, comparable EE value was obtained for PLGA503H and PLGA503 group (77.94 ± 2.43 and 83.42 ± 3.20%), PLGA502 and PLGA502H group (71.41 ± 2.39 and 68.10 ± 2.60%), but the EE value in the PLGA 502 group was almost 10% lower than that of the PLGA503 group.

Morphology of the LPPs prepared with different PLGA types is shown in Fig. 1(C, E, F and G). It was found that PLGA with free carboxyl group terminated in structure (503H and 502H) had smaller pore size than PLGA with ester terminated (503 and 502) ones. Specific surface area of the LPPs was measured, it was 2.62, 2.28, 2.37 and 2.74 m^2/g for PLGA 503H, 503, 502H, 502 based samples, respectively, implying comparable overall porosity despite of different PLGA type.

In vitro release profiles of cinaciguat loaded PLGA-LPPs with different PLGA type are shown in Fig. 2B. The burst release was influenced by both PLGA molecular weight and its end group composition. Lower burst release was observed for 503H and 503-LPPs (35.75 ± 3.10 and 31.37 ± 4.62%), when compared with low molecular weight 502H and 502-LPPs (59.54 ± 4.20, 50.30 ± 3.15%). After the burst release, cinaciguat was released from the LPPs in a controlled manner, with the release rate increase in the order 503 < 502 < 503H < 502H.

Taking cinaciguat loaded 503H-LPPs (20% PVP) as an example, morphology change of the LPPs during incubation with the release medium was studied and shown in Fig. 3. The surface pores were found to merge and become larger along with time, with the internal porous structure well exposed. This is consistent with the fast drug release in the later stage. Taking both the burst release and the later release profile into consideration, three cinaciguat loaded LPPs formulations (20% PVP-503H-LPPs, 40% PVP-503H-LPPs and 20% PVP-502H-LPPs) were selected for further investigation.

3.3. Solid state analysis of LPPs

Solid state of cinaciguat in the LPPs was characterized using both DSC and X-ray powder diffraction (X-RPD). As shown in Fig. 4A, for all the LPPs investigated, no drug endothermic peaks were observed (Fig. 4Af). Moreover, no endothermic event of PVP-k12 was observed at around 80 to 100 °C from the thermograms. The drug existing state in the LPPs was further validated by X-RPD assay (Fig. 4B). Strong characteristic peaks were observed for cinaciguat and its physical mixture with PLGA 503H. In contrast, for the LPPs investigated, a very weak diffraction peak could be observed at around 19.5° (2θ), where the strongest diffraction peak of cinaciguat showed. These studies demonstrated that cinaciguat existed mostly in the amorphous state in the LPPs, possibly with minor amount in the crystalline state, if at all.

3.4. In vitro aerodynamic properties

To achieve better lung deposition, the aerodynamic properties of the LPPs are of special importance. First of all, the theoretical mass mean aerodynamic diameter ($MMAD_t$) of the selected LPPs was calculated based on the measured tap density, as shown in Table 2. Compared to the Cina-lactohale powder blend, LPPs had a markedly lower density, and the tap density decreased with the increase of porogen content. Correspondingly, the calculated $MMAD_t$ value was in the range of 5–8 μm despite of their large geometric diameter (12–19 μm), another demonstration of their porous structure. The calculation of $MMAD_t$ was inapplicable to the powder blend due to the different particle sizes of micro-Cina, Lactohale LH 200 and Lactohale LH 300 in the formulation.

The experimental mass mean aerodynamic diameters ($MMAD_e$) and fine particle fraction (FPF%) of the selected LPPs were further determined by NGI. The aerodynamic particle size distributions are shown in Fig. 5. In stage 1, Cina-503H-LPPs (40% PVP) has comparable retention with that of Cina-lactohale powder blend, which is significantly higher than that of Cina-502H-LPPs (20% PVP) and Cina-503H-LPPs (20% PVP) groups ($P < 0.05$). In stage 2, Cina-lactohale powder blend has statistically higher retention than the three LPP groups tested ($P < 0.05$ or $P < 0.01$) with the retention ratio in the order Cina-503H-LPPs (40% PVP) > Cina-503H-LPPs (20% PVP) > Cina-502H-LPPs (20% PVP). No significant difference in drug retention in stage 3 was found. Cina-lactohale powder blend had higher retention than LPPs in stage 4 ($P < 0.05$), implying the existence of fine particles in the system. As listed in Table 2, all the LPP formulations tested have comparable $MMAD_e$ (5–6 μm). The FPF value of the 20% PVP-502H-LPPs was similar with 20% PVP-503H-LPPs. Increasing PVP concentration from 20% to 40% caused significant increase in FPF value ($P < 0.05$). In contrast, the powder blend of micro-cinaciguat with inhalable lactose presented lower $MMAD_e$ and higher FPF% values, implying better lung deposition. For the 20% PVP-502H-LPPs and 20% PVP-503H-LPPs, these two values are comparable, implying $MMAD_t$ can be used as a fast screening parameter in the early stage of formulation development.

3.5. In vivo residence of LPPs

To study the in vivo residence of the LPPs and to test whether prolonged drug retention or sustained release can be achieved with

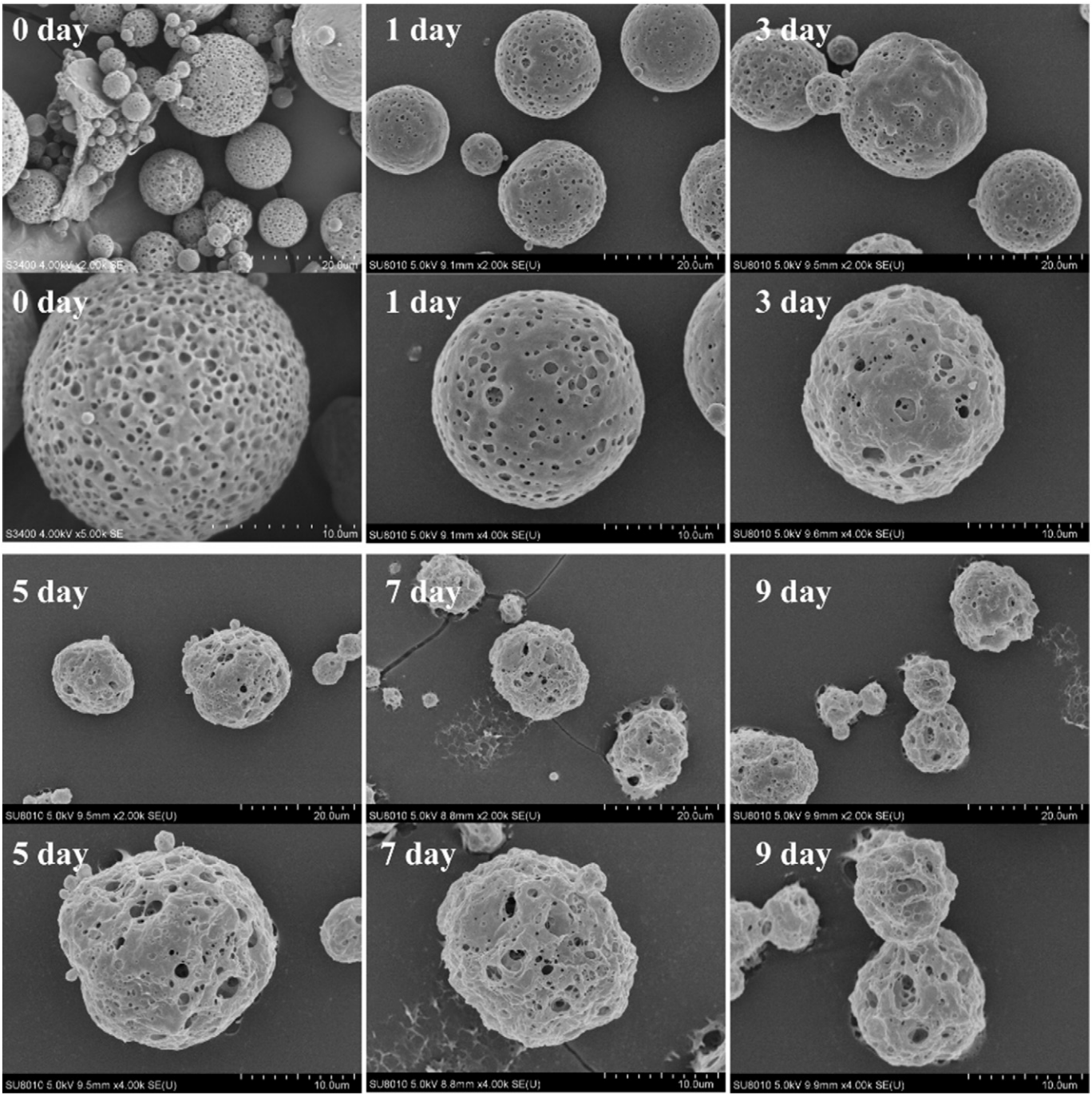

Fig. 3. Morphology of Cina loaded 503H-LPPs (20% PVP) incubated under release conditions.

these LPPs, the local content of cinaciguat in the bronchoalveolar lavage fluid of male SD rats was analyzed at different time points, the drug average recovery rates of these formulations at 0 h were within the range of 74.59 ± 7.18% to 83.81 ± 8.92%. As shown in Fig. 6, cinaciguat-lactohale powder blend had a retention time of only ~8 h (4.45 ± 3.01% of Cinaciguat residue at 8 h). In contrast, significantly prolonged drug retention in the BALF was observed for all the LPPs investigated ($P < 0.01$). In agreement with the in vitro release data, the longest retention time (up to 36 h, 10.15 ± 3.01% of drug residue) was observed with 20% PVP-503H-LPPs, followed by 20% PVP-502H-LPPs and 40% PVP-503H-LPPs, with retention time up to 24 h (9.38 ± 2.12% and 6.84 ± 2.62% of drug residue respectively). And 20% PVP-503H-LPPs showed significant higher cinaciguat residue than other two LPP formulations at time point of 24 h (17.49 ± 4.55%, $P < 0.05$). Therefore, 20% PVP-503H-LPPs was selected for further studies.

3.6. Macrophage uptake study

In this study, LPPs were designed and expected to decrease or even avoid pulmonary macrophages uptake in order to exert sustained drug release effect in the lung. To test whether or not the decreased macrophage uptake contributed to the prolonged retention of the LPPs in the lung, macrophage phagocytosis of the 20% PVP-503H-LPPs was studied in rats at 2 h, 4 h and 8 h after LPP administration and compared to the cinaciguat-lactose physical mixture, with micro-cinaciguat in the size range of 1–5 μm added on the lactose surface (Fig. 1J and K). The

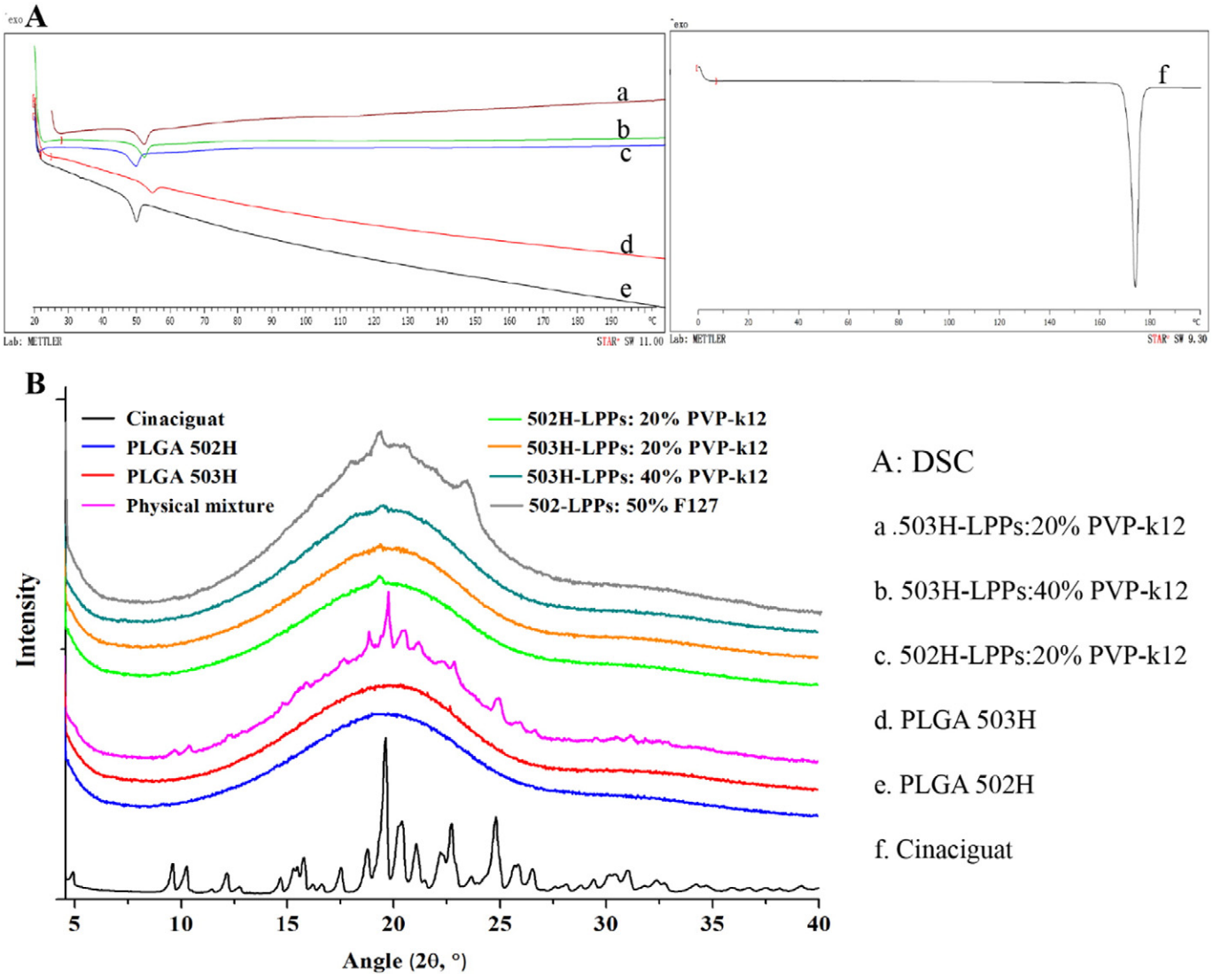

Fig. 4. (A) The DSC thermograms of PLGA, cinaciguat loaded PLGA LPPs and cinaciguat; (B) the X-RPD patterns of cinaciguat/PLGA physical mixture and cinaciguat loaded PLGA LPPs.

results are shown in Fig. 7. It was noted that micro-cinaciguat in the conventional physical mixture was rapidly phagocytized by the macrophages and the uptake amount increased with time. In contrast, significantly reduced macrophage uptake was shown for the LPPs developed in this study ($P < 0.05$), and no apparent increase in uptake was observed in the 8 h investigated ($P > 0.05$), which is consistent with the prolonged drug retention in the BALF (Fig. 6).

3.7. Pharmacodynamic effect of the LPPs in mini-pig model

Based on the above studies, 20% PVP-503H-LPPs were selected for in vivo pharmacodynamic studies using mini-pigs with established pulmonary arterial hypertension model, to test whether sustained release behavior can also be demonstrated in vivo. Data were compared to the Cina-lactohale powder blend. As shown in Fig. 8, pulmonary arterial blood pressure (PAP) as well as systemic arterial blood pressure (BP) was continuously monitored over 240 min after formulation delivery at a dose of 18.75 μg Cina/kg (Fig. 8A and B). Before the application of the cinaciguat formulations, pure matrix/formulation applications (blank LPPs and lactose) were performed to differentiate the vehicle effects from the drug effects. The significant decrease in the PAP was observed for the powder blend formulation 30 min after application and its effect diminished thereafter and lasted for <180 min. In contrast, the LPPs formulation decreased the PAP value for the entire duration of the experiment. With respect to the systemic effects on BP, a slight effect was observed for both formulations.

To further evaluate the difference between the two formulations, a three-fold higher dose (56.25 μg Cina/kg) was applied (Fig. 8C and D).

Table 2
Aerodynamic parameters of the LPPs and the physical blend.

Formulation	FPF (%)	$MMAD_e$ (μm)	$MMAD_t$ (μm)	ρ_t (g/cm^3)
Cina-502H-LPPs (20% PVP)	19.80 ± 1.28	5.73 ± 0.25	6.43 ± 0.13	0.237 ± 0.006
Cina-503H-LPPs (20% PVP)	21.52 ± 4.41	6.17 ± 0.33	5.81 ± 0.25	0.220 ± 0.019
Cina-503H-LPPs (40% PVP)	28.56 ± 1.91	5.78 ± 0.27	8.07 ± 0.20	0.184 ± 0.010
Cina-lactohale powder blend	36.00 ± 1.40	4.82 ± 0.08	–	1.103 ± 0.048

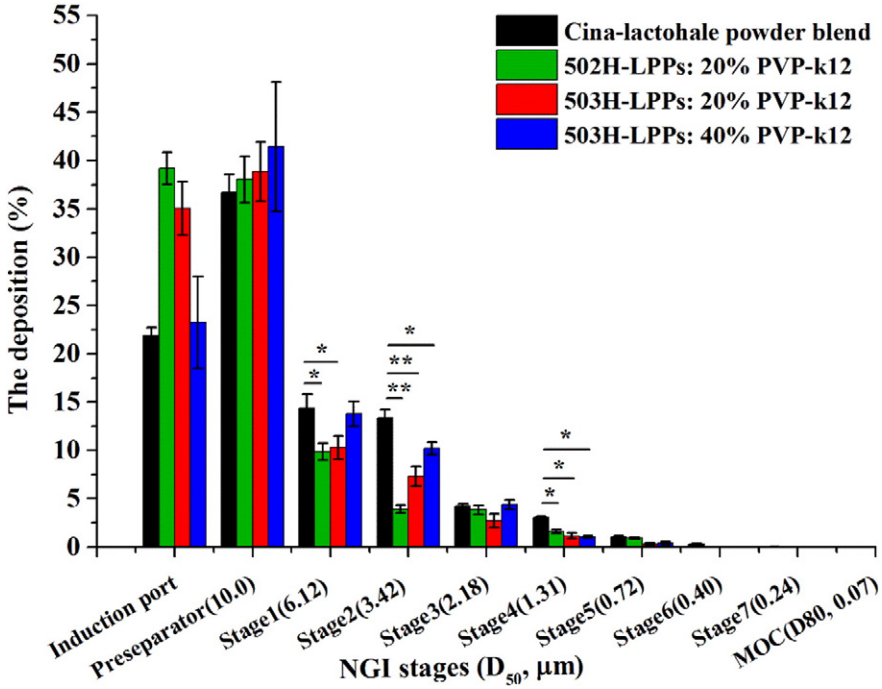

Fig. 5. In vitro aerodynamic diameter distributions of cinaciguat-lactose powder blend and cinaciguat loaded PLGA LPPs as measured by NGI. (**P < 0.01 and *P < 0.05, compared to the cinaciguat-lactose powder blend group).

Again the decrease in the PAP was diminished in its maximal effect and sustained longer for the duration of the experiment. With respect to the systemic effects on BP both formulations showed a similar effect.

Findings seen during histopathological evaluation in all animals of the study were some minimal focal inflammatory infiltrates in various organs/tissues, generally liver and kidney, or minimal focal pigment deposition. In the trachea and probably due to intubation (see Section 2.8), focal epithelial degeneration with beginning inflammatory reaction and intraluminal detritus and cells were seen. The majority of organs/tissues evaluated morphologically were without pathological findings.

4. Discussion

Large and highly porous particles based on PLGA (PLGA-LPPs) are considered as an efficient vesicle to deliver drugs to the lung with sustained drug release. These particles are light enough to be aerosolized and well deposited attributed to the low densities caused by their highly porous structure. Furthermore, large particles with geometric size larger than 5 μm are not easily captured by lung macrophages since they are too large to be phagocytized [12,28]. Therefore, LPPs can stay in the lungs for a longer period of time if well-aerosolized and properly inhaled.

However, at present, PLGA-LPPs are mainly prepared by double emulsion (w/o/w) method using ammonium bicarbonate as an effervescent porogen, which is more suitable for the encapsulation of hydrophilic drugs [29–31]. For the loading of hydrophobic drugs, double emulsion method was also used previously by dissolving the hydrophobic drugs in the organic phase of w/o/w emulsion, there the internal water phase is added only to play a role of pore-forming, with lower drug encapsulation efficiency reported [19]. Therefore, it should be more reasonable to prepare hydrophobic drugs loaded PLGA-LPPs with single emulsion method (o/w), with simplified preparation

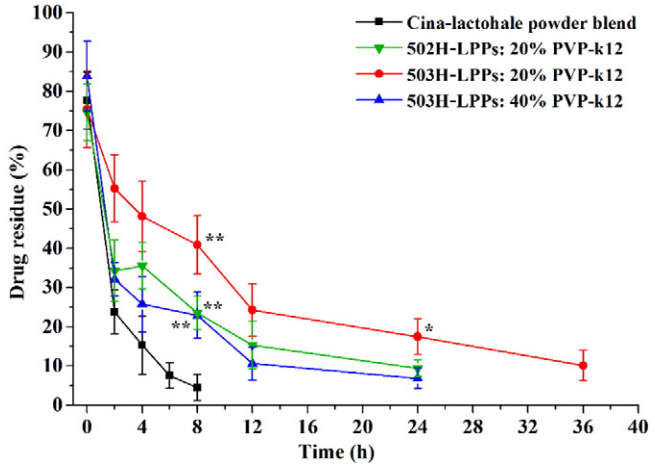

Fig. 6. In vivo residence of the cinaciguat-lactose physical mixture and the LPP formulations at different time points based on bronchoalveolar lavage fluid (BALF) analysis. (**P < 0.01, compared with drug residue of powder blend at 8 h; *P < 0.05, compared with drug residue of the other two LPP formulations at 24 h).

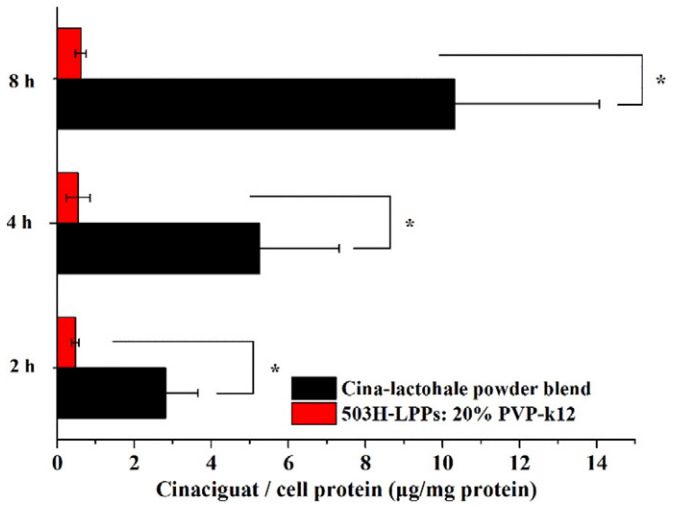

Fig. 7. Macrophage uptake efficiency of Cina-lactohale powder blend and Cina loaded PLGA LPPs at three time points (2 h, 4 h and 8 h). (*P < 0.05).`

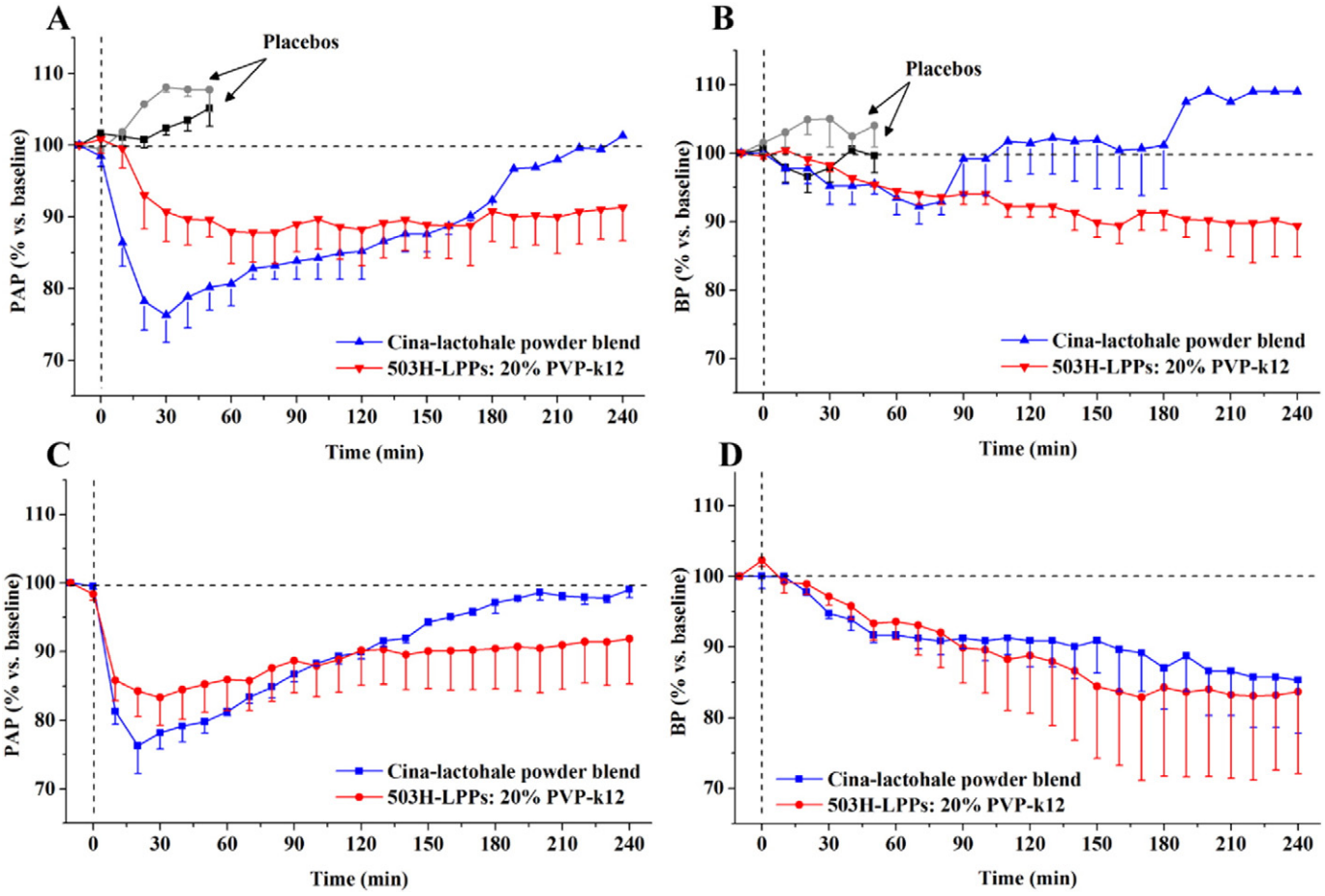

Fig. 8. Pharmacodynamic effect of PLGA503H-LPPs with 20% PVP and Cina-lactohale powder blend in mini-pig model: (A) pulmonary arterial pressure, (B) systemic blood pressure, (C) pulmonary arterial pressure with high dose application and (D) systemic blood pressure with high dose application.

process and increased drug loading. Then the prerequisite is to find an appropriate pore forming agent to construct the porous structure. For this objective, single emulsion (o/w) method combined with F127 as an extractable porogen has been investigated previously [21]. In our study, the feasibility of using F127 as porogen to prepare PLGA-LPPs was also investigated. Visible porous structure was formed when F127 concentration was increased to 50% (F127/PLGA, *w*/*w*), and the EE% value was extremely high (95.60 ± 4.00%). However, disappointingly, obvious agglomeration and many fragments were observed from SEM images (Fig. 1L). Moreover, drug crystals were detected by X-RPD analysis (Fig. 4B). Thus, F127 is not an appropriate porogen to produce PLGA-LPPs using single emulsion method.

PVP-k12, a non-ionic polymer with HLB 8.7, is widely used in the field of pharmaceutics for its good safety and fine solubility in both aqueous and most organic solvent. Besides, it is safe in the blood and its solution has been used as plasma volume expander in the clinic in the past [32–34]. In the present study, the feasibility of using PVP as the porogen to prepare PLGA-LPPs via single emulsion method was elucidated. The porosity of the LPPs was PVP concentration dependent and microparticles with good morphology were obtained (Fig. 1). The drug mainly existed in amorphous state in the microspheres (Fig. 4) with encapsulation efficiency in the range of 70%–80%. And more importantly, during the homogenization process, no apparent emulsification enhancement happened, leading to favorable particle size distribution and fine porous morphology. Besides, compared with the widely reported double emulsion method by using gas-forming agent as the pore forming agent, preparing the microspheres using the single emulsion method with extractable porogen not only simplified the preparation process but also improved the encapsulation efficiency of hydrophobic drugs.

In addition to encapsulation efficiency, the burst release is another major challenge for LPPs. For conventional PLGA microspheres, the burst release was attributed to not only the contact surface area, but also the presence of drug crystals on the particle surface [35]. Here, when introducing PVP into the preparation process, absence of endothermic events in DSC analysis and obvious X-RPD peaks in the resulted LPPs suggested that drug crystals adsorbed on the particle surface was very limited. Then the increased contact area of the microparticles with the release media, characterized by the specific surface area data, became the main reason for the increased burst release. In addition, PLGA type (different molecular weight and end-group property) also influenced the burst release (Fig. 2B). Interestingly, LPPs prepared with PLGA of different end-group also present different pore size on the surface (Fig. 1), suggesting the hydrophilic nature of PLGA probably played a role during the porous structure creation process because different end-group could impact the water-uptake capacity of the polymer [36, 37].

An acceptable burst release to reach a therapeutic dose followed by drug release in a controlled manner would be highly desirable. For non-porous PLGA microspheres, as long as 4–6 week drug release could be achieved by a 50:50 PLGA with low to medium molecular weight (502H, 502, 503H or 503) [35]. In contrast, the overall drug release behavior of PLGA-LPPs changed considerably, with an accelerated release after the burst (no lag time) (Fig. 2). This might be explained by the

enhanced degradation of PLGA upon increased contact area with the release medium attributed to the high porosity. As disclosed, the pore diameter increased steadily along with incubation time (Fig. 3). Excitingly, when PVPk12 was used as the porogen, the overall drug release from the LPPs can be tuned by varying the porogen content and PLGA type (Fig. 2), which provided multiple options for clinical application.

FPF of the LPPs showed lower value compared to the physical mixture, which can be explained by their aerodynamic diameter difference. Here, the developed LPPs formulations had a MMADe of appro. ~6 μm, which was larger than the powder blend (4.8 μm), and this might contribute to the reduction in FPF% value. According to the USP pharmacopeia, FPF > 15% is required for dry powder inhalation, all the LPPs tested in this study reached this requirement.

It was reported previously that increased microparticle size could reduce macrophage phagocytosis [38,39]. In this study, a significant reduction of LPP phagocytosis by macrophages was demonstrated in Raw 264.7 cells uptake study, which would be beneficial to the objective of sustained release in the lung.

Bronchoalveolar lavage (BAL) is widely reported and applied both in laboratory and clinic for pulmonary inflammation analysis and diagnosis [40,41]. In this study, modified BAL was carried out and drug content in the lavage fluid was analyzed to evaluate in vivo residence of cinaciguat loaded LPPs. LPPs were found to have extended retention time in the lung. 20% PVP-503H-LPPs had a sustained drug release profile in vitro and correspondingly, the obvious longer retention time in the lungs of SD rats was obtained. It is worth noting that almost the same retention tendency was observed between 20% PVP-502H-LPPs and 40% PVP-503H-LPPs despite of their different in vitro release behavior, implying that probably too high burst release and faster in vitro release is not beneficial to achieve sufficient sustained effect in vivo despite of the limited fluid in the lung.

On the base of the sustained in vitro release and prolonged retention in the rat lung, 20% PVP-503H-LPPs were selected for pharmacodynamic study in PAH mini-pig model with two doses. As anticipated, compared to the powder blend, significantly sustained effect on pulmonary arterial pressure was demonstrated for both low and high dose LPPs. Still, both LPPs and the powder blend had a slight effect on the systemic blood pressure, implying part of the drug was absorbed into the systemic circulation.

It is well known that FPF value is extremely important for pulmonary drug delivery, but the therapeutic effect is not only related to FPF but also retention time of the formulation in the lung. As shown in the study, although the physical mixture present higher FPF value (Fig. 5, Table 2), since they can be easily taken up by the macrophage (Fig. 7), it has shorter retention time in the lung (Fig. 6), therefore, its therapeutic effect can only last for a short period of time. In contrast, decreased macrophage uptake, longer retention time in the lung and sustained release of LPP formulations (Figs. 2, 6, 7) in combination with good lung deposition (Fig. 5) brought to improved therapeutic effect, as demonstrated by the pharmacodynamics study (Fig. 8).

Tracheal findings seen during histopathological evaluation in all animals of the study (e.g. epithelial degeneration and inflammation) are classified to be due to intubation. All other morphological findings detected in the organs/tissues evaluated, were assessed not to be related to the exposure to the test particles. Similar findings are known from controls and therefore regarded to be of spontaneous nature.

5. Conclusions

In this study, polyvinyl pyrrolidone, a new extractable porogen, combined with single emulsion (o/w) solvent evaporation method, was explored to prepare large porous cinaciguat-loaded PLGA particles for the treatment of pulmonary hypertension via inhalation. These porous particles presented desirable aerosolization properties, high drug encapsulation efficiency, and phagocytosis escapement characteristics. They were found to be able to deposit and retained in the rat lung for over 36 h. Sustained pulmonary drug release was also demonstrated in in vivo pharmacodynamic study. This simple PLGA LPPs engineering using single emulsion method with PVP as porogen may find extensive application for the pulmonary delivery of hydrophobic drugs to achieve sustained effect with good safety profile.

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