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Endotracheal Aerosolization Device for Laboratory Investigation of Pulmonary Delivery of Nanoparticle Suspensions: In Vitro and in Vivo Validation

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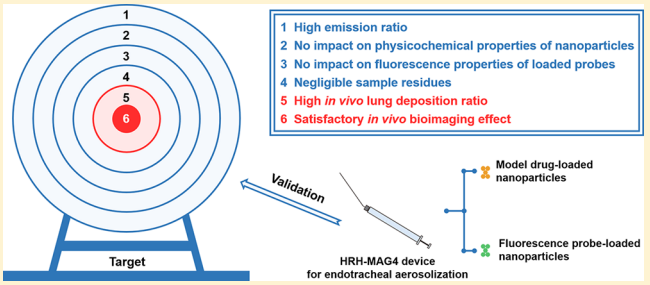
Supporting Information

ABSTRACT: The objective of this study was to perform the in vitro and in vivo validation of an endotracheal aerosolization (ETA) device (HRH MAG-4, HM). Solid lipid nanoparticle suspension (SLNS) formulations with particle sizes of approximately 120, 240, 360, and 480 nm were selected as model nanoparticle suspensions for the validation. The emission rate (ER) of the in vitro aerosolization and the influence of aerosolization on the physicochemical properties were investigated. A high ER of up to 90% was obtained, and no significant alterations in physicochemical properties were observed after the aerosolization. The pulmonary deposition of model drug budesonide in Sprague−Dawley rats was determined to be approximately 80%, which was satisfactory for pulmonary delivery. Additionally, a fluorescent probe with aggregation-caused quenching property was encapsulated in SLNS formulations for in vivo bioimaging, after excluding the effect of aerosolization on its fluorescence spectrum. It was verified that SLNS formulations were deposited in the lung region. The results demonstrated the feasibility and reliability of the HM device for ETA in laboratory investigation.



KEYWORDS: *pulmonary delivery, nanoparticle, aerosolization device, aggregation-caused quenching, bioimaging*

INTRODUCTION

Due to their behavior that results in escape from the endosomes in the lung region, a phenomenon termed "nanoescapology",[1] nanoparticles are considered to be a promising vehicle for pulmonary drug delivery. The application of two kinds of nanoparticles for pulmonary drug delivery has been reported: solid nanoparticles (solid state as a dry powder) and nanoparticle suspensions (mainly referred to aqueous suspensions). Generally, solid nanoparticles are employed dry powder inhalers,[2] whereas nanoparticle suspensions can be administered using nebulizers[3] or sprayers.[4] The authors believe that nanoparticle suspensions are the preferred delivery vehicles. This is because most nanoparticles are fabricated in liquid media,[5,6] and nanoparticle suspensions as an in situ delivery platform can prevent the particle-aggregation issues associated with the solidification process in solid nanoparticle preparation.[7,8] Until now, the laboratory investigation of nanoparticle suspensions for pulmonary delivery has gained extensive interest among researchers, and many exciting results, including improved pharmacokinetic and pharmacodynamic profiles, have been demonstrated.[9,10]

However, the emphasis of previous laboratory investigation has been the preclinical outcome, and fewer studies are focused on the pulmonary delivery approach of nanoparticle suspensions. This omission may lead to possible artifacts, since different pulmonary delivery approaches will result in different in vivo pharmacokinetic and pharmacodynamic profiles.[11] A proper approach should be unambiguously determined for the laboratory investigation to ensure the lack of such artifacts.

It is noteworthy, that the choices of methods for pulmonary delivery of nanoparticle suspensions in animals is limited, which remains a challenge that must be met.[12] Active inhalation is impractical in animal models, and semipassive/passive inhalation methods, such as nebulization,[13] oropharyngeal aspiration,[14] metered-dose inhalation,[15] intratracheal instillation,[16] and endotracheal aerosolization (ETA), are available. Nevertheless, some critical shortcomings are associated with these methods, except for ETA. Dose inaccuracy is often observed in nebulization, oropharyngeal aspiration, and metered-dose inhalation as the result of dose wastage. With the help of a

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nebulizer, nanoparticle suspensions can be nebulized into the air, and the drug-containing mist can be inhaled by the animals.[17] A considerable amount of the nebulized nanoparticle suspensions will remain in the atmosphere or may attach to the skin of animals and are therefore not inhaled.[18] Oropharyngeal aspiration refers to a procedure in which the nanoparticle suspensions are placed in the oropharyngeal region and inhaled by reflex.[19] The administered nanoparticle suspensions may be swallowed and transported into the gastrointestinal tract after oropharyngeal aspiration, which reduces the amount of nanoparticle suspensions that enter the pulmonary region.[20] Metered-dose inhalation is an actuation-dependent administration method, where nanoparticle suspensions are delivered with the propellant on actuation.[21] However, actuation-inhalation-coordination[22] cannot be assured in animals, which leads to exhalation or escape of the delivered nanoparticle suspensions and results in a low pulmonary deposition. For intratracheal instillation, the trachea is exposed and nanoparticle suspensions are directly instilled into the respiratory tract.[23] Although a satisfactory dose accuracy can be achieved, the surgery that is performed to expose the tracheal region causes relatively severe wounds.[11] These limitations may influence the laboratory investigation of pulmonary delivery of nanoparticle suspensions in animals.

In ETA, nanoparticle suspensions are directly aerosolized within the trachea and readily deposited into the pulmonary region.[24] A more satisfactory dose accuracy can be assured in ETA compared to nebulization, oropharyngeal aspiration, and metered-dose inhalation[12] because, theoretically, the entire dose of nanoparticle suspensions will be located in the trachea. Additionally, ETA is a noninvasive delivery method (if performed with the assistance of a laryngoscope), which is a more physiological intervention than intratracheal instillation.[11] Hence, ETA is an appropriate and promising method for the pulmonary delivery of nanoparticle suspensions in animals.

It is worth mentioning, that the success of ETA administration is dependent on the ETA device. A qualified ETA device should deliver the loaded nanoparticle suspensions with a high efficiency and should not alter the physicochemical properties of nanoparticle suspensions. There was only one robust and widely accepted ETA device on the market, Microsprayer,[25] and regrettably, its production ceased in 2015. HRH MAG-4 (HM, Huironghe Technology Co., Ltd., Beijing, China) is a recently developed ETA device for rats. It is designed to assist the laboratory pharmacokinetic and pharmacodynamic investigation of nanoparticle suspensions for rodent models. According to the clarification of the manufacturer, two characteristics differ between HM and Microsprayer. (I) The inner diameter of the delivery channel of HM is larger than that of Microsprayer. This means that samples with a larger particle size can be loaded and delivered, and a larger inner diameter will result in a lower aerosolization pressure (Bernoulli principle[26]). (II) The main body of HM is smaller than that of Microsprayer, which facilitates its manual use. Nevertheless, no publications up to the present have verified the feasibility and reliability of HM. The ability of HM to deliver a nanoparticle suspension with a satisfactory efficiency is uncertain, due to the lower aerosolization pressure of HM. Furthermore, whether ETA by HM will influence the physicochemical properties of nanoparticle suspensions remains unknown. These uncertainties limit the further application of HM as an emerging alternative for Microsprayer for academic and industrial use.

This work was aimed to perform the in vitro and in vivo validation of HM. Solid lipid nanoparticle suspensions (SLNS) were prospective nanoparticulate vehicles for pulmonary delivery due to their high safety and deep lung deposition.[27] Therefore, SLNS was selected as the model nanoparticle suspension. The physicochemical and morphological properties of the SLNS formulations were investigated, and in vitro aerosolization and ETA were conducted by HM. The in vitro emission ratio, influence of aerosolization on SLNS physicochemical properties, and sample residue were investigated in detail. Further, in vivo pulmonary deposition and bioimaging were performed. Noticeably, a versatile aggregation-caused quenching (ACQ) fluorescent probe with an aza-BODIPY fluorophore[28−30] was employed in the bioimaging examination. It was anticipated that the validation of HM could be accomplished by these in vitro and in vivo tests.

MATERIALS AND METHODS

Materials. Palmityl palmitate (PP) and dichloromethane (DCM) were purchased from Aladdin Industrial, Inc. (Shanghai, China). Polysorbate 80 (Tween 80), ethanol absolute, and ethyl acetate were obtained from Damao Chemical Reagent Factory (Tianjin, China). Budesonide (BUD) was supplied by Hubei Jusheng Technology Co., Ltd. (Hubei, China). Triamcinolone acetonide (TAA), the internal standard for the in vivo studies, was obtained from the National Institutes for Food and Drug Control (Beijing, China). Acetonitrile (HPLC grade) was purchased from Honeywell International, Inc. (Morristown, NJ). Ultrapure water was obtained using the PureLAB option water purification system (ELGA Lab Water, Inc., High Wycombe, United Kingdom).

The ACQ probe, coded P4, was designed and synthesized by Wei Wu et al. in Fudan University (Shanghai, China) according to the published method.[31] Detailed information regarding P4 can be found in the literature.[32,33]

Animals. The animals involved in this study were Sprague−Dawley (SD) rats. Male SD rats weighing approximately 180 g were supplied by Guangdong Medical Laboratory Animal Center (Guangdong, China). The SD rats were housed 5 per cage under a condition of 23 ± 2 °C, 55 ± 5% relative humidity, and noise ≤60 dB. Food and water were provided ad libitum until 1 day before dosing. All protocols were approved by the Animal Ethical and Welfare Committee at Sun Yat-sen University (approval IACUC-DD-17-1108).

Pretreatment of PP for the Fabrication of SLNS. PP, the lipid employed for SLNS fabrication, was pretreated. The PP was successively washed with water and ethanol absolute and then vacuum-dried under 25 °C (Bluepard BPZ-6210-2, Shanghai Yi Heng Scientific Instruments Co., Ltd.). The washed PP was melted using a 65 °C water bath and immediately filtered through a preheated 0.22 μm nylon-66 membrane (Membrana GmbH, Wuppertal, Germany). The filtrate was vacuum-dried at 25 °C, and the obtained PP was collected and stored in a sealed container in a dark environment at 20 °C.

Production of SLNS. PP was melted using a water bath (lipid phase), and 50 mL of an aqueous solution of Tween 80 was heated to the same temperature (aqueous phase). For the BUD- or P4-containing formulations, 1.5 mL of BUD or P4 stock solution was added to the melted PP. These stock solutions were prepared by dissolving BUD or P4 in DCM at concentrations of 40 μg/mL or 100 μg/mL, respectively. The DCM in the lipid phase was allowed to evaporate while the

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solution was stirred. The lipid phase was then poured into the aqueous phase, and the system was subjected to high-shear emulsification (FA25, FLUKO Equipment Shanghai Co., Ltd.). The obtained crude emulsion was subjected to hot high-pressure homogenization (EmusiFlex-C3, Avestin, Inc., Ottawa, Canada). Three batches of each SLNS formulation were produced. A scheme of the preparation procedure is provided in Figure 1A.

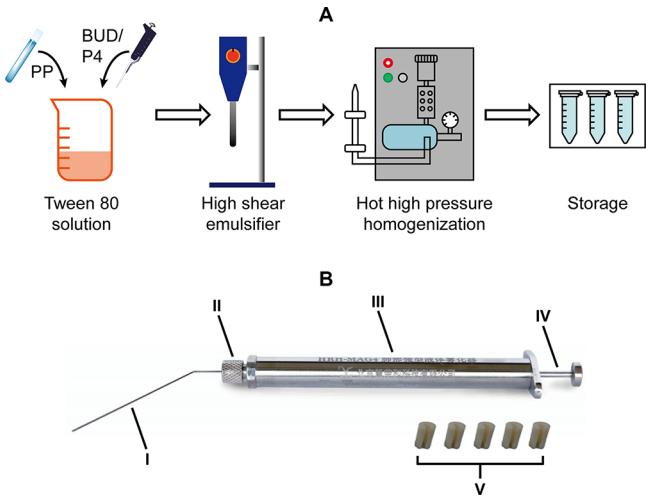

Figure 1. A graphical representation of the preparation procedures of SLNS formulations (A) and a typical image of the HM device monotype (B). (I) Delivery tube, (II) micronebulizer, (III) sample cell, (IV) piston, and (V) accessories volume-fixer. Abbreviations: HM, HRH-MAG 4 device.

The related formulation details and processing parameters are summarized in Table 1. For simplicity, the blank, BUD-loaded, and P4-loaded SLNS formulations were coded as b-SLNS, BUD-SLNS, and P4-SLNS, respectively; the SLNS with different particle sizes were denoted as SLNS1−SLNS4.

Physicochemical Characterization of SLNS. *Particle Size Analysis and Zeta-Potential (ZP) Examination.* The particle size and ZP of the SLNS were determined using a Zetasizer Nano ZS90 (Malvern Instruments Ltd., Worcestershire, U.K.). The SLNS was diluted by 120-fold with ultrapure water prior to the tests to ensure a proper count rate (ca. 300 kcps). The particle size and ZP were analyzed at 37 °C. Ten scans and 20 scans were performed for each measurement of the particle size analysis and ZP determination, respectively, and three parallel measurements were conducted.

Density (ρ) Determination. Ultrasonication (15 s) was performed on the SLNS formulations prior to ρ determination. One hundred microliters of the SLNS formulations was precisely weighed, and ρ was calculated by the weight divided by the volume. The measurement was performed in triplicate.

Drug Loading (DL) and Encapsulation Efficiency (EE) Determinations. The prepared SLNS consisted of the suspended nanoparticles and the bulk aqueous phase.[34] A certain fraction of the drugs was distributed in the bulk aqueous phase because of loading failure and/or leakage of nanoparticles during the fabrication and storage process.[35] Thus, the DL and EE of both the suspended nanoparticles (termed as DL_{SLN} and EE_{SLN}) and the entire SLNS formulations consisting of the suspended nanoparticles and the aqueous phase (DL_{SLNS} and EE_{SLNS}) were determined. Ultrafiltration tubes (Merck KGaA, Darmstadt, Germany) were employed in the assay. The details of the measurements of the DL_{SLN}, EE_{SLN}, DL_{SLNS}, and EE_{SLNS} can be found in the Supporting Information, Section 1.

Dynamic Viscosity Evaluation. The dynamic viscosity of b-SLNS, BUD-SLNS, and P4-SLNS was investigated using a Kinexus Lab$^+$ Rheometer (Malvern Instruments Ltd., Worcestershire, U.K.). For each sample, approximately 4 mL was applied to the sample stub. The viscosity was recorded within the shear rate of 10−100 s^{-1} at 25 °C according to a standard operation procedure provided by the rSpace operation software (Malvern Instruments Ltd., Worcestershire, U.K.). The cone used in the tests was CP 4/60 (60 mm diameter, 4° cone angle, and 0.03 mm gap truncation).

Transmission Electron Microscopy (TEM). The freshly produced SLNS formulations, including b-SLNS1−4, BUD-SLNS1−4, and P4-SLNS1−4, were subjected to examination by TEM. The samples were carefully placed on copper grids and then negatively stained with a 1% (w/v) phosphotungstic acid solution. The excess solution was absorbed prior to being examined by a 100 kV JEM-100 CX II transmission electron microscope (JEOL Ltd., Tokyo, Japan).

In Vitro and in Vivo Validation of the HM Device. The HM device was utilized for the in vitro aerosolization and ETA of SLNS. Detailed information about the composition and application of HM was as follows.

A typical image of HRH-MAG4 is shown in Figure 1B. It includes four parts, i.e., a delivery tube (I), a micronebulizer (II), a sample cell (III), and a piston (IV), with an accessory volume-fixer (V). The delivery tube was placed in the liquid sample, and then the piston was used to generate a difference in pressure for sample loading. Ultrasonication (15 s) was performed on the samples prior to sample loading. The piston was held for at least 15 s for a sufficient loading. Three volume-fixers were installed on the rod of the piston. One, two, or three volume-fixers could be removed in order to aerosolize 50, 100, or 150 μL of sample (50 μL per volume-fixer).

In Vitro Emission Ratio (ER) Determination. The weight of 50 μL of each sample (m), viz. b-SLNS, BUD-SLNS, and P4-SLNS, was calculated on the basis of the determined ρ values. Subsequently, 150 μL samples were successively loaded into the HM. The initial weight of the loaded device was denoted as m_{150}. Eppendorf tubes with the opening covered with paraffin film were used as the receptor for the aerosolized samples. The paraffin film was carefully penetrated by the

Table 1. Formulation Composition and Processing Parameters for SLNS Formulations

formulation[a]	PP amount (g)	Tween 80 content (%, w/v)	Tween 80 volume (mL)	homogenization temperature (°C)	homogenization pressure (bar)	homogenization cycle
b-SLNS1	1.5	2.0	50	70	1250	20
b-SLNS2	3.5	0.5	50	70	750	20
b-SLNS3	7.5	0.5	50	70	750	35
b-SLNS4	9.5	0.5	50	80	750	55

[a]UD-SLNS and P4-SLNS were produced by adding BUD or P4 stock solution into b-SLNS1−b-SLNS4.

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delivery tube of HM, and continuous aerosolization was performed in one step. After the in vitro aerosolization of the 50, 100, and 150 μL samples, the device was weighed, and the weights were recorded as m_{100}, m_{50}, and m_0. The ER for each aerosolization volume (ER_{50}, ER_{100}, and ER_{150}) was obtained by eq 1:

$$ER_x = \frac{m_{150} - m_y}{m} \times 100\% \quad (1)$$

where ER_x represents ER_{50}, ER_{100}, or ER_{150}. For ER_{50}, ER_{100}, or ER_{150}, $m_y = m_{100}$, m_{50}, or m_0, respectively.

Influence of in Vitro Aerosolization on the Physicochemical Properties of the SLNS Formulations. The formulations of the SLNS that had been aerosolized in vitro were carefully collected. Their physicochemical properties including particle size distribution, ZP, DL, and EE were evaluated as described previously. Negligible influence of aerosolization by HM could be confirmed if no distinct change in physicochemical properties was observed.

Influence of in Vitro Aerosolization on the Fluorescence Spectrum of P4. The P4-SLNS formulations before and after in vitro aerosolization were dissolved in acetonitrile in the 70 °C water bath. Sample dissolution was accomplished within a sealed container to avoid evaporation of the acetonitrile. The resulting solution was filtered through a 0.22 μm nylon-66 membrane, and the fluorescence spectrum was scanned (Fluoromax-4, Horiba Instruments, Inc., Albany, NY) under ambient conditions. The scanning was carried out using a slit-width of 2 nm. The excitation wavelength was set at 651 nm, and the emission wavelength range was 350−850 nm with 1 nm step-size. An acetonitrile solution of P4 was selected as a reference for this test.

Sample Residues Assessment. The residual amount of BUD-SLNS and P4-SLNS in the sample cell of HM was quantified. After in vitro aerosolization, the device was rinsed thoroughly with 10 mL of hot ethanol absolute (c.a. 70 °C). The resulting solution was vacuum-dried, and the residue was reconstituted in 200 μL hot acetonitrile (c.a. 70 °C). Sample dissolution was completed within a sealed container to avoid evaporation of the acetonitrile. The supernatant was filtered through a 0.22 μm nylon-66 membrane and then assayed. Thereafter, the processed device was extracted by ethanol absolute for a second time and chromatographically quantified to determine whether any residue remained after one time of washing-off.

In Vivo Pulmonary Deposition Determination. BUD-SLNS1−BUD-SLNS4 were administered to SD rats (n = 5). Briefly, SD rats were anesthetized with 5 mL/kg 20% urethane (w/v) i.p. and immobilized before ETA. The trachea was visualized with the help of a mini laryngoscope for rats (Huironghe Technology Co., Ltd. Beijing, China). The delivery tube of HM (BUD-SLNS-loaded) was carefully introduced into the trachea anterior to the first bifurcation. The BUD-SLNS formulations (100 μL) were aerosolized into the respiratory tract of the SD rats. The animals were immediately sacrificed by cervical dislocation, and the lungs were surgically excised. The visible bronchi were carefully removed.

The BUD in harvested lungs was assayed as described in the In Vitro and in Vivo Validation of the HM Device section. On the basis of the determined pulmonary BUD concentration, dose recovery was calculated, including total recovery and emission recovery.

$$\text{Total recovery (\%)} = \frac{\text{Recovered BUD amount in the lung}}{\text{Loaded BUD amount}} \times 100\% \quad (2)$$

$$\text{Emission recovery (\%)} = \frac{\text{Recovered BUD amount in the lung}}{\text{Emitted BUD amount}} \times 100\% \quad (3)$$

Fluorescence Bioimaging Study. Fluorescence bioimaging was conducted using a NightOWL II LB983 instrument (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany). Photographic images of the SD rats before and after ETA were acquired. For the in vivo bioimaging, the SD rats were administered the P4-SLNS1−P4-SLNS4 by the same method used in section 2.6.4. The abdominal hair of SD rats was removed by hair removal cream (Veet, Reckitt Benckiser Group, Plc., Hubei, China) to reduce the autofluorescence. The lungs of the administrated SD rats were excised, and ex vivo bioimaging was performed. The parameters for the bioimaging were set as follows: exposure time 0.25 s for in vivo bioimaging and 0.10 s for ex vivo bioimaging, high gain (feedback) for in vivo imaging and low gain (feedback) for ex vivo imaging, excitation wavelength 630 nm, emission wavelength 680 nm, sample size 160 mm, and sample height 22 mm. The quantification of the fluorescence signal was conducted using the instrument-associated Indigo software.

Chromatography. *Instrumentation and HPLC Conditions.* Quantification of BUD and P4 was performed using a Dionex Ultimate 3000 instrument (Dionex Solution GmbH, Germering, Germany) that was equipped with a variable wavelength detector (VWD). A Luna C_{18} column (250 mm × 4.6 mm, 5 μm, Phenomenex, Inc., Torrance, CA) was utilized for separation. The mobile phase was water−acetonitrile (40:60, v/v) for BUD and water−acetonitrile (18:82, v/v) for P4. The detection wavelengths for BUD and P4 were 240 and 651 nm, respectively. The flow rate was set to 1.0 mL/min, and the injection volume was 20 μL. The validation of this method is shown in Tables S1 and S2.

Pretreatment of Lung Samples Containing BUD. The harvested lungs were washed with cold saline to remove the contaminating blood. Then, the lungs were cut into pieces, and saline (5 mL per 1 g of lung) was added. The suspended tissue was then homogenized (IKA-Werke GmbH, Deutschland, Germany). The resulting homogenate was pretreated as follows.

Three hundred microliters of acetic acid (0.1 mg/mL) was added to 1 mL of the homogenate for deproteinization. The solution was then spiked with the internal standard, 200 μL of a 5 μg/mL TAA solution in methanol. The BUD was extracted twice with 2 mL of ethyl acetate. The supernatant was collected after 5000 r/min centrifugation for 15 min at 4 °C (GL-20C, Anting Scientific Instrument Factory, Shanghai, China), and then vacuum-dried. The residue was reconstituted with 250 μL of the mobile phase. Finally, the solution was filtered through a 0.22 μm nylon-66 membrane and assayed. Vortex-mixing was conducted when necessary.

Data Expression and Statistics. The data are reported as the means ± SD where applicable. If necessary, grouped data were analyzed by one-way ANOVA with Bonferroni multiple comparisons using SPSS 19.0 software (IBM Corporation, Armonk, NY). In all cases, $p < 0.05$ was recognized as statistically significant.

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