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Confocal imaging of rat lungs following intratracheal delivery of dry powders or solutions of fluorescent probes

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Abstract

The overall pulmonary disposition of various fluorescent probes was viewed by confocal imaging following intratracheal delivery in the rat in vivo. The green fluorescent dyes, coumarin-6, a 350 Da lipophilic molecule; calcein, a 623 Da hydrophilic molecule; or FITC–albumin, a 65 000 Da hydrophilic molecule; were insufflated as a dry powder or instilled as a solution in the lungs of rat in vivo. Immediately, 2 or 24 h following delivery, the lungs were colored with sulforhodamine and fixed by vascular perfusion. The lungs were then removed, grossly sliced and examined by confocal laser scanning fluorescence microscopy. Coumarin-6 diffused within minutes across the trachea, airways and alveolar tissue but was also retained for hours in type II alveolar epithelial cells. The diffusion of calcein across the tissue was fast as well, with no particular affinity for specific cells. FITC–albumin slowly permeated the tissue. It remained in the airspaces for hours and was intensively captured by alveolar macrophages. Compared to the powder, the solution bypassed dissolution and therefore shortened the lag time for diffusion and cellular capture. The technique allowed to obtain an overview of the fate of fluorescent probes locally in each region of the lungs and highlighted the strong dependence of the localization behavior on physico–chemical properties of molecules as well as a capture by particular cells of the pulmonary tissue.

Keywords: Pulmonary disposition; Fluorescent markers; Pneumocytes and macrophages; Confocal microscopy; Dry powder

1. Introduction

Unique physiological characteristics make the lung an attractive port of entry to the systemic circulation for the administration of drugs. (i) The alveoli present a large surface for absorption (100 m^2), immediately accessible to drugs; (ii) a very thin diffusion path separates the airspace from the bloodstream, i.e., the alveolar epithelium, the vascular endothelium and their respective basal membranes are less than 0.5 μm thick; (iii) the high blood flow (5 l/min) of the pulmonary circulation rapidly distributes molecules throughout the body, without first-pass hepatic metabolism; (iv) the metabolic activity locally in the lungs is relatively low [1,2]. These features, together with the design of new inhalers that have a high efficiency and reproducible delivery, have led systemic drug delivery by inhalation to successful clinical development [3–5].

Although systemic bioavailabilities following pul-

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monary drug administration can achieve values close to 100% in the case of small molecules, they can also be as low as a few percents for peptide and protein therapeutics due to significant biological losses occurring in the lungs [3,6–8]. Drug losses due to passage through the lungs can be quantified based on pharmacokinetic studies and pulmonary deposition data collected in vivo. For instance, since inhaled insulin reaches 10% bioavailability in clinical trials [9], and given an average pulmonary deposition of 60% of the nominal dose [3,6–8], it follows that only one or two molecules of insulin out of 10 deposited in the human lungs reach the bloodstream. The local losses of therapeutics that occur in the lungs and decrease systemic absorption have not been ascribed to any precise mechanism up to now [3,8].

The aim of our study was to obtain an overview of the pulmonary disposition of molecules, as a first step towards the identification of the mechanisms involved in the local drug losses. The fate of small molecular weight compounds of various solubility in the lungs of rats was compared to that of a model protein, FITC–albumin, using confocal imaging. The impact of the physical state of the inhaled aerosol, dry powder or solution, on overall pulmonary disposition was assessed as well.

2. Materials and methods

2.1. Chemicals

Human and rat serum albumin (fraction V, 96–99% albumin), sulforhodamine, 96% ethanol and cacodylate sodium were obtained from Sigma (st. Louis, USA), α-lactose monohydrate from Acros Organics (New Jersey, USA) and dipalmitoylphosphatidylcholine (DPPC) from Lipoid (Lipoid GMBH, Ludwigshafen, Germany). High purity calcein (>95%) was purchased from Molecular Probes (Leiden, The Netherlands) and coumarin-6 from ICN (ICN Biomedicals Inc, Ohio, USA). The percent of lipophilic contaminants in calcein was found negligible by thin layer chromatography [10].

Formaldhehyde was bought from Merck Eurolab (Leuven, Belgium), glutaraldehyde from Fluka (Neu-Ulm, Switzerland). Maclura pomifera agglutinin biotinylated lectins and Texas Red streptavidin were obtained from Vectors Laboratories (Burlingame, CA, USA) and Hank's balanced salt solution (HBSS) without Ca^{2+}, Mg^{2+} and phenol-red from Life Technologies (Merelbeke, Belgium).

2.2. Fluorescence labeling of albumin with FITC

Fluorescein isothiocyanate (FITC) Isomer I is among the most widely used fluorescent labeling reagents due to the fluorophore's high quantum efficiency and conjugate stability. FITC reacts with free amino groups of proteins to form stable conjugates.

20 μl of FITC solution (100 mg/ml in dimethylsulfoxide) was added drop-wise per 1 ml of albumin solution (10 mg/ml in bicarbonate buffer, pH 8.8, 0.1 M). The solution was incubated 3 h at room temperature with gentle stirring and protected from light. It was then placed in a dialysis tubing (cut-off 10 000 Da; Snakskin Dialysis Tubing; Perbio Science, Erembodegem-Aalst, Belgium) in order to eliminate the largest part of the unconjugated FITC. The labeled albumin (FITC–albumin) was further purified from the unconjugated FITC by a Sephadex G-25M column (Amersham Pharmacia Bioteck Benelux, Roosendaal; The Nederlands). The FITC–albumin solution was then dialyzed against ultrapure water for salt elimination. Finally, the solution was lyophilized to obtain a powder of FITC–albumin. In this way, human and rat albumin were labeled with 1 and 3 mol FITC per mol albumin, respectively. A sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) carried out on FITC–albumin and native albumin indicated that the molecular weight of albumin was intact following the labeling procedure.

2.3. Formulation of the dry powders

Dry powders were prepared with albumin, lactose, DPPC (20/20/60 w/w/w) and different green fluorescent dyes, by spray-drying [11,12]. Albumin and lactose were dissolved in ultrapure water. The pH was adjusted to 7 by addition of NaOH (Merck Eurolab, Leuven, Belgium). DPPC was dissolved in 96% ethanol. The two solutions were then combined

to form an 80% ethanolic solution of 0.1% w/v total powder concentration.

Coumarin-6 was dissolved in ethanol with DPPC and incorporated in the formulation at a load of 1% w/w. Calcein was dissolved in the aqueous solution with albumin and lactose, and incorporated at a load of 0.5% w/w in order to obtain a similar fluorescence intensity as that of the coumarin-6 powder. For the studies with FITC–albumin, the unlabeled albumin was replaced with the protein custom-labeled with FITC (see Section 2.2 above) and powders of composition FITC–albumin/lactose/DPPC (20/20/60 w/w/w) were prepared. The fluorescence intensity per mg FITC–albumin powder was only a 1/35 fraction of that of the coumarin and calcein powders.

The powders were produced using a LabPlant laboratory-scale spray-dryer (Lab-Plant Limited, Huddersfield, UK). The solutions were pumped into the drying chamber at a rate of 20 ml/min and pneumatically atomized through a two fluids external mixing 0.5 mm nozzle using compressed air at 1.5 bar. The inlet temperature was established at 110 °C; in these conditions, the outlet temperature was worth approximately 50 °C. The powders were collected and stored in a dessicator (at room temperature and 25% relative humidity; [12]). A SDS-PAGE carried out on FITC–albumin released from the particles indicated that the molecular weight of albumin was intact following spray-drying [13].

2.4. Particle size, density and aerosolization properties of the powders in vitro

The particle diameter (d) was measured by laser diffraction in the dry state following dispersion with compressed air at a pressure of 3 bar (Sympatec GmbH, Clausthal-Zellrfelg, Germany; [14]). The powder density (ρ) was determined by tap density measurements, i.e., following 1000 taps which allowed the density to plateau [15]. The theoretical aerodynamic diameter of the individual particles, d_{aer}, was calculated based on the following definition:

$$d_{aer} = \sqrt{\frac{\rho}{\rho_1}}d, \quad \text{with } \rho_1 = 1\ \text{g/cm}^3\ [16].$$

The pulmonary deposition of the dry powders was assessed in an Andersen cascade impactor equipped with a USP induction port (1 ACFM eight stage non-viable cascade impactor, Graseby Andersen, Atlanta, GA) using a Spinhaler™ device (Fisons, Bedford, MA) at a flow rate of 28.3 l/min (flowmeter type GMTX, Platon, Hampshire, UK). The trays of the impactor were coated with an hydroxypropylmethylcellulose gel (22.5% w/v in water). The powder deposited on the different levels was recovered by immersing each tray and the stage below in 80% ethanol. The powder deposited in the throat and pre-separator was also collected. After dissolution of the particles, the fluorescence of each solution due to coumarin, calcein or FITC–albumin was determined using a Perkin Elmer Luminescence Spectrometer LS50B [12].

The emitted dose was determined as the percent of total powder mass exiting the capsule. The cumulative mass of powder less than the stated size of each stage of the Andersen impactor was calculated and plotted on a log probability scale, as percent of total mass recovered in the impactor against the effective cut-off diameter. The experimental mass median aerodynamic diameter of the particles is defined from this graph as the particle size at which the line crosses the 50% mark and the geometric standard deviation (GSD) as $\text{GSD} = \sqrt{\frac{\text{Size } X}{\text{Size } Y}}$, where size X is the particle size for which the line crosses the 84% mark and size Y the 16% mark. The fine particle fraction was calculated from the same plot as the fraction of powder emitted from the inhaler with an aerodynamic diameter ≤ 5 μm [17].

2.5. Intratracheal delivery in vivo and lung preparation for microscopy

Wistar male 7–10 week-old rats (200–350 g; Iffa Credo, Saint Germain les Arbreles, France) were anesthetized using ketamine/xylazine (90/10 mg/kg) intraperitoneal injection. An incision was made in the ventral cervical region, and the proximal trachea exposed. A small metal tube (18 G*15 mm; Terumo, Leuven, Belgium) was loosely inserted between two cartilaginous rings. Two milligrams of the powder was placed in the inhalation port of a Harvard Ventilator (Harvard Apparatus, Ltd.; Edendridge, UK) and insufflated in the lungs through the tube; or a solution (100 μl) of the probe in PBS

(calcein and FITC–albumin) or in 80% ethanol (coumarin-6) was intratracheally instilled. In order to study the effect of the FITC–albumin dose, FITC-labeled human albumin from Sigma, 10 times more labeled than our custom-labeled albumin, was instilled in the lungs at a dose of 40 μg.

At various pre-determined times following intratracheal delivery (0–1 min, 2 and 24 h), an abdominal incision was made and the posterior vena cava cannulated with a 16-gauge catheter (Ohmeda, Helsingborg, Sweden) connected to a double reservoir containing two solutions: (1) phosphate buffered saline (PBS; 138 mM NaCl, 8.1 mM Na_2HPO_4, 1.1 mM KH_2 PO_4, 2.7 mM KCl, pH 7.4) containing sulforhodamine (0.1%); and (2) a fixative solution (0.6% formaldhehyde–0.9% glutaraldehyde solution in cacodylate buffer, pH 7.4 75 mM) with 0.1% sulforhodamine. Both carotids and jugulars were cut, and solution (1) was perfused through the vasculature at a flow rate of 10–15 ml/min for 5 min. Lung fixation via the pulmonary vasculature was then carried out using solution (2) at a flow rate of 4 ml/min for 5 min [18]. The thoracic cavity was opened and the lungs removed for analysis by confocal laser scanning microscopy. The total time for perfusion and lung preparation for microscopy was $\leq$30 min. Each experimental condition was repeated at least three times.

All experimental protocols in rats were approved by the Institutional Animal Care and Use Committee of the Université catholique de Louvain.

2.6. Confocal laser scanning microscopy

Confocal Laser Scanning Microscopy (CLSM) was chosen to localize coumarin-6, calcein and FITC–albumin in the pulmonary tissue as this technique allows localization of fluorescent molecules without distortion of the living tissue. Slices ($\pm$2 mm) of the lobes and trachea were directly placed in a sample holder and covered with a coverslip glass. The confocal microscope was a BioRad MRC 1024 confocal unit equipped with an argon–krypton laser and mounted on a Zeis Axiovert 13SM inverted microscope. Laser excitation wavelengths of 488 nm and 568 nm were used individually to scan lung tissue, and fluorescent emissions from coumarin-6, calcein, FITC–albumin (emission λ=515–545 nm), and sulforhodamine (emission λ=589–621 nm) were collected using separate channels. Images were acquired with a Zeiss Plan-Neofluor 40$\times$ oil immersion and 10$\times$ lens. Grayscale images (obtained from each scan) were pseudo-colored green (coumarin-6, calcein, FITC–albumin) and red (sulforhodamine) and then overlaid (Zeiss LSM confocal software) to form a multicolored image.

To assess the autofluorescent properties of the pulmonary tissue, samples were examined with the confocal microscope in the absence of administration of fluorescent markers. The autofluorescence of the rat lung in the green channel was found to be very low with the confocal settings used in this study, except in peripheral regions where fiber networks and cells exhibited high autofluorescence (data not shown). In order to avoid these areas of high fluorescence background, confocal images were only collected in regions at least 10 μm below lung surface.

2.7. Bronchoalveolar lavage

The capture of FITC–albumin by alveolar macrophages was assessed by analyzing the cellular components of bronchoalveolar lavage (BAL). The airways and lungs were washed with Hank's balanced salt solution (HBSS) without Ca^{2+}, Mg^{2+} and phenol-red, in order to minimize cell clumping. The BAL was obtained by slowly injecting 10 ml of HBSS into the trachea, waiting for 30 s and then withdrawing the liquid from the lungs. The lavage procedure was repeated with four additional 10 ml aliquots until a total volume of 50 ml was injected. The BAL was centrifuged at 700 *g* for 10 min at 4 °C. The supernatant was removed and the cells were resuspended in 1 ml HBSS. A few droplets of the cells suspension were directly placed in a sample holder and covered with a coverslip glass for analysis by CLSM.

2.8. Lectin staining

Coumarin-6 appeared to accumulate specifically in particular cells of the alveolar tissue, which were suspected to be type II alveolar epithelia cells. In order to precisely identify these cells, the lungs were

stained with a lectin, Maclura pomifera agglutinin (MPA) which binds specifically to the surface of alveolar epithelial type II cells [19]. Thirty minutes after inhalation of the powder incorporating coumarin-6, the airways and lung were washed twice with HBSS (10 ml BAL) in order to remove the macrophages, and then intratracheally instilled with 5 ml of MPA biotinylated lectin (50 μg/ml in HBSS). After 1 h of incubation, the lungs were rinsed three times (BAL with 10 ml HBSS) and intratracheally injected with 5 ml Texas Red steptavidin (20 μg/ml in HBSS). After 1 h incubation, the lungs were rinsed three times (BAL with 10 ml HBSS) and removed for visualization by confocal microscopy.

3. Results

Coumarin-6, calcein or FITC–albumin were delivered as inhalation dry powders or solutions to the lungs of rats in vivo. At pre-determined times following intratracheal delivery, the lungs were colored with sulforhodamine, a red fluorescent dye, fixed, removed and grossly sliced for visualization by confocal laser scanning microscopy.

The dry powders were prepared by spray-drying using albumin, lactose and dipalmitoylphosphatidylcholine and contained one of the above green markers [11,12]. They presented original physical characteristics which made them particularly suited to deposition in the deep lungs, that is, an average particle geometric size of 4.5 μm, an average bulk tap density of 0.08 g/cm^3 and an average primary particle aerodynamic diameter of 1.2 μm. The aerosolization behavior of the dry powders was estimated in the Andersen cascade impactor operated at 28.3 l/min using a Spinhaler™ inhaler device. The emitted doses ranged between 65 and 79%, the fine particle fractions between 41 and 59%, the experimental mass median aerodynamic diameters (MMAD) between 4.3 and 5.9 μm and geometric standard deviations between 1.4 and 2.0. The MMADs were 4-fold larger than the primary aerodynamic diameters, indicating that the powder only partially deaggregated at this low airflow rate using the Spinhaler™ device.

3.1. Pulmonary disposition of coumarin-6

Coumarin-6, a 350 Da lipophilic molecule (log $P = 5.43$, as calculated by the Rekker method [20]), diffused across the trachea, airways and alveolar tissue within minutes after insufflation of the dry powder (Fig. 1a–c). Additionally, it appeared to accumulate in particular cells of the alveoli (Fig. 1c). The diffusion across the trachea was almost completed within 2 h 30 min after delivery. Some particles of coumarin-6 remained on the surfaces of the airways for a few hours, but 24 h following delivery, no more green fluorescence was visible in the airspaces or the tissue of the bronchioles (data not shown). In the alveoli, coumarin-6 was apparent in the airspace fluid and tissue for a few hours as well, but the cells capturing the dye still showed some green fluorescence at 24 h (data not shown). Overall, the same pulmonary disposition was observed following instillation of a coumarin-6 solution (Fig. 1e), indicating that the physical state of coumarin-6 as well as the excipients of the dry powder had no significant impact on its pulmonary fate.

Fig. 1. (opposite, top) Confocal imaging of the trachea (a), a bronchiole (b) and alveoli (c) 30 min following the pulmonary administration of a dry powder containing coumarin-6 (green) in vivo. The tissue is labeled in red with sulforhodamine. (d) Same picture as (c) but only the green channel. The plain arrows indicate particular cells of the alveolar tissue that captured coumarin-6; the dotted arrows show that coumarin-6 did not permeate the cell nuclei. (e) Alveoli 30 min after intratracheal instillation of a solution (100 μl) of coumarin-6. (f) The tissue staining with sulforhodamine and fixation were not carried out following coumarin-6 delivery; instead, the surfaces of the type II alveolar epithelial cells were labeled in red with Maclura pomifera agglitinin lectin. The red fluorescence from the lectin (double line arrows) was superimposed on the cells capturing coumarin-6 (plain arrows). Scale bars are 100 or 50 μm.

Fig. 2. (opposite, bottom) Confocal imaging of the trachea (a), a bronchiole (b) and alveoli (c) 30 min following the pulmonary administration of a dry powder containing calcein (green) in vivo. The tissue is labeled in red with sulforhodamine. (d) Same picture as (c) but only the green channel. (e) Alveoli 30 min after intratracheal instillation of a solution (100 μl) of calcein. (f) Calcein disposition in the alveoli was viewed without tissue staining with sulforhodamine and fixation. Scale bars are 100 or 50 μm.

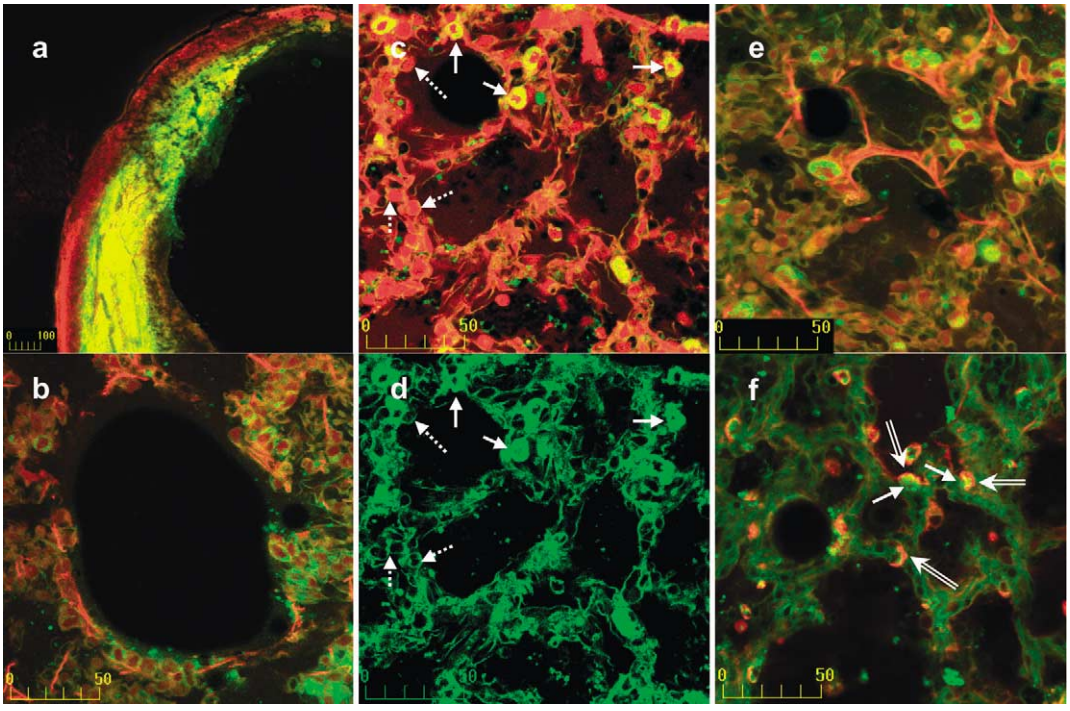
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Fig. 1.

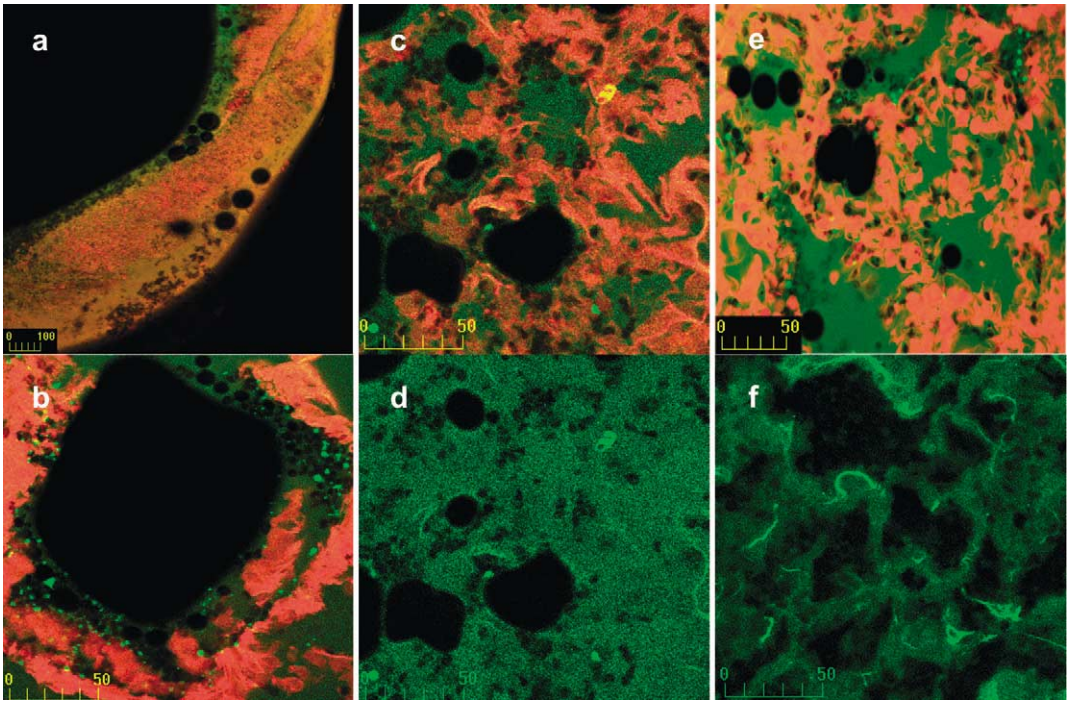
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Fig. 2.

The diffusion of coumarin-6 in the airways and alveolar tissue was not homogeneous. Sharp fluorescent margins surrounded a non-fluorescent dark interior, suggesting that coumarin-6 did not penetrate in the nuclei of the cells (Fig. 1c,d). In addition, some cells appeared more brightly illuminated than others, the cytoplasm displaying intense punctate fluorescence (Fig. 1c–e); these cells were cuboidal, and located in the corners of the alveoli where type II alveolar epithelial cells are found.

In order to confirm the identity of the cells that accumulated coumarin-6, type II alveolar epithelial cells were specifically labeled using Maclura pomifera agglutinin lectin [19]. The red fluorescence from the lectin was superimposed on the cells highly emitting in the green channel, demonstrating that the cells capturing coumarin-6 were principally the type II alveolar epithelial cells (Fig. 1f).

3.2. Pulmonary disposition of calcein

The pulmonary disposition of calcein, a 623 Da hydrophilic molecule (log $P = -1.1$, according to Rekker), was totally different from that of coumarin-6, the lipophilic dye. Although transport was as fast in the trachea, airways and alveoli following dry powder insufflation, the dissolution of calcein was more rapid -no particles were visible in the airways or alveoli 2 h 30 min following delivery—and its diffusion within the tissue was homogeneous, that is, calcein penetrated uniformly in the cytoplasm and nucleus of the cells and no preference for particular cells of the tissue was apparent except for a capture by rare alveolar macrophages (Fig. 2 and data not show). No modifications to calcein disposition were observed following delivery as a solution instead of a powder (Fig. 2e).

In order to verify that the preparation of the tissue for confocal microscopy did not alter the localization behavior, calcein disposition was visualized in the tissue without staining with sulforhodamine and fixation. The diffusion of calcein was fast and homogenous in the tissue and mucus as well (Fig. 2f).

3.3. Pulmonary disposition of FITC–albumin

In contrast to coumarin-6 and calcein, the pulmonary disposition of FITC–albumin, a hydrophilic macromolecule of 65 000 Da, occurred over several hours (Fig. 3). Thirty minutes following dry powder delivery, FITC–albumin appeared as clusters of particles and/or in soluble form on top of the tissue surfaces in the trachea, airways and alveoli (Fig. 3a–c). The dissolution and/or diffusion of FITC–albumin from the particles was completed only after 2 h 30 min (Fig. 3d–e). The majority of the dissolved labeled protein was then located in the airspace fluid and an intense capture by macrophages took place (Fig. 3e). The penetration of FITC–albumin in the tissue was low and took several hours (Fig. 3f). Twenty-four hours following delivery, FITC–albumin remained apparent only in macrophages in the airspaces of the airways and alveoli (data not shown).

The intratracheal instillation of a FITC–albumin solution bypassed the steps of dissolution and diffusion that were observed in the case of the dry powder and, as a result, the capture of the protein by alveolar macrophages initiated earlier. This is shown in Fig. 3g where, 30 min after delivery, the FITC–albumin instillate appeared homogeneously spread in the airspace with some protein already loaded in alveolar macrophages. Some particulate FITC–albumin was also visible in the airspace (Fig. 3g).

The capture of FITC–albumin by alveolar macrophages was substantiated and the influence of the dose and species source of albumin on this capture investigated as follows. A large number of macrophages collected by bronchoalveolar lavage 2 h following delivery of the FITC–albumin dry powder were stained in green (Fig. 3h). In our experiments, 400 μg of FITC-labeled human albumin was delivered as inhalation dry powder, while the normal albumin content in the lungs of rats ranges between 500 μg and 1 mg [21,22]. Therefore, the dose of albumin administered was perhaps too important and elimination processes took place. However, the instillation of 40 μg of FITC–albumin induced the same capture by alveolar macrophages. Finally, the influence of the species source of albumin was investigated. Alveolar macrophages appeared as intensively fluorescent following instillation of FITC–albumin from rat as from human, indicating that the origin of albumin had no obvious effect on the phenomenon (Fig. 3i).

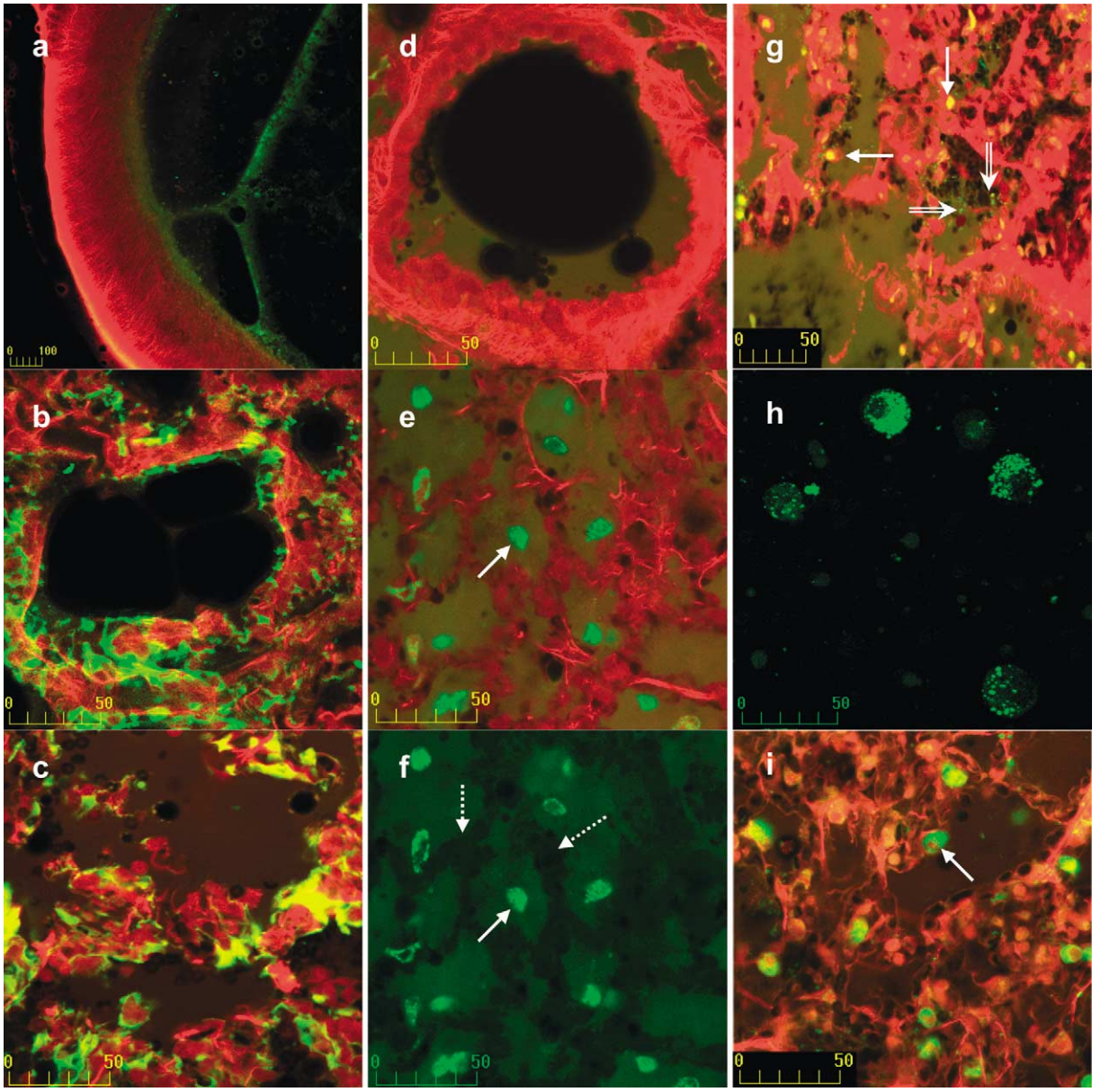

Fig. 3. Confocal imaging of the trachea (a), a bronchiole (b, d) and alveoli (c, e) 30 min (a, b, c) or 2 h 30 min (d, e) following the pulmonary administration of a dry powder containing FITC–albumin (green) in vivo. The tissue is labeled in red with sulforhodamine. (f) Same picture as (e) but only the green channel. The plain and dotted arrows indicate alveolar macrophages including significant FITC–albumin and the slight permeation of FITC–albumin in the tissue, respectively. (g) Alveoli 30 min after intratracheal instillation of a solution (100 μl) of FITC–albumin. The plain and double line arrows indicate alveolar macrophages loading FITC–albumin and particulate FITC–albumin in the airspaces, respectively. (h) Macrophages recovered by bronchoalveolar lavage 2 h after insufflation of the FITC–albumin powder. (i) Alveoli 2 h 30 min after instillation of 40 μg FITC-labeled rat albumin (100 μl instillate); the plain arrow shows a macrophage accumulating rat albumin. Scale bars are 100 or 50 μm.

4. Discussion

In this article, we present a confocal imaging technique that allows us to obtain an overview of the pulmonary disposition of fluorescent probes following delivery to the lungs of rats in vivo. In contrast to the confocal microscopy studies performed previously by Pohl et al. [23,24], we viewed the disposition of the fluorescent compounds in the trachea, bronchi as well as alveoli, investigated the fate of a large protein and studied the impact of delivering molecules as inhalation dry powders or solutions. Additionally, in comparison with other microscopy techniques, our method of tissue preparation was rapid (less than 30 min; [18,25], did not involve micro-dissection [18] or tissue incubation in various solutions, embedding, freezing and/or dehydration [25], which might lead to profound disruption of the native milieu of the lungs and significant loss of the probe. Finally, in comparison with in vitro models of alveolar and airway epithelial cell monolayers where pulmonary transport across one cell type at a time is investigated [26], we simultaneously assessed the involvement of all cell types in the fate of molecules locally in the lungs. This permitted us to specifically highlight the uptake of molecules by particular cells of the respiratory tract.

Significant differences in the localization behavior and clearance from the lungs between the diverse fluorescent probes were evident (Figs. 1–3). The small molecules, coumarin-6 and calcein, diffused within minutes across the trachea, airways and alveolar tissue, while FITC–albumin remained for hours in the airspaces and displayed only a low penetration in the tissue. This agrees with previous pharmacokinetic studies that report a low (5% absolute bioavailability) and slow (16–24 h time to peak serum concentration) systemic absorption of instilled albumin in rats [27], in contrast to the complete and rapid (as fast as intravenous injection) absorption observed in the case of inhaled opioïds [6,7]. Differences appeared also in the interaction of molecules with the pulmonary cells. Coumarin-6 and FITC–albumin were captured preferentially by type II alveolar cells and alveolar macrophages, respectively. However, calcein did not exhibit any specific affinity for particular cells.

Coumarin-6, the small lipophilic dye, diffused within minutes across the pulmonary tissue but was also retained for hours in type II pneumocytes (Fig. 1c–e). Pohl et al. observed peripheral regions of intact unfixed rat lungs by confocal laser scanning fluorescence microscopy following intratracheal instillation or vascular perfusion of a rhodamine B solution, a lipophilic fluorescent marker [23]. Similarly to coumarin-6, rhodamine B brightly illuminated type II alveolar epithelial cells more than other cell types. Type II cells of the alveolar epithelium synthesize, store and secrete the pulmonary surfactant, a mixture of 90% neutral and anionic phospholipids and 10% proteins. Prior to secretion, this material is stored in vesicles called lamellar bodies and lipophilic molecules are likely to partition into such regions [28]. This phenomenon might also be related to some sustained-release effect and/or degradation, depending on its quantitative significance as well as the intra-cellular destiny of the molecule.

Unexpectedly, calcein permeated the pulmonary tissue through the cells without distinction of subcellular compartments (Fig. 2). Calcein carries four negative charges at physiological pH and is therefore highly polar. It has been shown to not permeate passively the highly ordered lipid bilayers of the stratum corneum, the skin outermost layer, as well as to not spontaneously escape from the interior side of liposomes [10,29]. The location behavior of calcein in the lungs was not due to contamination of calcein by lipophilic entities (see the Materials and methods section) or to tissue treatment before microscopy, since intact lung showed the same pictures of homogeneous diffusion (Fig. 2f). Pohl et al. viewed the peripheral alveoli following intratracheal instillation of 6-carboxy-fluorescein, a 376 Da hydrophilic probe, in rats [24]. Although penetration in the interstitium only through paracellular pathways was claimed, the confocal images displayed some passage of the green dye into alveolar cells. The transport pattern of calcein was additionally supported by sulforhodamine, a 607 Da hydrophilic molecule with a -1 charge, which exhibited the same transcellular diffusion as calcein following vascular perfusion (Figs. 1–3).

FITC–albumin was slowly absorbed by the pulmonary tissue, probably due to restricted diffusion of

the 7 nm globular protein through the limited number of large pores (>3 nm [30]) found in the alveolar epithelium, but also due to the ineffective active transport at the dose used [26]. The continuing presence of albumin in the airspaces that results renders it more susceptible to local degradation processes. The confocal images indicate that these processes can involve a capture by alveolar macrophages (Fig. 3e–i), some agglomeration of soluble protein (Fig. 3g), as well as simple mucociliary clearance. Accumulation of albumin in macrophages has been previously reported both in vivo and vitro and related to the phenomenon of pinocytosis, or endocytic uptake of fluid [25,31]. Hastings et al. [25] observed the alveolar region of rabbit lungs by light and electron microscopy following instillation of human albumin in vivo and showed that both type I and type II pneumocytes endocytosed albumin, but that alveolar macrophages took up protein earlier and more avidly than the epithelial cells. Ehrenreich et al. [31] showed that mouse peritoneal macrophages cultivated in vitro continually and vigorously engulfed ^{125} or ^{131}I-labeled human serum albumin dissolved in the medium and that the products of digestion were excreted primarily as ^{125} or ^{131}I-monoiodotyrosine. Here, pinocytosis was supported by the fact that the phenomenon occurred independently of the type of albumin formulation (powder or solution), albumin dose as well as species source (Fig. 3i). However, phagocytosis of particulate protein could also contribute to the uptake by macrophages (Fig. 3g).

No major differences in the pulmonary disposition of the three probes resulted from their physical state and/or from the excipients of the dry powder, except in the speed of disposition as illustrated by FITC–albumin (Fig. 1c,e; Fig. 2c,e; Fig. 3c,e,g). Delivering albumin as a solution bypassed the steps of dissolution, accelerated diffusion within the airspace fluid, and shortened the lag time for uptake by macrophages (Fig. 3c,e,g).

It is our intention to apply the present method to therapeutic peptides and proteins and to combine it with quantitative analysis of pulmonary disposition in order to precisely identify the most significant obstacles that the drug faces before systemic absorption and thereby develop adequate formulation strategies to increase bioavailabilities.

5. Conclusion

The fate of various probes in the lungs of rat was observed following in vivo delivery as inhalation dry powders or solutions. Coumarin-6 diffused within minutes across the trachea, airways and alveolar tissue but was also retained for hours in type II alveolar epithelial cells. The diffusion of calcein across the tissue was fast as well, with no particular affinity for specific cells. FITC–albumin slowly permeated the tissue. It remained in the airspaces for hours and was intensively captured by alveolar macrophages. The physical state of the probe did not cause any qualitative changes in pulmonary disposition; however, the additional step of dissolution of the powder significantly delayed molecular diffusion as well as the capture by alveolar macrophages in the case of FITC–albumin.

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