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In vitro evaluation of lysophosphatidic acid delivery via reverse perfluorocarbon emulsions to enhance alveolar epithelial repair

Diane L. Nelson^{a,*}, Yutong Zhao^b, Mario L. Fabilli^c, and Keith E. Cook^a

^aDepartment of Biomedical Engineering, Carnegie Mellon University, 5000 Forbes Avenue, Scott Hall 4th Floor, Pittsburgh, PA, 15213, USA

^bDepartment of Medicine, University of Pittsburgh, Division of Pulmonary, Allergy and Critical Care Medicine, East 1200A Biomedical Science Tower, 200 Lothrop Street, Pittsburgh, PA, 15213, USA

^cDepartment of Radiology, University of Michigan, 3226A Medical Sciences Building I, 1301 Catherine Street, Ann Arbor, MI, 48109, USA

Abstract

Background: Alveolar drug delivery is needed to enhance alveolar repair during acute respiratory distress syndrome. However, delivery of inhaled drugs is poor in this setting. Drug delivery via liquid perfluorocarbon emulsions could address this problem through better alveolar penetration and improved spatial distribution. Therefore, this study investigated the efficacy of the delivery of lysophosphatidic acid (LPA) growth factor to cultured alveolar epithelial cells via a perfluorocarbon emulsion.

Methods: Murine alveolar epithelial cells were treated for 2 h with varying concentrations (0–10 μ M) of LPA delivered via aqueous solution or PFC emulsion. Cell migration was evaluated 18 h post-treatment using a scratch assay. Barrier function was evaluated 1 h post-treatment using a permeability assay. Proliferation was evaluated 72 h post-treatment using a viability assay.

Results: Partially due to emulsion creaming and stability, the effects of LPA were either diminished or completely hindered when delivered via emulsion versus aqueous. Migration increased significantly following treatment with the 10 μ M emulsion ($p < 10^{-3}$), but required twice the concentration to achieve an increase similar to aqueous LPA. Both barrier function and proliferation increased following aqueous treatment, but neither were significantly affected by the emulsion.

Conclusions: The availability and thus the biological effect of LPA is significantly blunted during emulsified delivery *in vitro*, and this attenuation depends on the specific cellular function examined. Thus, the cellular level effects of drug delivery to the lungs via PFC emulsion are likely to vary based on the drug and the effect it is intended to create.

*Corresponding author: dlnelson@andrew.cmu.edu.

Disclosure

All authors have materially participated in the research and/or article preparation and have approved the final article.

Conflicts of interest

None.

Keywords

Acute respiratory distress syndrome; Partial liquid ventilation; Perfluorocarbon; Lysophosphatidic acid

1. Introduction

Despite recent advances, improved treatments are needed for Acute Respiratory Distress Syndrome (ARDS). Acute Respiratory Distress Syndrome is characterized by severe inflammatory damage to the lung alveolar epithelium and capillary endothelium, resulting in the build-up of proteinaceous edema in the alveoli and the decrease in normal gas exchange [1]. Therefore, the ideal treatment for ARDS would both support the respiratory needs of the patient while also enhancing regeneration of the damaged alveolus and recovery of normal alveolar barrier function [2].

Low-volume, lung protective ventilation remains the primary beneficial advance in sustaining respiratory function and improving survival in ARDS by reducing lung damage [3,4]. To date, no pharmacological treatments, including delivery of intrapulmonary lung surfactant or systemic delivery of anti-inflammatory medications, have demonstrated significant reductions in mortality among adults [5]. However, potential therapeutic benefits might be achieved if drugs could be delivered directly to the injured alveoli where they are needed, achieving greater alveolar drug concentrations and lower systemic concentrations [6]. Unfortunately, inhaled drug delivery to alveoli is difficult, even in healthy adults, with only a small fraction of drug penetrating to the alveolar level [7]. ARDS further impedes inhaled delivery since the damaged, edematous regions of the lungs are poorly ventilated, making it even more difficult to deliver drugs to injured airways.

To remedy this, we propose delivering drugs to the alveolus during ARDS using a reverse water-in-perfluorocarbon (PFC) emulsion [8–12]. These emulsions contain a dispersed aqueous phase (< 2.5% by volume) that is emulsified within the liquid PFC. Any water-soluble drug can then be dissolved within the aqueous phase for delivery. The resulting emulsion is delivered to the alveolus by partially filling the lung with the emulsion and then ventilating with a standard gas ventilator over the emulsion. In a similar fashion to partial liquid ventilation [13–16], the perfluorocarbon would wash exudate from the alveoli and towards the conducting airways due to its low surface tension and high density [16–21] and enhance gas exchange. Unlike liquid ventilation, however, the emulsion would deliver drugs to actively enhance alveolar barrier function and repair.

The optimal drugs for this purpose are not yet known, but could potentially include epithelial and endothelial growth factors, anti-inflammatory drugs, antibiotics, and pulmonary surfactant. The purpose of this paper is to examine this concept through the delivery of one growth factor, lysophosphatidic acid, from PFC emulsion to lung epithelial cells. Lysophosphatidic acid (LPA), a serum-derived, phospholipid growth factor, induces epithelial cell migration and proliferation, and, most importantly, enhances barrier function [22,23]. After the inflammatory exudate has been washed up and suctioned out following treatment with PFC emulsions, an increase in barrier function would slow the influx of

edema and neutrophils, allowing alveolar cells an opportunity to migrate into the wounded area and proliferate to reestablish functional tissue.

Delivery of antibiotics to biofilms via PFC emulsions has been shown to be effective in previous studies [24,25], but delivery of a growth factor to affect cellular repair and inflammation has never been attempted using a water-in-PFC single emulsion. In the current study, the effects of delivering LPA to alveolar epithelial cells via water-in-PFC emulsions were compared to that of aqueous LPA at similar concentrations using migration, proliferation, and barrier function assays in vitro.

2. Material and methods

2.1. Materials

1-oleoyl (C_{18:1}) LPA and fluorescein isothiocyanate–dextran (FITC-dextran; 4 kDa) were purchased from Sigma–Aldrich (Milwaukee, WI, USA). The alamar blue cell viability reagent was purchased from Life Technologies (Carlsbad, CA, USA). Bovine serum albumin (BSA) was purchased from New England Biolabs (Ipswich, MA, USA). Crystal violet (0.1% aqueous) was purchased from Ward's Science (Rochester, NY, USA). Luria-Bertani (LB) agar powder was purchased from ThermoFisher Scientific (Waltham, MA, USA). Similar to previous work with antibiotic-loaded, water-in-PFC emulsions [24,25], the PFC used was perfluorocycloether/perfluorooctane (FC-770) purchased from 3 M Inc. (St. Paul, MN, USA) and the fluorosurfactant used ("FSH-PEG") was perfluoropolyether (Krytox 157FSH oil: 7 kDa, n = 41)-polyethelene glycol (PEG: 1 kDa, m = 22)- Krytox 157FSH (i.e., FSH-PEG-FSH) tri-block copolymers synthesized from Krytox 157FS oil purchased from Dupont (Wilmington, DE, USA) [26]. See Fig. 1 for the molecular structure of the FSH-PEG copolymer.

2.2. Cell culture

The murine lung epithelial cell line MLE-12 cells were purchased from A.T.C.C. (Manassas, VA, USA) and were maintained in HITES medium (Dulbecco's modified Eagle's medium/F-12 medium) complemented with 10% fetal bovine serum in a 37 °C incubator in the presence of 5% CO₂.

2.3. Preparation of aqueous solutions

Aqueous LPA solutions were prepared fresh in Dulbecco's serum-free medium with 1% BSA before experiments. Appropriate volumes of 5 mM LPA (in ethanol) were added directly into the serum-free medium + BSA to prepare 0, 1, 5, 10, 200, and 400 μM aqueous solutions. Uniform dispersion was ensured by vortexing for 2 min. A pH meter was used to ensure aqueous droplets would not be harmful to cells. Aqueous crystal violet solutions were either undiluted (i.e. 100%) or diluted to 2.5% v/v in sterile water.

2.4. PFC emulsion preparation

LPA-loaded, water-in-PFC emulsions were prepared as previously described in 5 mL batches [24]. Briefly, a mixture of PFC, aqueous LPA, and fluorosurfactant was emulsified via sonication (Model VCX 130, 20 kHz, 3.2 mm diameter microtip; Sonics & Materials,

INC., Newtown, CT, USA) at 200 W cm^{-2} for 60 s. All emulsions had the same aqueous volume percent (2.5%) and aqueous concentration of fluorosurfactants ($C_{fs} = 2 \text{ mg/mL}$ of water). Aqueous LPA solutions of 0, 40, 200, and 400 μM were emulsified within the PFC to yield total concentrations of 0, 1, 5, and 10 μM LPA emulsions, respectively. Emulsion formulations are summarized in Table 1. The 0 μM emulsion, used to determine the effect of fluorosurfactant on cell behavior, was prepared the same way with a 0 μM LPA aqueous solution (serum-free media + BSA). Crystal violet-PFC emulsions were prepared by emulsifying 250 μL undiluted crystal violet in 9.75 mL of PFC and fluorosurfactant to yield a total concentration of 2.5% (v/v) emulsion.

2.5. Emulsion viscosity

The dynamic viscosities of pure PFC and the 0 μM emulsion were measured using a cone and plate rheometer (model DVII + Pro; Brookfield Engineering Laboratories, Middleboro, MA) at 37°C as previously described [24]. Briefly, samples were interrogated at 200 s^{-1} . Three repeated measurements were taken.

2.6. Emulsion surface tension and aqueous interfacial tension

The surface and interfacial tension of pure PFC and the 0 μM emulsion were measured using a DuNouy ring tensiometer with a platinum-iridium ring (Cenco Model 70545; 6 cm circumference; CSC Scientific Company Inc., Fairfax, VA) as previously described [24]. Briefly, 0 μM emulsion and pure PFC were allowed to reach room temperature ($22\text{--}25^\circ\text{C}$) before measurements. Interfacial tension measurements, deionized, filtered water was introduced on top of the 0 μM emulsion or pure PFC. Three repeated measurements were taken for each condition.

2.7. Delivery of crystal violet to aqueous surface

To determine how emulsion creaming (separation of the less dense aqueous droplets to the top of the emulsion) affected delivery to an aqueous surface (cells, lung epithelium, etc.), LB agar (100 μL) was allowed to gel at the bottom of 2 mL microcentrifuge tubes and then exposed to 2 mL of either 2.5% aqueous or 2.5% emulsified crystal violet solutions for 2 h. The microcentrifuge tube was oriented such that the gel sat beneath, above, or adjacent to the media (see Fig. 2). After exposure, the media was removed, and the gel was placed in a clean tube and melted with 1 mL water. The absorbance of 100 μL samples was measured at 590 nm in triplicate.

2.8. Scratch assay for cell migration

ARDS is characterized by areas of denuded epithelium. Thus, alveolar repair requires epithelial cells to migrate into these regions. A scratch assay was performed to determine epithelial cell migration. The procedure followed the method of Zhao, et al. [27]. Briefly, cell monolayers were seeded in 6-well plates, scratched with a 10 μL pipette tip, washed to remove non-adherent cells and cellular debris, and digitally photographed using a phase contrast microscope. Cells were then incubated for 2 h with 1 mL of either i) 0 μM aqueous control, ii) 1 and 5 μM aqueous LPA (positive control), iii) pure PFC (PFC control), iv) 0 μM emulsion containing no drug (fluorosurfactant control), or v) 5 and 10 μM LPA

emulsions. Next, cells were incubated 18 h in serum-free medium and the final image was taken. ImageJ was used to quantify the area occupied by cells and thus, the extent of cell migration (see Fig. 2). The amount of migration is calculated using Eq. (1)

$$\text{migration} = \frac{\text{pre migration area} - \text{post migration area}}{\text{length of scratch}} \quad (1)$$

, where “pre migration area” is the area of the wound before treatment and “post migration area” is the area after treatment and incubation (see Fig. 2). This data is then presented as the percent increase in migration normalized by cells treated with 0 μM aqueous control, Eq. (2)

$$\% \text{increase in migration} = \frac{\text{migration}_{\text{treatment}} - \text{migration}_{\text{control}}}{\text{migration}_{\text{control}}} \times 100 \quad (2)$$

2.9. Epithelial permeability assay with measuring dextran leak

Epithelial barrier disruption leads to the edematous alveoli that is characteristic of ARDS. A rapid increase in barrier function will slow the influx of exudate, enabling epithelial cells to repair. Dextran leak was utilized to determine the effect of aqueous and emulsified LPA (5 and 10 μM) on pulmonary epithelial barrier function. The procedure was followed according to Strengert and Knaus [28]. Briefly, MLE-12 cells were plated at 100% confluence on permeable inserts containing 0.4 μm pores (Corning, Kennebunk, ME, USA). For 2 h, the top chamber was treated with 300 μL of either i) 0 μM aqueous control, ii) 5 and 10 μM aqueous LPA (positive control), iii) pure PFC, iv) 0 μM emulsion, or v) 5 and 10 μM LPA emulsions. The bottom chamber was filled with 450 μL serum-free medium. After treatment, the cells were washed with Hank’s buffered salt solution (HBSS). FITC-dextran (150 μL) was added to the top chamber at 0.5 mg/mL in HBSS for 1 h; the bottom chamber was filled with 450 μL HBSS. To further ensure this specific PFC (FC-770) had no lasting effect on barrier function, a separate experiment was conducted. Cells were treated with 200 μL of blank medium or PFC for 2 h and then exposed to a final mixture of dextran (0.5 mg/mL) and aqueous LPA (5 or 10 μM) in HBSS for 1 h.

For both experiments, fluorescence levels in the top and bottom chamber were measured in triplicate by a fluorescence microplate reader with excitation at 490 nm and emission at 520 nm and were then converted to a dextran concentration ($\mu\text{g/mL}$) using a standard curve. The concentration of dextran that leaked into the bottom chamber ($\mu\text{g/mL}$) is indicative of the barrier function, with less dextran leakage implying greater barrier integrity. Dextran leak was normalized by the dextran leak of cells treated with 0 μM aqueous control (Eq. (3)), and the data is depicted as percent increase in barrier function (Eq. (4)).

$$\text{normalized dextran leak} = \frac{\text{dextran leak}_{\text{treatment}}(\mu\text{g/mL})}{\text{dextran leak}_{\text{control}}(\mu\text{g/mL})} \quad (3)$$

$$\%increase\ in\ barrier\ function = 100 - (normalized\ dextran\ leak * 100) \quad (4)$$

2.10. Alamar blue assay for proliferation

Alveolar type II epithelial cells must proliferate in order to repair the denuded alveolar wall and re-establish functional tissue. Proliferation was determined using the alamarBlue assay, per manufacture's specifications. Cells (10,000 cells/well) were seeded in 24-well plates and, after an 8 h incubation, were starved overnight in serum-free medium. Cells were then treated for 2 h with 300 μ L of either i) 0 μ M aqueous control, ii) complete HITES medium with 10% FBS (positive control), iii) 5 and 10 μ M aqueous LPA, iv) pure PFC, v) 0 μ M emulsion, or vi) 5 and 10 μ M LPA-PFC emulsions. Cells treated with emulsions were then quickly washed with PFC to remove any remaining FSH-PEG. All cells were incubated for 72 h in medium (5% FBS); the positive control cells were incubated in complete medium (10% FBS). alamarBlue in serum-free medium (10% by volume) was added to each well (225 μ L) and incubated an additional 4 h. Reduction of alamarBlue was determined by measuring well absorbance in duplicate at 570 and 600 nm (λ). Within an acceptable range of cell density and incubation time, the level of reduced alamarBlue linearly increases with the number of living cells; thus, percent difference in reduction in alamarBlue between treated and control cells will be presented as percent increase in proliferation (Eq. (5))

$$\%increase\ in\ proliferation = \% difference\ in\ reduction$$

$$\%increase\ in\ proliferation = \%difference\ in\ reduction \quad (5)$$

$$= \frac{\epsilon_{600} * A_{570} - \epsilon_{570} * A_{600}^{treated}}{\epsilon_{600} * A_{570} - \epsilon_{570} * A_{600}^{control}} * 100$$

, with $\epsilon_{600} = 117,216$ and $\epsilon_{570} = 80,586$ as the molar extinction coefficients for Alamar Blue at different wavelengths and A_{λ} being the absorbance values from each wavelength.

2.11. Statistical analysis

Statistical differences ($p < 0.05$) in all variables were determined in SPSS (IBM Corporation, Armonk, NY) using a one- or two-way ANOVA with multiple comparisons applying Tukey's method and the Sidak correction. All values are represented as mean $\pm$ standard error unless stated otherwise.

3. Results

3.1. Emulsions maintained similar physical characteristics as PFC

To determine if the use of the more stable and less cytotoxic FSHPEG fluorosurfactant had any effect on emulsion characterization compared to previous work, the viscosity and surface tension were measured [Orizondo et al., 24, Orizondo et al., 29]. There was no significant difference in viscosity between pure PFC (mean $\pm$ standard deviation: 2.30 ± 0.09 cP) and the 2.5% emulsion (2.41 ± 0.09 cP). The surface tension of the emulsion (mean $\pm$

standard deviation: 15.6 ± 0.3 dyn/cm) slightly increased compared to that of pure PFC (14.1 ± 0.09 dyn/cm; $p < 10^{-3}$). The aqueous-emulsion (16.3 ± 5.4 dyn/cm) significantly decreased compared to aqueous-PFC interfacial tensions (35.6 ± 4.4 dyn/cm; $p = 10^{-3}$), respectively.

3.2. Availability of crystal violet was decreased in emulsions

To determine the effect of emulsion creaming on drug availability to an aqueous surface, the uptake of aqueous or emulsified crystal violet into an agar gel was measured in three orientations. Fig. 2 shows that although emulsions deliver the same payload of dye, the availability of that dye to be taken up in an aqueous surface is (1) orientation dependent and (2) generally decreased in emulsified form (normalized to dye delivered in aqueous form). Emulsions took up 60% less dye than aqueous solutions in both the bottom and side orientations ($60 \pm 8\%$; $p = .99$). However, the emulsion achieved only a 19% reduction in uptake compared to aqueous exposure to the gel on top ($19 \pm 7\%$; $p = 10^{-4}$ compared to side and bottom orientations).

3.3. LPA emulsion induced epithelial cell migration

To determine if the emulsion delivered enough LPA to retain its effect on epithelial cell migration as seen with aqueous LPA, cells were treated with both aqueous (1 and 5 μ M) and emulsified (5 and 10 μ M) LPA during a scratch assay. Fig. 3 pictorially demonstrates typical results of experimental groups, with greater migration into the scratch after 5 μ M aqueous treatment.

Fig. 4 presents the percentage increase in epithelial cell migration vs. the 0 μ M aqueous control. Migration increased with LPA concentration, both in purely aqueous form, as researchers have previously shown [Zhao et al., 27], and when delivered via PFC emulsion. Thus, the emulsion was capable of delivering LPA to the cells. For aqueous delivery, percent increase in migration following treatment with 1 μ M LPA ($12.3 \pm 6.6\%$) was not significantly greater than that following treatment with 0 μ M control ($0 \pm 2.9\%$), but percent increase in migration following treatment with 5 μ M LPA ($27.6 \pm 8.3\%$) was significantly greater than that of the 0 μ M control ($p < 0.05$). For emulsified delivery, the 10 μ M emulsion ($26.2 \pm 6.1\%$) had significantly greater percent increase in migration than the 0 μ M aqueous control ($p < 10^{-3}$), but the 5 μ M emulsion did not ($p = .1$). However, the 10 μ M emulsion was not significantly different than the 5 μ M emulsion ($14 \pm 2.3\%$; $p = .5$). Treatment with pure PFC alone ($-15.8 \pm 2.1\%$) significantly reduced migration compared to 0 μ M aqueous control ($0 \pm 2.9\%$; $p < 0.05$), however, there was no difference between the 0 μ M emulsion ($-8.1 \pm 4.1\%$) and 0 μ M aqueous control ($p = .6$). Comparing the performance of aqueous to emulsified LPA, there was no difference between the aqueous LPA and emulsified LPA groups. However, approximately twice the concentration of emulsified LPA (10 μ M emulsion) was required to achieve the same ~30% increase in migration as demonstrated in 5 μ M aqueous.

3.4. LPA emulsion did not enhance epithelial barrier function

To determine if emulsified LPA retained its effect on epithelial cell barrier function as seen with aqueous LPA, cells were treated with both aqueous (5 and 10 μ M) and emulsified (5 and 10 μ M) LPA during a dextran permeability assay. Fig. 5 presents the extent of dextran

leak across the epithelium as a percentage of that seen in the 0 μM aqueous control. These results confirm that aqueous LPA increases barrier function [30]. In Fig. 5A, the 5 and 10 μM aqueous LPA ($33 \pm 3\%$ and $42 \pm 4\%$, respectively) increased barrier function (reduced normalized dextran leak) compared to the 0 μM aqueous control ($0 \pm 0.6\%$; $p < 10^{-5}$ and $p < 10^{-6}$, respectively), but were not significantly different from each other ($p = .1$). However, the emulsified LPA had no effect on barrier function compared to the 0 μM aqueous control ($p > 0.95$ for all groups). In addition to Fig. 5A showing pure PFC alone had no effect on barrier function ($p = .99$), Fig. 5B also shows that pre-exposure to pure PFC did not affect subsequent cellular response to aqueous LPA treatment ($p = .99$). There was no difference in barrier function between LPA treated cells with or without pre-exposure to pure PFC ($p > 0.99$).

3.5. LPA emulsions did not induce cell proliferation

To determine the effect of LPA on proliferation, alveolar epithelial cells were treated with both aqueous and emulsified LPA. Fig. 6 shows increasing proliferation with increasing aqueous LPA concentration, but not to the extent of the positive control (HITES medium). The HITES control had significant growth with a mean of $84 \pm 10\%$ ($p < 10^{-6}$), whereas the 5 and 10 μM aqueous LPA had percentages of $16 \pm 5\%$ and $28 \pm 5\%$, respectively. The 10 μM aqueous significantly increased proliferation compared to the 0 μM aqueous control, but the 5 μM aqueous did not ($p < 0.05$ and $p = .3$, respectively). There was no significant difference in proliferation after treatment with PFC or LPA emulsions compared to the 0 μM aqueous control ($p > 0.95$ for all groups).

4. Discussion

Lowering the mortality of ARDS requires both supporting the respiratory function as well as enhancing alveolar repair. This second requirement is difficult to fulfill as researchers have yet to discover a drug or combination of drugs that promotes alveolar repair in vivo [5]. In any case, once the optimal drug cocktail is developed, the delivery vehicle must uniformly apply drugs in the diseased regions and either penetrate or remove any edema that has pooled in those regions. Our previous research has already shown that antibiotics are delivered to the lungs after filling with PFC emulsions [24,25,29]. In addition, others have been able to washout exudate from the lungs of ARDS patients during PFC ventilation [16]. The objective of this paper was to determine the effectiveness of a PFC emulsion containing one growth factor, LPA, when delivered to epithelial cells.

For this emulsion to be effective, it must first preserve PFC's viscosity and low interfacial tensions that allow for effective ventilation and retain a level of functionality for alveolar repair. There was no difference in emulsion viscosity compared to that of pure PFC, which is expected given the low aqueous volume percent (2.5%). The surface tension of the emulsion slightly increased from that of neat PFC, whereas the aqueous-emulsion interfacial tension was considerably less than the aqueous-PFC interfacial tension. This is likely due to the surfactant present in the emulsion. Their hydrophilic and fluorophilic groups preferentially accumulate at the emulsion-aqueous interface, causing a decrease in the interfacial tension. Thus, emulsions maintain their ability to penetrate down to the alveolar level. In addition,

the lower aqueous interfacial tension (compared to pure PFC) should more effectively penetrate fluid-filled airways and enhance fluid removal [31,32]

Second, the emulsion must be able to deliver drug effectively. Previous research has determined that the optimal drug delivery occurs at an intermediate fluorosurfactant concentration [29]. Below this concentration, the emulsion is too unstable to be prepared and delivered prior to phase separation. Above this, the emulsion droplets are too stable and, thus, do not coalesce with the aqueous surface of the lung. As such, the emulsions are purposely made to be unstable, and thus the emulsions are not sufficiently stable to measure droplet size and number density. Droplet dimensions for more stable versions of these emulsions are published elsewhere [25], demonstrating average droplet diameters of $1.9 \pm 0.2 \mu\text{m}$ over a 24-h period and droplet number density of $3.5 \pm 1.7 \times 10^9$ droplets/mL that exhibited a nearly half-log decrease over 24 h.

Regarding emulsions retaining a level of functionality for alveolar repair, the effect of emulsified LPA was either diminished or completely hindered in all cases. This was not due to sonication possibly disrupting LPA bioactivity since aqueous LPA sonicated at the same intensity and duration settings as that used to make the LPA emulsion prompted the same cellular response for migration, barrier function, and proliferation as un-sonicated aqueous LPA (data not shown). Thus, the reasons for emulsified LPA's diminished activity are multifactorial, involving both differences in drug availability and direct effects of emulsion components on the cells.

Previous work has demonstrated 55% drug availability when delivering tobramycin from a similar emulsion to an agar well [25]. Fig. 2 showed LPA's availability to bind with cellular receptors is likely reduced by emulsion creaming in this experimental set-up: the PFC is 1.8 times more dense than water, and thus the aqueous droplets rise up and away from the epithelial layer at the bottom. This explains why dye uptake increased with orientations that increased exposure to the aqueous droplets from the emulsion. Furthermore, as drug is delivered from the aqueous emulsion droplets to other aqueous media (e.g. agar, cells layers), fluorosurfactant is also delivered to the PFC-aqueous interface. Over time, this stabilizes the interface and hinders further delivery of aqueous droplets [25]. This process likely accelerates with PFC evaporation. Accordingly, the ability of LPA to modify epithelial function is certainly hindered to some degree by drug availability in this experiment. This latter effect would be less of an issue in vivo. There, droplet buoyancy is as likely to bring droplets into contact with airway walls as it is to float the droplet out of the alveolus.

Even if LPA is available to the cell, contact with the other emulsion components could perturb other cellular properties such as receptor binding affinity or subsequent signaling pathways that could limit the effect of LPA. The cellular level effects of PFC and the fluorosurfactant are not well understood, but we have previously shown that there are no toxic effects from this emulsion formulation nor pure PFC on epithelial cells [Orizondo et al., 29]. This is confirmed here in our proliferation and barrier function results. In addition, there was no significant difference between the PFC only group and the $0 \mu\text{M}$ emulsion group in any of the assays. However, PFC has a direct, negative effect on migration in this in vitro setting. A PFC with more documented in vivo safety has been developed and would be

used for eventual clinical translation (perfluorooctyl bromide, Liquivent, Origen Biomedical, Austin, TX). The fluorosurfactant, however, is custom made and further formulations must be developed to decrease cytotoxicity and increase in vivo clearance. LPA, media, fluorosurfactant, and PFC are all being delivered to the cell surface simultaneously via the emulsion. The final effect on the cell is the sum of all these effects.

Despite this, LPA was successfully delivered from the emulsion and was sufficiently available to stimulate epithelial migration. Pure PFC significantly stunted migration; however, the 0 μM emulsion did not. This is likely due to the 0 μM emulsion providing some polar, aqueous environment to the cell surface, such that the cells do not contact the PFC as directly [33–35]. Researchers have shown PFC to interfere with the attachment of various cell types to various surfaces, leading to less spreading [36]. Although cells are only exposed to PFC for 2 h, there may be some residual PFC that disrupts cellular anchorage and migration. However, the emulsified LPA was able to recover and stimulate migration when the LPA concentration was increased by 50%. This was expected taking into account the 60% loss in drug availability we predict based on the crystal violet experiments.

Unlike with migration, the loss in functionality of emulsified LPA on barrier function cannot be attributed to some hampering effect from PFC or 0 μM emulsion. We have shown that our PFC has no effect on barrier function, as seen elsewhere [37]. In addition, PFC has no after-effect on barrier function, as treatment with aqueous LPA directly following exposure to pure PFC exhibited a similar increase in barrier function as seen when treated with aqueous LPA only. Taking into account the 60% loss in drug availability we predict based on the crystal violet experiments, the effect of LPA is likely blunted by lesser LPA availability from the emulsion, as is seen with migration. However, the barrier function is not recovered after a 50% increase in concentration. This could be due to some unknown interference with the downstream signaling pathways from the PFC and/or the fluorosurfactant.

Lastly, LPA has not previously been shown to increase alveolar epithelial cell proliferation, specifically. However, it has been shown for other cell types including fibroblasts and mesenchymal stem cells [38–40]. For alveolar epithelial cells, we see a dose-dependent increase in proliferation with aqueous LPA. The effect is small, however, and not seen at all with the emulsion. As with barrier function, neither PFC nor 0 μM emulsion decreases proliferation and thus reduced drug availability is the predominant issue. Taking into account the 60% loss in drug delivery we predict based on the crystal violet experiments and the small effect LPA has on proliferation, it is possible higher concentrations are needed. However, these effects on barrier function and proliferation might be different in vivo given the greater surface area to volume ratio and the lessened effect of droplet creaming on drug availability.

5. Conclusions

In summary, emulsions can successfully deliver bioactive LPA growth factor. However, the availability and thus the biological effect of LPA is significantly blunted by delivery via the emulsion in vitro, and the extent of the attenuation depends on the specific cellular function examined. Thus, the cellular level effects of drug delivery to the lungs via emulsion are

likely to vary based on the drug and the effect it is intended to create. In some cases, the benefit of direct delivery to the lungs while ventilating the patient may outweigh the reduced effect or it may be possible to simply increase emulsion concentrations to achieve the desired effect.

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Fig. 1.

A. Molecular structure of Krytox 157FS oil B. FSH-PEG-FSH triblock copolymer ($n = 41$, $m = 22$).

Fig. 2. Concentration of dye in agar gel after 2-h exposure to 2.5% emulsified crystal violet solution normalized to agar concentration after exposure to 2.5% aqueous solution. Error bars are standard deviation ($n = 3$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 3.
Scratch assay of MLE cells. Scale bar equals 15 μm .

Fig. 4. Percent increase in proliferation post 2 h treatment and 72 h incubation. Error bars are standard error. There was an $n = 5$ for all groups.

Fig. 5.

Percent increase in barrier function using dextran leak assay. (A) 2 h treatment followed by 1 h exposure to FITC-dextran (0.5 mg/mL). (B) 2 h pre-exposure to serum-free medium or PFC followed by 1 h simultaneous treatment with aqueous LPA (5 or 10 μ M) and FITC-dextran (0.5 mg/mL). Error bars are standard error. There was an $n = 6$ in panel A and $n = 4$ in panel B.

Fig. 6. Percent increase in proliferation post 2-h treatment and 72-h incubation. Error bars are standard error. There was an $n = 5$ for all groups.

Table 1

Emulsified LPA formulation.

Emulsion Formulations ($C_{fs} = 2$ mg/mL water)	Aqueous volume percent [%]	Aqueous drug concentration [μ g/mL water]	Total emulsion drug concentration [μ g/mL emulsion]
0 μ M	2.5	0	0
1 μ M	2.5	40	1
5 μ M	2.5	200	5
10 μ M	2.5	400	10