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Can pulmonary RNA delivery improve our pandemic preparedness?

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ABSTRACT

The coronavirus pandemic has changed our perception of RNA medicines, and RNA vaccines have revolutionized our pandemic preparedness. But are we indeed prepared for the next variant or the next emerging virus? How can we prepare? And what does the role of inhaled antiviral RNA play in this regard? When the pandemic started, I rerouted much of the ongoing inhaled RNA delivery research in my group towards the inhibition and treatment of respiratory viral infections. Two years later, I have taken the literature, past and ongoing clinical trials into consideration and have gained new insights based on our collaborative research which I will discuss in this oration.

1. Introduction

1.1. How RNA formulation and delivery research fell into my lap

When I started to work on RNA formulation in 2004, it was like a dream come true for me. Ever since I had studied genetics and nucleic acids in my biology course in high school, I was mesmerized by the beauty and intelligence of biology and code. I have always been a nerd, and after teaching myself HTML from a book, I built my own website about the genetic code in 1999 where I stated back then that I wanted to work in genetics or clinical pharmacy one day – notably with a tiled image of a double-helix as the background of my page kept in transparent pastels to allow for the text to be decipherable. Twenty-some years later, I am not a geneticist and don't exactly work in clinical pharmacy, but I am doing exactly what I had envisioned before graduating high school: I am combining the beauty of biology and code of nucleic acids for therapeutic purposes. I believe I just didn't know back then how to phrase it. But developing medicines based on nucleic acids is what I vaguely had in mind. Sometimes in life it is all about being in the right place at the right time, and therefore, I am glad until this day that I chose to study pharmacy in Marburg (much to my parents' regret who would have had it easier to support five children throughout their university years had I accepted a full ride chemistry scholarship). It is probably not to the surprise of the readership of the Journal of Controlled Release that I, once again, found fascination in one particular subject during my university studies, namely Pharmaceutical Technology taught by Professor Thomas Kissel at the time. The interdisciplinary approach of pharmaceutical sciences was what had led me to the decision to study pharmacy rather than chemistry in the first place. Therefore, Prof. Kissel's engaging lectures appealed to my interest in applied physical chemistry for therapeutic purposes in combination with biopharmacy, the interaction between physiology and physics. It wasn't much later that his PhD students asked me to join the group as a student assistant, and during the interview a few weeks later, I thought I was just applying for the assistantship when Prof. Kissel said he had heard I wanted to do a PhD. From that day on, I believe I was part of the group. I started my student assistantship and learned a bit about gene delivery and polyplexes, and everyone knew I would come back after graduation for one half of my pharmacy intern year in the Kissel group. In the early 2000s, RNA delivery was still in its infancy, and when I heard my own project would be about siRNA formulation, I believe only a dozen papers were to be found online that even described what siRNA was. With my fascination for therapeutic nucleic acids that had grown over the years, I was enthusiastic about this opportunity and chance to start this specific research area in the Kissel lab. To connect the dots, I eventually started my siRNA delivery and formulation project as part of my pharmacy intern year in Prof. Kissel's lab in 2004, and I have been stubborn enough to focus on siRNA delivery for over 17 years now, despite dozens of rejected grant proposals on the topic and lots of unpleasant reviewers' comments.

1.2. RNA therapeutics today

When I made my first baby steps towards RNA formulation and delivery research in 2004, RNA therapeutics were praised the medicine of the future. But this future has become our reality in the meantime [1].

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With currently two messenger RNA (mRNA) vaccines on the market, four short interfering RNA (siRNA)-based therapeutics and five antisense oligonucleotide (ASO)-based drugs [2], a literal RNA revolution has started which is only thwarted by one major hurdle, that is delivery [3]. RNA requires efficient protection from degradation by ubiquitous RNases which can be achieved by nucleic acid chemical modification or by nanoformulation. Most ASO-based drugs, for example, are heavily stabilized by 2′O-methoxyethyl-stabilized or phosphorodiamidate morpholino ribose modifications with or without phosphorothioate backbone modification [2]. Both approved mRNA vaccines contain N1-methylpseudouridine nucleobases to increase effectiveness [4]. On top of stability, delivery across membranes is an important barrier for macromolecules of course. Therefore, the approved siRNA therapeutics either contain minimal chemical 2’-*O*-methylation when encapsulated in lipid nanoparticles (LNPs) for delivery, as in case of patisiran [5], or they are maximally stabilized by 2’-*O*-methyl- and 2′-fluoro-groups when they are administered as *N*-acetylgalactosamine (GalNac)-conjugates, as realized for givosiran, lumasiran, and inclisiran [6]. For both mRNA vaccines, LNP formulations were chosen, confirming the need for efficient delivery of macromolecules [7]. LNP formulations have advanced from liposomes over LNPs for small molecules to siRNA loaded LNPs prepared by rapid mixing, which have paved the way for the fast development of the mRNA vaccine formulations in 2020 [8]. While LNPs are currently the only approved nanocarriers for siRNA and mRNA, they also bear disadvantages such as their immunogenicity, their passive targeting to the liver [9], and their rather low drug loading with just short of 4% *w*/w mRNA per lipid in Comirnaty®, for instance [7]. Therefore, in the area of RNA formulation and delivery science, it seems imperative to develop delivery alternatives using materials other than lipids or by combining characteristics of lipids and other materials, such as polymers [10].

1.3. Development of polymer based RNA nanocarriers

While RNA researchers around the world felt affirmed, relieved and maybe a bit proud at the same time when the first COVID-19 vaccines were approved in 2020 and their many years of research finally paid off for the good of humanity, the impact of the broad administration of LNP-based COVID-19 vaccines during the current pandemic needs to be taken into consideration for future treatments with LNP-based therapeutics. Considering that Comirnaty® and Moderna COVID-19 Vaccine both employ LNP formulations which are surface modified with polyethylene glycol (PEG), it is expected that after several vaccine booster shots, a large abundance of immune response towards PEG will be observed in the general public [11]. Therefore, nanocarriers that do not rely on PEGylated lipids will become essential in coming years to ensure effectiveness of therapeutic RNA delivery and to avoid the so-called “accelerated blood clearance” (ABC) phenomenon of PEGylated nanocarriers after production of IgM against PEG [12–14]. Polyamine-based nanocarriers could overcome these shortcomings and additionally offer the advantage of particularly high RNA drug loading with a drug-to-excipient ratio up to one in contrast to lipid-based nanocarriers which require larger weight-based excesses of excipient in order to efficiently encapsulate small RNA [9]. Polyamine-based RNA nanocarriers such as polyethylenimine (PEI) bear other drawbacks, however, such as their inherent toxicity, particularly in cases of very high density of positively charged amino groups [15,16] which has been addressed by many polymer modifications in the past years [17–19].

The approach my lab chooses to decreasing toxicity and increasing *in vivo* biocompatibility is the design and synthesis of biodegradable polyspermines which are based on the endogenous polyamine spermine [20]. The other drawback of polyamine-based RNA nanoformulations in contrast to solid lipid nanoparticles is their dynamic state. Polycations such as polyamines self-assemble with polyanionic nucleic acids due to electrostatic attraction into so-called polyelectrolyte complexes (short polyplexes). Such polyplexes have a less defined morphology and composition which has been reported to lead to polydisperse particle size distribution and poorly reproducible formulations [21]. By microfluidic assembly of polyplexes, both disadvantages can be addressed efficiently, however, for the formulation of defined and reproducible RNA nanocarriers [22]. In the context of electrostatic self-assembly of polyplexes, instability problems have also been observed *in vivo* where RNA was released prematurely after intravenous injection before the target site was reached [23]. Since electrostatic polyplex formation is characterized by high enthalpic gain accompanied by a lower and unfavorable entropic loss with an overall gain in absolute free energy ΔG [24], the formation of polyplexes and their stability depend on the parameters of their environment, including the ionic strength and dielectric constant of the solvent they are dispersed in [25]. Hence, optimization of polyplex RNA nanoformulations always must include enhancing their stability. In the past years, we have learned that hydrophobic modifications of polyamines critically contribute to polyplex stability [24,26,27] and enable efficient endosomal release of siRNA nanocarriers after endocytosis [22]. Therefore, we have developed new poly(beta- amino ester) (PBAE) brush copolymers with spermine- and fatty acid-side chains (Fig. 1) which can readily be synthesized and modified in a tailormade manner for a defined hydrophilic/hydrophobic ratio and with saturated or non-saturated fatty acids to accommodate the specific needs for RNA formulation and delivery with respect to RNA encapsulation, polyplex size and zeta potential, formulation reproducibility and polydispersity, cellular delivery, endosomal escape and therapeutic efficacy.

1.4. Predicting molecular interaction between carrier and RNA for rational nanocarrier design

Polymer synthesis is a bottom-up approach that, depending on the synthetic route chosen, can yield large amounts of material. However, the approach of the past years where libraries over libraries of differently modified polymers were synthesized to learn later on that maybe one or two materials were well-suited for their intended purpose seems a bit antiquated in the times of artificial intelligence. Machine learning has become a common tool in drug discovery in the past years already [28,29] but also finds more and more applications for drug delivery purposes. Brad Pentelute's group at the MIT, for instance, reported the identification of cell penetrating peptides *via* machine learning algorithms [30], and Theresa Reineke's group at the University of Minnesota recently established algorithms to correlate polymer and polyplex characteristics with their *in vitro* fate and efficacy for plasmid and ribonucleoprotein (RNP) complex delivery [31]. Beyond the aforementioned approaches, my group additionally engages molecular dynamics (MD) simulations to better predict molecular interaction between carrier materials and RNA for the rational design of enhanced RNA nanocarriers.

This area of research particularly resonates with my fascination for code and combines areas I am very interested in, including computer language, genetic code, synthetic chemistry and formulation science.

Based on our previous studies on RNA and DNA formulation, it has become obvious that nucleic acids behave differently during polymer encapsulation depending on their structural features. Even though polyplex formation is an electrostatically-driven process, supercoiled double-stranded DNA interacts differently with polycations in comparison to rigid and short double-stranded siRNA [24,32,33], and long single-stranded mRNA is expected to behave differently from the former two. We have therefore previously applied molecular dynamics simulations for energetic and structural analyses of nucleic acid-polycation interactions on the atomic level between differently modified polymers and dendrimers and RNA to compare thermodynamic data with experimentally obtained thermodynamic assembly results measured by isothermal titration calorimetry [33]. Accordingly, we have correlated computational data with polyplex characteristics [32] and their *in vitro* and *in vivo* efficacy [24] as a function of chemical modification to

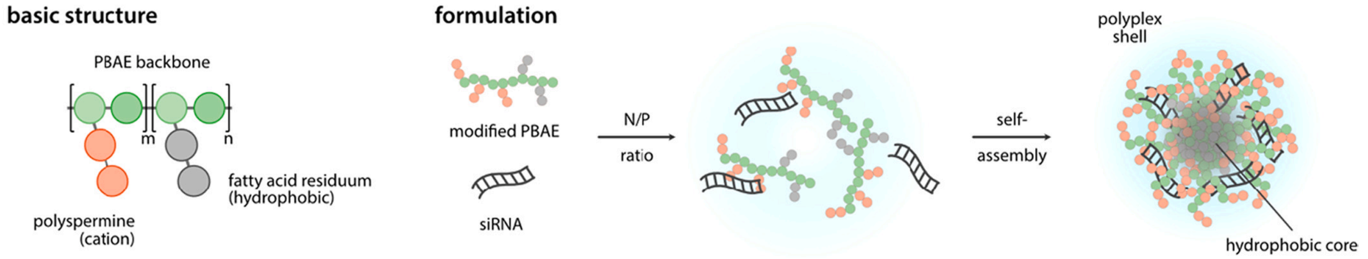

Fig. 1. *Polyplex formation with amphiphilic poly(beta- amino ester)s.* siRNA molecules assemble with modified PBAEs due to electrostatic and hydrophobic interactions. The hydrophobic chains of the polymers assemble into a hydrophobic core of what can be called a micelleplex. Hydrophilic parts of the polymer are oriented towards the interface with an aqueous dispersant.

determine conducive nanocarrier characteristics (Fig. 2). Two main conclusions were drawn based on our previous work, namely the need for flexible polymer structures for efficient encapsulation of rigid, short double-stranded RNA, and the need for amphiphilic materials whose hydrophobic cores increase polyplex stability [24]. Therefore, we currently assess flexible brush *co*-polymers with readily exchangeable side chains for optimizing RNA formulation and delivery and the prediction of their molecular interaction with RNA depending on side chain modifications.

In contrast to the all-atom MD simulations performed in my previous projects, my group currently develops coarse grained (CG) models for mapping atomic structures of the brush copolymers to MARTINI beads based on the recently upgraded MARTINI forcefield [34] (Fig. 3). Even though details from all-atom simulations are partially lost in CG models, the advantage of the reduced amount of degrees of freedom is the ability to simulate far larger molecules over longer time frames. Considering that both nucleic acids as well as our synthetic polymers are made of repeating units, the new bead types and subtypes that came with the MARTINI upgrade are expected to support efficient mapping of the complex structures we deal with. The advantage of the brush *co*-polymers is their linear polymer structure, for which models can be generated rather easily in MD simulations by multiple tools to simulate the dynamic behavior of large co-polymers. For branched polymers, different individual polymers can be combined to block-copolymers [35] as shown in the preliminary work simulating a blend of PCL-PEG and PEI-PCL-PEI (Fig. 3).

The biggest advantage we see in this computer-aided approach is that based on a Design-of-Experiment setup only a small set of polymers rather than an entire library needs to be synthesized experimentally [36]. After correlating their chemical composition with simulated and experimentally determined parameters, assumptions can be made on optimized chemical structure based on *in-silico-in-vitro* simulations. After establishing machine learning algorithms for the rational design and prediction of material characteristics, individual polymers which show enhanced properties *in silico* can be synthesized later for *in vitro* evaluation and to assess the simulations' ability to predict lead polymer compositions. With this rational design of materials, we can decrease synthetic efforts to use our resources more sustainably and to avoid synthesizing materials that will sit on shelves until the next generation of PhD students needs the space.

1.5. *RNA delivery to the lung*

As discussed above, nanocarriers optimized for payload protection and delivery of RNA tend to accumulate in the liver upon intravenous injection [23]. To develop approaches for RNA delivery beyond the liver, alternative administration routes have been investigated in numerous clinical trials [37]. Clearly, intramuscular delivery of mRNA has been proven effective in billions of humans in the past 12 months [38]. With the success of intravitreal (fomivirsen) and intrathecal (nusinersen) injections, other local administration routes such as intranasal or pulmonary delivery have been tested in clinical trials, but so far no approved product has evolved [39].

Having trained in the Kissel lab, myself as well as Juliane Nguyen at UNC Chapel Hill, Lea Ann Dailey at the University of Vienna and Dagmar Fischer at the University of Erlangen have kept an interest in pulmonary drug delivery. Speaking for myself, I have focused my work mainly on local administration routes, with the strongest attention to nasal and pulmonary delivery.

The lung, indeed, offers a variety of currently undruggable targets which could potentially be treated with RNA therapeutics, while all the marketed siRNA drugs target the liver. With more attention to the development of inhalable formulations, local, pulmonary delivery of RNA nanoparticles could potentially finally enable delivery beyond the liver.

In fact, delivery to the lung was the aim of one of the first siRNA clinical trials with Alnylam's ALN-RSV01, which showed remarkable effects in humans [40–42]. As discussed recently [39], the nasal spray

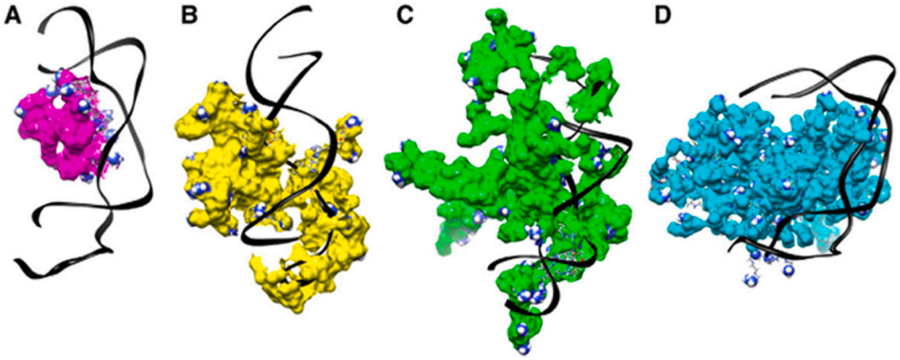

Fig. 2. *Molecular Dynamics Simulations.* Interactions of siRNA with differently modified dendrimers (A-C) and PEI (D). Reproduced with permission. Reproduced with permission. Copyright 2011, Elsevier.

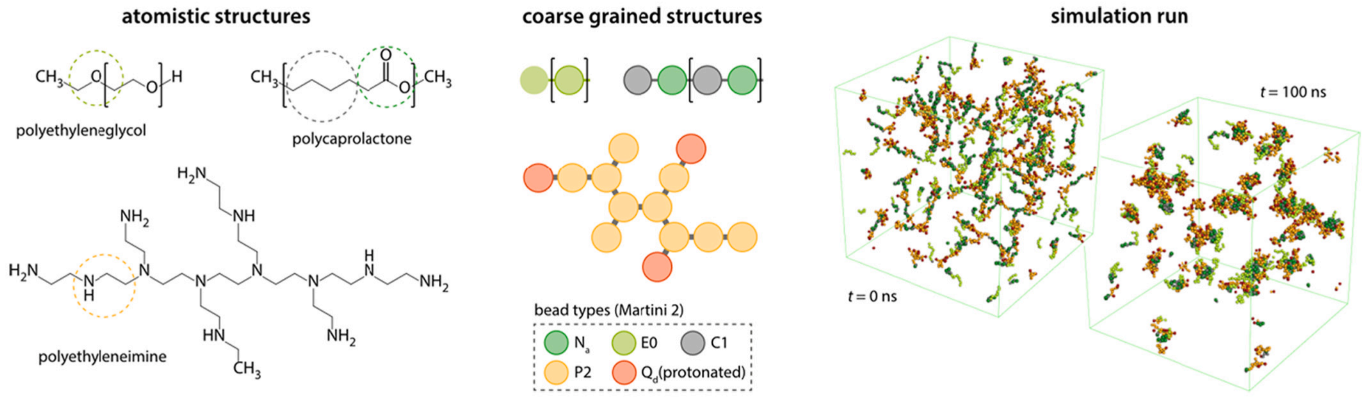

Fig. 3. *Molecular Dynamics Simulations.* Preliminary Martini 2 simulations with PEI-based block copolymers grafted with polycaprolactone (PCL)-polyethylene glycol (PEG) chains.

containing naked siRNA administered to adults that were experimentally infected with *wild-type* RSV decreased the number of infections by 38% [42], but the reduction of progressive bronchiolitis obliterans in lung transplant patients, set as primary endpoint in the following phase 2b study, was not met, unfortunately. In my personal opinion, this failed study is a result of two shortcomings in the study plan. First, only one single-sequence siRNA molecule was used. Considering how fast SARS-CoV-2 escape mutations are observed when treated with shRNA [44], it seems likely that drug resistant RSV variants emerged under therapy. Therefore, in upcoming anti-viral siRNA trials, a mixture of siRNA sequences should be delivered in parallel [45]. The second aspect I believe could be improved is the delivery route. While nasal administration of siRNA leads to large parts of drug dose being swallowed and degraded in the gastrointestinal tract, inhalation formulations require more pharmaceutical development but lead to more quantitative lung delivery [46].

In my opinion, the failure of ALN-RSV01 reflects an overlooked area of formulation science that is highly important for pulmonary RNA delivery. While pulmonary delivery of nucleic acids in general is the focus in at least a dozen academic groups around the world, development of clinically relevant dosage forms is not considered by many. Without knowing the exact picture in industry, it seems that aerosol medicine development and characterization is not a top priority for most RNA companies. Understanding and optimizing lung deposition and developing formulations that patients will accept, is imperative, however, if inhalation clinical trials are expected to deliver.

Inhalation devices currently available (Fig. 4) include atomizers and soft mist inhalers (SMIs) for liquid formulations, pressurized metered dose inhalers (pMDIs) which are most commonly used for small molecule inhalation, and dry powder inhalers (DPIs). Development of pMDI [47] and DPI [48] formulations of nucleic acid nanoparticles requires far greater development effort, however, compared to liquid formulations. Therefore, liquid formulations are generally developed, particularly for *in vitro* assessment. To mimic the nebulization process *in vitro,* the ALICE Cloud exposure system can be used to directly nebulize drug formulations onto cell culture wells [49]. As rodents are obligate nose breathers [50], inhalation exposure is in principle also possible in preclinical experiments. However, the exposed drug dose is subject to strong inter-individual variation after nose-only exposure [50], and therefore, in preclinical pulmonary administration studies with potent drugs, intubation and intratracheal nebulization with devices such as the Penn-Century microsprayer is commonly chosen [51,52]. For potential clinical administration, various types of nebulizers are available, with vibrating mesh nebulizer offering advantages for the nebulization of macromolecules [39].

My group has also taken on the challenge of developing dry powder formulations from RNA loaded nanosuspensions for DPI development by spray-drying [48,53]. The challenge in this endeavor is in fact not only the spray-drying process itself but also the question if the dry powder redisperses into nanoparticles with unchanged parameters in comparison to the freshly prepared formulation after spray-drying. Since DPIs also offer important advantages, the quest is worth the effort, however. In an inhalable solid dosage form, chemical instabilities and microbial contaminations are significantly limited, and physical instabilities such as sedimentation, aggregation, coalescence or creaming are avoided [54]. Development of DPIs requires process engineering and optimization [48,53]. But in the light of the required ultra-low temperature storage requirements of Comirnaty®, for example, the development of a spray-drying platform technology for a range of different RNA nanoformulations seems very attractive for improved storage and transport conditions, particularly in developing countries. Spray-dried powders offer the opportunity for inhalation administration but can as well be redispersed into suspensions for other administration routes, of course. In contrast to freeze-drying (lyophilization) where small volumes of

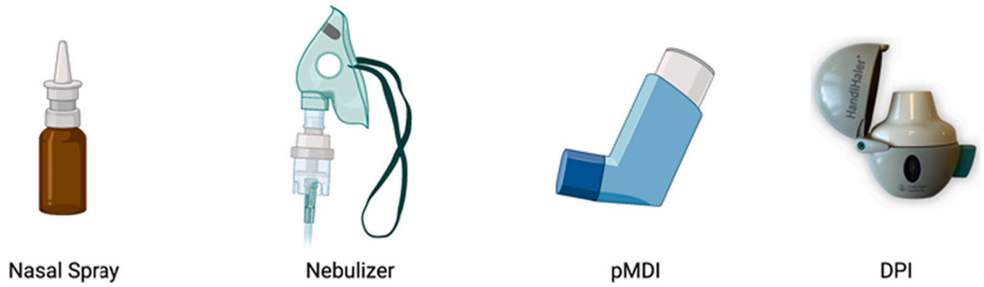

Fig. 4. Administration devices for RNA delivery to the lung. Nasal sprays are easy-to-handle devices but don't allow for quantitative delivery to the lung. Nebulizers are less handy, but development of nano suspensions for aerosolization is more straight-forward than development of pMDIs and DPIs. Figure created with BioRender.com

liquids are converted into lyo-cakes, spray-drying allows for continuous manufacturing in combination with microfluidic assembly of the RNA nanoformulation and continuous feed of the spray-dryer to reduce variability between batches [55]. Additionally, spray-drying is less energy consuming and thus cheaper and more sustainable than freeze-drying.

Two globally-acknowledged groups, namely Camilla Foged's group at the University of Copenhagen [56], and Jenny Lam's group at Hongkong University [57], also focus on developing inhalable RNA dry powder formulations. TranslateBio (recently acquired by Sanofi) recently disclosed a patent application describing their efforts on LNP spray-drying [58]. But so far, successful spray-drying with RNA-loaded LNPs without polymer encapsulation has not been reported in the literature. Our collaboration with Dominik Witzigmann and Pieter Cullis at the University of British Columbia has resulted in a platform technology we recently protected [59], and we are looking forward to optimizing this platform further.

For the sake of completeness, I also want to mention pMDI formulations of nucleic acid nanoparticle suspensions. Their biggest advantage is that patient compliance of pMDIs is highest amongst all inhalers. Unfortunately, their development in liquefiable gasses is particularly challenging and often results in poor dosage uniformity [47]. Sandro da Rocha at Viginia Commonwealth University has a long-standing interest in this research area and has successfully developed pMDI formulations with siRNA-dendrimer conjugates, for example [60].

As discussed in a recent review article, the aerodynamic properties of aerosol medicines need to be optimal for quantitative deposition at their site of action [39]. For deep lung deposition, the ideal aerodynamic diameter of the aerosol is expected to be between 1 and 5 μm. In contrast, smaller particles can be easily exhaled, and larger particles are deposited in the upper airways mouth and throat depending on their aerodynamic size [61,62]. This size does not refer to the nanoparticles within a nebulized suspension, however, but rather to the droplet size produced by the nebulizer. Other parameters that are important in the development of RNA aerosol medicines are the influence of heat, interfaces and shear stress on the macromolecule during nebulization [63], as well as RNA integrity and recovery in order to optimize engineering processes, surface materials, and to determine therapeutic doses [48].

If all of these parameters are optimized, there is just one final hurdle, which is stability and diffusivity of RNA nanoformulations in the lung environment [64].

1.6. Mimicking conditions of the lung in vitro and identifying therapeutic targets

Even though the lung is directly accessible *via* inhalation, its barriers are manifold. Many reviews describe the branched anatomy, the presence of mucus, the stratified epithelium, cellular and immunologic factors as main hurdles for nanoparticulate drug delivery to the lung [46,64–66]. Therefore, we recently discussed *in vitro* models of the lung cultured at air-liquid interface (ALI) for drug delivery and disease modeling [49]. In sophisticated ALI models, lung epithelial cells grow on porous membranes into multilayered, polarized and differentiated epithelium [68] after removing the medium from the apical chamber (Fig. 5). Many ALI-cultured cell lines and primary cells additionally produce mucus (Fig. 5) to mimic yet another relevant aspect in drug delivery but also in disease modeling.

For our research, ALI cultures have become indispensable. While we can also measure mucus diffusion in simple cell-free setups using artificial or donor mucus in porous membrane chambers [69] or by fluorescence correlation spectroscopy [70], for example, only the actual cell culture experiment reflects the changes nanoformulations undergo in the presence of mucus, which may affect their cellular uptake. Even though pulmonary mucus does not contain serum, the formation of a protein corona in mucus has been discussed [71], and instabilities have been observed [72]. But another aspect has become evenly important for our work in anti-viral siRNA delivery to the lung, which is the fact that many respiratory viruses cannot multiply in medium-covered 2D cell cultures [73]. This observation is underlined by our finding that ALI-cultured Calu-3 cells express high levels of ACE-2 receptor on their apical side leading to very high titers after SARS-CoV-2 infection [71], while very low receptor levels are found in 2D-cultured Calu-3 cells which are not differentiated and do not have an apical side.

ALI models with different lung cancer and healthy lung epithelial as well as nasal epithelial cell lines have been established in my lab and routinely serve as models for assessing siRNA and drug delivery with nanoparticles through the mucus layer and into the epithelial cells and for the assessment of therapeutic gene silencing in ALI culture [69,71]. In this regard, my lab has mainly worked with monocultures, even if co-cultures with macrophages, dendritic cells or other cell types can of course add value in ALI models to better mimic the *in vivo* conditions [49].

The intrinsic disadvantage of ALI cultures, however, is their static nature, whereas the lung epithelium *in vivo* is supplied by blood flowing in the underlying capillaries. Donald Ingber at Harvard University has therefore developed powerful organ-on-a-chip models, including lungs-on-a-chip [74], and we are currently developing co-culture models on an ibidi microfluidic chip for ALI culture (https://ibidi.com/content/409-ibidi-prototypes-for-virology-research).

While we mainly focused on anti-inflammatory siRNA delivery in the past and have recently reported on anti-NFkB siRNA delivery in a cystic fibrosis project in collaboration with Francesca Ungaro at the University of Napoli [69], we dove into new endeavors in the summer of 2019 and started collaborating with a young and upcoming virologist, Thomas Michler at TU Munich. At the time, he was interested in antiviral siRNA delivery to the lung to work on treatments of RSV. But only about six months later, it became obvious that we had to shift gears and focus on a different respiratory virus: SARS-CoV-2. The past years have been eye-opening for me as a pharmaceutical technologist with very little virology background. But the synergism between Thomas Michler's expertise in molecular biology, virology, anti-viral RNA and clinical science and our approaches of formulating RNA for aerosol medicine has been a great joy for all lab members on both sides. Initially, the Michler

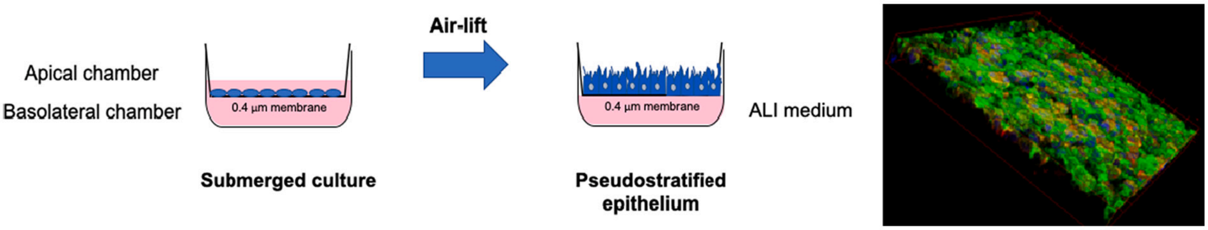

Fig. 5. ALI cell culture. Shown schematically using the example of Calu-3 cells (mucus stained with FITC-WGA Wheat-Germ agglutinin, green), cytoskeleton red, cell nucleus blue). Reproduced with permission. Copyright 2021, Wiley. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

group worked very hard on identifying targets in the coronaviral genome that would be conserved and accessible to RNA interference, and after lots of bioinformatic and *in vitro* screening, ORF1 was identified as a promising target in SARS-CoV-2 [75]. In the next step, we collaborated on developing *in vitro* [71] and *ex vivo* [76] models of infection. For the *in vitro* model, we chose an ALI mono-culture of Calu-3 cells, considering that we had observed high levels of ACE-2 receptor on their apical membrane, which allowed for high titer infection with the Wuhan strain [71]. Most importantly, however, in collaboration with Suzie Pun at the University of Washington in Seattle, we used her Virus-inspired polymer for endosomal release (VIPER), which we had successfully used before for *in vivo* pulmonary siRNA delivery [52], and were able to show efficient mucus diffusion of the VIPER/siRNA nanoparticles as well as significant reduction of viral replication in the Calu-3 infection model [71]. For the *ex vivo* approach, we obtained ethics approval through LMU's biobank to use human peritumor lung tissues from lung biopsies to produce human lung explant sections, so-called precision-cut lung slices (PCLS) [76]. The beauty of PCLS is that they not only reflect the complex interplay between cell types in the lung parenchyma but even preserve mobile cells, such as macrophages, dendritic cells, T cells and many more. Therefore, in contrast to cell culture, they really reflect in a three-dimensional manner the architecture and cellular setup of the lung as well as changes to the extracellular matrix when tissue is obtained from diseased patients [77]. After confirming the dose-dependent infect ion of the human tissue with the Wuhan strain [71], we showed over 90% reduction of SARS-CoV-2 replication after prophylactic transfection with an optimized siRNA sequence [76]. The Michler group has in the meantime screened over 300 siRNA sequences after initial sequences showed very high conservation of the target sequence as well as high activity after chemical modification [76].

I consciously chose the title of this oration to be a question. And by no means do I have an answer. It is probably wishful thinking to some extent because we are all tired of this pandemic. We miss human interaction, socializing, we miss normal school days, play dates and birthday parties for our kids, we are sick and tired of video calls and online conferences. We cannot deal with yet another quarantine, and we need to lose some weight to get out of our sweat pants and into dress pants again. It is a challenging time, and we were so full of hope when the vaccines became available, but we are dealing with yet another rough winter. So, can pulmonary siRNA delivery improve our pandemic preparedness? Is there light at the end of the tunnel?

The good news is that the siRNA developed by the Michler group targets a sequence that is highly conserved (>99%) in all variants of interest and all variants of concern of SARS-CoV-2 [76] as well as in other coronaviruses. Considering the high risk that coronaviruses could cross the species limits from animal reservoirs again [78], pandemic preparedness means to some extent that we need to make an educated guess of what to expect. If we can target conserved regions of the coronavirus genome that also potential future human-pathologic coronaviruses will contain, we will be a step ahead next time. But the question is: Can escape mutations arise also for these highly conserved regions, or would the virus lose viability or other critical characteristics if this region were to mutate? A recent study shows how fast escape mutations arise upon AAV-mediated shRNA transduction [44]. Therefore, there is still work to do for us.

1.7. Pulmonary delivery in vivo

What is also still missing in our quest for a potential inhalable siRNA based therapy against respiratory viruses is compelling *in vivo* efficacy and safety data. Even though the conditions in the lung can be mimicked in cell culture models [49], only *in vivo* experiments account for biodistribution, clearance and physiologic phenomena [72], which cannot entirely be incorporated into sophisticated cell culture models. Pulmonary siRNA delivery can be investigated in a number of disease models, including respiratory virus infection models [79]. But in the beginning of the pandemic, wildtype mice were considered unsuited because murine angiotensin-converting enzyme 2 (ACE2) receptor, the functional host receptor on pulmonary epithelium for both SARS-CoV-1 and SARS-CoV-2 [80–82], had very little affinity with the receptor-binding domain (RBD) in SARS-CoV-2. The wildtype virus (now Wuhan strain) could only be used in animal models of hamsters, ferrets, humanized hECE2 mice [83] or higher animals [84]. With the arrival of the various variants, however, it has been shown that new RBDs have appeared that do indeed have high enough affinity towards murine ACE2 that wildtype mice can conveniently be infected [85]. As all these animal models can only be employed under biosafety level 3 (BSL3) conditions, however, alternative models using BSL2 viruses have gained interest in the past months. With our goal of targeting conserved regions of the coronavirus genome, endemic coronavirus strains (229E, HKU1, NL63, OC43) which instigate symptoms similar to the common cold and can infect wildtype mice [86–88] could therefore serve as models for SARS or MERS infections.

In our next steps, we therefore need to confirm efficacy of Thomas Michler's siRNA sequences against different coronavirus strains *in vitro* to further assess *in vivo* therapeutic efficacy of pulmonary siRNA delivery in an endemic infection model. Our goal is to translate our *ex vivo* prophylactic setup into *in vivo* efficacy and safety data to optimize doses and dosing regimens. Afterwards, the treatment of a manifest infection can be investigated. To ensure delivery to the right cell types in the lung, we recently confirmed that siRNA polyplexes are efficiently taken up by epithelial cells, such as Type II pneumocytes, club cells and ciliated cells [71], which are the main target cells for SARS-infections [89,90]. And to keep an eye on safety, we measured cytokine levels in the lung lavage and serum of treated mice, where no elevated levels were observed [71].

2. Conclusions

In conclusion, I believe that pandemic preparedness is a combination of aspects. Having efficient antivirals that are specific enough to display a favorable therapeutic range but broad enough to be effective against potential newly emerging viruses is key. But also the ability to produce and store such antivirals affects our preparedness. The combination of identifying antiviral siRNA against conserved areas of the coronavirus genome with developing safe nanomedicines and the production of dry powders for inhalation and enhanced storage stability puts us in a good position for developing RNA therapeutics – the dream I already had in high school without being able to put it in words. And to return to the central thread, we also aim to eventually correlate results from our *in vivo* studies with *in silico* and *in vitro* parameters to determine if efficient siRNA nanocarriers can be predicted by computational approaches.

My personal goal for the coming years is the establishment of a machine-learning algorithm for predicting effective RNA nanocarriers to enable clinical trials with RNA therapeutics more quickly in the future.

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