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A comprehensive screening platform for aerosolizable protein formulations for intranasal and pulmonary drug delivery

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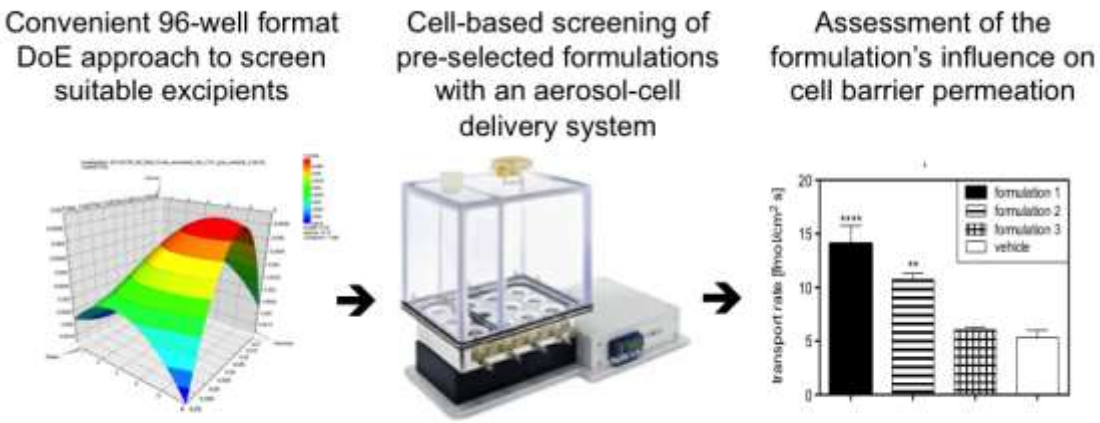
Graphical Abstract



This manuscript includes 1 graphical
abstract, 3 figures and 1 table.
Colour should be used in print for all figures.
A file containing the supplemental data is for review purposes at the end of the manuscript.

Abstract

Aerosolized administration of biopharmaceuticals to the airways is a promising route for nasal and pulmonary drug delivery, but – in contrast to small molecules - little is known about the effects of aerosolization on safety and efficacy of biopharmaceuticals. Proteins are sensitive against aerosolization-associated shear stress. Tailored formulations can shield proteins and enhance permeation, but formulation development requires extensive screening approaches. Thus, the aim of this study was to develop a cell-based *in vitro* technology platform that includes screening of protein quality after aerosolization and transepithelial permeation. For efficient screening, a previously published aerosolization-surrogate assay was used in a design of experiments approach to screen suitable formulations for an IgG and an antigen-binding fragment (Fab) as exemplary biopharmaceuticals. Efficient, dose-controlled aerosol-cell delivery was performed with the ALICE-CLOUD system containing RPMI 2650 epithelial cells at the air-liquid interface. We could demonstrate that our technology platform allows for rapid and efficient screening of formulations consisting of different excipients (here: arginine, cyclodextrin, polysorbate , sorbitol, and trehalose) to minimize aerosolization-induced protein aggregation and maximize permeation through an *in vitro* epithelial cell barrier. Formulations reduced aggregation of native Fab and IgG relative to vehicle up to 50% and enhanced transepithelial permeation rate up to 2.8-fold.

Abbreviations

ALI	air-liquid interface
ALICE-CLOUD	System for aerosolized delivery of liquid drugs to cells cultured at the ALI

Röhm *et al.* 2017; formulation platform for protein aerosols; Int J Pharmceutics

CNS	central nervous system
DoE	design of experiment
ELISA	enzyme linked immunosorbent assay
Fab	antigen binding fragment
FBS	fetal bovine serum
Fc	crystalizable fragment
FcRn	neonatal Fc receptor
FITC	fluorescein isothiocyanate
HBC	(2-Hydroxypropyl)-β-cyclodextrin
HPLC	high performance liquid chromatography
IgG	immunoglobulin G
KRB	Krebs Ringer buffer
mAb	monoclonal antibody
m_M	molecular mass
P_{app}	permeability coefficient
PS20	polysorbate 20/Tween® 20
RT-PCR	reverse transcription - polymerase chain reaction
SD	standard deviation
SEC	size exclusion chromatography
SE-HPLC	size exclusion high performance liquid chromatography

Röhm *et al.* 2017;	formulation platform for protein aerosols;	Int J Pharmceutics
SEM	standard error of the mean	
TBS	Tris-buffered saline	
TEER	transepithelial electrical resistance	

Keywords : Antibodies; scaffolds; aggregation; stability; aerosolization; nebulizer; airways; air-liquid interface; permeation

1. Introduction

Drug delivery to the airways using intranasal or inhalative aerosols represents a comfortable and non- to minimally-invasive way of self-administration. Although these administration routes are well established for small molecules, very little is known about the safe and effective administration of biopharmaceuticals in the airways. Most experience with aerosolized biopharmaceuticals has been gained for the delivery of desoxyribonuclease to the lung for the treatment of cystic fibrosis (Pressler, 2008), cytokines or cytokine agonists for the therapy of asthma (Thipphawong, 2006), but also with inhaled insulin (Siekmeier and Scheuch, 2008). Intranasal delivery with nasal sprays of the peptides desmopressin and calcitonin is well established for over 20 years (Ozsoy et al., 2009). As intransal delivery is also suitable to target the CNS, numerous studies delivered the peptide oxytocin (1 kDa) to treat social disorders (Hurlemann et al., 2010) and likewise the small protein insulin (5.8 kDa) that is able to improve the outcome of some cognitive tasks (Craft et al., 2012; Stützle et al., 2015). However, for larger and more complex proteins such as antibodies only few data are available demonstrating their feasibility as aerosols (Dellamary et al., 2004; Patton and Platz, 1992; Schüle et al., 2008).

Depending on aerosol size and inhalation maneouver the aerosolization system allows targeted aerosol deposition in the nasal cavity (Engelhardt et al., 2016), nasal sinuses (Moller et al., 2014) and pulmonary respiratory tract (Coates, 2008). However, generation of micron sized aerosol droplets can have tremendous effects on proteins: complex proteins like monoclonal antibodies (mAbs) are

very susceptible towards shear and mechanical stress experienced during aerosol formation, which can result in aggregation and unfolding of proteins (Maa and Hsu, 1997; Respaud et al., 2014). For instance, immunoglobulins have hydrophobic domains that can be adsorbed and enriched at the surface of liquid droplets where they interact with gas phase components. As a consequence, proteins are known to unfold aggregate, degrade and denature (Bosquillon et al., 2004; Couston et al., 2012; Yu et al., 2006) resulting in decreased biological activity and immunological side effects (Rombach-Riegraf et al., 2014). The extent of aggregation is extremely dependent on protein structure, molecular mass, structural motifs, charge and hydrophobicity (Chiti, 2004).

Nebulizers are convinient devices for atomization of liquid drugs as they provide a moderate continuous flow rate and a constant aerosol size distribution in the optimal range for pulmonary or nasal delivery (Brun et al., 2000). Choosing a nebulizer exerting low shear forces during aerosol generation can reduce aggregation and loss of bioactivity (Andrew R Martin & Warren H Finlay, 2015; Hertel et al., 2015). Likewise, it is well known that protein stability during aerosolization can be further improved by a suitable drug formulation (Shoyele and Slowey, 2006). Even though mAbs share the majority of the sequence in their constant regions, each mAb is unique and needs a tailored formulation (Frokjaer and Otzen, 2005; Wang, 1999; Wang et al., 2007). Interestingly, formulations can additionally have a positive effect on protein permeation through the mucosa and increase thereby bioavailability (Aungst, 2012; Patton and Byron, 2007; van der Lubben et al., 2001). Optimization of formulations consisting of various excipients typically requires systematic testing of a large number and combination of excipients. As nebulization and subsequent sample collection for each of these mixtures is very time consuming, an alternative surrogate method with high throughput capability is desirable. Recently, Hertel *et al.* (Hertel et al., 2014a) have shown that agitation of protein solutions can be used as surrogate for the stress caused by energy input during nebulization.

In a previous study, we have investigated the impact of aerodynamic particle diameter and flow rate on intranasal deposition using a vibrating mesh system (Engelhardt et al., 2016). The purpose of the

present study was to develop a comprehensive and fast screening platform for protein aerosols assessing 1) the effect of excipients and formulations on protein stability during aerosolization and 2) the permeation of formulated, aerosolized proteins through an epithelial airway cellular model. For this study, an IgG and its antigen-binding fragment (Fab) were used. The number of required experiments for selection of an optimized formulation consisting of five different excipients is kept at a manageable level by using a previously published aerosolization-surrogate screening method (agitation instead of nebulization) combined with the statistical design of experiments (DoE) tool in a high throughput approach (Hertel et al., 2014a). Subsequently, transepithelial permeation of the best suited drug formulation was determined after aerosolized application onto nasal epithelial cells cultured under physiologic conditions at the air-liquid interface (ALI) using the ALICE-CLOUD aerosol-cell exposure system.

2. Material/ Methods

2.1. *Proteins*

The mAb HIRMab 83-14 (designated here as IgG) is an agonistic antibody that binds to and activates the human insulin receptor (Krook et al., 1996; McKern et al., 2006). In ongoing studies, we use this IgG and its Fab as a powerful tool to compare intranasal bioavailability and efficacy of intranasal immunoglubulins with intranasal insulin, which is well described for intranasal delivery (Born et al., 2002; Craft et al., 2013). IgG and its Fab were produced and analyzed as recently described (Röhm et al., 2016a). The hybridoma cell line producing this antibody was kindly donated by Ken Siddle (Cambridge University, UK). The antigen of IgG was absent in all studies to avoid interfering effects of antigen binding (data not shown). If not stated otherwise, samples were used at equimolar concentrations of 30 µM (1.4 mg/mL for Fab; 4 mg/mL for IgG).

2.2. Aerosol generator

A vibrating mesh nebulizer (Aeroneb Pro, Aerogen Inc., Galway, Ireland) was used in this study. The principles of operation of this device is depicted and described in figure 1A. Liquid passing though a vibrating membrane is dispersed into droplets with a mass median aerodynamic diameter of 2.5 –

6.0 µm. This clinically proven nebulizer is widely used due to its high liquid output rate of 0.3 – 0.8 mL/min and freely selectable air flow rate (air-less aerosol generation) (Longest et al., 2013). Here, 1 mL of the formulations was nebulized and aerosol droplets were either collected in a 15 mL Falcon tube and analyzed for monomer content or deposited onto epithelial cells for permeability measurements as described below.

2.3. SE-HPLC for the quantification of soluble and sub-visible aggregates

Size exclusion chromatography (SEC) was used for the detection and quantification of protein aggregates (Den Engelsman et al., 2011; Mahler et al., 2009). Non-nebulized, nebulized and agitated samples were analyzed using an UltiMate 3000 (Thermo Scientific, Langenselbold, Germany) high-pressure liquid chromatography (HPLC) system equipped with a MAbPac™ SEC-1 size exclusion chromatography column (4 x 300 mm) combined with a precolumn MAbPac™ SEC-1 (5 µm, 300 Å, 4x50 mm; Thermo Scientific). The quantification of the monomer peak was normalized to a non-nebulized reference and displayed as percentage of monomer recovery [%].

2.4. Design of Experiment (DoE)

To determine aggregate formation during nebulization in a small scale and rapid format, agitation was used as surrogate for nebulization as previously described (Hertel et al., 2014a). Briefly, 125 µL/well of formulated protein solution was transferred to a 96-well plate and constantly agitated with 900 rpm for 15 min at 30°C in an orbital shaker (HLC, Ditabis, Pforzheim, Germany). For identification of an optimized stabilizing formulation, a central-composite-face centered design was used with five factors (excipients), which enables an estimation of linear and quadratic terms as well as first order interactions. (Eriksson, 2008). Three concentration levels for each factor were investigated where the maximum, minimum and centre point concentrations are referred to as 1, -1, and 0, respectively. The investigated excipients were L-arginine, (2-Hydroxypropyl)-β-cyclodextrin (HBC), polysorbate 20 (PS20, Tween® 20), sorbitol, and trehalose (all purchased from Sigma Aldrich, Munich, Germany). The model contained 96 experimental runs (see Supplementary data). Six additional controls were implemented to better constrain the model by setting each factor to zero,

while the other factors were held constant at their centre points and one control with all factors at their centre points. Monomer recovery [%] was determined by SE-HPLC as response factors and the data were fitted with the software MODDE 9 (MKS Data Analytics Solutions, Umeå, Sweden). The mathematical model was evaluated using partial-least square regression. Single, quadratic and interaction effects of the excipients were analyzed and non-significant coefficient factors (95% confidence interval includes zero) were removed (displayed in grey in the Supplementary table). The model was fitted until R^2 (model fit) and Q^2 (model prediction power) were optimal. The results were modelled with a polynomial equation (see supplementary data).

2.5. Cell culture of nasal epithelial cell line RPMI 2650

RPMI 2650 cells, a carcinoma from squamous epithelium obtained from a human nasal septum (Moorhead, 1965), were cultivated in T-Flasks with Minimum Essential Medium supplemented by 10% foetal bovine serum (FBS), 1:100 non-essential amino acids, 4 mM L-glutamine and 10 u/mL penicillin (all purchased from Sigma-Aldrich, Munich, Germany) and 10 µg/mL streptomycin (Gibco, Darmstadt, Germany) at 37°C in a humidified atmosphere of 5% CO_2. Throughout this study, 24-well format transwells were used (0.334 cm^2, translucent, 0.4 µm pore size and 1 x $10^8/cm^2$ pore density; Greiner, Frickenhausen, Germany), which were coated with rat-tail collagen (0.05 v/v%; Sigma-Aldrich, Munich, Germany) prior to seeding 2 x 10^5 cells per insert. 600 µL medium was given to the abluminal (lower) and 100 µL to the luminal (upper) compartment and the cells were cultured for 24 h under submerged conditions and then cultured for additional 24 h at the air-liquid interface (ALI) by removing the luminal medium from the inserts.

For immunofluorescence, cells were washed with PBS, fixed with 4% formaldehyde, permeabilized with 0.5% Triton X-100 and blocked with 10% FBS for 1.5 h. Cell staining was performed with anti-human zona occludens-1 and anti-human E-cadherin (BD biosciences, Oxford, UK) antibodies, which were detected with anti-mouse Alexa Fluor 546 and anti-rabbit Alexa Fluor 488 (Life Technologies, Darmstadt, Germany), respectively. Nuclei were stained with Hoechst 33342. The transwell insert membrane was then detached from its housing with a scalpel and placed onto a

microscope slide with cells facing up and mounted with ProLong® Gold Antifade Reagent (Life Technologies). All samples were analyzed with a Zeiss LSM710 (Zeiss, Jena, Germany) confocal laser scanning microscope equipped with a LASOS® Ar-Ion laser.

2.6. ALICE-CLOUD System

The ALICE-CLOUD System (Lenz et al., 2014) is a refined verion of the Air-Liquid Interface Cell Exposure (ALICE) system (Lenz et al., 2009) utilizing the unique properties of cloud motion for efficient aerosol-cell delivery. Here, we used the commercially available version, the VITROCELL-CLOUD 12 system (VITROCELL Systems GmbH, Waldkirch, Germany) with adapters for the use of 24-well transwell inserts (Lenz et al., 2014). This system is equipped with an Aeroneb Pro vibrating mesh nebulizer, which generates a dense cloud of aerosol into an exposure chamber containing up to nine 24-well transwell inserts. One of the key performance parameters of the ALICE-CLOUD is the so-called deposition factor, which inidicates the fraction of the "invested" aerosolized drug volume (here 1 mL) deposited on the bottom of the exposure chamber. For no loss of drug, one would expect a deposition factor of unity, *i.e.* 100% of the "invested" drug deposits on the bottom of the exposure chamber. The deposition factor for each formulation in the ALICE-CLOUD system was experimentally determined for 1 mL vehicle spiked with FITC-dextran (500 µg/mL; Sigma Aldrich) with an average molecular mass of 4.0 kDa. Samples were taken from the wells and the FITC-dextran concentration was determined by fluorescence spectroscopy (excitation/emission at 490 nm/520 nm) using a microplate reader (SpectraMax M5, Molecular Devices LLC., Sunnyvale CA, USA). The deposition factor was determined as previously reported (Lenz et al., 2014) accounting for differences in nebulized volume, area of the wells (3.631 cm^2) and area of the bottom plate of the exposure chamber (137 cm^2).

2.7. Determination of permeation across cell layer

Permeation experiments were performed both under ALI and submerged cell culture conditions. For the latter, 100 µL of the fluorophore tracer FITC-dextran (500 µg/mL; Sigma-Aldrich, Munich, Germany) was pipetted onto the transwell insert. Samples were taken from the abluminal

compartment and the permeability coefficient (P_{app}) was calculated as follow: $P_{app}\left(\frac{cm}{s}\right) = \frac{\Delta[C]_A \cdot V_A}{A \cdot [C]_L \cdot \Delta t}$

where $\Delta[C]_A$ is the change of the abluminal FITC-dextran concentration at the sampling time (relative to initial conditions of 500 µg/mL), $V_A$is the abluminal volume (100 µL) , A is the insert surface (0.334 cm^2), $[C]_L$ is the luminal concentration and Δt is the incubation time.

In addition, permeation experiments with aerosolized proteins and aerosolized FITC-dextran were performed with RPMI 2650 cells cultured at the ALI. Prior to aerosolization, cells on transwell inserts were rinsed carefully with Krebs-Ringer-Buffer (KRB, pH 7.4) and transferred to the ALICE-CLOUD system. 1 mL samples of 30 µM formulated proteins or FITC-dextran were nebulized and cells were exposed to the aerosol in the ALICE-CLOUD system. After 90 min or 240 min incubation time, the protein concentration was determined in the abluminal medium by either ELISA or fluorescence spectroscopy, respectively. Permeation of aerosolized proteins or FITC-dextran is displayed either as transport rate $\left[\frac{fmol}{cm^2 \cdot s}\right]$ or as transported fraction [%]. 100% is equal to the average amount of aerosolized protein or FITC-dextran deposited luminally per insert, which was determined as the product of the "invested" drug volume (1 mL), the deposition factor and the cell-covered area per insert (0.334 cm^2) normalized to the area of the ALICE-CLOUD exposure chamber (137 cm^2).

Direct ELISAs were performed for the quantification of IgG and Fab. Samples and standards were diluted in coating buffer (50 mM carbonate-bicarbonate, pH 9.6) and incubated for 1 h at 37°C in 96-well plates (Brand immunograde, Brand GmbH, Wertheim, Germany). Wells were blocked with 2% (w/v) skim milk powder in Tris-buffered saline (TBS; 1 h at RT), incubated with an anti-murine kappa-chain antibody conjugated with horseradish peroxidase (1 µg/mL; 1 h at 37°C), and 3,3',5,5'-tetramethylbenzidine (Sigma-Aldrich, Munich, Germany) used as substrate for 45 min at room temperature. Absorbance at 640 nm was analyzed with a plate reader (SpectraMax). All washing steps were performed with TBS supplemented with 0.1% Tween® 20. All ELISA assays detect the native protein only (data not shown); hence denatured and aggregated proteins cannot be appropriately detected by the ELISA antibodies.

2.8. Statistical analysis

A one-way analysis of variance (ANOVA) and a Bonferroni's Multiple Comparison test was performed to compare the differences between individual groups and an unpaired Student's t-test was applied when comparing two sets of data using GraphPad Prism (Version 5.01, La Jolla, CA, USA). P values of 0.05 were considered to represent a significant difference. All data were expressed as mean ± standard error of the mean (SEM) or standard deviation (SD) as indicated.

3. Results

3.1. Screening formulations using a surrogate assay for aerosolization and Design of Experiment (DoE)

The excipients polysorbate 20 (PS20), trehalose, sorbitol, L-arginine, and (2-Hydroxypropyl)-β-cyclodextrin (HBC) were chosen for the formulation screen, since they are known to either prevent surface adsorption of proteins or to have beneficial effects on protein stability. Trehalose and sorbitol are both FDA-approved osmolytes and are reported to improve storage stability and reduce the formation of aggregates (Kamerzell et al., 2011; Maury et al., 2005). L-arginine is known for its solubilizing and refolding potential and to suppress protein-protein interaction, which can lead to the formation of high molecular mass aggregates (Baynes et al., 2005; Tischer et al., 2010). The influence of HBC on enhanced drug absorption and flux was reported by several researchers (Challa et al., 2005; Salem et al., 2009; Serno et al., 2010). Concentration ranges for DoE were chosen according to FDA-approved antibody formulations (Wang et al., 2007).

In order to screen protein formulations time- and cost-efficiently, a previously reported surrogate (Hertel et al., 2014a) was used in a statistical experimental design. We have verified in our lab the validity of agitation as a surrogate for nebulization with a vibrating mesh nebulizer (figure 1A) with the above-mentioned excipients (data not shown) and adapted it to a 96-well format with off-line determination of monomer recovery (figure 1B). As in our preliminary stability studies, Fab was rather unstable and showed easily statistically significant low levels of monomers (unpublished data), Fab was chosen as protein to be formulated.

For DoE, the five excipients were varied according to their typical individual concentrations in FDA-approved biopharmaceutical drug products as follows: trehalose (0.5 to 6%), sorbitol (0.5 to 5%), L-arginine (0.5 to 5%), PS20 (0.005 to 0.05%) and HBC (0.35 to 3.5%). As centre points for DoE analysis were defined: 3.25% trehalose, 2.75% of both sorbitol and arginine, 0.027 % of PS20 and 1.92% of HBC. All formulations were based on PBS (pH 7.2), denominated as vehicle. Sub-visible and soluble aggregates were investigated with SE-HPLC (displayed as % monomer recovery) as response factor. The full DoE approach can be found as Supplementary data.

The regression coefficient analysis revealed the impact of the excipients on monomer recovery. Sorbitol and L-arginine were identified as beneficial excipients in the coefficient plots (figure 1C). The optimal concentrations of L-arginine were in the range of 2.1 to 5%. Consequently, a response contour plot was generated to visualize the impact of the excipients, which had a significant positive impact on monomer recovery (sorbitol and arginine) with the other factors held constant at their centre points (figure 1D).

Based on the DoE, suitable concentrations of those five excipients were identified to be used as formulations for protein aerosols. The red and orange area in figure 1D represent the highest protein monomer recovery. Three different sorbitol and arginine combinations were chosen for three different formulations (F1-F3, see table 1) to be further characterized. Since increasing levels of trehalose showed no protective effect, a low concentration of trehalose (1%) was chosen for formulation 1 (F1). However, trehalose is one of the most frequently used protein stabilizers and is reported to preserve the protein's native conformation particularly under thermic stress that can occur during aerosol generation (Hertel et al., 2014b). Therefore, also higher trehalose concentrations (6%) were tested in F2 and F3.

3.2. Confirming the effect of selected formulations on protein aerosols

As the three different formulations (F1-F3) were identified based on a surrogate method for nebulization, they were now tested for their ability to preserve protein stability of Fab and IgG during aerosolization.

The formulated protein solutions were nebulized at equimolar concentrations (30 µM) and monomer content was analyzed before and after aerosolization. The non-aerosolized sample was set as 100% monomer content. In the vehicle control, the monomer abundance decreased by roughly 25% and 10% during aerosolization for Fab and IgG, respectively (figure 1E and figure 1F). In contrast, all formulations showed a stabilizing effect on at least one of the proteins. F1 was the most potent formulation (90.3±2.8% vs. 77.9±1.0% (vehicle); mean ± SEM; n=2; figure 1E) for Fab. It was significantly superior over F2 (Student's t-test; $^{\S\S}p<0.01$), but failed significance over F3 ($p = 0.051$). While F3 significantly protected Fab against aggregation relative to vehicle control (**p<0.002), F2 failed statistical significance as compared to vehicle (p=0.057).

For the rather stable IgG, F1 and F2 provided protection with over 95% monomers detected (98.4±1.2% (F1) and 97.3±0.7% (F2) vs. 90.3±2.0% (vehicle); **p<0.001, for both F1 and F2; figure 1F). The effect of F3 on nebulized IgG revealed a tendency but failed statistical significance. These results confirm earlier studies suggesting that the protective effect of formulations is not transferable from one protein to another, even if one protein (Fab) is a derivative of another one (IgG) and shares identical domains (Wang et al., 2007).

It is noteworthy that our Fab DoE data using the surrogate indicated that higher levels of sorbitol (F1 - F3) combined with lower levels of arginine (F2 and F3) may reduce aggregation more efficiently. However, this could not be confirmed by our aerosolization experiments as all formulations were active and F1 with the highest arginine levels had a tendency to be superior over F2 and F3.

3.3. *Establishing an in vitro exposure system for permeation studies with aerosols*

The nasal epithelial cell line RPMI 2650 is well characterized in the literature and was used in several studies as model for the respiratory epithelium of the nasal cavity and the bronchus (Bai et al.,

2008a; Kreft et al., 2014a; Moore and Sandberg, 1964). Immunoreactivity for cell junction proteins such as tight junctions (*zona occludens*) and adhesion junctions (E-cadherin) confirmed the formation of a confluent cell layer 48h after seeding (figure 2A-C). The occurrence of cell junction molecules was accompanied by an increase of transepithelial electrical resistance (TEER; data not shown), a good indicator for monolayer integrity (Lehr, 2003). However, as typically observed, TEER values of RPMI 2650 are rather low (50 - 200 Ω x cm^2) and hence, less conclusive with respect to permeability as compared to other cell lines such as Caco-2 cells (Kreft et al., 2014b; Markowska et al., 2001). In addition, FITC-dextran was used as a fluorophore tracer at 125 µM to determine the permeation from the luminal (upper) to the abluminal (lower) compartment of the transwell. Previous studies suggest that cell lines, which are derived from epithelial cells differentiate at the interface to ambient air (ALI), form a tighter barrier than non-differentiated cells (Bai et al., 2008b; Gruenert et al., 1995). Accordingly, the P_{app} values decresed from 1.6 x 10^{-5} cm/s for submerged cultures to 1.2 x 10^{-5} cm/s for RPMI 2650 cultured at ALI when seeded at 4 x 10^5 cells/insert. Although, FITC-dextran permeability observed here was similar to what was reported previously (Hoogstraate et al., 1994; Wengst and Reichl, 2010), it should be noted that RPMI 2650 form a rather loose barrier.

In accordance with earlier reports (Bai et al., 2008b), we observed slight multi-layer formation occurring at seeding densities of 4x10^5 cells and higher. Hence, 2 x 10^5 cells/insert were chosen as this resulted in a confluent monolayer after 24h submerged and 24 h ALI conditions.

As mentioned above, the VITROCELL-CLOUD 12 system used in this study is the recently introduced commercial version of the prototype ALICE-CLOUD system presented by Lenz *et al.* (Lenz et al., 2014). To explore its aerosol delivery performance, the aerosol deposition factor and insert-to-insert variability of the insert-delivered aerosol volume were investigated. The three formulations and the vehicle were spiked with FITC-dextran (125 µM) and 1 mL was nebulized with the vibrating mesh nebulizer positioned at the top of the ALICE-CLOUD system (figure 2E). Each formulation was nebulized in triplicates into the exposure system and a total time of 7 min was allowed for

nebulization and aerosol settling. This is more than the 3 to 5 min of exposure time used by Lenz *et al.* (Lenz et al., 2014) since 1 mL instead of 0.2 mL was nebulized here.

The deposition factor for each formulation and vehicle was calculated based on the recovered FITC-dextran from the wells (figure 2F), normalized to the amount of substance used - the so-called "invested amount" - , taking into account that the wells represent only a fraction of the total area of the bottom inside the exposure chamber. For all protein formulations (F1, F2, and F3), the deposition factor was unity within experimental uncertainties indicating that no significant loss of substance occurred in the ALICE-CLOUD system. For the vehicle, the deposition factor was 16% lower (0.84) and nearly identical to what was reported previously (Lenz et al., 2014). This deviation was accompanied by an about 4-fold higher aerosol output rate as inferred from the shorter nebulization time of just 1 min for the vehicle (1.0 mL/min) as compared to about 4 min for each volume of F1 to F3 (0.25 mL/min). This phenomenon, which is probably due to the different viscosity and/or surface tension of formulation versus vehicle (Beck-Broichsitter et al., 2014a), can account for the moderate differences in deposition factor. For all four cases, the repeatability (%CV) of the well-delivered aerosol dose for different nebulizations was high (<5%) and the insert-to-insert variability of the well-delivered dose was less than 10% of the mean dose over all wells (5.4% for F1; 3.1% for F2; 6.8% for F3 and 8.3% for vehicle). Hence, in spite of the high output rate of the nebulizer for the vehicle, which leads to a slightly lower deposition efficiency, there was no statistically signficant change in repeatability and spatial uniformity of the aerosol cloud in the ALICE-CLOUD system. These differences in deposition factor, which resulted in slightly different aerosol volumes deposited per 24-well insert (2.44 µL and 2.05 µL for deposition factor of 1.0 (F1-3) and 0.84 (vehicle), respectively) were accounted in the permeability studies described below.

3.4. Influence of protein formulations on transport rates and abluminal recovery

To investigate the influence of formulations on transport across the airway epithelial cell line RPMI 2650, 30 µM of IgG, Fab and FITC-dextran were prepared in the various formulations and the vehicle.

Samples of 1 mL were nebulized and RPMI 2650 cells were exposed to the aerosol in the ALICE-CLOUD system for 7 minutes. After either 90 min or 240 min incubation the abluminal volume (figure 3F) was recovered for protein analysis. For the determination of abluminal recovery as prediction for local protein absorption, SE-HPLC is not suitable as it can hardly distinguish between IgG, Fab and proteins derived from the presence of RPMI 2650 cells. Therefore, IgG and Fab in the abluminal compartment were identified via standardized ELISA that recognizes the Kappa light chain. We have verified that the two ELISAs used here were selective to the native proteins only, *i.e.* aggregated and/or degraded proteins were not detected (data not shown). Hence, the reported protein transport rates refer to native protein only. However, we cannot rule out a minor disaggregating activity of the ELISA antibodies as observed for therapeutic antibodies (Moreth et al., 2013) that could result in a slight overestimation of the protein concentration. The determined amount of protein was corrected for differences in deposition factor and presented either as transport rate or percentage of protein recovered from the abluminal volume.

F1 had a remarkably positive influence on Fab transport and recovery after 240 min incubation: Compared to vehicle, 2.8-times higher levels of native Fab were found in the abluminal volume when F1 was used (figure 3A and figure 3C, ****$p<0.0001$). F2 also revealed a 2.1-fold improved transport rate as compared to vehicle (figure 3A, **$p<0.01$), while F3 did not significantly alter the transport rate as compared to the vehicle.

Although Fab is the proteolytic derivative of IgG, F1 had no significant effect on transport rate of IgG as compared to vehicle (figure 3B and figure 3D). Instead, F2 and F3 revealed improved transport rates of the native IgG protein (1.8-fold for F2, ***$p<0.001$ and 1.4-fold for F3, **$p<0.01$) as compared to vehicle.

F1 and vehicle were additionally investigated after 90 min of incubation and displayed in figure 3C-D as percentage of protein transported to the abluminal compartment relative to the total deposited protein per well. F1 demonstrated a time-dependent increase on Fab transport (figure 3C), but no effect at all on IgG transport (figure 3D). In figure 3E the transport rates for the formulations with the

strongest effect on permeation for each of the proteins are displayed, *i.e.* F1 for Fab and F2 for IgG are plotted against the protein's molecular mass (m_M). FITC-dextran (4 kDa) was used in F1 and vehicle as control for effects associated with permeability and not with stability. As expected for diffusion-driven processes, the transport rate increases with lower molecular mass according to a power law relationship ($\sim m_M^{-b}$; $b_{vehicle} = -0.62$; $b_{formulation} = -0.38$).

A potential positive bias on protein transport rates may have been arisen from inadvertent excipient-induced membrane permeabilization as reported for *e.g.* HBC (Fujii et al., 2013; Loftsson et al., 2005; Sintov et al., 2010; Tiwari et al., 2010) at concentrations of 20% in nasal epithelium (Abe et al., 1995). As seen from figure 3E this effect was negligible in the present study, since there was no significant effect of formulation F1 (HBC concentration below 1%) on FITC-dextran transport rates. This indicates that HBC and all other excipients had apparently no effect on tight junctions or permeability at the concentrations used in F1. Hence, it is likely that the observed increased abluminal protein recovery is predominantly due to formulation-induced protein protection during nebulization and subsequent barrier permeation, since denatured or aggregated proteins were not detected by ELISA. The protective effect during barrier penetration is evident from the enhanced abluminal protein recovery as compared to monomer recovery after nebulization (figure 1E-F).

4. Discussion

Aerosolized drug administration offers a broad application spectrum for the treatment of different pulmonary and systemic diseases (inhalation (Darquenne et al., 2016a)) but also of neurological disease such as neurodegeneration (intranasal (de la Monte, 2013)). Several intranasal studies are currently under investigation and further novel medical devices can be expected to reach the market in the near future (Touitou and Illum, 2013). Currently, a wide variety of small molecule drugs are clinically available, but the development of aerosolizable biopharmaceuticals peptids, proteins and cells is hampered by the delicate nature of biologics resulting in decreased efficacy and/or enhanced toxicity due to degradation during the aerosolization process (Frokjaer and Otzen, 2005; U.S. FDA,

2014). From vaccine development it is known that large protein assemblies with repetitive arrays of antigens are usually the most potent for inducing immune responses (Muttil et al., 2010). In particular, the airways harbour many immune-competent cells triggering immunological responses to drugs as it is well documented for intranasal vaccinations (Pabst, 2015). In particular, protein aggregates have a risk to induce immunogenicity representing a potential threat for the use of protein aerosols in the airways (Rombach-Riegraf et al., 2014). Luckily, suitable formulations can reduce the occurrence of protein aggregates, but established physiologic pre-clinical testing methods are lacking. Therefore, our aim was to provide a comprehensive screening platform with high-throughput potential for the development of formulations optimized for both protein stability during aerosolization and transepithelial permeation, the pre-requisite for drug delivery beyond the epithelial barrier.

An important requirement of this study was to provide the manufactured proteins in a high quality without detectable aggregates. This was achieved by optimizing the manufacturing yield of IgG and Fab, respectively, and by adding an additional polishing step as previously reported (Röhm et al., 2016b). Moreover, experimental drugs are often very expensive and in limited supply. Therefore, the screening platform was designed with stringent requirements on material-saving that included the use of the proteins at 30 µM (4 mg/mL for IgG), which is roughly 5-times lower than in a typical formulation for intravenous administration. The clinical relevant administered concentrations for airway delivery are quite diverse: while rather high concentrations are needed for intranasal insulin Nasulin™ (Leary et al., 2008), relatively low amounts of an Erythropoietin-Fc-fusion protein demonstrated clinical activity (Dumont et al., 2005). Relying on low concentrations for pre-clinical testing is not only advantageous in terms of cost-effectiveness, but also in terms of biodegradation testing, since proteins and other biotherapeutics are known to be more vulnerable in low concentrations, i.e. towards oxidation, deamidation or fragmentation affecting the heterogeneity and finally its safety and efficacy.

For this study, well established excipients for biopharmaceuticals were selected according to the US and European Pharmacopeia (Rowe et al., 2009). The addition of surfactants, like PS20 is important to reduce evaporation within the aerosol particle (Arun Rasheed, 2008; Shire, 2015). Also, it has been reported that PS20 can form a protective film at the ALI that may shield proteins (Jamur and Oliver, 2010) and HBC has demonstrated to reduce shear stress at the ALI during agitation (Serno and Carpenter, 2009), which presumably is advantageous for protein aerosolization. Furthermore, trehalose and other polyols are able to protect proteins against thermal unfolding (Kaushik and Bhat, 1998; Liu et al., 2010) that was reported to occur in vibrating mesh nebulizers (Hertel et al., 2014b).

An *in vitro* platform to assess the influence of different formulations on protein quality after aerosolization and transepithelial absorption is a prerequisite for the intranasal or inhalative delivery of proteins to the airway interstitium, where important effector cells for various diseases such as myofibroblasts for pulmonary fibrosis are residing (Fernandez and Eickelberg, 2012). Typically, this kind of permeation studies is performed under non-physiological, submerged cell culture conditions *i.e.* the drug is pipetted (not aerosolized) into the cell culture medium, which completely covers the cells (Darquenne et al., 2016a). This method is prone to provide biased results of drug transport, since it does not reflect the clinical conditions with respect to aerosolized drug delivery and ALI conditions of epithelial cells (Darquenne et al., 2016b; Lenz et al., 2013). For instance, a burst-like permeation kinetics with a large initial transport rate followed by a rapid decline was found for aerosolized drug delivery to ALI cells, while this was not the case for *in vitro* testing with standard submerged cell culture systems, where the drug is pipetted (not aerosolized) into the cell culture medium. This was recently demonstrated for liposomal cyclosporine (Schmid et al., 2017). While ALI epithelial cell cultures have been available for quite some time, easy-to-use and yet sufficiently efficient and dose-controlled, aerosol-cell delivery systems were lacking. In 2009, the ALICE technology was introduced (Lenz et al., 2009) and employed successfully for toxicological studies to deliver aerosolized saline and nanoparticle suspensions to ALI cell cultures (Brandenberger et al., 2010a; Brandenberger et al., 2010b; Endes et al., 2014; Lenz et al., 2009). Recently, the ALICE-CLOUD

system, a refined version of the ALICE technology, was described and employed in a proof-of-concept study for inhalation drug screening focusing on small molecules (Lenz et al., 2014). This technology has the potential to become the state-of-the-art method for characterizing the performance of inhalation products and preclinical testing of innovative drug formulations (Lenz et al., 2014). In the present study, the commercial version of the ALICE-CLOUD technology (VITROCELL-CLOUD) is introduced, its performance is described and applied for the first time to proteins. For nebulization of the vehicle, the repeatability of the delivered dose, the insert-to-insert variability and the deposition factor agreed with the corresponding values reported by Lenz *et al.* for a 6-well prototype version of the ALICE-CLOUD system (Lenz et al., 2014). Similarly, no significant differences were observed for the formulations except for the deposition factor, which was higher (mean of all three formulations = 1.04) than the value reported for the vehicle (0.84). It is important to note that the enhanced deposition factor is associated with a 4-fold lower liquid/aerosol output rate of the nebulizer (0.25 and 1.0 mL/min for protein formulations and vehicle, respectively). Hence, it is likely that this phenomenon is due to differences in viscosity and/or surface tension of the nebulized substance, which are known to affect the liquid output rate of vibrating mesh nebulizers (Beck-Broichsitter et al., 2014b). We conclude that the performance of the VITROCELL-CLOUD system for protein formulations is as good as the previously described prototype of the ALICE-CLOUD system (Lenz et al., 2014).

Surprisingly, the tendency on protein stability displayed in figure 1E-F was not indicative for abluminal recovery (figure 3) and the difference between vehicle and “best” formulations in these two experiments increased from roughly 30% observed for monomer recovery to 300% displayed in transport rate. A possible explanation for this might be the different experimental conditions *e.g.* the exposure time to ALI: For monomer analysis the formulated protein samples were nebulized into a 15 mL tube, where the wall-deposited droplets gathered quickly to a larger amount of fluid and were stored in a closed tube without gas exchange until analysis. By contrast, in the ALICE-CLOUD system less than 2.5 µL of the mist reached the transwell insert with an area of 0.334 cm^2 forming an circa

7.5 µm thick liquid film on the luminal side of the cells. During the 90 minutes to 4 hours incubation time in the incubator, an intensive interaction with air and in particular oxygen can be assumed. Although evaporation is mitigated due to the almost (water-)saturated conditions in the exposure chamber and the incubator, even small evaporation and re-condensation rates may not be beneficial for the structural integrity of the proteins. Since similar conditions are likely to occur on during inhalation therapy on the pulmonary epithelium, the conditions in the exposure system are likely to be closer to the *in vivo* conditions than in the highly artificial conditions during the nebulization experiments for monomer analysis. For future studies, these effects should be taken into account for monomer analysis by nebulizing the protein samples into the transwell inserts (e.g. with the ALICE-CLOUD) and including the prolonged incubation times for the permeability assay. This may improve the predictability of the stability screen for the permeation properties of protein formulations.

In this study, the Fab was less stable than the IgG it was derived from. However, it cannot be concluded that Fabs are always less stable than full-length IgGs, since manufacturing conditions or even concentration changes can speed up protein unfolding or aggregation process (Yano et al., 2009).

In the present study, a clear negative correlation (power law) between molecular mass of the studied proteins and their transport rates was observed. This is consistent with results previously reported for peptides (Föger et al., 2008). Hence, the use of Fab or even smaller fragments instead of the full IgG promises better permeation through the airway tissue. However, also other aspects should be considered for transepithelial delivery of IgG to secondary organs such as the brain, which can be accomplished either through the lung (via blood circulation) or targeting through the nose (via olfactory bulb). Neonatal Fc receptors (FcRn) transport IgG via their Fc domain from the mucosal surface to the blood, which can be a very favourable uptake mechanism (Bitonti and Dumont, 2006). By contrast, FcRn also transport Fc-bearing molecules from the brain to the blood (Cooper et al., 2013). A permeation of 11% through *ex vivo* porcine nasal mucosa after 2.5 h was reported for the clinical product Avastin® (Samson et al., 2012) containing the mAb bevacizumab formulated for

parenteral use with 0.6 % trehalose, 0.0004 % PS20 in phosphate buffer (Warne, 2011). In general, the percentage of transported drug is hardly comparable from one study to another as it differs dramatically when altering the experimental conditions. Therefore, we calculated from the published data (Samson et al., 2012) an absolute transport rate of roughly 65 $\frac{fmol}{cm^2 \cdot s}$, which is about 10-times higher than what we achieved here for IgG. This indicates that despite the high molecular mass of this mAb and although this formulation was not developed for nasal administration, a very efficient transport was evident, which might be related to FcRn. Recently, the presence of FcRn was confirmed in nasal epithelium (Heidl et al., 2015). Hence, Fc-bearing proteins as IgG and Fc-fusions may be advantageous for mucosal drug delivery when targeting the central compartment, and accordingly Fc-domain free antibody fragments and scaffolds may be more suitable for intranasal CNS delivery.

It is well known that IgG (HIRMab 83-14) is transcytosed by its antigen, the insulin receptor (Pardridge et al., 1985). To exclude transcytosis of IgG in RPMI 2650, expression of human insulin receptor was tested by immunofluorescence, flow cytometry and RT-PCR (unpublished data). This analysis revealed that these cells hardly express any significant amount of insulin receptor. Therefore, we can exclude that transcytosis of IgG or Fab via the insulin receptor plays a relevant role in the RPMI 2650 model (Di Guglielmo et al., 1998). Currently, we perform a study that compares active and passive transport in the RPMI 2650 model and the kinetics observed in the present study clearly indicates passive transport mechanisms. So, it is conceivable that no other transcytosis mechanism is involved in this model.

5. Conclusion

With this study, we present an efficient screening platform for rapid and reliable evaluation of candidate formulations intended for high protein stability and efficient transepithelial transport of aerosolized drugs by combining and refining two automation-friendly technologies: a throughput-

optimized aerosolization-surrogate assay for protein stability and the ALICE-CLOUD technology. This platform approach enables an efficient, high-throughput, and material-saving development of formulations for inhalative and intranasal delivery of aerosolized drugs. In addition, we conclude that – in spite of the dominating effect of the permeation assay for the proteins investigated here – a comprehensive formulation platform for protein aerosols should include both, evaluation of protein aggregation and effects on mucosal permeation to assess the full spectrum of a protein formulation.

Declaration of interest

The authors declare no commercial or financial conflict of interest.

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Legends to figures

Figure 1: **Screening and confirming formulations for protein aerosols**

(**A**): A vibrating mesh nebulizer oscillates a perforated membrane at a high frequency (100,000 times/second) to generate an aerosol. This nebulizer was used in this study.

(**B**): Agitation was used as surrogate for nebulization as it is suitable to run 96 samples in parallel for a fast and cost-efficient formulation screen. Aggregation of proteins was detected by SE-HPLC.

(**C**): High-throughput DoE screening of excipients stabilizing Fab during agitation. Normalized model regression coefficients are displayed with a 95% confidence interval (± SD) for monomer recovery. Only regression coefficients are shown, which revealed a significant impact on the model.

(**D**): Response contour plot showing the impact of arginine and sorbitol with red colour for high monomer content and blue for low monomer content. Concentrations of other excipients were constant at trehalose (3.25%), HBC (0.35%) and PS20 (0.0275%). Three formulations (F1-F3) were selected (closed circles) for further investigation.

(**E**): Monomer content of Fab after aerosolization relative to non-aerosolized protein to verify the protective effect of formulations selected in the DoE screen. All experiments were conducted at least in duplicates (mean ± SEM). For statistical analysis, an unpaired two-tailed Student's t-test was applied and differences were considered significant at *$p<0.05$ vs. vehicle, **$p<0.01$ vs. vehicle, $^{+}p<0.05$ vs F3, and $^{\S\S}p<0.01$ vs F2. (**F**): As (E), but for IgG.

Figure 2: **Cellular exposure system to screen the influence of formulations on aerosolized protein quality and mucosal permeation.**

(**A-D**) Adhesion protein immunoreactivity in RPMI 2650 seeded at 2 x 10^5 cells per insert incubated for 48 h. Representative confocal microscopy images stained for the nucleus (Hoechst, blue), tight junctions (*zona occludens*, green), adhesion junctions (E-cadherin, red),

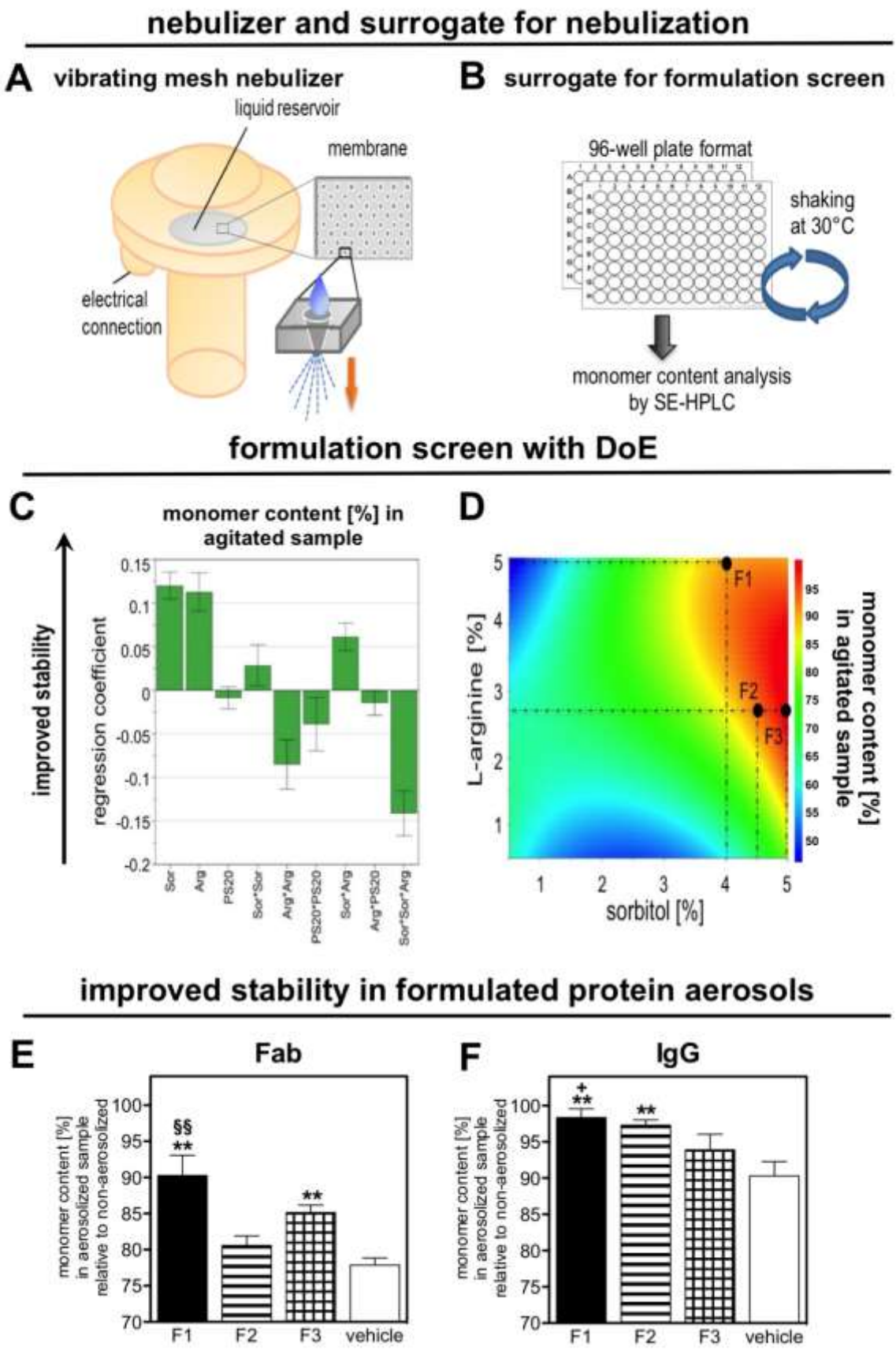
and a merge of all three fluorescence channels. Cellular contacts are important for the formation of a tight barrier-like epithelium. Scale bars = 10 µm.

(E) Experimental setup of the ALICE-CLOUD system (VITROCELL-CLOUD 12) with nebulizer, insert support rings and transwell inserts. The main exposure chamber offering space for 9 transwell inserts is used for aerosol-cell exposure. Only the 6 wells indicated by the arrows were used in the present study.

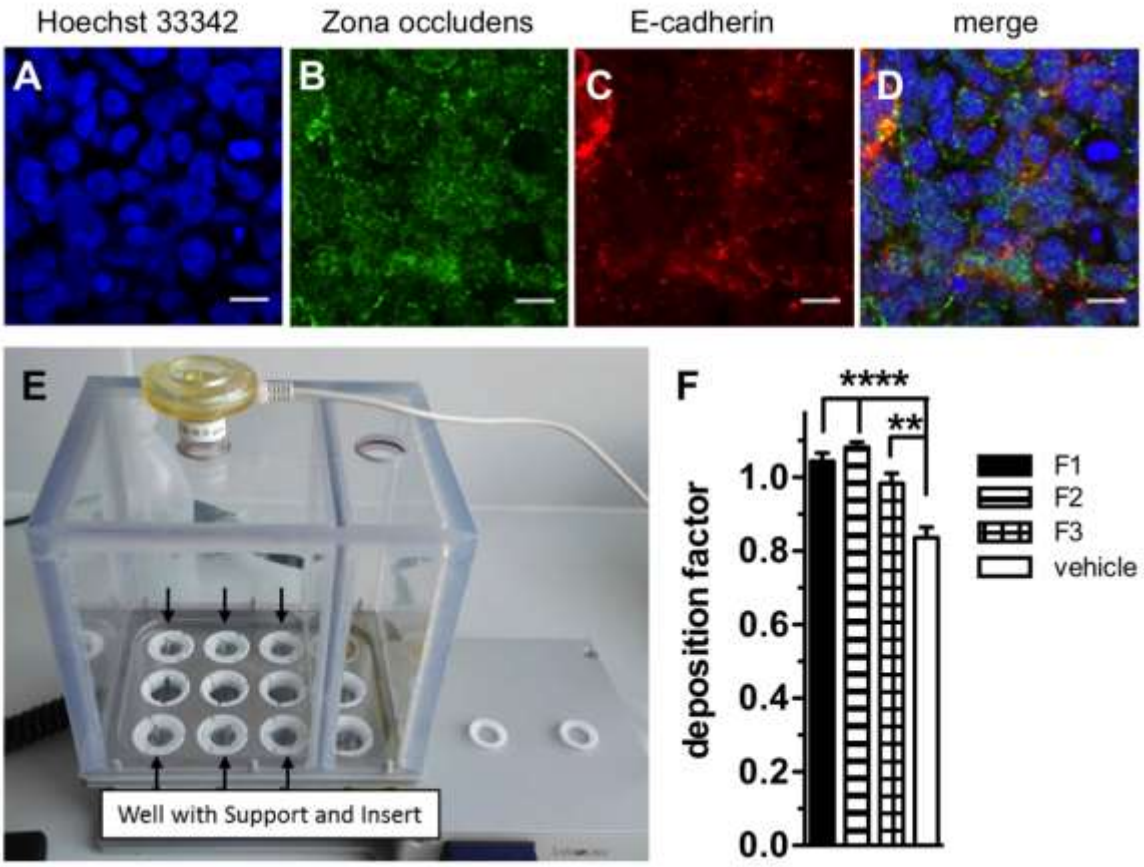
(F) The deposition factor represents the percentage of aerosol mist reaching the bottom of the exposure chamber after 7 min settling time (relative to the "invested amount of liquid" (1 mL)). It was measured using FITC-dextran as a fluorescent tracer. Values close to unity indicate almost no substance loss in the ALICE-CLOUD system (n = 3 independent experiments, mean ± SEM; one-way ANOVA with a Bonferroni post-hoc test; **p<0.01 and ****p<0.0001).

Figure 3: **Permeation of aerosolized proteins through the RPMI2650 cell layer to the abluminal transwell compartment**. 30 µM Fab, IgG and FITC-dextran were dissolved in the three different formulations or vehicle (FITC-dextran only in F1) and nebulized with the vibrating mesh nebulizer placed on the ALICE-CLOUD chamber.. (A-B) Transport rates of the different formulated protein solutions after 240 minutes. (C-D) Kinetics of permeation for formulation 1 and vehicle. (E) Dependence of transport rate at 240 min on molecular mass and formulation (best formulation is displayed, *i.e.* formulation 1 and 2 for Fab and IgG, respectively). (F) Setup of transwell inserts in the ALICE-CLOUD wells. All aerosol experiments were performed with n=6 per formulation and incubation time. (mean ± SEM; **p<0.01, ***p<0.001, ****p<0.0001, One-way ANOVA).

Figr-1



Figr-2



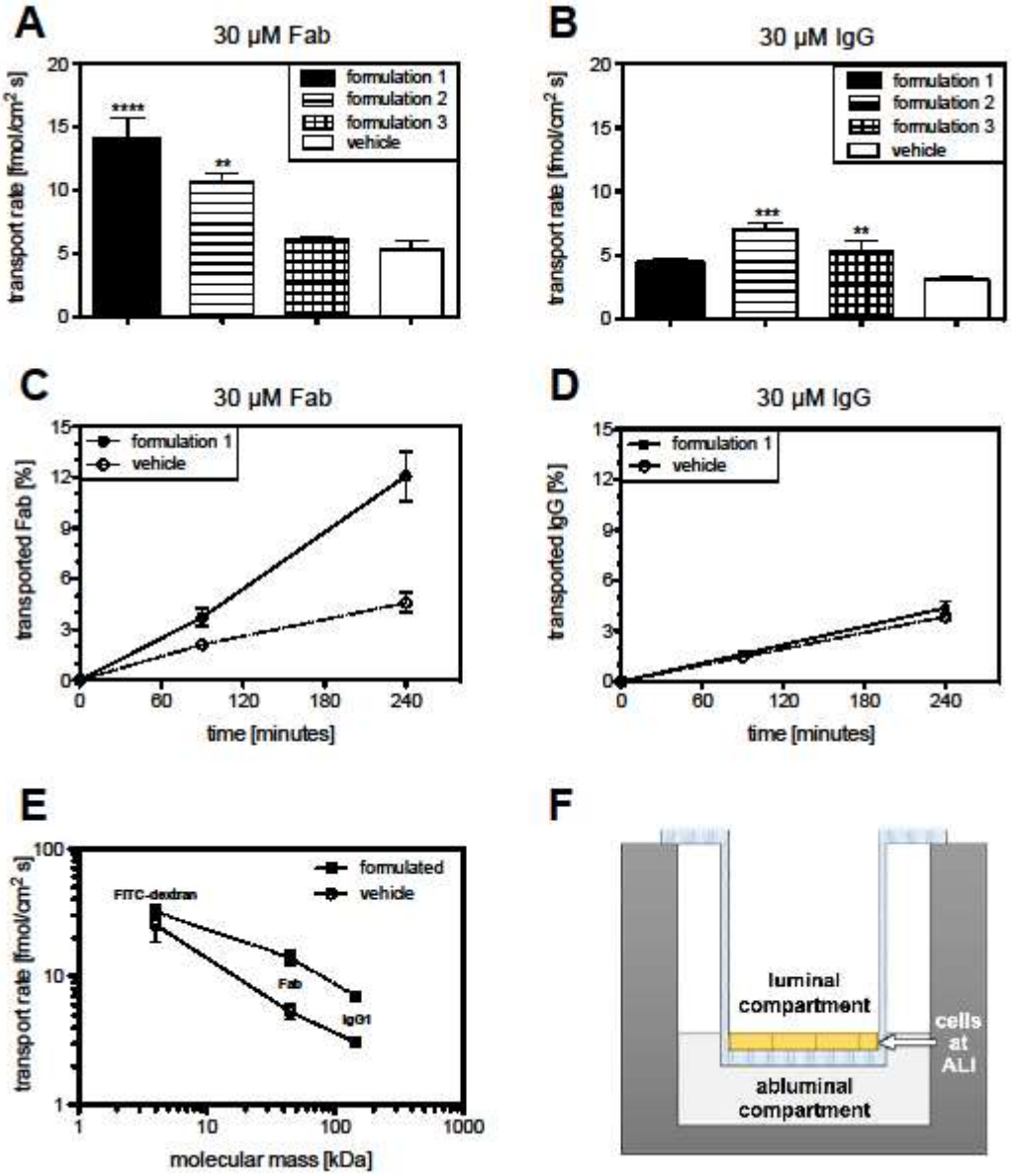


Table 1: **Composition of three different protein formulations (plus vehicle control) that were tested for protecting protein integrity during aerosolization and for effects on permeation.** F1-F3 contain different concentrations of trehalose, sorbitol, arginine, PS20 and HBC. Density of formulations was calculated according to the volumetrically weighted densities of the individual excipients.

	compounds [% w/v]						density
formulation	trehalose	sorbitol	L-arginine	PS20	HBC	PBS	g/cm^3
F1	1	4	5	0.0275	0.35	1x	2.0461
F2	6	4.5	2.75	0.0275	0.35	1x	2.3711
F3	6	5	2.75	0.03	0.2	1x	2.4064
vehicle	-	-	-	-	-	1x	1.0081