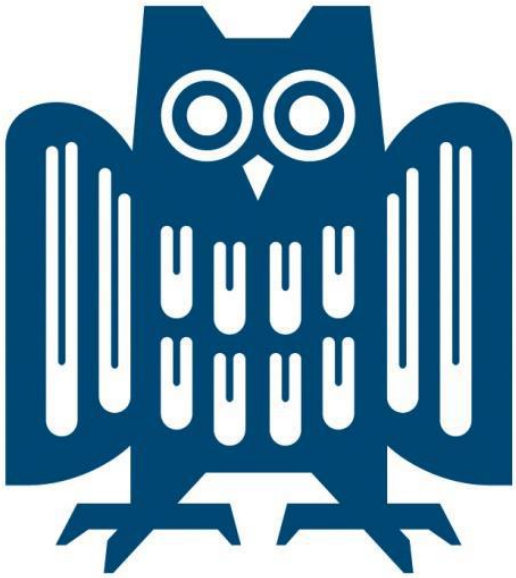
Preparation of Novel Multifunctional Formulations Intended for Pulmonary Delivery



Dissertation zur Erlangung des Grades

des Doktors der Naturwissenschaften

der Naturwissenschaftlich-Technischen Fakultät

der Universität des Saarlandes von

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Saarbrücken 2019

Tag des Kolloquiums: ……25.07.2019……

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Die vorliegende Arbeit wurde von April 2015 bis Juni 2018 an der Universität des Saarlandes unter Betreuung von Prof. Dr. Marc Schneider angefertigt.

If you see it in your mind,

You can hold it in your hand.

Steve Harvey

Dedicated to my beloved

Syria and my family

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Summary

The eradication of pathogenic bacteria in cystic fibrosis (CF) lungs is often unsuccessful because of the poor permeability of the drug through the thick respiratory mucus barrier and biofilm. Nanoparticles are promising carriers due to their ability to cross these barriers and to deliver the loaded drug into the site of action. Nanoparticles exhibit a poor lung deposition; therefore, they need to be transformed into microparticles, which are suitable for dry powder inhalers and thus can be applied pulmonary.

In this work, PLGA NPs loaded with Curcumin were produced using microfluidic technology. While using different factors during the manufacturing, their effect on the size and the encapsulation efficiency of the drug was evaluated. Also, the particles showed a fast permeability through the mucus barrier. Furthermore, a novel microparticulate matrix (composed of an antibiotic and N-acetylcysteine) was developed using a spray-drying technique in order to embed the NPs within it and to add even more functionality to the formulation.

The aerodynamic properties of microparticles were tested using NGI. Then, their impact on the viscosity of mucus was tested. Additionally, a solid-state characterization and investigations regarding the physical stability of the obtained DPI formulations were performed. Finally, their effect against biofilm formation and inflammation was tested. Summarizing, the formulations represent a promising approach for the future treatment of pulmonary infections in CF.

Kurzzusammenfassung

Oft misslingt die Eradikation pathogener Bakterien in Atemwegen von Mukoviszidose Patienten. Grund ist die schlechte Permeabilität des Arzneistoffes durch den pulmonalen Mukus und den Biofilm. Nanopartikel sind hierfür vielversprechende Trägersysteme, da sie diese Barrieren durchdringen und den geladenen Arzneistoff an den Wirkort bringen können. Aufgrund der geringen Deposition von Nanopartikeln in der Lunge ist die Umformulierung der Mikropartikel nötig, damit sie pulmonal appliziert werden können.

In dieser Arbeit wurden durch Verwendung von Mikrofluidik Kurkumin beladene PLGA Nanopartikel hergestellt. Der Einfluss verschiedener Herstellungsfaktoren auf Partikelgröße und Verkapselungseffizienz wurde bewertet. Die Partikel zeigten eine rasche Permeation in den Mukus. Mittels Sprühtrocknung wurde eine neuartige Matrix (aus Antibiotikum und N-Acetylcystein) entwickelt, die die Einbettung der Nanopartikel erlaubt und die Funktionalität der Formulierung weiter steigert.

Die aerodynamischen Eigenschaften der Mikropartikel wurden mittels NGI untersucht und die Verflüssigung des Mukus wurde nach Anwendung gezeigt. Die Formulierungen für den Pulverinhalator wurden im festen Aggregatzustand charakterisiert und ihre physikalische Stabilität getestet. Schließlich wurde ihre biologische Wirkung (gegen Biofilme und Entzündungsreaktionen) untersucht.

Die Formulierungen stellen einen vielversprechenden Ansatz für die künftige Behandlung von pulmonalen Infektionen bei Mukoviszidose dar.

1. Introduction

1.1 Cystic fibrosis

Cystic fibrosis (CF) is the most typical severe chromosomal recessive disorder; it recorded among Caucasians with an incidence of one case per 2500 births [1, 2]. It is also very well known as multi-organ disease as it influences the lungs, small intestine, gall and pancreas. The most prominent reason behind this disease is a mutation in a single gene on chromosome number (7), followed by the formation of the translated protein called Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) which works as a cAMP-regulated chloride channel [1, 2]. Hence, the mutation in CFTR results in the misfolding or malfunction of proteins which fail to reach the apical cell membrane. Furthermore, altered CFTR causes unregulated chloride and subsequently Na+ transport which thus leads to improper water absorption in response to the net transport of NaCl [3, 4]. This improper content of water in lungs leads to dehydration of the airway surface liquid (ASL) which is giving rise to build-up viscous mucus which is as well triggers impaired mucociliary clearance (MCC) [3]. As a result, the cilia cannot beat efficiently in such drastic condition, resulting in the entrapment of bacteria in the viscous airway surface liquid (ASL) for a prolonged period of time. Such prolonged infections increase the risk of life-threatening lung infections and inflammation [3, 4]. Persistence of such condition may eventually initiate tissue damage as it is illustrated in the cycle of lung disease in (figure 1) [5]. It has been shown that CF patients have a higher risk of bacterial infections such as *Haemophilus influenza* and *Staphylococcus aureus* which always happen in an early stage of the disease [6]. Nevertheless, *Pseudomonas aeruginosa (P. aeruginosa)* is the major infective pathogen which influenced the lung of patients in CF and the treatment for P. aeruginosa infection is very difficult to be cured, due to the mucoid growth of the bacteria [6, 7]. The thick mucus and as well the chronic bacterial infection with well-protecting biofilm matrix is considered the main challenge for efficient therapy in CF lungs [2].

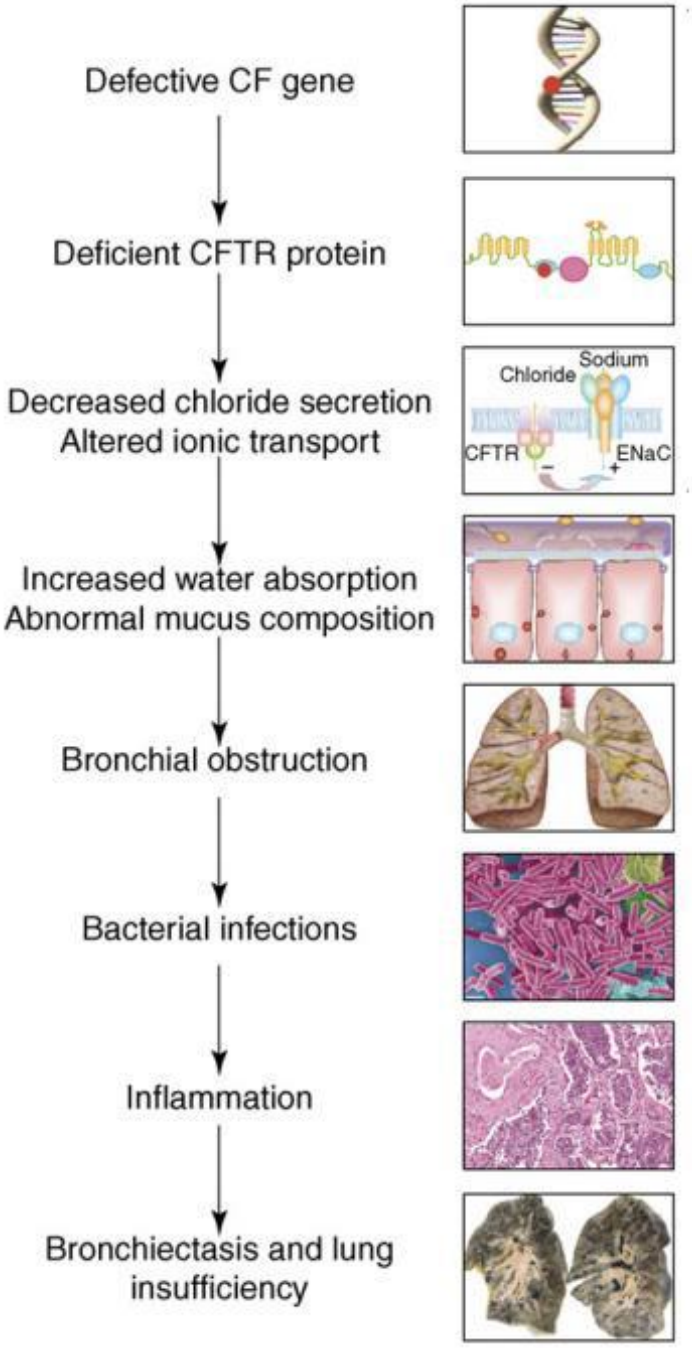

Fig. 1: *Trend of CF dysfunction. Image was taken from ref [8].*

Nonetheless, a successful treatment for curing the CF diseases would rely on providing a gene therapy or CFTR correctors. Although research is still under investigation particularly, gene therapies are still far away from being available as a product on the market [8]. For CFTR correction, Ivacaftor (oral drug) is the only and first CFTR potentiator available on the market. Unfortunately, only 4% of patients who carry the specific mutations, which responsd to Ivacaftor treatment, can benefit from it [9]. Due to lacking gene therapies, the whole concept of the treatment mainly relays on symptomatic therapy in order to increase the life expectancy of the patient and improve their quality of life. In the last few years, an increase in the life expectancy of CF patients from 18 to 31 years was observed (1995-2012) indicating the success of general treatment [2, 5, 9, 10]. Despite all these improvements in the treatment, eradication of pathogenic bacteria is still facing several challenges such as developing a resistance towards antibiotics after a long time of treatment. Therefore, improvements in the treatment of CF are still needed to ensure a better quality of life and prolong the life expectancy of the patients.

1.2 The strategy for the treatment of cystic fibrosis disease

As it is illustrated in table 1, the concept of the treatment in CF disease is mainly based on symptomatic therapy such as using high doses of antibiotics to counter microorganism infections and manifestation of biofilms. For a sufficient treatment against bacteria, co-therapy treatment such as a combination of orally antibiotics (P.O.) with intravenously (i.v.) or pulmonary (inhalation) applied antibiotics is more appropriate for evading the resistance of bacteria towards antibiotics [10]. Even though the systemic therapies afford a successful treatment, their sturdy adverse effects in gastrointestinal tracts like nausea, diarrhea, and vomiting are the main restraint for not being the first choice [11]. Therefore an alternative route of administration of antibiotics is needed. Taking antibiotics in the inhalation dosage form is more convincing, due to the requirement of fewer doses in comparing to the oral dose. Furthermore, according to the last guideline for treating CF disease, it is always recommended to take a mucolytic agent such as N-acetylcysteine (NAC) or other inhalation drugs like dornase alfa and saline prior to antibiotics dose [2, 12]. The aim of this combination therapy is to fluidize the abnormal viscous mucus by the mucolytic agents and it is also intended to aid the antibiotics to diffuse faster through the less viscous mucus and indirectly facilitate also mucociliary clearance [13]. Moreover, D-Mannitol (an osmotic material) has gained a great deal of attention within the scientific community, since it works as an osmotic agent within the respiratory organs [14]. The mechanism of action works by permitting the water to accumulate within the lung lumen resulting in decreasing the consistency of the thick mucus barrier [14].

In addition, to slow the progression of airway inflammation in CF lung disease, a dose of corticosteroids and nonsteroidal anti-inflammatory drugs like Ibuprofen (NSAIDs) is highly recommended. Moreover, Ivacaftor as CFTR corrector drug can be used in CF patients, but only in case the patient can respond to the ivacaftor treatment [9]. At the final stage of disease, lungs transplantation would be the last option.

Table1: *Guideline of the treatment in cystic fibrosis.*

Promote airway clearance • Physical techniques (like physical therapy, high–frequency chest wall oscillation) • Pharmaceutical approaches (like Dornase alfa, hypertonic saline, albuterol) • Exercise
Treat bacterial infection • Systemic antibiotics • Aerosolized antibiotics like aztreonam for inhalation, tobramycin inhalation solution
Modify CFTR • Ivacaftor
Reduce inflammation • Ibuprofen • Steroids
Replace damaged lungs • Lung transplantation

1.3 Overcoming biological barriers using nanoparticles

In the light of nanotechnology science, nanoparticles (NPs) are represented as an attractive drug delivery system because NPs have the potential to overcome the thick mucus and biofilm barrier in CF patients (Fig 2) [15].

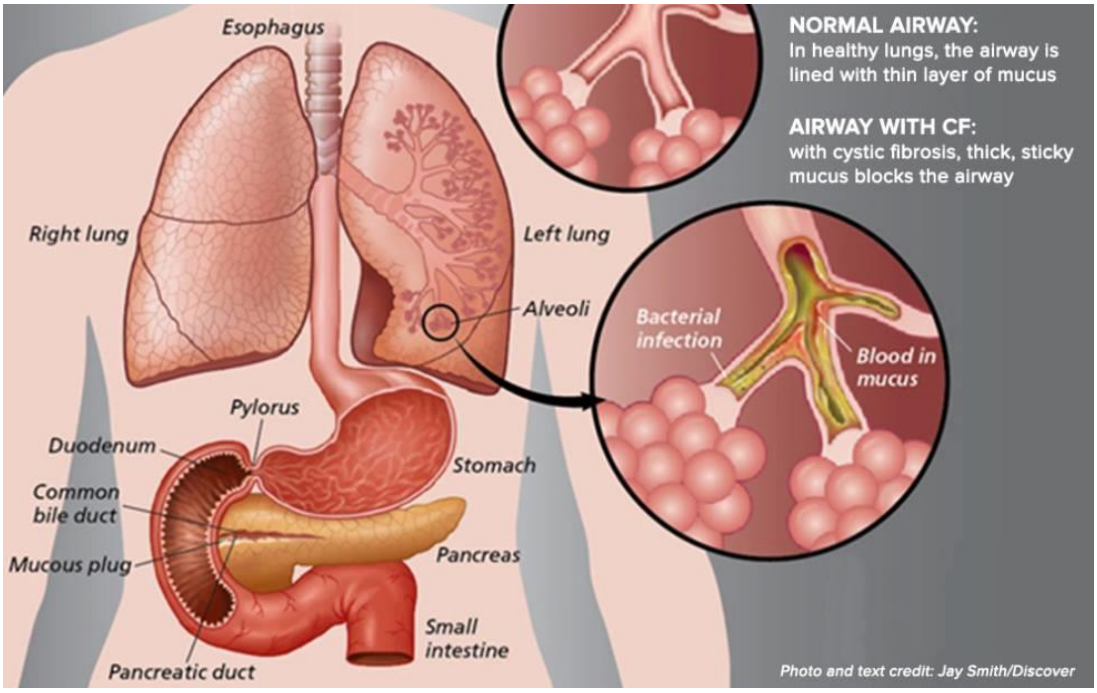

Fig. 2: *Normal and CF airways. Source www.discovermagazine.com*

The advantages of using nano-carrier systems in CF are (1) permeation of drug through the thick mucus in CF, (2) aiding the drug for a better permeability through biofilm matrix, (3) protection of the drug from inactivation inside the mucus and biofilm, (4) offering a controlled and sustained drug release profile [15, 16]. The combination of these advantages can result in decreased toxicity and at the same time improved effectivity of the used drug as compared to traditional formulations. Generally, to enable the particles to move freely through mucus without being immobilized, NPs should be designed with specific characteristics to evade their interaction with the viscous mucus barrier in CF [17].

For the development of muco-penetrating nanoparticles, understanding of the filtering mechanisms of particulates within the mucus barriers is indispensable. Normally, the filtering of particles through the mucus barriers can occur in two steps, biophysical (size filtering) or biochemical (interaction filtering) mechanism [18]. As it is very well known that the size of the mesh structure of mucus differs depending on the state of disease, for instance, in CF disease, the pores size is in range of 150 ± 50 nm. Thereby, submicron particles with a size less than 100 nm are needed for avoiding their immobilization within the pores, ensuring of delivering drugs and across the mucus barrier [15, 18]. Regarding the interaction filtering mechanism, several interactions (e.g. H-bond interaction, electrostatic interaction; hydrophobic interaction, etc.) could occur [18]. Avoiding such kind of interactions can be accomplished by engineering the surface chemistry of particulate (charge) because it plays a decisive role in determining the level of interaction of this particulate with biological barrier. After conducting comprehensive studies on the way of NPs-mucus interaction, it was shown that using neutral or slightly negatively particles such as PEG-PLGA NPs diffuse more easily without restriction through mucus barrier in comparison to positively charged particles such as chitosan NPs [19, 20]. The positively charged chitosan NPs tend to interact mostly with the negatively charged mucins via electrostatically interactions; therefore Chitosan NPs are categorized as muco-adhesive particulates [21]. On the flip side, the neutral or slightly negatively charged particles are categorized as muco-penetrating particles due to that the particles are mostly repelled from mucus barrier [19].

For this reason, the neutral particles exhibit faster permeability to cross the thick mucus barrier in CF. As mentioned before, negatively charged NPs with a diameter of ~ 100 nm such as PLGA-PEG NPs, have freely diffused without being immobilized in the pores of the thick mucus barrier, in contrast to the behavior of NPs with a diameter ~500 nm which have been retained within the mucus pores without crossing the mucus barrier (Fig. 3) [22-24].

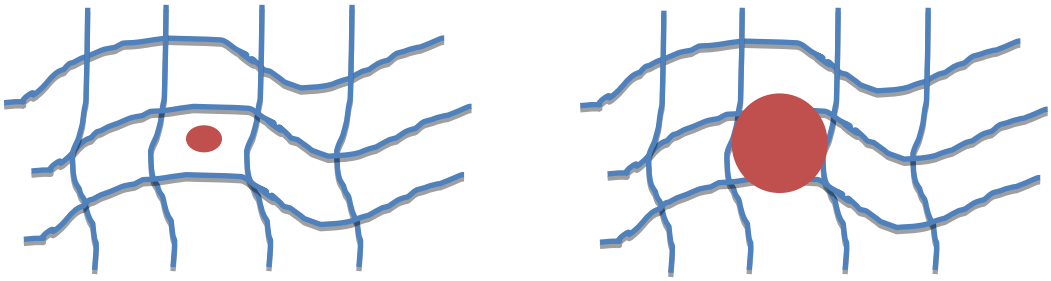

Fig. 3: *Size filtering mechanism of particles through the network of mucus.*

Apart from facilitating the permeability of NPs, coating or shielding the surface of NPs would add benefit in term of enhancing the stability of the formulation and might be as well contribute to hinder hydrophobic interactions [25].

Bacterial biofilm is also considered as the main challenging barrier for NPs to penetrate. The bacterial biofilm is an extracellular matrix containing several components such as polysaccharides, proteins, lipids, and nucleic acids which enrich the biofilm with negative charges [26]. The same as in the thick mucus in CF, the size exclusion and electrostatic interactions take place as well in the bacterial biofilm [18]. NPs have shown a better penetration in bacterial biofilm as well as a higher local concentration in the biofilms, due to the fact that the drug is perpetually released inside the viscous-mucus and not at once before being penetrated into the mucus barrier [16, 27]. To conclude, advanced drug delivery systems such as nanoparticles represent an ideal transport and protection carrier for drugs, with a point of view to improve the efficacy of the drug and to lower toxicity effect.

1.4 Obstacles in delivering nanoparticles to the lungs

The lungs are an appealing area for drug delivery *(air-to-lung-delivery)* as it provides high solute permeability for the drug, its large surface vicinity for absorption and its restricted proteolytic activity [28]. The functional structure of the lungs is divided into 2 regions. The airways area which is including trachea, bronchi, and bronchioles and the second region called the alveoli which are for gas exchange. The respiratory system consists mainly of the trachea which is dividing into two branches called bronchi and then the bronchi still continue to branch into minuscule bronchioles where finally, the terminal bronchi are finishing with what is called the alveolar sacs (Fig.4) [29, 30]. In CF disease, it's most ideal that inhaled particles will be deposited in bronchioles as it is normally the disease process conventionally begin there and then reach out toward the bronchi area [29].

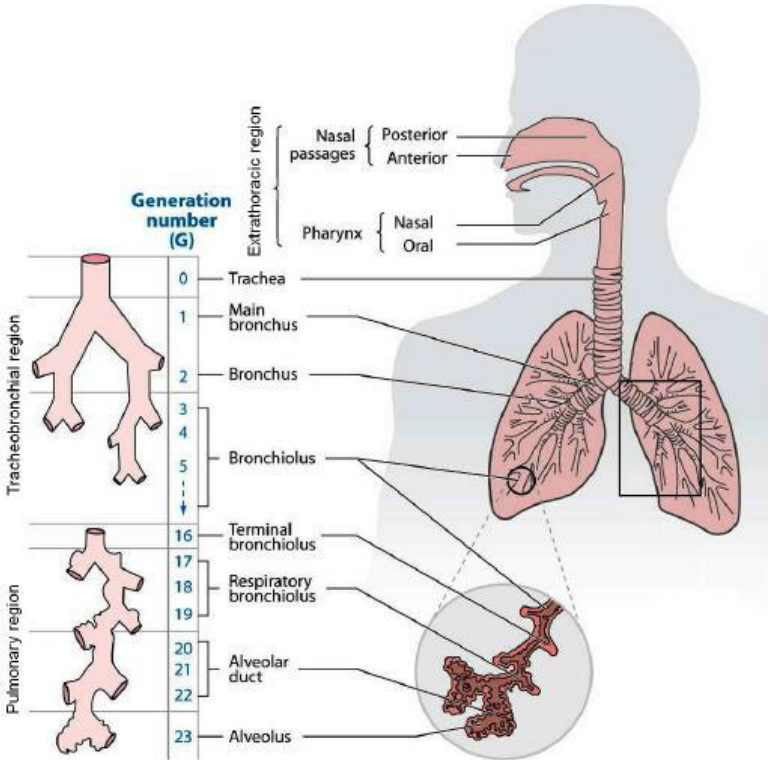

Fig. 4: *Anatomical structure of airways according to the ICRP (International Commission on Radiological Protection) model [31].*

For treating the lung, inhalation therapy has shown superiority compared to other routes of administration, for instance, oral in term of [32]:

- Non-invasive route of administration.
- Enhanced drug bioavailability.
- Rapid onset of action.
- The ability of drug to avoid the first pass effect due to the fact that the drug metabolizing enzymes has very low concentrations within the lungs than in the digestive system and also the liver [33].
- A higher local drug concentration in comparison to systemic application as the inhalable drug reaches directly into the lung epithelium.
- Straightforward route of administration and very useful for the patient who needs to take the drug regularly or patient who has liver or kidney problem.

In respiratory research, it is still not straightforward for applying nanoparticles (NPs) directly into the lungs because of the chance of being removed during the exhalation process. For developing a drug formulation being applicable by inhalation, some key factors should be taken into consideration like breathing pattern, airways geometry, humidity, and particles morphology (MMAD, particles size, and particles shape), as these factors have a profound influence on the particles' deposition inside the lungs of the patient [29, 34, 35]. The researchers have reported that the ideal MMAD of the particles is 1-5 µm for sufficient pulmonary delivery [35]. After ensuring the particles deposition, the physio-chemical properties of drug molecules would have an influence on the absorption rate such as molecular weight and lipophilicity. When inhalable particles are applied into lungs, they can be subject to diverse kind of deposition mechanisms such as inertial impaction, gravitational sedimentation, interception, Brownian diffusion and electrostatic precipitation (Fig.5). The sizes, density and shape of particles are the main factor which impacts their deposition inside the lung.

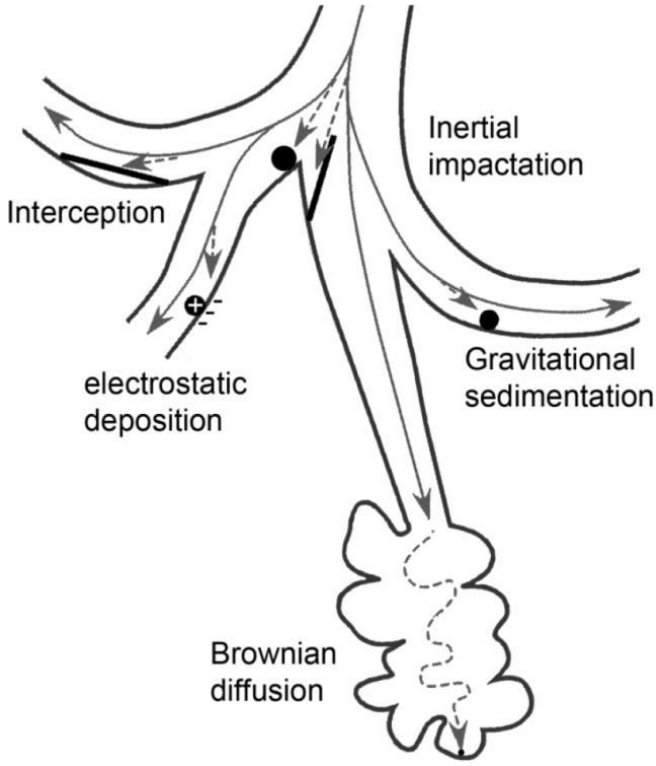

Fig. 5: *Particle deposition mechanisms in the lung [36].*

Impaction phenomena happen in the extra-thoracic airways exactly at the point where right angle curvature of the pharynx and at airway bifurcations. Once particles have an excessive amount of inertia because of their size or velocity and also the airstream is shifting in direction, it might cause that the particles will hit the airway wall instead of following the flow. Additionally, an increase in total cross-sectional area would lead to a profound decrease in the flow velocity while particles go into lungs, impaction occurs more often in upper airway rather than in the lower airway [29, 34, 35].

Gravitational sedimentation phenomena are more prominent in the lower part of an airway and also in the alveolar region, due to the fact that airstream velocity is lessened there. For the reason of the gravity phenomena, particles can settle down during holding breathing or slow breathing. The rate of sedimentation is almost 3 second of breath-holding which is almost sufficient to induce settling of a 5 μm particle size.

For submicron inhalable particles, a random Brownian motion is a driving force for their deposition into the lungs and thereby the small particles will be the first and the fast one to move for reaching inside the lung [29, 34, 35].

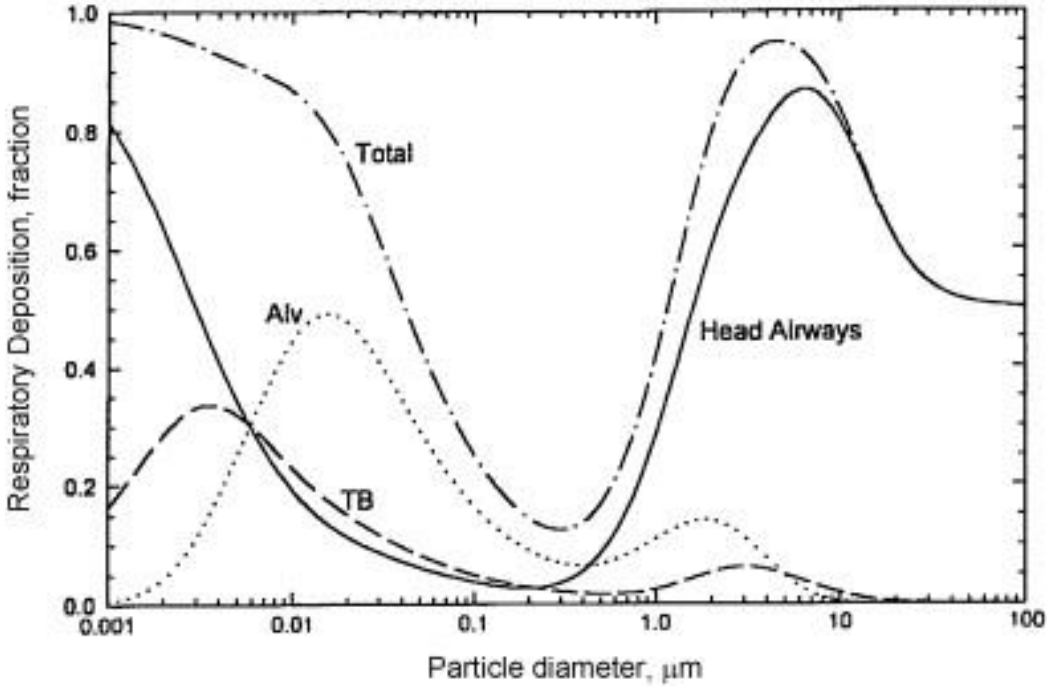

Fig. 6: *Total and regional deposition based on the ICRP (International Commission on Radiological Protection) deposition model [35].*

Diffusion of particles happen when the particles are below 1µm, whilst particles with an aerodynamic diameter between 1-10 µm tend for sedimentation or impaction [29]. These findings are in agreement with deposition model from the ICRP (International Commission on Radiological Protection) (Fig 6) where the total deposition is represented in two peaks. The first peak in the lower nanometer range has represented the deposition of inhaled particles by diffusion. The second peak represents the particles with an aerodynamic diameter 1-10 µm and their deposition through sedimentation and impaction mechanism.

1.5 Nebulization in comparison with dry powder inhalers and metered dose inhalers

Despite the fact that nanoparticles can be applied via nebulization for inhalation purpose, the nebulization technique is suffering from some drawbacks such as microbiological contamination, particles aggregation and lower stability and required a specifics storage condition like a fridge [37]. From the viewpoint of patients, nebulization is a complex technique to be used because of its less portable and time-consuming due to device preparation, maintenance and cleaning which will increase the risk of microbiological contamination and poor reproducibility of the aerosolized droplet [37, 38]. Moreover, there is not always a possibility to nebulize or spray drying virtually any drug because of some issues with their chemical stability. To successfully deal with these issues, using nanoparticles as a carrier system for delivering a drug is a promising technology. Overall, for treating a pulmonary disease, formulating the drug as a dry powder inhaler DPI is more preferable in comparison to nebulization. The formulated drug as DPI has superior properties because of its easy usage, the ability to deliver a high dose of the drug compared with nebulization, no contamination risk and its more stable under the storage conditions [38, 39].

1.6 Techniques of interest

In the upcoming sub-chapter, selected preparations and analytical methods which have been used for the work in this thesis will be presented.

1.6.1 Nanoparticles using nanoprecipitation method

In this work, two of the most common methods to manufacture polymeric nanoparticles (PLGA NPs) were used, namely the conventional nanoprecipitation and the microfluidic nanoprecipitation technique. Generally, nanotechnology has gained a huge interest in the last decades, especially in the pharmaceutical field. The growing interest is related to their unique advantages in comparison to the preparation of drug in classic techniques [40]. The advantages of the nanoparticles are listed below:

- A lower dose is required in comparison to the classic formulation of a drug
- The power of reducing the unwanted side effect due to using of a small dose
- Providing a protection for the drug from *e.g*. degradation
- Enhancing the solubility of poorly soluble drugs
- Offering the opportunity to control the release profile of drugs which would be helpful to avoid delivering of high dose
- Allowing some drugs to have an alternative route of administration for example by solving the solubility issue

Due to these unique advantages of NPs, enormous research studies were conducted for manufacturing different kinds of nanoparticles with a different method. Moreover, the influence of adjusting some factors during the manufacturing process of NP was studied, in order to evaluate the effect on the properties of NPs such as size. Among all of the manufacturing methods, nanoprecipitation (solvent displacement) is the most favourable method. This is because it is very fast, simple method and required a low energy.

The principle of nanoprecipitation relies on mixing two miscible solvents, from which one is a solvent containing a polymer and the other one a nonsolvent containing a stabilizer. Hence, the polymer solution (solvent) is gradually added into the nonsolvent solution. As a consequence, the polymer is precipitated and forms NPs [41, 42]. Although so many methods for manufacturing NPs were developed, their chances of being applicable in pharmaceutical industries are unrealistic. These limitations in their use are related to the lacking reproducibility (variation from batch to batch). Recently, microfluidic technologies were developed as a promising technique to solve this issue. The microfluidic technique is based on mixing two reagents under laminar flow condition in a very short time less than a minute. As a consequence of that, controllability of the NPs properties is achieved [43, 44]. Currently, microfluidic is pointed out as the most ideal method for being adopted in pharmaceutical industries [44].

1.6.2 Microparticles preparation for inhalation therapy

Formulating drugs in an inhalation dosage form is still quite challenging. One of the most challenge points is to formulate the drug with appropriate sizes, to enable their deposition inside the lungs. For formulation an inhalation dosage form, there is a possibility to use different approaches like milling “Top-down” or “bottom-up” such as precipitation by supercritical fluids process (e.g. spray-drying and freeze-drying) [45]. In this work I will focus on the technique which was mainly used: spray drying.

1.6.2.1 Spray drying

Spray drying “bottom-up” technique is a drying process in which liquid or suspended formulations converts into solid dried formulations. Also, it is a well-known technique in pharmaceutics as well as in the food industry. The process of spray drying consists of three fundamental operations process, namely atomization, drying and separation. The feed solution containing active pharmaceutical ingredients and the excipients is atomized by a nozzle or vibrating mesh into very small droplets, then, those droplets expose their large surface immediately to a heated gas flow. Thereby, a rapid evaporation of the liquid inside the droplets occurs, resulting in a solid dry formulation. Afterward, the dried formulation is separated from the drying gas by a cyclone or electrostatic precipitator (Fig 7) [45, 46].

The important factors in the spray-drying process are gas flow, spray gas flow, inlet and outlet temperature, feed rate, as well as the concentration and composition of the spraying liquid. Thereby, a little change in these factors would have an influence on the size of dried droplets, shape, and density, which will also influence their aerodynamic properties.

In the normal case, the most of sprayed particles have a spherical shape, but it is also possible to have an irregular structure like raisin shape or hollow and this is depending on used spray-drying factors and the composition of the formulation [45].

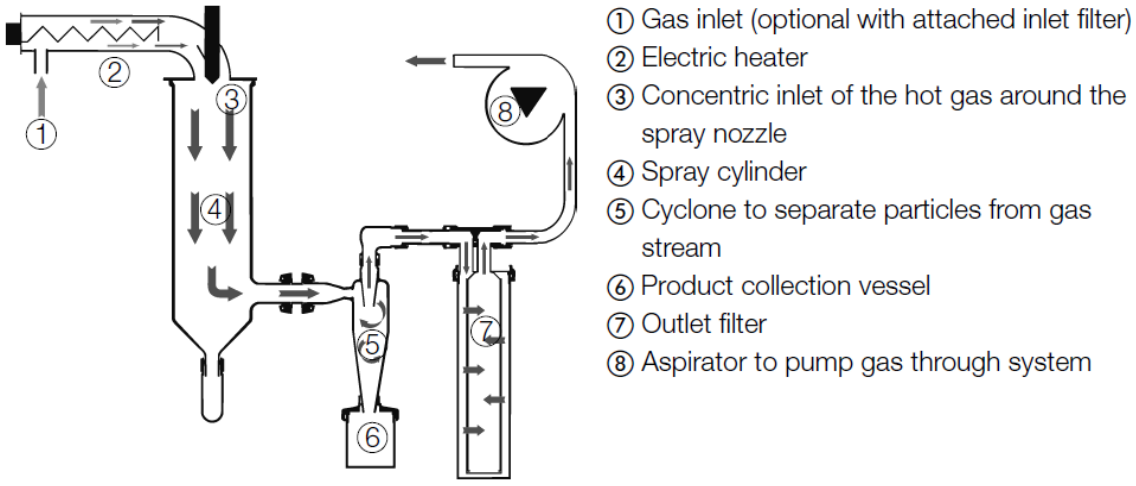

Fig. 7: *Diagram of a spray dryer, Source: BÜCHI B-290 Manual.*

"Formulation is all about playing with the excipient, factors of the preparation method and choosing the right technology"

Moreover, low-density particles are very well suited for pulmonary treatment. In general, spray-drying (SD) technique is an appropriate preparation method to formulate microparticles intended for pulmonary application [47].

2. Aim of the work

Recently, there is a new trend to formulate the antibiotics in inhalation dosage form for treating chronic pulmonary infections such as cystic fibrosis. Therefore, there is growing interest for using nanotechnology, to formulate inhalable particles by taking the advantage of this carrier. Principally, nanotechnology is playing a pivotal role as a smart technology to carry the drug. Thanks to NPs of the faced problems in developing drugs, like poor-solubility and poor permeability to cross the thick mucus barrier, could be solved.

The overall objective of this thesis was to develop a multifunctional formulation intended for pulmonary application. Ultimately, these multifunctional formulations have the ability to permeate through the challenging thick mucus barrier and deliver their payload into the lungs.

This work is subdivided into the following 3 parts:

I. Manufacturing of small-size polymeric nanoparticles (PLGA NPs) of less than 100 nm using nanoprecipitation in a microfluidic system, and comparing the effect of different preparation techniques on the encapsulation efficiency of the loaded drug.

II. Development of a multifunctional microparticle matrix in the form of dry powder for the purpose of inhalation. The matrix is composed out of different types of drugs, using a spray drying technique. Further, the inhaled formulation will be characterized.

III. Embedding of the PLGA-NPs into a multifunctional microparticle matrix for inhalation, using the spray drying technique.

The first aim focuses on the manufacturing of a small polymeric nanoparticle (PLGA NPs) of a size below ~100 nm, using a microfluidic technique. Further, we tested the influence of changing some factors during the preparation on the NPs' size and their encapsulation efficiency of the loaded drug. Then, we studied the permeability of the NPs through the mucus barrier. Furthermore, the impact of using different approaches during preparation on the encapsulation efficiency of the loaded drug into the nanoparticles was evaluated.

The second aim focuses on formulating a multifunctional microparticle matrix composed out of multiple drugs (mucolytic agent and antibiotics). The multifunctional microparticle was achieved based on the salification process. Then, the liquid formulation containing the mucolytic agent and antibiotics was formulated into a dry microparticle formulation using a spray dry technique. The resulted dry microparticle formulation showed a proper aerodynamic property and a quick disintegration behavior which is suitable for pulmonary application. The main goal was to take the benefit of a mucolytic agent, to fluidize the thick mucus in CF, therefore allowing the other drugs to penetrate through the mucus barrier without being entrapped within the pores of the mucus barrier.

The third aim focuses on incorporating the PLGA NPs loaded with an anti-inflammatory drug into the multifunctional matrix (nano-embedded microparticles). Then, the influence of NPs on the aerodynamic properties was tested. Moreover, the stability of PLGA NPs within the multifunctional matrix of microparticles was evaluated.

*Preparation of size-tunable PLGA nanoparticles by microfluidics intended for mucus penetration**

*** This chapter is prepared for publication as a journal article:**

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3. Preparation of size-tunable PLGA nanoparticles by microfluidics intended for mucus penetration

3.1 Abstract

Great challenges still remain for drugs to penetrate biological barrier like mucus in cystic fibrosis and also for the treatment of bacteria residing in biofilms embedded in mucus. Drug carrier systems such as nanoparticles (NPs) need to have a proper surface chemistry and small sizes to ensure their permeability through those hydrogel-like systems. This holds also true for mucus in cystic fibrosis showing increased barrier properties. Exclusively, we have employed a microfluidic system to fabricate coated PLGA nanoparticles with muco-penetrating stabilizer (Pluronic), with hydrodynamic sizes ranging from 40 nm up to 160 nm. The preparation based on the nanoprecipitation method was successfully performed using LabSmith microfluidic system. The size dependence was evaluated by varying different parameters during preparation, namely polymer concentration, stabilizer concentration, solvent nature, the width of the focus mixing channel, flow rate ratio and total flow rate. Furthermore, the influence of the length of the focus mixing channel on the size was evaluated in order to understand the nucleation-growth mechanism. Surprisingly, the channel length was revealed to have no effect on particle sizes. Finally, the permeability of muco-penetrating PLGA NPs through pulmonary human mucus was assessed; small NPs with sizes less than 100 nm showed a fast permeation.

3.2 Introduction

In the last decades, application of nanotechnology has gained significant attention especially in the biomedical field for vaccine delivery [48, 49], in anticancer therapies [50, 51] as well as for gene delivery [52, 53]. Owing to the nanoparticles' unique physicochemical properties, a specific modification on the surface of nanoparticles could be designed to meet the needs of the desired application [54, 55]. This holds also true for protecting the drug carriers from being inactivated by avoiding the interaction with the non-cellular mucus [56]. Nanoparticles (NPs) have shown a tremendous effect in terms of facilitating the diffusion of drug through such biological barriers for e.g. thick mucus in cystic fibrosis [23, 57]. Notably, only particles with sizes below 200 nm seemed to permeate well through mucus without being entrapped by size filtering mechanisms [18, 23]. Furthermore, modifying the surface chemistry of NPs is beneficial for avoiding the interaction filtering mechanisms such as H-bond interaction and electrostatic interactions [58-60]. Moreover, nanoparticles as carrier system have the ability to protect the drug from inactivation, to reduce unwanted side effects and to enhance the efficacy of the active pharmaceutical ingredient (API) such as solubility and bioavailability [7]. Among a large number of polymers for the preparation of polymeric nanoparticles, poly (lactide-co-glycolide) (PLGA) as a very benign material is considered as a highly potent material for all routes of administration. Thus, PLGA NPs have been extensively studied in the pharmaceutical field, relying on PLGA's biodegradability and its FDA approval in some products [61, 62]. Besides that, PLGA has the ability to incorporate various active pharmaceutical ingredient (API), showing a high encapsulation efficiency for hydrophobic drugs and also contribute to control the release of the incorporated drug [63, 64]. Many different methods were designed to prepare PLGA NPs such as double emulsion and nanoprecipitation [65, 66].

Among many other techniques, nanoprecipitation has been adopted very quickly to prepare submicron particles, because it's a simple and straightforward technique, without any chemicals additives being involved and also does not require harsh formulation parameters like energy input, mechanical shear stress as induced e.g. by sonification [67, 68]. The mechanism of nanoprecipitation is based on the mixing of a solvent solution for the polymer (organic phase) with a non-solvent solution (aqueous phase). Thus, full miscibility initiates PLGA precipitation and spontaneous formation of nanoparticles [69]. Nonetheless, preparation of submicron NPs in a conventional "bench-top" nanoprecipitation method is still facing several critical challenges which restrict them from being adopted in a pharmaceutical industry such as lacking reproducibility [70, 71]. This problem holds especially true for NPs below 200 nm which are more preferable in order to penetrate faster through biological barriers. Although several methods for the preparation of NPs below 200 nm were shown, all these methods are still facing a major challenge such as poor reproducibility (batch to batch variations) which makes them undesirable techniques. These issues were mainly attributed to poor control of the mixing time. An improved control on mixing time can be achieved utilizing impinging jets or microfluidic systems, as they allow to control mixing time in millisecond instead of a minute [72, 73]. The group of Farokhzad has explored that mixing time plays the major role to fabricate monodisperse colloids with the desired size. This can be manipulated by adjusting the flow rate ratio of the organic phase to the aqueous phase and changing the channel width. As well, fast mixing (as in microfluidics) has shown a variety of advantageous over conventional methods (bench-top) on the physicochemical and encapsulation properties of the nanoparticles [74].

LabSmith system (LabSmith, Inc, Livermore, USA) was implemented for manufacturing PLGA NPs using the nanoprecipitation method. This system offers stable conditions to produce monodisperse particles with small sizes. At the same time it offers the possibility to vary key parameters during preparation such as channel diameters and channel length [74]. For successful permeation of the particles through mucus, particles as small as possible are needed. Furthermore, the encapsulation of a lipophilic model drug (Curcumin, a nonsteroidal naturally anti-inflammatory drug) was assessed comparing different preparation approaches. The anti-inflammatory drug was selected as a potential drug for loading into PLGA NP which would be meaningful to be used in e.g. cystic fibrosis, to address strong and continuous inflammatory responses [75]. Particle diffusion-related sizes were characterized by dynamic light scattering (DLS) whereas, the morphology was visualized by scanning electron microscopy (SEM). Finally, the penetration of muco-penetrating PLGA NPs through Pulmonary human mucus was assessed utilizing confocal laser microscopy (CLSM).

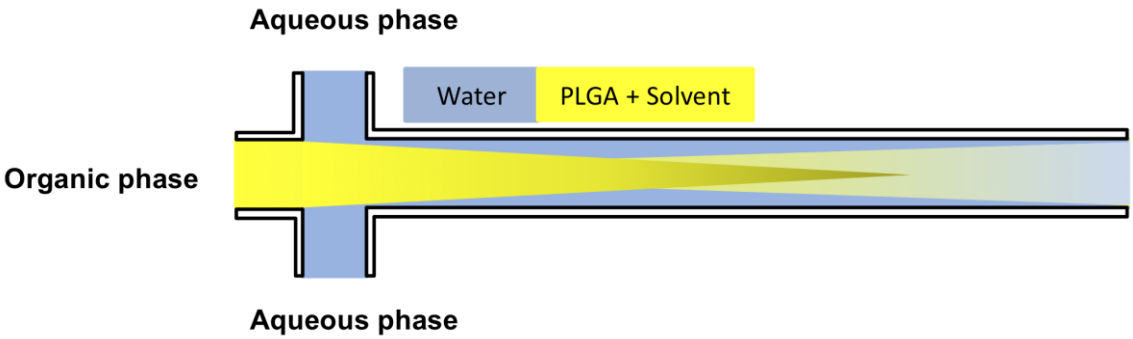
3.3 Materials and methods

3.3.1 Materials

Porcine mucin, Curcumin and acetonitrile (ACN) were purchased from Sigma-Aldrich (Chemie GmbH, Steinheim, Germany) and poly lactic-co-glycolic acid (PLGA) (Resomer RG 503 H, 50:50 ratios, average Mw = 24.000–38.000 Da) was obtained from Evonik Industries (Darmstadt, Germany). Amphiphilic block copolymer Poloxamer (Pluronic F68) was a kind gift from BASF SE (Ludwigshafen, Germany). Pulmonary human mucus was collected by the endotracheal tube method after informed consent from patients (Winterberg hospital, Saarbrücken, Germany). AlexaFluor-WGA–(wheat germ agglutinin) was purchased from (Invitrogen, Oregon, USA). All materials employed in the preparation of nanoparticles were of HPLC grade.

3.3.2 Manufacturing of PLGA nanoparticles in a microfluidics system

Microfluidic system was assembled using a cross-channel microreactor design, connected via glass capillaries (180µm ID and 300 OD, Labsmith, Livermore, USA). Monodispersed PLGA NPs coated with Pluronic (F68) on the surface were manufactured as illustrated in (Sketch 1).



Sketch 1: *Design of microfluidic system.*

In brief, the stock solution of stabilizer containing Pluronic F68 (0.1%) was dissolved in water and injected into the side channels (B) of the microfluidic reactor using a syringe pump (Harvard Apparatus PHD 2000 Syringe, Holliston, USA). In parallel, the organic phase containing 3 mg of PLGA in 1ml acetonitrile (ACN) was pumped into the middle channel (A) using another syringe pump (Multi Programmable Syringe Pump, Sarasota, USA). The flow rate ratios (FRR) of the two phases were varied from (0.05 up 1). The two liquids were brought together in the mixing channel and the PLGA started to precipitate and form NPs. PLGA NPs sample was collected from the outlet of the channel. Then, PLGA NPs suspension was left overnight under stirring to evaporate the organic solvent. Finally, PLGA NPs suspension were washed twice using centrifugation (30 min at 10,000 g at 4°C) and redispersed with MilliQ water to get rid of excess stabilizer. The experiments were conducted under the same condition in triplicate for all formulations.

3.3.3 Characterization of colloidal PLGA NPs

3.3.3.1 Measurement of size distribution

The colloidal properties of PLGA NPs such as size and size distribution (PDI) were measured utilizing a Zetasizer Nano ZS90 (Malvern Instruments, Worcestershire, UK). Briefly, nanoparticles were suspended in 10 mM NaCl solution in order to measure the zeta potential based on the electrophoretic mobility. All measurements were performed at least in triplicate under the same conditions.

3.3.3.2 Morphology with scanning electron microscopy (SEM)

The morphology of the PLGA NPs was visualized utilizing a scanning electron microscope (SEM) EVO HD 15 from Zeiss (Jena, Germany). 10µl of PLGA NP suspension was dropped on a silica wafer, and then it was left for overnight drying at ambient conditions. Next day prior to visualization, NPs were sputtered with a thin gold layer (~10 nm), utilizing a sputter coater Quorum Q150R ES (Quorum Technologies Ltd, East Grinstead, UK).

3.3.4 In vitro assessment the interaction of NPs with mucin as a simple model

Mucin (1%) was dissolved in water containing 1 % NaCl and it was left overnight under stirring at room temperature to form a kind of artificial mucus (AM). The sample was stored in the fridge (4°C) until usage. AM was incubated with the suspension of PLGA NPs stabilized with different types of Pluronic in 1:1 volume ratio at ambient conditions for pre-defined time intervals. Afterwards, the nano-suspension was centrifuged at 5000 g for 10 min to separate the NPs from mucin prior to analysis. As reference, PLGA NPs without stabilizer were measured. The properties of the NPs after incubation with AM were measured using DLS to determine whether; NPs size will be increased as a response to strong interactions with AM or rather remain unchanged.

3.3.5 The permeability of tunable muco-penetrating PLGA-NPs through pulmonary human mucus

The permeation of Rhodamine-B labelled PLGA NPs coated with 0.1% Pluronic (F68) was confirmed by 3D time laps imaging utilizing confocal laser scanning microscopy (CLSM), (LSM710, Zeiss, Jena, Germany). Each 40 µl of pulmonary human mucus without air bubbles was labelled with 1 µl of AlexaFluor-wheat germ agglutinin. Afterwards, the stained mucus was placed in an imaging chamber made by nail polish on a cover slip resulting in an equally thick mucus layer [76]. At time zero, PLGA NPs were added on the top of mucus, z-stacks within the mucus sample were obtained at constant distance from the bottom of the slide. PLGA NPs permeability through mucus was tracked by the change of the fluorescence signal. This approach allowed us to study the size-dependent permeation of PLGA NP through pulmonary human mucus. One day before the experiments, frozen native pulmonary human mucus samples were left to thaw in the fridge at 4 °C. Rhodamine-B labelled PLGA NPs of 60, 140 and 400 nm size were dispersed in MQ water at 0.1% w/v. 5 µl of the nano-suspension was added on top of the mucus. Then, the time-dependent vertical penetration was observed by a 40×/1.1 objective at 37 °C utilizing humidified and temperature-controlled air in an incubation chamber (Zeiss, Jena, Germany) in order to avoid drying. The labelled pulmonary mucus was detected with λ_{ex} = 488 nm and a detection between 467−554 nm. PLGA NPs were excited at λ_{ex} = 561 nm and the signal were collected between 624−707 nm. The permeability of NPs within mucus was assessed from 0 min up to 1 h after their application. All experiments were accomplished in triplicate.

3.3.6 Evaluation of drug encapsulation efficiency using different NP preparation approaches

To evaluate the encapsulation efficiency of Curcumin (EE-Cur) into PLGA NPs, the influence of using different technique was investigated. The EE-Cur after adding the organic phase into the aqueous phase by hand, using a syringe pump or using the microfluidic system was compared. In brief, (9:1) ratio of PLGA to Curcumin with a final concentration of 3 mg/ml was dissolved in 1ml of acetonitrile (ACN).

Then, the organic phase was precipitated in an aqueous phase containing 0.1% Pluronic F68 as stabilizer. First, to prepare a conventional nanoprecipitation, a plastic syringe was used to inject the organic phase containing (PLGA-Curcumin)-mixture into the aqueous phase by hand. In parallel to this, the second approach was carried out using a syringe pump (Harvard Apparatus PHD 2000 Syringe, Holliston, USA) with a flow rate setting of 0.1mL/min, while in the third approach, an organic phase flow of 20 µL/min and an aqueous phase flow of 200 µL/min were used in an microfluidic setup. The resulting flow rate ratio of 0.1 was used as a standard for NP preparation. All experiments were carried out at least in triplicate.

3.3.7 Determination of the encapsulation efficiency of curcumin

For analysing the encapsulation efficiency of Curcumin (EE-Cur), 1 – 3 mg of the prepared nanoparticles (PLGA NPs) was dissolved in 2 ml acetonitrile. 200 µl of each solution was then transferred to a solvent resistant plate reader plate. The plate was placed in a Tecan plate reader (Tecan, Männedorf, Switzerland) and analysed using an excitation wavelength of 460 nm and an emission wavelength of 515 nm. A calibration curve for curcumin was prepared using acetonitrile (ACN) as solvent. Using the calibration curve, the amount of curcumin inside the sample solution was determined as [Drug encapsulated]. The stock solution for NP preparation contained in 10 mL acetonitrile 0.1 mg of curcumin for each 0.9 mg of PLGA [Drug used]. With this and the analysed amount of curcumin in the sample solution, the encapsulation efficiency (EE) was determined using following formula:

EE (Drug) %= ([Drug encapsulated] / [Drug used])*100 %.

3.4 Results and discussion

3.4.1 Influence of different parameters on the size of NPs

Smaller nanoparticles (NPs) are proven to have a better diffusion through mucus, owing to their smaller size, which helps them to avoid the size filtration mechanism [23]. Furthermore, the surface chemistry of NPs plays a crucial role in facilitating their penetration through mucus [56]. In this context, we have used microfluidics to produce tunable sizes of poly (lactic-co-glycolic acid) (PLGA) NP coated with muco-penetrating stabilizer (F68). To gain more insight about the factors influencing nanoparticle sizes, various parameters such as flow rate ratio, PLGA concentration, nature of the solvent, diameter of the mixing channel and stabilizer concentration were tested. Furthermore, as our system allow us to cut different lengths of the mixing channel, the impact of the length of the mixing channel on the nucleation-growth mechanism of the nanoprecipitation was evaluated.

3.4.2 Effect of flow ratio and total flow speed

Flow rate ratio was calculated as low rate ratio= (flow of organic phase)/ (flow of a queous phase). We have varied the flow rate ratio (FRR) of the organic phase to the aqueous phase from (0.05-1) and the flow speed of the aqueous phase was varied from 10µl to 100µl while adapting the organic phase volume accordingly. At a flow rate ratio of 0.05, a substantial reduction on the NPs size from 160 nm to 60 nm with narrow size distribution (PDI) below 0.05 was obtained (Fig. 1). The reduction in NPs size is attributed to the rapid and efficient mixing process as it was demonstrated in the literature [73]. Further, Ostwald ripening phenomena could be avoided at fast mixing times [77]. Additionally, it was observed that an increase in FRR above 0.2 (by adjusting only the organic phase flow rate) bigger NPs were obtained.

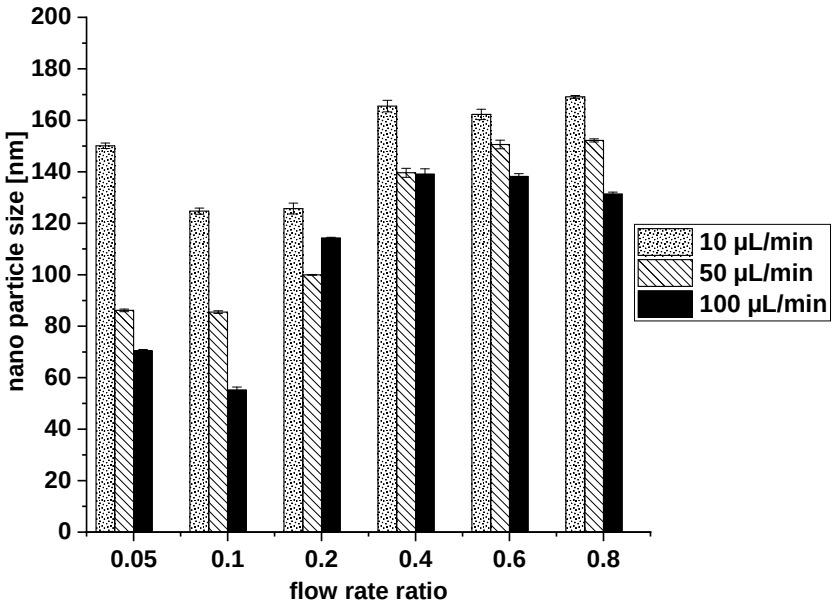
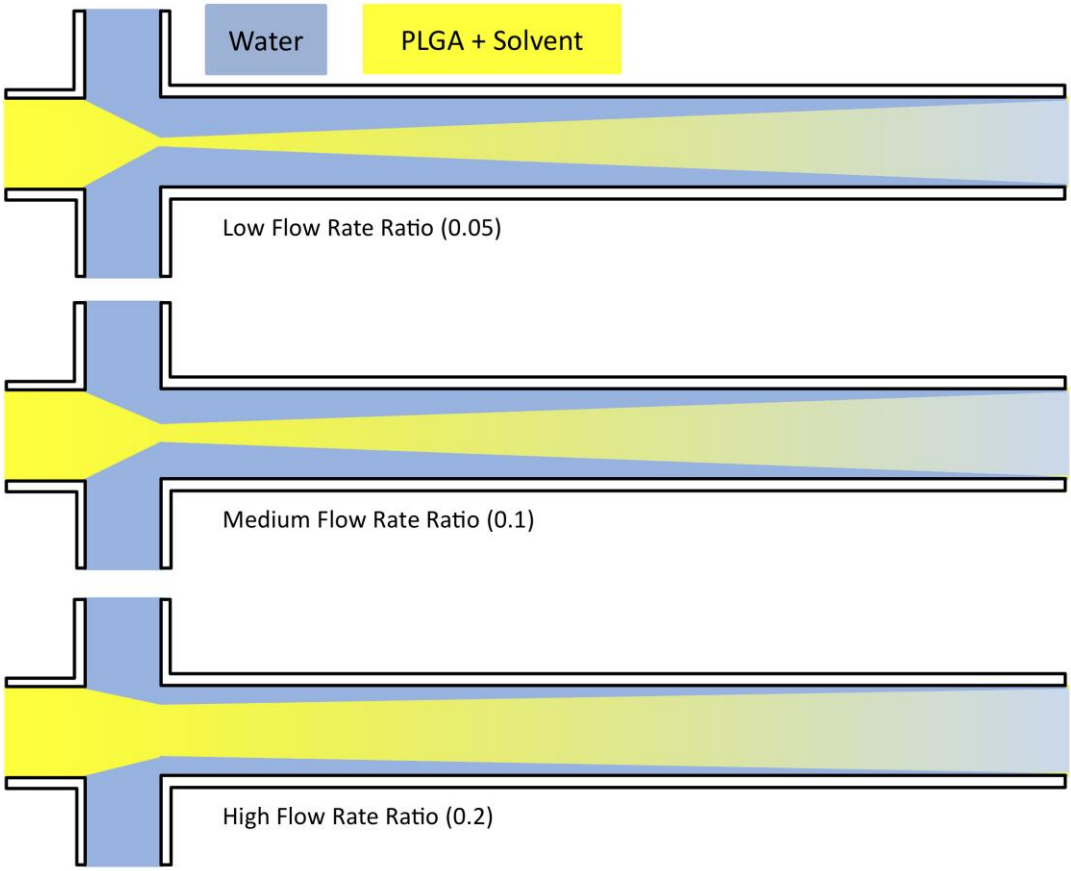


Fig. 1: *Effect of flow rate ratio and total volume of the aqueous phase on NPs size.*

This implies that an increase in the width of the focus point of the organic phase occurred as a result of the higher FRR as illustrated in sketch 3 [78]. For this reason, a longer time was required for the diffusive material to be mixed. Another scenario would be that an increase in NPs size at higher FRR is related to the use of larger amounts of solvent causing swelling of the NPs as proposed by Wang et al. [79]. Afterward, the next step was to test the influence of increasing the flow speed of the aqueous phase on the size of NPs. Therefore we have varied the aqueous phase flow from 10-100 µl/min while keeping the flow of the organic phase constant at 10µl/min. It was observed, an increase in the flow speed of the aqueous phase from 10 µl/min - 100 µl/min has led to a reduction in the mean diameter of the NPs from 160 nm to 60 nm (Fig 1).



Sketch 2: *Illustration the impact of increasing the flow ratio on the mixing pattern.*

This evidence points to the fact that most likely the polymer concentration decreases by increasing the aqueous phase flow rate (a large volume of the aqueous phase is available), thus small NPs were obtained. Another reason could be that the rate of the NPs to grow got decreased as well [80, 81]. Markedly, the tendency of the NPs for aggregation decreased at a higher flow aqueous phase due to the usage of the large volume of water which prevents particle encounter. Moreover, the particles are easier to redisperse and more stable within aqueous suspension.

3.4.3 Effect of polymer concentration

Nanoprecipitation mechanism is predicted to be primarily ruled by the Marangoni effect where the concentration gradient and the concentration of the polymer play a role in influencing the colloidal properties [69]. For this purpose, the influence of polymer concentration (PLGA) on mean diameter of NPs was tested.

It can be observed by varying the polymer concentration (PLGA) from 1 mg up to 10 mg that, a higher concentration of PLGA is related to an increase in mean particle size from 60 nm up to 170 nm (Fig. 2).

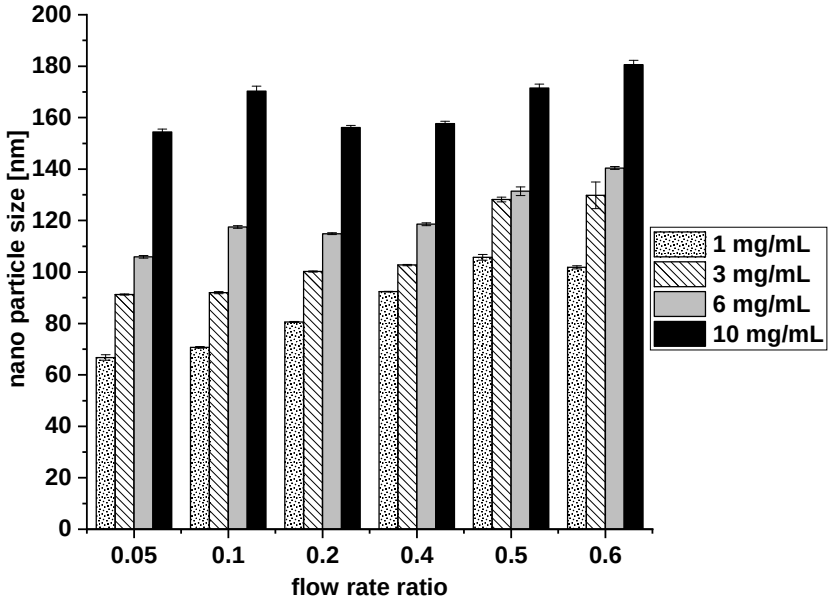


Fig. 2: *Effect of PLGA concentration on NPs size.*

This influence is most likely coming from increasing the viscosity of the organic phase which led to impeding the diffusivity of the organic phase in the aqueous phase, subsequently, a longer mixing time resulted. Also, it appears that a large number of nuclei is formed and the high concentration of polymer per unit volume promotes particle growth, therefore bigger particles were obtained [80]. Notably, using higher concentrations of PLGA than 10 mg resulted in clogging of the mixing channel because of agglomeration. For concentrations less than 1 mg we were not able to produce monodisperse PLGA NPs; several peaks ranging from 20 nm up to 100 nm were observed in DLS. As indication of the lacking monodispersity a PDI > 0.7 can be considered [81]. In literature, the formation of micelles from the stabilizer was discussed as potential reason for the very small particles [79].

3.4.4 Effect of the diameter of the focus channel

Besides the flow rate, flow rate ratio and polymer concentration, another important factor is the channel geometry which also has an impact on NP sizes. This holds true especially for the width of the focus channel in which the formation of NPs take place. Literature has demonstrated that the key parameter to modulate NPs properties is the mixing time (τ^mix). The mixing time depends on the geometry as described by [73].

$$\tau^{mix} \sim \frac{w_f^2}{4D} = \frac{w^2}{9D\left(1+\frac{1}{FRR}\right)^2}.$$

Where D is diffusivity of the used solvent, wf is the width of the focus channel, w is the width of the other channels, and FRR is the flow rate ratio of the organic phase to the aqueous phase. According to this equation modulating the width of the focus channel wf will modulate the flow rate of the organic phase to the aqueous phase. As our microfluidic system (Lab smith, Livermore, USA) has an unparalleled trait of the possibility to choose different diameters for the focus channels from 20 µm up to 300 µm, we have elucidated the influence of the channel width on the colloidal particles. In Fig. 3 can be seen that NPs size was reduce from 130 to 70 nm as a result of modulating the width of the mixing channel from 280 µm to 100 µm due to shortened mixing times.

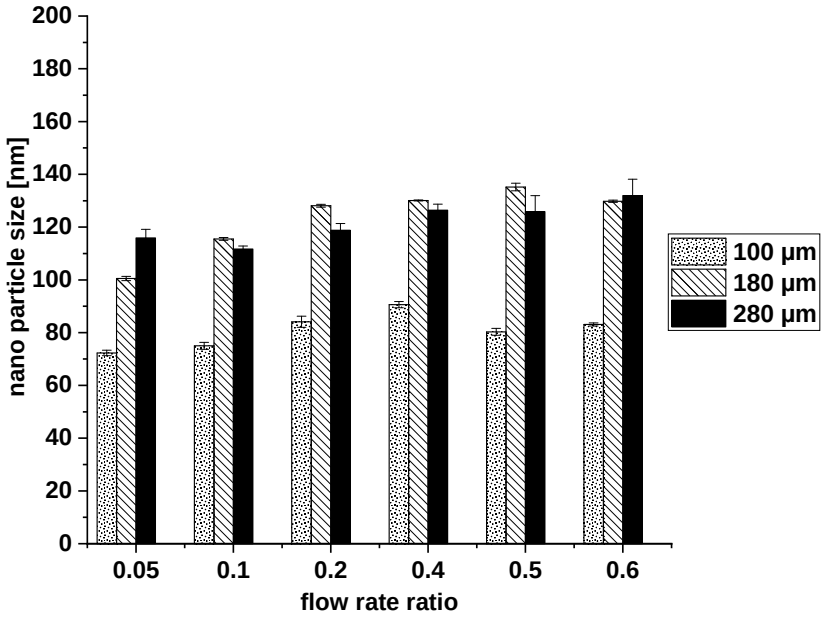


Fig. 3: *Effect of diameter of focusing channel on NPs size.*

Due to the reduction of the channel diameter the mixing time was minimized at flow rate ratio (FRR = 0.05) from 2.19 to 0.27 ms. Additionally, a focus channel of 50 µm was tested but unfortunately it resulted in a multimodal size distribution of NPs which is most likely due to a non-stable flow pattern (turbulent flow instead of laminar flow).

3.4.5 Effect of stabilizer concentration

Also, to aid the NPs to permeate successfully through the mucus without being trapped, coating the PLGA NPs with a muco-inert stabilizer must be taken into consideration. Using the right stabilizing molecules would reduce the interaction with mucus and it would also at the same time foster stability of the colloidal system, minimizing agglomeration of NPs and nucleation growth [77]. To gain more insight about the influence of the stabilizer on the colloidal size, Pluronic (F68) as a stabilizer was chosen.

It has a good safely profile [82] and was proven to be a muco-inert material [83]. Using Pluronic (F68 0.1%) as stabilizer, has shown a slight increase in NPs size from 70 nm to 90 nm (Fig. 4).

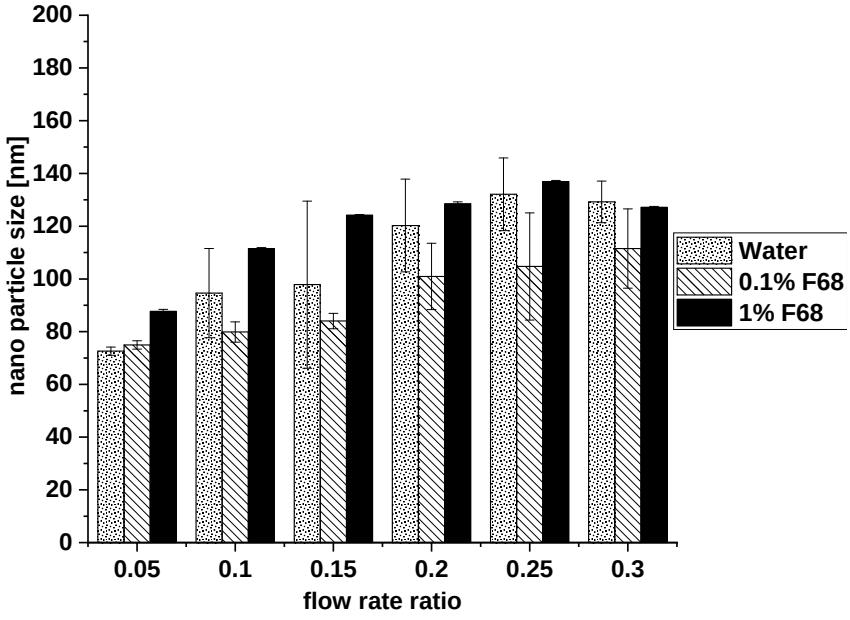


Fig. 4: Effect of stabilizer concentration on NPs size.

Our observation is consistent with results shown from Panagiotopoulos group indicating that the stabilizer has nearly no impact on the mean diameter of NPs [84]. The slight effects are in accordance with the expectation where adding stabilizer is thought to increase the viscosity of the aqueous phase and thus prolonged the mixing time. The small sizes of particles at low concentrations of Pluronic might be due to the dominating effect of the surface tension over the change in viscosity [85]. For low concentrations of Pluronic the viscosity might not be strong enough to play a crucial role whereas the surface stabilization is already occurring and influencing the growth of nuclei. These results were in accordance with results from the conventional methods (data not shown).

3.4.6 Effect of solvent nature and solvent mixture

The solubility of drugs fundamentally relies on the solvent. Therefore, assessing the influence of the nature of solvent on the colloidal properties of our drug carrier would be meaningful. Different solvents were used to elaborate their impact on NP size namely, DMSO, acetonitrile and acetone. We can see that there is no correlation between the viscosity of the used solvent and the final size of NPs (table 1)

***Table 1**: Physicochemical properties of the used solvents*

	MW	Density [g/cm^3]	Molar volume	Heat of evaporation [J/mol]	Hildebrand solubility parameter $MPa^{1/2}$
Acetonitrile	41.05	0.786	52.23	33225	24.28
DMSO	78.13	1.1	71.03	52900	26.65
Water	18.01	1	18.01	44000	48.03
Acetone	58.08	0.784	74.08	31300	19.74

But the Hildebrand solubility parameter correlates with the formation of small NPs (table 1) [86]. When DMSO was used as solvent, the size of NP was minimized from 120 nm to 40 nm in comparison to acetone and ACN as can be seen in Fig. 5. This data underlines that the nature of the organic phase plays a decisive role controlling the diffusion of the organic phase to the aqueous phase which induces as well a change in the mixing time [87, 88].

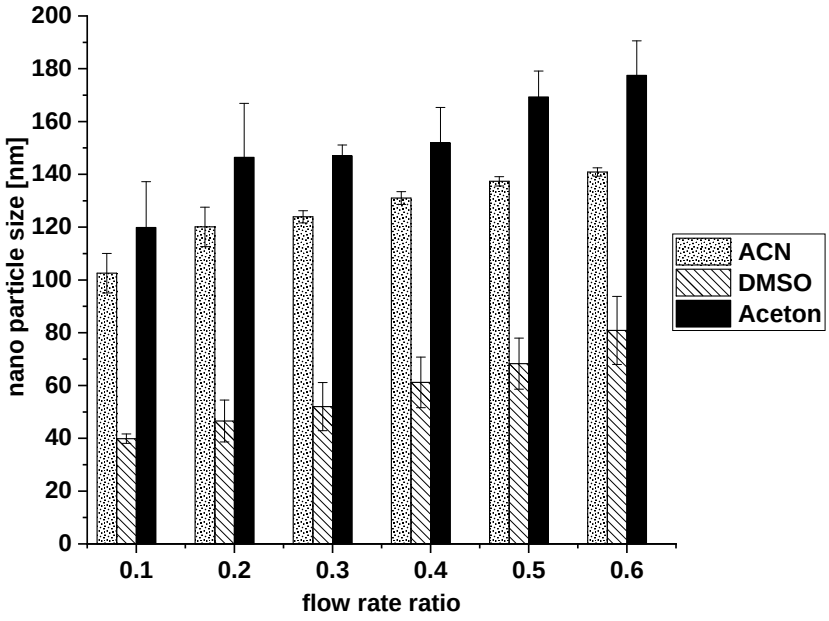


Fig. 5: Effect of solvent nature on NPs size.

3.4.7 Effect of the length of the focus mixing channel

Attempts have been made to explain the mechanisms of nanoprecipitation in order to have a better control over the kinetics of the colloid formation. To the best of our knowledge, no comprehensive study presented relevant experimental evidence enabling more insight into the nanoprecipitation mechanism. Our microfluidic system has a unique feature allowing us to select different lengths of the mixing channel. The impact of the length of the mixing channel was investigated choosing 1 cm, 3 cm, and 5 cm long mixing channels.

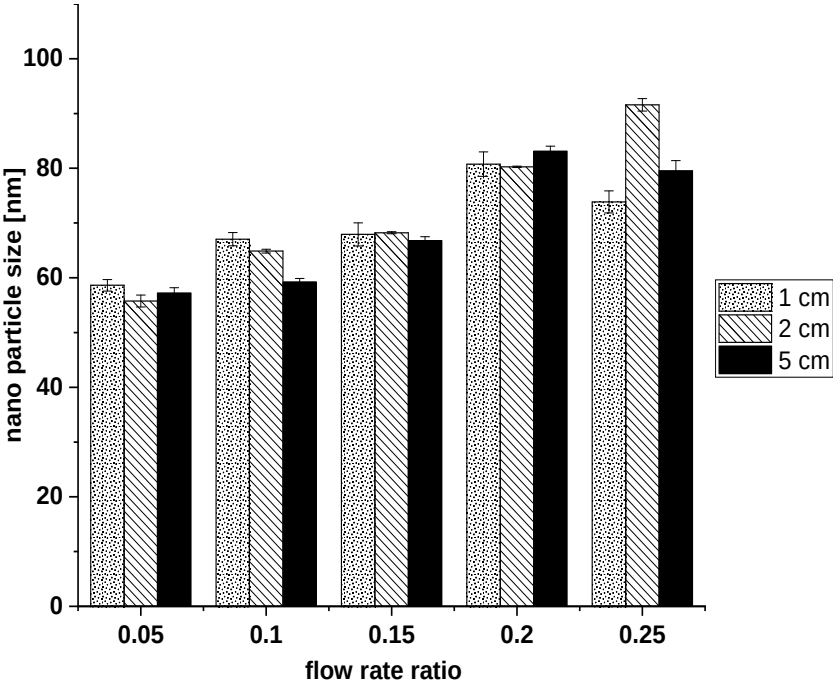
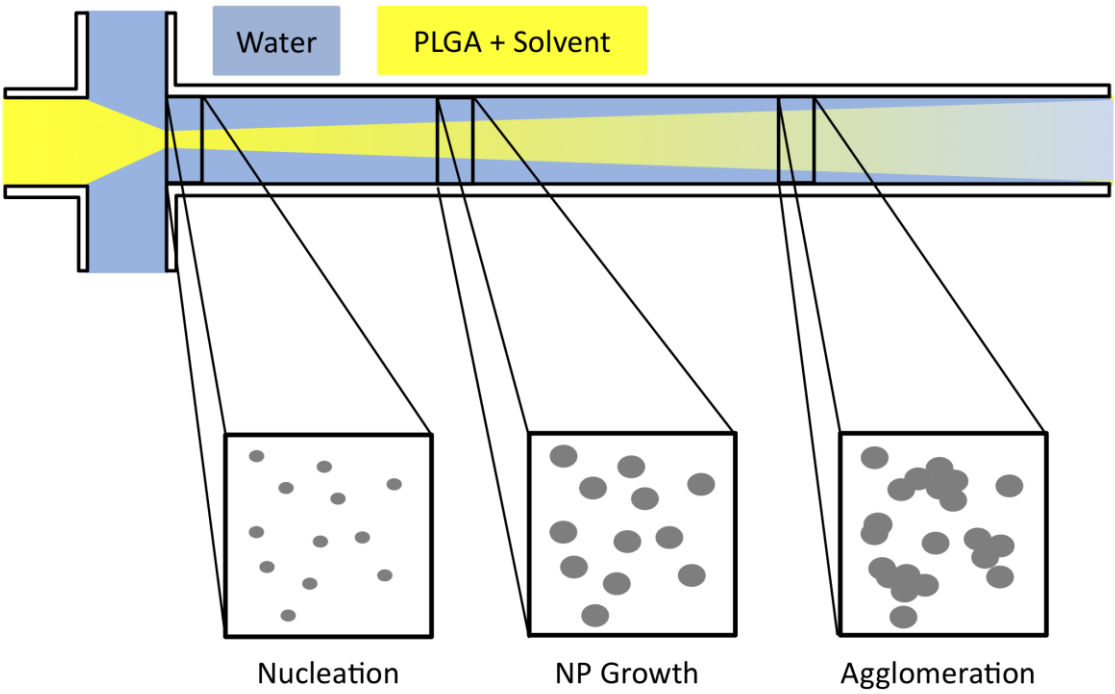


Fig. 6: *Effect of length of the focusing mixing channel on NPs size.*

Fig. 6 displays that the length of the mixing channel has no impact on particles size. It can be seen at (0.05 flow rate ratio) that for all channel lengths the NPs size was 50-60 nm. Also for other flow rate ratios no influence of the channel length could be observed (Fig. 6). According to literature, nanoprecipitation is more linked to nucleation and growth which consist of three stages: nucleation, growth, and aggregation as illustrated in (sketch 2). Based on the theoretical description of the mechanism, the particle formation is dependent on the time available for growth and agglomeration. Aggregation is assumed to happen after the initial formation and it is assumed to depend on the length of the mixing channel [36]. Therefore, adjusting the length of the mixing channel so that its length does only allow nuclei formation should, allow to avoid the growth of NPs, ensuring that only the nuclei (small NPs) would be collected. Overall, separation of the three stages could be meaningful to understand their respective influence on the colloidal size. We can conclude that on a length scale of > 1cm the process is completed.



Sketch 3: *Illustration of nucleation and growth mechanism of nanoprecipitation along the focus mixing channel in microfluidic system.*

3.4.8 Encapsulation of curcumin into PLGA NPs using different techniques

Finally, after evaluating the parameters which are relevant to have an influence on the colloidal properties, incorporation of drug into the nanocarriers was addressed. The goal was to compare the encapsulation efficiency of curcumin into PLGA NPs while using different approaches (microfluidics, injected by hand or using a syringe pump). As can be seen from Fig. 7, loading the PLGA NP using microfluidics resulted in particle increase from 70 nm up 100 nm in comparison to unloaded PLGA. In contrast, loading the PLGA NPs using injection by hand or using a syringe pump, exhibited in a larger increase in size up to 145 nm. Thus, microfluidic is an optimal method for producing small, loaded nanoparticles with a good reproducibility and small variability.

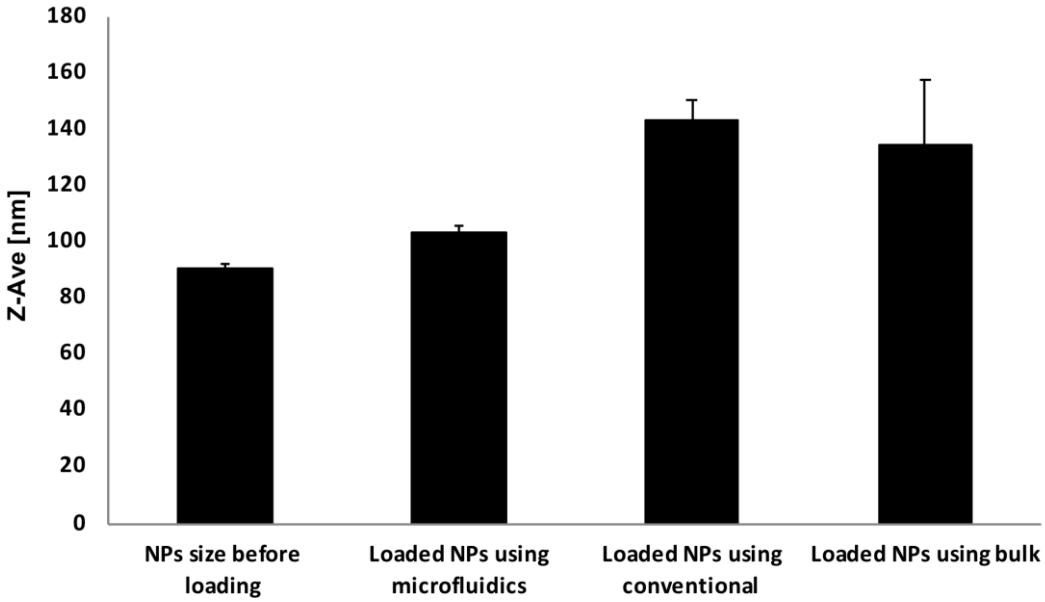


Fig. 7: *Comparison of the size of NPs after encapsulation using different approach.*

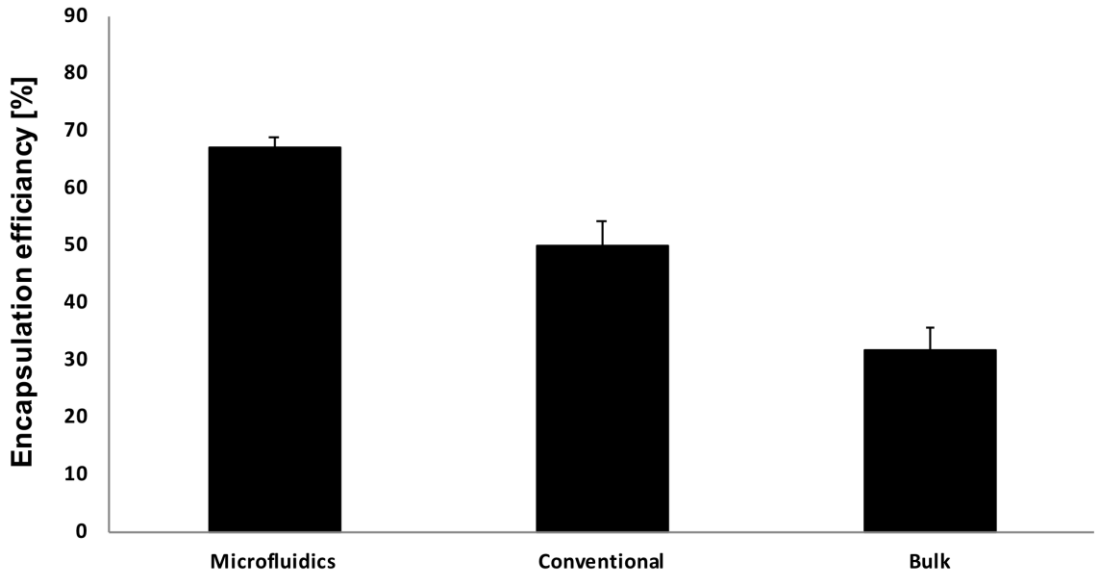


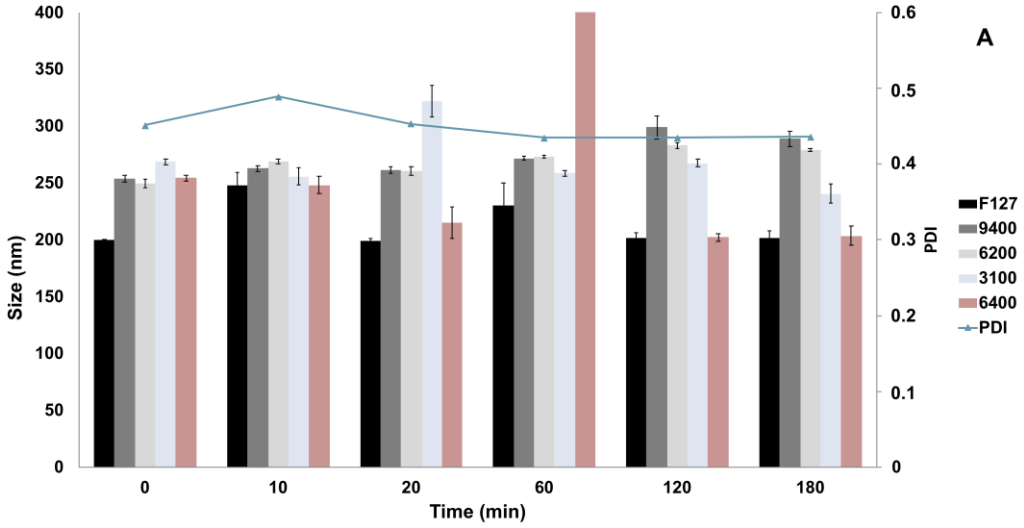
Fig. 8: *Encapsulation efficiency of curcumin using different approaches.*

This is associated with the ability of microfluidics to mix solutions under laminar flow conditions ensuring controlled precipitation and short mixing times. Moreover, the microfluidic approach revealed a higher encapsulation of the lipophilic curcumin into PLGA. Using microfluidics 67.15% of the drug was encapsulated while the average encapsulation was around ~55% using the conventional approach with a syringe pump (Fig. 8).

Finally, the encapsulation efficiency of curcumin was only ~12% for NPs prepared by hand injection, due to lacking laminar flow conditions. This was in accordance with previous reports on drug encapsulation [73]. Overall, it was shown that microfluidics offer a reliable way for encapsulation without much variability in size (small standard deviation) and succeeded in reaching higher encapsulation efficiencies for curcumin.

3.4.9 Nanoparticles' interaction with mucin

Stability of NP within biological fluids is an essential factor with respect to aggregation and potential biological effects [89]. This holds especially true for the interaction of the particles with mucus. To estimate this, mucin as a simple model was chosen for assessing the interaction of surfactant coated PLGA NP with mucin in comparison to surfactant-free PLGA NPs [83, 84]. Fig. 9 indicates that PLGA NP stabilized with Pluronic F68 or Pluronic 10500 showed no interaction with mucins from 0 to 3 h.



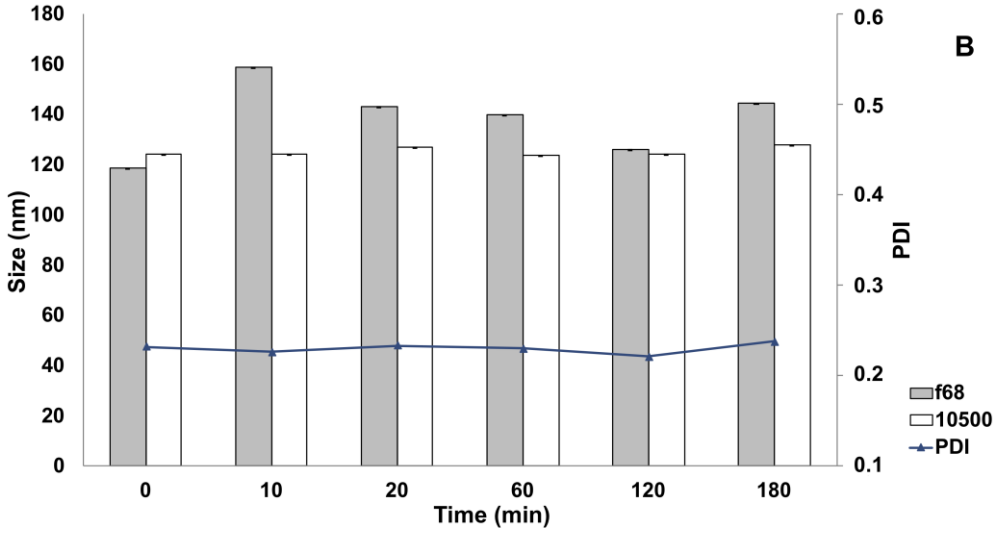


Fig. 9: *Nanoparticles' without surfactant and stabilized with different types of Pluronic and their interaction with mucin*

This is reflected by the absence of any size change, indicating that the stabilizer shielded NP from adsorption or bridging. Contrarily, surfactant-free PLGA NPs and other types of Pluronic (F127, 9400, 6200, 3100 and 6400) have directly shown aggregation (from t = 0 min on), connected to an increase in the mean size up to 400 nm. A considerable shift in PDI from 0.026 up to 0.3 was also noticed. Thus as expected, the coating of the particles is for such an application indispensable. The increase in size and the broad size distribution is related to the interaction of the hydrophobic polymer with mucins which has led for agglomeration, hampering the ability of the particles to serve as efficient drug delivery system.

3.4.10 Permeability of NPs through pulmonary human mucus

Besides specific surface chemistry to avoid the interaction with mucins, manufacturing NPs smaller than the pore size of mucus should be beneficial to avoid size filtering mechanism and thus facilitated their penetration through biological barriers [23, 52]. A CLSM-based set up was used to study the penetration of NPs through pulmonary human mucus [57]. Different sized PLGA NPs (60 nm, 120 nm, 400 nm) were synthesized using microfluidics (also labelled with rhodamine B). NPs in aqueous suspension were added on top of a thin layer of human pulmonary mucus.

Then, the penetration kinetics was analysed by scanning a defined volume at a fixed distance from the objective at different time points. As illustrated in Fig. 10, stabilizer-coated 60 nm PLGA NPs permeated through the human mucus directly when applied.

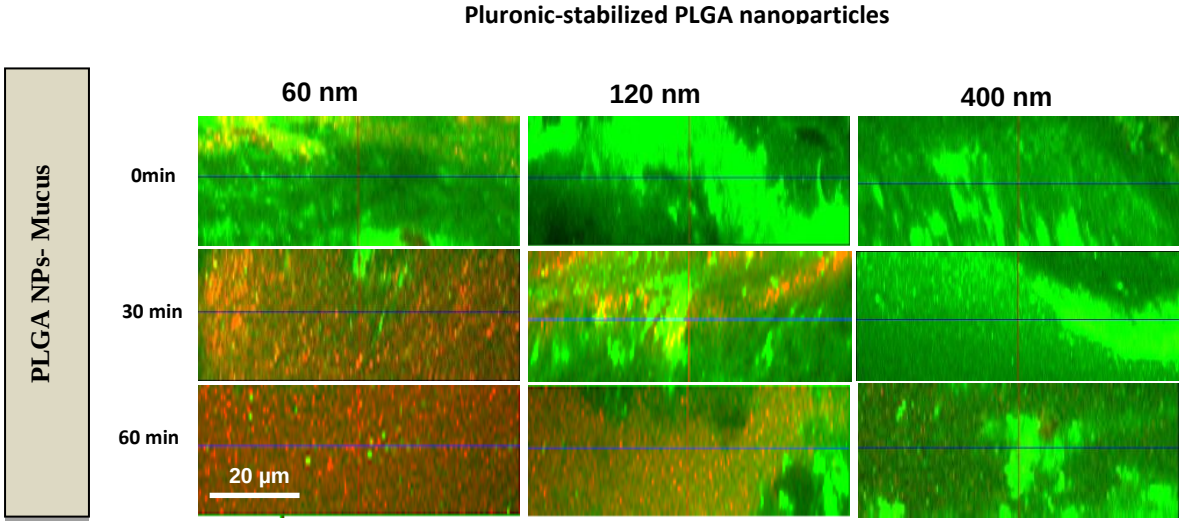


Fig.10: *Confocal laser scanning microscopy study of the penetration of 60,120 and 400 nm red-fluorescent coated PLGA NPs with Pluronic F68 through 40 µm of pulmonary human mucus at predetermined time interval. Mucus was stained with wheat germ agglutinin (Green fluorescent). Scale bar = 20 µm*

Penetration was observed up to 1h after the application. In contrast, 120 nm PLGA NP reached the detection volume after 30 min, whereas, 400 nm PLGA NPs were not observed in the respective volume, as an indication of no or very slow penetration. The observed behaviour can be attributed to the different sizes of the particles as all particles were coated with Pluronic F68 which was described to facilitate mucus penetration [23].

3.5 Conclusion

The ability of microfluidics to precisely mix reagents at short mixing times under laminar flow conditions, has allowed to generate monodisperse PLGA NPs with tunable sizes. After varying different factors during the preparation, the results showed that the most dominating influence on the NPs size was governed by controlling the mixing time. Furthermore, we could show that the formation of particles is not influenced by a particle growth mechanism due to the diffusion in the mixing channel for a certain time (as the length of the channel has no impact). In addition, small particles produced this way were perfectly suited to diffuse through mucus as a biological barrier without being immobilized. NPs around 60 nm have shown an improved penetration through pulmonary human mucus. In contrast, NPs of 400 nm were trapped in mucus as it was expected due to the size filtering interaction.

This well controllable preparation of small particles using microfluidic in combination with a specific muco-inert surface chemistry led to a promising drug delivery system which showed enhanced mucus penetration. Moreover, a higher encapsulation efficiency of curcumin ~ 67.15% was obtained while using microfluidic in comparison to conventional nanoprecipitation methods.

*Spray-drying for generation of an inhalable, multifunctional matrix for the treatment of biofilms as formed in cystic fibrosis**

*** This chapter is prepared for publication as a journal article:**

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4. Spray-drying for generation of an inhalable, multifunctional matrix for the treatment of biofilms as formed in cystic fibrosis

4.1 Abstract

Cystic fibrosis (CF) a serious lung disease is commonly susceptible to Pseudomonas aeruginosa colonization and this pathogenic bacterium is hard to be treated under the respective circumstances. The thick mucus in CF together with biofilm formation is the main reason for limiting the permeability of drugs for reaching their site of action causing of failure in the treatment of the bacterial infection. Beside the treatment with antibiotics, N-acetylcysteine to fluidize mucus is always recommended as it allows for facilitated coughing. Although several formulations have been developed for being used as inhalation therapy in cystic fibrosis disease, still there is no comprehensive study on the role of combining antibiotics with N-acetylcysteine (NAC) in one formulation as multifunctional formulation (DPI). Using such a multifunctional formulation might contribute to ameliorate the treatment. In this work, we developed an innovative pulmonary dry powder formulation based on electrostatic interactions between NAC and antibiotics. NAC and different classes of antibiotics were prosperously spray-dried without using any organic solvent. The obtained mass median aerodynamic diameters were in the range for inhalation (< 5.0 µm). This approach allows potentially incorporating nanoparticles which will result in adding more functionality to the formulation. The multifunctional microparticles have shown synergistic effects against bacteria in biofilm assays due to NAC's property of reducing viscosity allowing for a better way to reach the bacterial targets in the mucus and biofilm. The combination of antibiotics to treat bacterial infections and NAC to facilitate diffusion of drugs and coughing as a convenient treatment option might also enhance treatment compliance, and thereby therapeutic effects in CF.

4.2 Introduction

Cystic fibrosis (CF) is a congenital lethal chronic disease in which thick and sticky mucus is secreted in different body organs such as the pancreatic duct, intestine, and lungs [90]. This abnormal thickness of the mucus causes problems like intestinal blockages and a reduced clearance of the airways. Further, bacterial infections come along with the disease and cause severe problems, for instance, the infection by Pseudomonas aeruginosa that colonizes into a resistant biofilm in the lung in the later stages of the disease. According to recent statistics, approximately 70,000 to 100,000 children and adults around the world suffer from CF [91].

The strategies of the treatment in CF partly rely on the application of inhaled antibiotics in high doses using nebulizers for the treatment of early infections and inhibition of biofilm formation as monotherapy or in addition to oral/IV antibiotics as “multiple therapy” [13]. Meanwhile, the guidelines related to treatment in CF, recommend taking a mucolytic agent to fluidize the mucus and osmotic material like Mannitol to preserve the hydration of the airway [92]. This allows the antibiotics to diffuse into mucus and biofilms to reach the pathogenic bacteria. Overall, the key advantage of inhalation approaches of antibiotics is to reduce systemic side-effects by avoiding their systemic circulation [93]. Currently, only (Tobi®, Novartis AG, Switzerland), aztreonam (Cayston®; Gilead Sciences, USA), and colistimethate sodium (Colomycin®, Promixin®) are available on the market for application via nebulization [94-96]. Nebulization technique is still suffering from several drawbacks such as the majority of nebulizers could be a source of bacterial infection due to the contamination of equipment, losing drug during aerosolization process, long time of administration is required and a reduced performance of the machine over time [97-101]. Some sort of nebulizers would also need a longer time to complete aerosolization resulting in non-optimal aerosols [102, 103]. In contrast to nebulization, dry powder inhalation (DPI) has the traits to reduce expenses of the treatment and it is more convenient to be used.

In addition, there is no need for storage of the formulation in the fridge as for nebulizers, lower contamination probability as it is less influenced by hygienic threats and higher dose delivery in comparison to nebulizers is possible [104].

Presently, also Bayer and Novartis are investigating in dry powders using an emulsion based spray–drying technique called PulmoSphere technology, to create porous particles with a sponge-like morphology. Using this technology, tobramycin as DPI has already been approved from FDA and ciprofloxacin as DPI is in Phase III clinical development [105]. Nevertheless, there is an urgent need to have new classes of inhaled antibiotics, since resistance to antibiotics is inevitable. Different classes of antibiotics might serve in a better way, as bacteria resistance towards antibiotics could be reduced [106]. Over and above, not only antibiotics but also N-acetylcysteine (NAC) has been proven to have bactericidal and antimicrobial effectiveness by decreasing biofilm formation and diminishing the production of extracellular polysaccharide matrix whilst fostering the disruption of the mature biofilm [107]. Furthermore, NAC has a mucolytic effect by cleaving the disulfide bonds in mucins leading to a reduced mucus viscosity [108]. Typically, NAC is administered in CF via nebulizers demanding more application time and entailing extended cleaning efforts. For this reason, manufacturing NAC as (DPI) for inhalation simplifies the application for the patient in comparison to nebulizers and can greatly enhance adherence to the therapy and therefore increase the effectivity [109]. The CF disease is a very complicated illness and requires using different drugs at the same time. These include antibiotics, anti-inflammatory agents, mucolytic agents and osmotic material [110, 111]. The essential need to combine different drugs for a sufficient therapy has inspired us to have all these drugs together in one formulation, with the ultimate goal of achieving an extra functional drug application that could potentially decrease symptoms and the severity of CF in patients. Recognizing this, we have developed a novel, interesting approach to prepare a dry powder formulation (DPI) composed of antibiotics and NAC by utilizing a spray drying technique. Based on that, the incorporation of additional drug carriers in the inhalable formulation could add another aspect for the treatment of CF such as addressing chronic inflammation as commonly established in CF [111].

In this regard, the extension of the pulmonary MP system by incorporating a carrier for an inflammatory drug might be meaningful (figure 1).

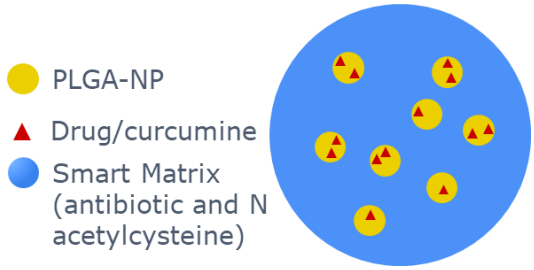

Fig. 1: *Sketch of the anticipated design and composition of the microparticles.*

As an example, PLGA NPs loaded with Curcumin as an anti-inflammatory drug could be loaded into the matrix which is a mixture of antibiotics and N-acetylcysteine. We hope that such a system could ultimately enhance the future treatment for cystic fibrosis. Overall, we have described the mechanism of complex formation of NAC/antibiotics. This knowledge will give an opportunity to use a variety of drugs to be in an inhaled form as (DPI). This could be achieved by exchanging the anionic or the cationic partner with different substances that exhibit desired properties. Besides the successful formation of a powder formulation for inhalation (next generation impactor) was used to evaluate their flying study. Also, their impact on biofilm and mucus were tested. The effect of the formulation on the viscosity of mucus was evaluated using pulmonary horse mucus. Furthermore, disintegration of MPs was assessed together with the activity of the antibiotic and the effect on the biofilm.

4.3 Material and methods

4.3.1 Materials

D-mannitol (≥98%) (MW= 182.172 g/mol), N-Acetylcysteine (MW= 163.19 g/mol), L-Leucine (MW= 131.18 g/mol), curcumin (MW= 368.39 g/mol), agarose, azithromycin (MW= 748.98 g/mol), ciprofloxacin (MW= 331.346 g/mol) and tobramycin (MW= 467.515 g/mol) were obtained from Sigma Aldrich (Steinheim, Germany). Nitrocellulose membrane was a gift from Fisher Scientific GmbH (Schwelten, Germany). Pulmonary horse mucus was obtained after BAL of horses. (Pferdeklinik Altforweiler, Germany).

4.3.2 Preparation of multifunctional microparticles

The multifunctional microparticles were produced by using a BÜCHI B-290 Spray Dryer from Büchi (Flawil, Switzerland). N-Acetylcysteine (NAC) was dissolved in Milli-Q water and one of the antibiotics was also dissolved under magnetic stirring in Milli-Q water, then NAC solution was added slowly into the antibiotics solution in molar ratios. The mixed solution was left for 10 min until the solution became clear (Table 1) and L-Leucine was also added.

Table 1: *Amount of NAC and antibiotic in the spray drying solution with the needed percentage of L-Leucine.*

Formulation	NAC [mol]	Antibiotic [mol]	L-Leucine [%]
AziNAC	2	1	5
CiproNAC	1	1	10
TobraNAC	5	1	10

The amount of L-Leucine was determined in preliminary studies. The mixed solution was adjusted to gain an overall concentration of all solid substances together of 1 wt. % for spray drying. The spray dryer was assembled and the settings were adjusted to fit the spray drying conditions. The gas rotameter was set to 50 mm which translates to an actual air volume flow of roughly 1050 L/h, peristaltic feeding pump was set to 10% (~ 3 mL/min of spray drying solution feed) and the aspirator was set to 100% which stands for an approximate gas flow rate of 35 m^3/h.

All mixtures were spray dried with compressed air. After spray drying, the obtained powders were collected and stored in a desiccator at room temperature. All formulations were spray dried in triplicate.

4.3.3 Morphology of the multifunctional microparticles

For morphology analysis, a scanning electron microscope EVO HD 15 from Zeiss (Jena, Germany) was used. Each of formulation was spread on an individual carbon disc and sputter-coated with a 10 nm gold thin layer using a Quorum Q150R ES sputter coater (Laughton, UK).

4.3.4 Determination of in vitro aerodynamic behavior

For examination of the aerodynamic properties, the (DPI) formulations were applied on a Next Generation Impactor (NGI) from Copley Scientific (Nottingham, UK). Before the experiment, the impactor pans were coated with a Brij-Coating consisting of 4 parts 15% Brij 35 in Ethanol in 6 parts glycerol; 10mL of Milli-Q water was filled in the pre-separator. For every experiment, a hard gelatin capsule (size 3) was filled with approximately 20 mg of formulation. The capsules were placed in a HandiHaler (Boehringer Ingelheim, Ingelheim, Germany) and pierced. The air flow for application was set to 60 L/min and controlled by a M1A flowmeter (Copley Scientific, Nottingham, UK). The active time of the vacuum pump (Erweka, Heusenstamm, Germany) and the critical flow controller (Erweka, Heusenstamm, Germany) was 4 seconds. After running the experiment, the powder contents in the NGI pans were dissolved in a defined amount of water and the fluorescence signal of Rhodamine B was analyzed using a Tecan reader infinite 200 (Tecan, Männedorf, Switzerland). 100 µL of a 5 mg/mL Rhodamine B-ethanol solution was added for each 100 mg of dry substance in the feeding solution for spraying. For each formulation, an individual calibration curve was prepared and the whole formulation was analyzed using an excitation wavelength of 565 nm and an emission wavelength of 625 nm. All experiments have been carried out in triplicates under the same conditions.

4.3.5 Disintegration behaviors of multifunctional microparticles

0.1% Agarose gel was prepared by dissolving it in hot water for 30 min and pouring the solution into Petri dishes. The gel was left overnight to cool down. Spray-dried microparticles (SD-MP) were spread on top of a nitrocellulose membrane by using a Penn-Century device (Wyndmoor, USA) in order to obtain a better distribution over the filter. Then, the nitrocellulose membrane pieces were placed on top of the gel pad for a pre-determined amount of time (0 min, 10 min, and 30 min) to determine the disintegration behavior of the matrix over time. After the experiment, the membranes were removed from the gel pads. Then, it was analyzed through using SEM after sputter coating (EVO HD15, Zeiss, Jena, Germany). All experiments were executed in triplicate.

4.3.6 Determination of the rheological properties of mucus after multifunctional microparticles application

The viscoelastic property of pulmonary horse mucus in the presence of formulations was determined by a rotational viscometer Kinexus (Malvern, UK). Pure pulmonary horse mucus (Pferdeklinik Altforweiler, Germany) without treatment with the MPs was measured as a control. 500 µL of mucus was treated with 100 µl solution of formulations after adjusting the content of NAC inside the formulation as (0.1%) for 30 min followed by measuring the viscous behavior using cone-plate geometry with 1° and 0.5° angle (C60/1, C60/0.5) at different shear stress rates range of (0.02 – 2 Pa). Measurements were performed at room temperature in triplicates.

4.3.7 Stability of the multifunctional microparticles

For stability analysis, a scanning electron microscope EVO HD 15 from Zeiss (Jena, Germany) was employed. For each formulation, 3 batches were prepared. One was stored in a desiccator in presence of silica sand at room temperature and one was stored in the lab at room temperature and in the dark. These storage conditions were chosen to enable a comparison between microparticles that were stored at dry conditions and at ambient/humid conditions. The formulations were investigated regarding their stability directly after fresh preparation, after 3 weeks and 3 months.

4.3.8 Influence of the multifunctional microparticles against Pseudomonas aeruginosa biofilm assay

Determination of MPs' effects on the overall biofilm mass was performed using the crystal violet (CV) assay according to reported procedures with a slight modification [26, 27]. P. aeruginosa PA14 strain was cultivated in 96 well plates using PPGAS medium. 2 µL of the MPs stock solution was added to 198 µL of the bacterial culture to give 200 µL total volumes (final concentration equals 1% of stock solution concentration). DMSO 1% was used as a control. Experiments were performed at least in duplicates. Graphical illustrations of the results represent the mean values and error bars denote the standard deviation.

4.3.9 Extracellular DNA (eDNA) assay

The impact of MPs on eDNA was assessed as previously reported [112] [113] via incubation of biofilm with propidium iodide solution (0.05 mg/mL) at 37 °C for 24 h and detection of specific fluorescence at 620 nm after a thorough washing step with 18MΩ H2O.

4.3.10 Antibacterial assay

Determination of growth inhibitory effects and minimum inhibitory concentrations (MICs) for the MPs and the antibiotics were performed as described in details elsewhere [29].

4.4 Results and discussion

4.4.1 Manufacturing and characterization of the spray-dried multifunctional microparticles

The research focus was to produce an inhalable dry powder (DPI) formulation with a functional matrix for microparticles using N-Acetylcysteine (NAC). The combination of drugs and drug carriers with the mucolytic property of NAC seemed to be an interesting target. The reduced mucus viscosity shall facilitate penetration through mucus. However, due to the high hygroscopicity of NAC, spray drying it into microparticles was not achieved yet [109]. Spraying of the pure NAC resulted in strongly agglomerated particles (Fig. 2).

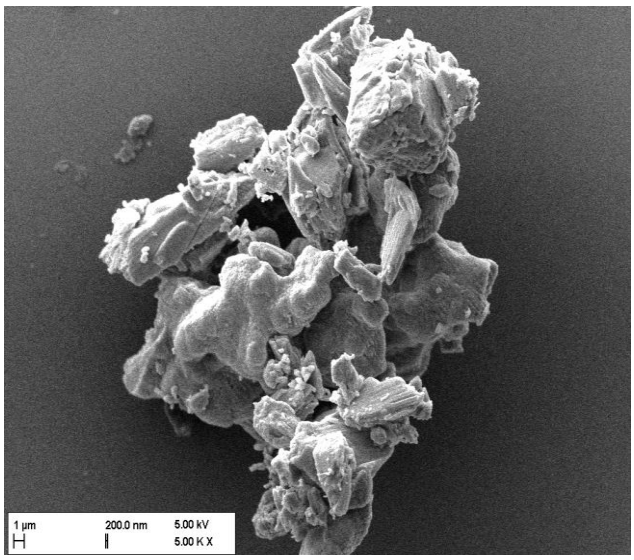

Fig. 2: *SEM micrograph of pure spray-dried NAC, Magnification 5.000x.*

Already, the collection of the spray-dried microparticles of the pure NAC was complicated because it solidified as a hard and sticky layer-like structure on the surface of the spray-dryer's collector glass. The second attempt was to have microparticles composed out of NAC and Mannitol. Mannitol (a sugar alcohol with osmotic characteristics) is one of the most commonly used excipients with respect to the treatment of pulmonary infections in CF disease [114] [115]. It is FDA approved as mucolytic drug (Bronchitol®) as add-on remedy in adult in CF patients. The applied DPI formulation has shown an osmotic effect increasing the liquid content of the mucus layer. Subsequently, the viscosity of the mucus is decreased and the mucociliary clearance is enhanced [115].

NAC and Mannitol were spray-dried in different ratios. As a reference, pure Mannitol was also spray-dried under the same conditions. Looking at their morphology under SEM, it was observed that the obtained formulations with a Mannitol:NAC ratio higher than 12:1 showed a better morphology with distinct spherical microparticles (Fig. 3).

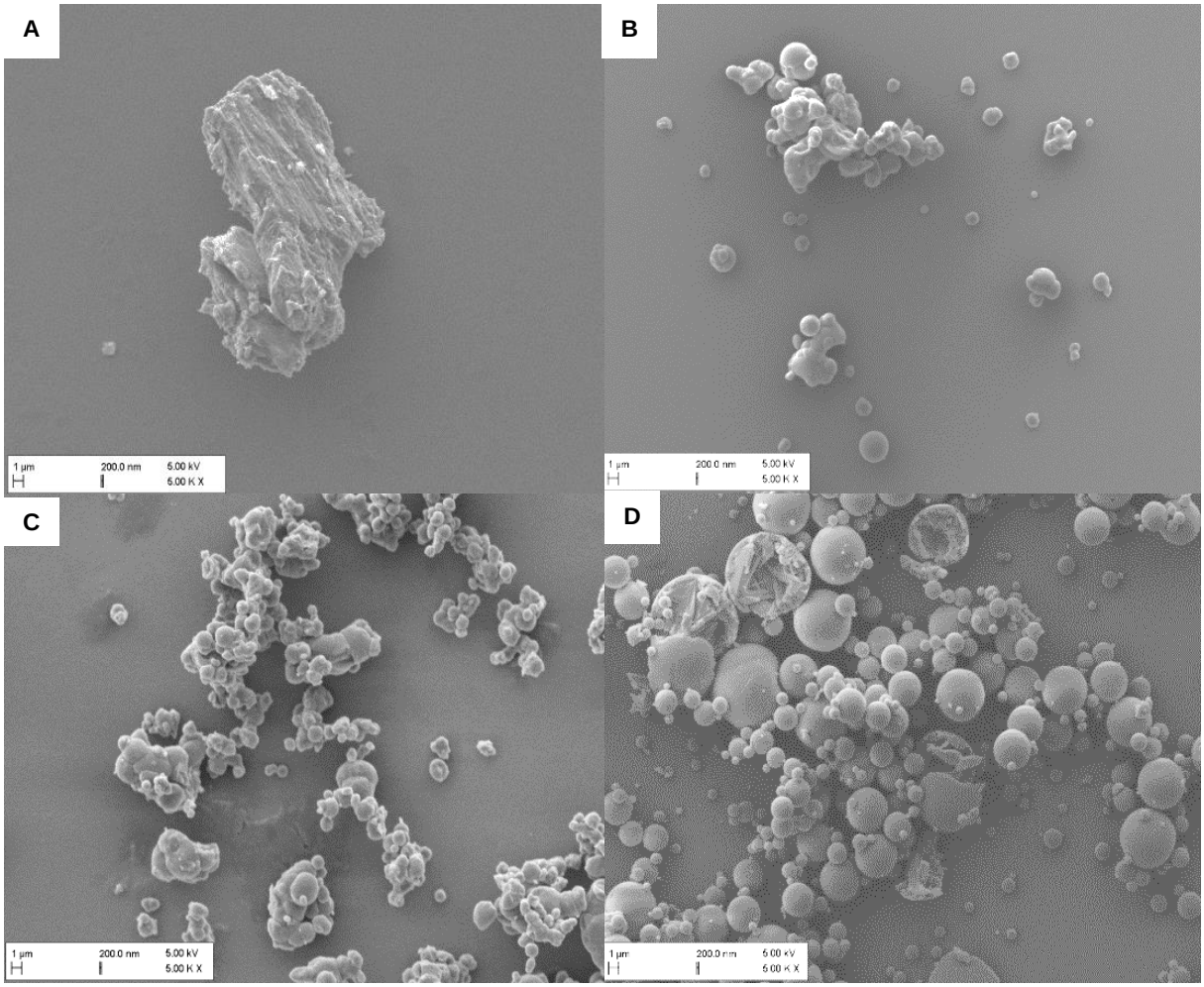

Fig. 3: *SEM micrographs of spray-dried Mannitol/NAC microparticles. 1:1 ratio (A), 12:1(B), 25:1 (C) and pure Mannitol as reference (D) at magnification 5.000x.*

In addition, the samples were easy to collect and to handle while storing in the desiccator. This could be due to the increase of the glass transition temperature of the whole formulation by increasing the mannitol content [109]. Several preliminary studies were conducted, to understand NAC's interaction with different chemical properties to increase its concentration. However, the only real improvement was observed while mixing NAC with the weak electrolyte chitosan (CS).

Hypothetically, the ionic interaction of the cationic amino groups of CS with the anionic carboxylic acid group of NAC might be responsible for this. A second drug used for the treatment in CF offering cationic groups such as several antibiotics seemed promising. Hence, this unique approach would combine NAC's mucolytic properties with the microbicide effect of antibiotics. However, it was unclear how NAC interacts with the amino groups of the antibiotics and how the interaction affects the solubility of the antibiotics. Therefore, NAC was combined with Azithromycin (an antibiotic with two free amino groups which has a low solubility in water) and dissolved in water. Our preliminary study showed that using an equimolar amount of NAC and azithromycin, the aqueous solution was highly turbid and the azithromycin was still mostly undissolved. Using a molar ratio of 1:2 of azithromycin to NAC the solution became clear shortly after adding everything together in water (Fig. 4).

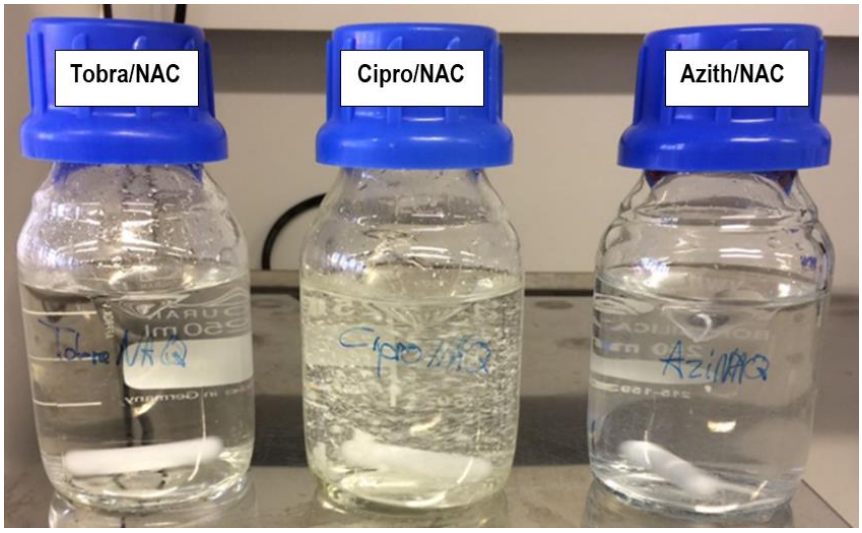

Fig. 4: *The dry substances of Tobra/NAC, Cipro/NAC & Azi/NAC respectively, show differences in dissolving speed upon contact with water. Cipro/NAC takes longer to completely dissolve than the other two formulations. Finally, all formulations were clear solutions. Picture was taken 1min after contact.*

The two ionizable amino groups and the charge ratio of the mixture support the hypothesis of the interaction process between the charged groups. To further proof this hypothesis, two different antibiotics with different amounts of amino groups were chosen namely ciprofloxacin (Cipro) (an antibacterial fluoroquinolone) and tobramycin (Tobra) (aminoglycoside antibiotic) also representing different classes of antibiotics.

This means for ciprofloxacin that only an equimolar amount of NAC was necessary due to the fact that only the secondary amino group in the piperazine heterocycle is an amino group that can be ionized. The other two nitrogen atoms are in a vinylogic interaction with the keto groups inside the molecule, for this reason, vinylogic amides are hard to protonate. For tobramycin, an already water-soluble antibiotic with five amino groups that are easily protonated, a ratio of 1:5 of tobramycin to NAC was chosen. This was done to achieve an optimal concentration for salification of NAC within the formulation. These equinormal ratios should also allow for stable formulations of the spray-dried powder balancing the high hygroscopic potential of NAC. Even though, it would have been possible to spray dry tobramycin with less NAC. The ionization of both components together as illustrated on the example of NAC interaction with antibiotics in (Fig. 5) should also improve the solubility necessary for spray drying from aqueous solution.

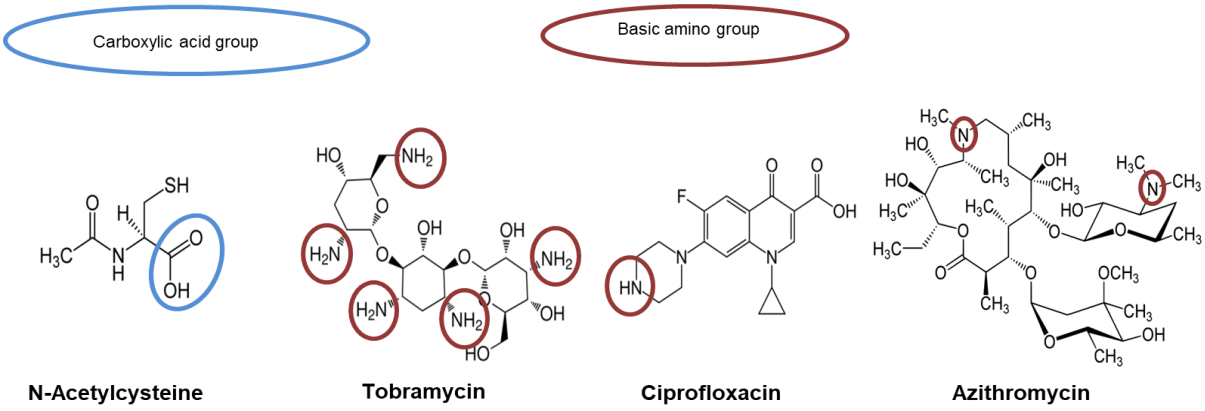

Fig. 5: *Ionization site of antibiotics and NAC for the spray-dried combination*

Having the antibiotics in a salt form would also increase its solubility in water and accordingly the efficacy of the treatment since it will allow the drug to increase the local concentration at the site of deposition [116]. Furthermore, these antibiotics and NAC formulations will be available as DPI formulation for inhalation. To counterbalance the hygroscopic properties of NAC, L-Leucine was added into the formulations. It showed already the ability to protect hygroscopic dry powders from wetting and therefore early disintegration [117].

This effect can be explained by the lipophilic tert-butylic tail of L-leucine which strengthens the hydrophobic properties of formulations, prevents water to adsorb at the particle surface and to penetrate in the deep layers of the particles. In addition, L-leucine showed that it is able to optimize the microparticle morphology by smoothing the surface and reducing wrinkles and cracks [117]. The feeding solutions for each antibiotic/NAC formulation were prepared as described in Tab. 1. For each amino group, one mole of NAC was added to one mole of antibiotic in order to achieve a saturation of all amino groups. The powder mixtures were all dissolved in Milli-Q water. Tobramycin in combination with NAC was easily dissolved hence it has good water solubility. Besides the low solubility of Ciprofloxacin (Cipro), the stoichiometric mixture with NAC (1:1) could be fully dissolved in 1h. For all formulations, the same standardized spray drying conditions were used (as indicated previously). All three formulations of antibiotic/NAC displayed similar handling properties with respect to collecting and storing. This implies low moisture content and low aggregation. As illustrated in (Fig. 6).

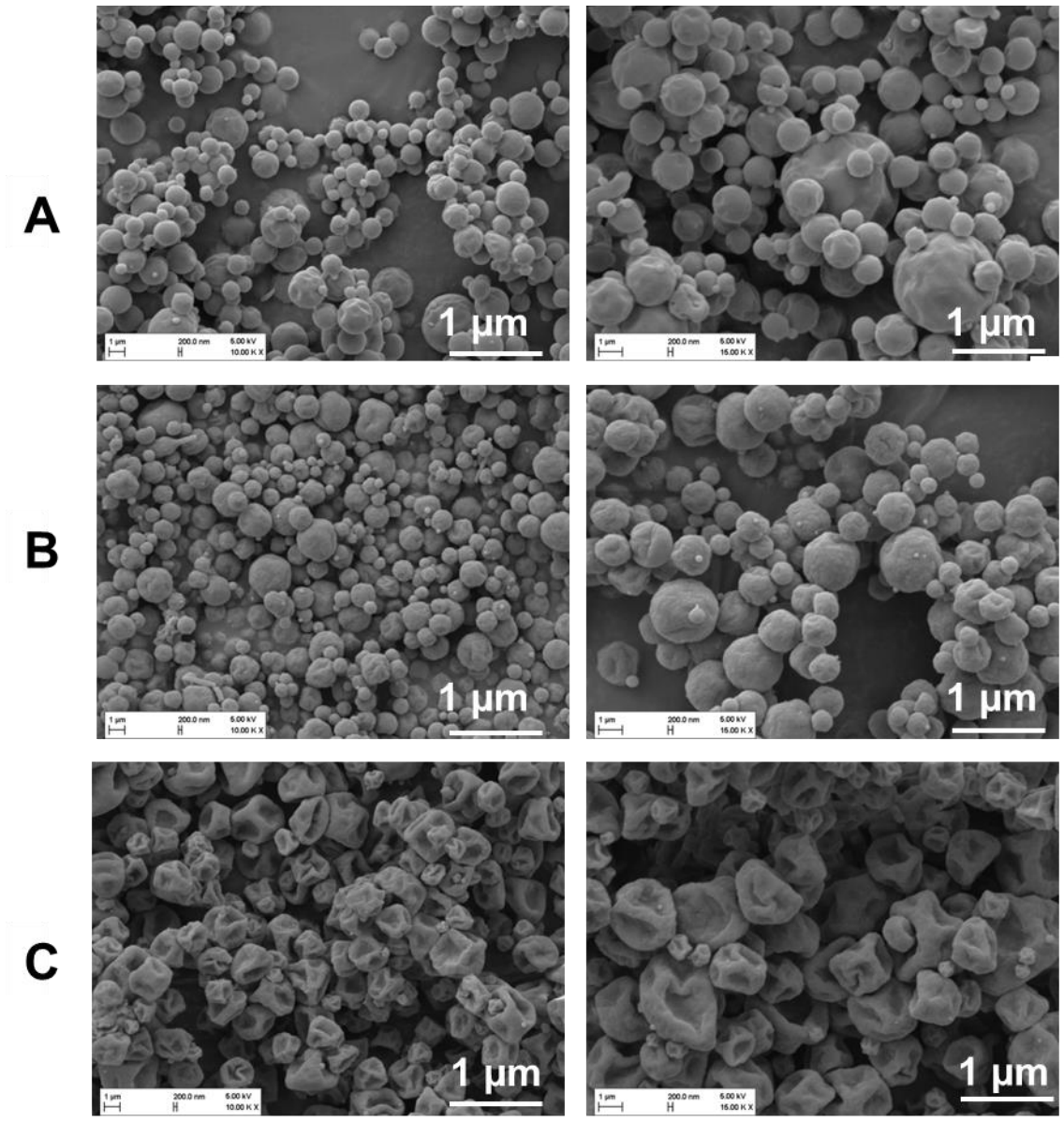

Fig. 6: *SEM micrographs of spray-dried antibiotics/NAC microparticles. Azithromycin/NAC (A) and Ciprofloxacin/NAC (B), Tobramycin/NAC at, 10.000x and 15.000x magnification respectively.*

All formulations were consisting of mostly distinct particles. Cipro/NAC and Azith/NAC formulations exhibited smoother surfaces than Tobra/NAC, showing a raisin-like shape. Finally, as our formulations were spray-dried completely out of pure water without any organic solvent, it was also possible to incorporate PLGA NPs and make it more functional. The ongoing study is running regarding this.

4.4.2 In Vitro Aerodynamic Properties

The flying properties of particles are an extremely important criterion when developing a novel formulation for inhalation. Particles with a diameter bigger than 5 µm mainly deposited in the oropharynx and large conducting airways, whilst particles ranging from 1 to 5 µm would be deposit in the small airways and alveoli. Thus, the aerodynamic properties of the powder formulations were investigated using the Next Generation Impactor (NGI). The data obtained such as the mass median aerodynamic diameter (MMAD), the geometric mean (GSD) and the fine particle fraction (FPF) are depicted in table 2.

Table 2: *Results of the NGI evaluation of the spray-dried powders, SD is provided in brackets.*

	Cipro/NAC	Tobra/NAC	Azi/NAC
MMAD [µm]	2.40 (± 0.02)	2.16 (± 0.33)	2.63 (± 0.03)
GSD	1.88 (± 0.008)	1.53 (± 0.06)	1.64 (± 0.13)
FPF [%]	61.60 (± 5.23)	97.52 (± 15.36)	67.40 (± 9.41)

All formulation containing antibiotics/NAC showed a proper FPF of at least 60% and comparable MMAD values ranging between 2.16 up to 2.63 µm. The innovated pulmonary microparticles have exhibited appropriate aerodynamic properties for a treatment of chronic lung infections in cystic fibrosis.

4.4.3 Disintegration behavior of the multifunctional microparticles in humid air

For microparticles intended for inhalation purpose, an appropriate disintegration rate of the matrix is crucial. Upon deposition into the lungs, a rapid disintegration in the small available amount of fluid is desired [114]. The combination of the antibiotics with NAC revealed already for spray drying the positive effect of the complex formation as also the drugs with low solubility (azithromycin and ciprofloxacin) became water soluble after spray drying. To investigate the dispersibility, DPI formulations were applied onto a filter membrane in 100% rH; then the membrane was placed on an agarose gel and stored at 37 °C.

The fate of the particles on the membrane was visualized using SEM. As a control, microparticles without placing on agarose pads were examined. As can be seen in (Fig. 7), the microparticles started to dissolve immediately (t =0 min) after placing them on the agarose gel while being mounted on the nitrocellulose membrane.

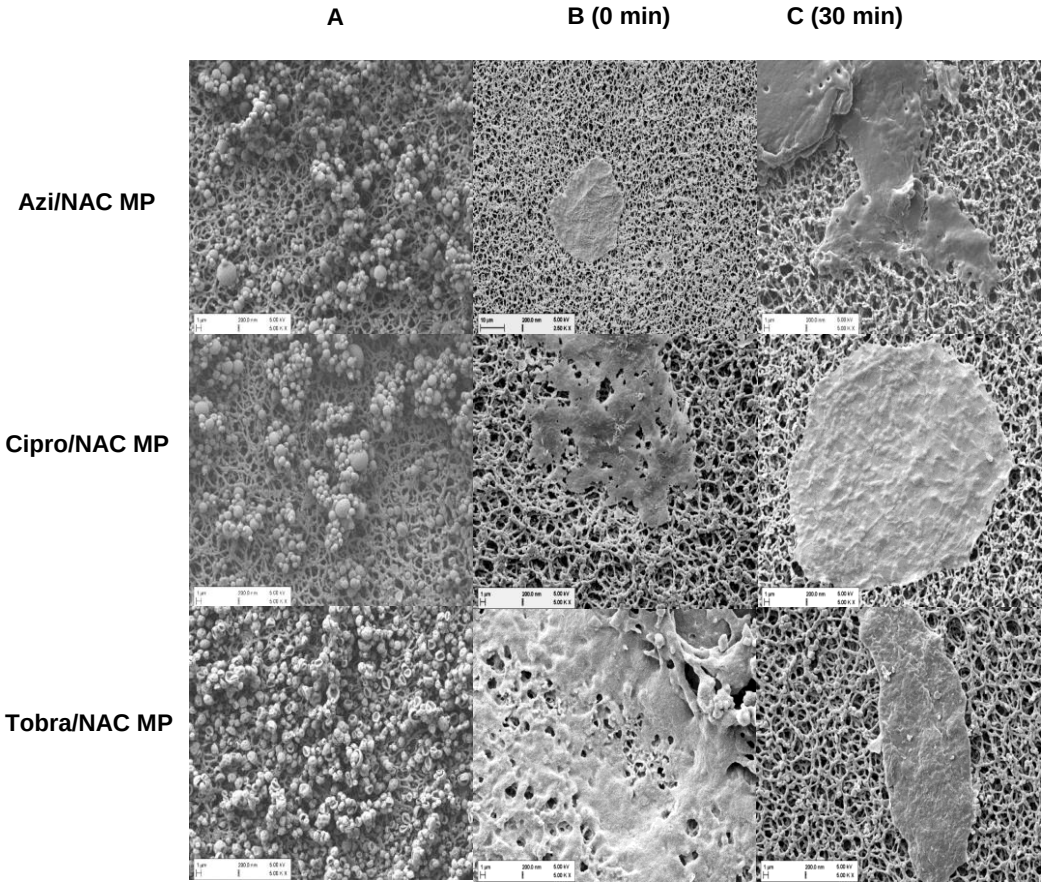

Fig. 7: *SEM micrographs of spray-dried microparticles deposited on top of a nitrocellulose membrane (A) formulation without placing ontop of agarose Pads. (B) antibiotics/NAC at 0 min when got placed on top of Agarose pads, antibiotics /NAC at 30 min (C) at 5.000 x,10.000 and 15.000x magnification respectively*

These results confirmed that the presence of NAC inside the matrix allowed improving solubilisation of the antibiotics based on the salification process [116, 118]. Also, in this case, the microparticles were much quicker disintegrating in the presence of NAC.

4.4.4 Effect of NAC-containing multifunctional microparticles on mucus

Mucus and especially the viscous mucus in CF reduce the permeability of drugs and drug carriers. Therefore, a mucolytic agent such as NAC is used to fluidize the thick mucus and enhance the permeation [119]. The viscosity of pulmonary horse mucus before and after the treatment with a solution of the multifunctional microparticles was measured, to assess NAC's influence on the rheological properties of mucus. Applying the antibiotic/NAC microparticles to mucus assuming complete dissolution as investigated before reveals altered rheological properties of mucus (Fig. 8). Mucus was diluted with pure water in the same amount of solution of microparticles which would be applied later in order to avoid the dilution effect. The diluted mucus in water was taken as control. Then, Pure NAC as a solution was added to undiluted mucus, to assess the effect of the NAC alone on the rheological properties of mucus.

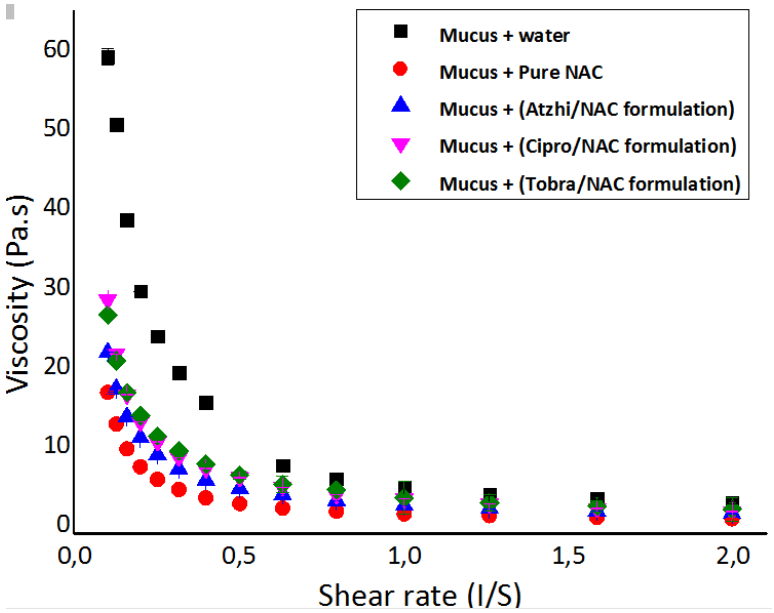

Fig.8: *Effect of the formulations containing NAC on the viscosity of mucus.*

It was shown that presence of pure NAC, led to a pronounced reduction in the viscosity from 60 Pa.s to 18 Pa.s. Due to the fact that NAC cleavage the disulfide bond in mucin within mucus [107]. Final steps were to assess the effect of NAC within multifunctional microparticles. Each formulation after dissolving it was added to mucus. Results showed that the presence of NAC inside the formulation, displayed also a reduction in the viscosity of mucus from 60 to 19 Pa.s after the treatment by the multifunctional microparticles. The effect of the formulation was approximately the same as pure NAC, confirming the efficacy of NAC as a mucolytic agent inside multifunctional microparticles.

4.4.5 Stability of spray-dried multifunctional microparticles

Particle stability under storage conditions is crucial when preparing a dry powder formulation. Agglomerated powders, sticky materials or wetted formulations greatly decrease the proper application and have a huge effect on the therapy efficiencies. The stability of antibiotics/NAC formulations were tested by visualizing them in SEM after several time steps (freshly prepared, after 3 weeks and 12 weeks) to determine, whether the formulations are stable and distinct particles still remain or start to show any sign of agglomeration. As can be seen in (Fig. 9)

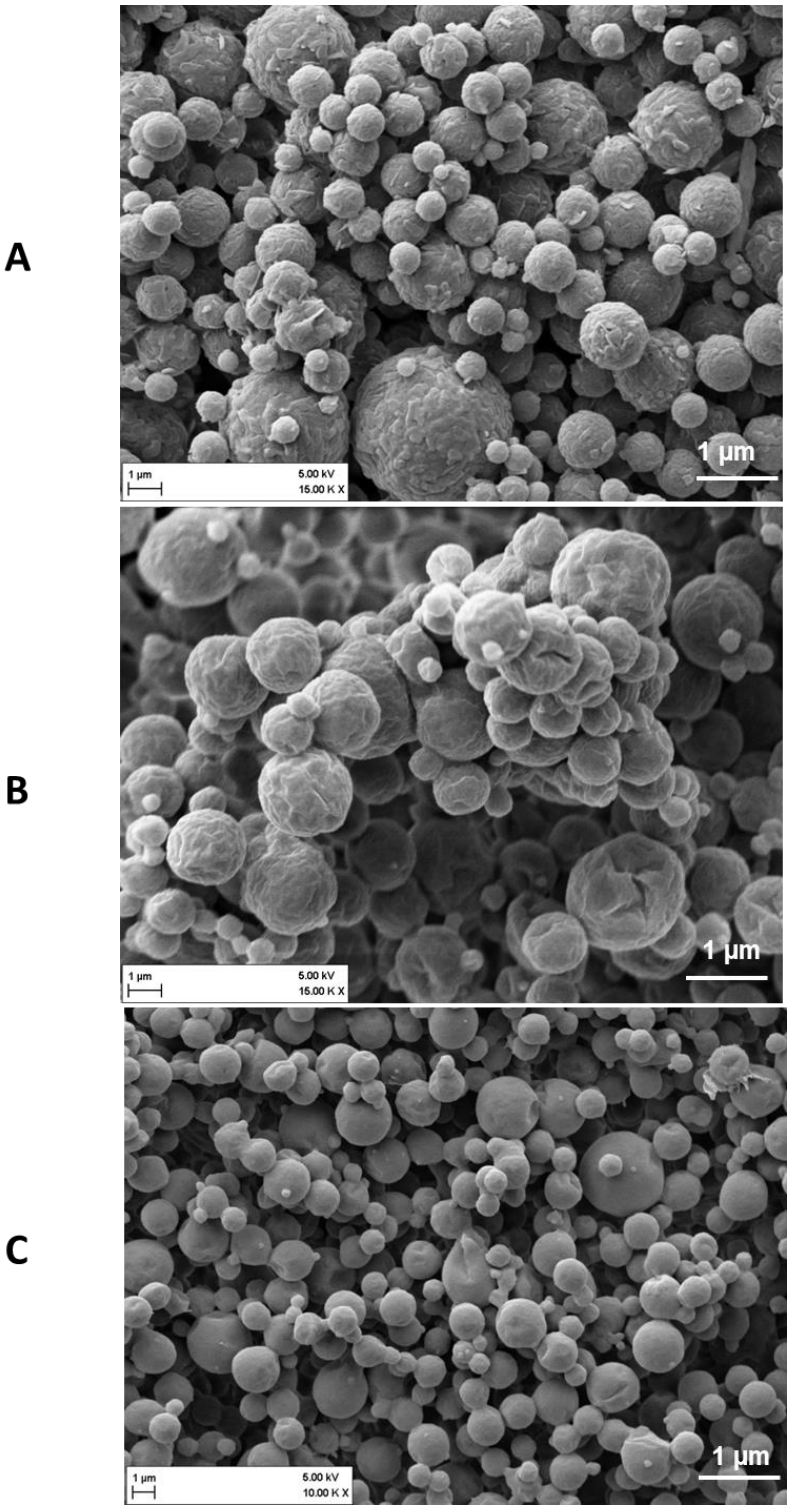

Fig. 9: *SEM micrographs of spray-dried of (A) Cipro/NAC, (B) Azith/NAC, (C) Tobra/NAC after 3 weeks at 15.000 magnification.*

Azthi/NAC and Tobra/NAC formulations were stable under both storage conditions (in the desiccator and in a drawer), with distinct particles and smoothly spherical shape. Surprisingly, in the case of Cipro/NAC, a rough shell structure as from recrystallization appeared on the MP. Which might be related to the presence of NAC and it is perhaps because of a kinetic instability of ciprofloxacin. The structure gives the impression of being the NAC started to separate from the ciprofloxacin and diffuse out on the surface of the MP as illustrated in (Fig. 9 A). The same scenario was observed after 3 weeks up to 12 weeks. No further changes could be monitored. As azithromycin and tobramycin were stable and did not show any change in the morphology (Fig. 9). Although a change in surface morphology could be seen (due to recrystallizing) of Cipro/NAC, still distinct microparticles with no agglomeration can be observed (Fig. 9 A).

4.4.6 In vitro evaluation of the effect of multifunctional MP against biofilm

A key parameter of the microparticles formulation (MP) is to have a decisive role against pathogenic bacteria. *Pseudomonas aeruginosa* biofilm is a community of bacterial cells embedded in a mixture of biopolymers, *e.g.*, polysaccharides, proteins, lipids, and extracellular DNA (eDNA) forming the biofilm matrix [26]. These extracellular polymeric substances (EPS) have structural, nutritional, and protective functions in the biofilm. Extracellular DNA is a main component of *P. aeruginosa* biofilm matrix. It plays an important role in biofilm formation, virulence as well as antibiotic resistance [120-122]. Antibiofilm activity of the MP formulations against the highly virulent clinical isolate *P. aeruginosa* PA14 was evaluated by two methods: crystal violet (CV) biofilm assay [123] [112] and eDNA assay [112, 113]. Azithromycin-NAC MPs showed a synergistic effect on biofilm (25% inhibition) at a concentration of 0.3 µg/mL, while neither azithromycin nor NAC separately showed inhibition at the same concentration (Fig. 10A).

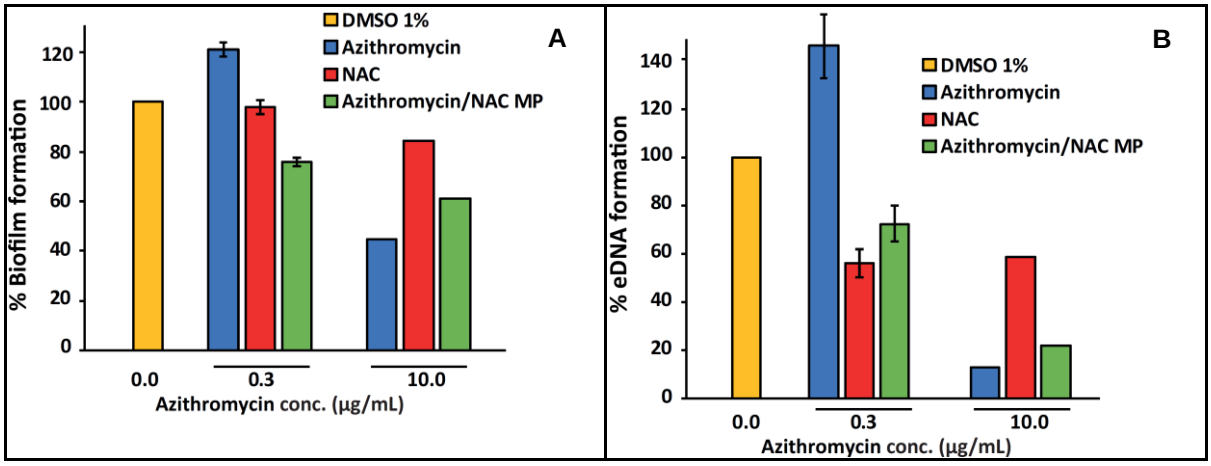

Fig.10: *Effects of azithromycin, NAC, and azithromycin-NAC MP at concentrations of 0.3 and 10.0 µg/mL on biofilm (A) and eDNA formation (B) in P. aeruginosa PA14.*

At a concentration of 10 µg/mL, comparable inhibitory effects of azithromycin-NAC MPs and azithromycin alone were observed (Fig. 10A). This can be attributed to the antibacterial effect of azithromycin at 10 µg/mL concentration. Similar results were obtained from eDNA assay. Combination of azithromycin and NAC in the MPs resulted in 30% inhibition of eDNA formation at 0.3 µg/mL, whereas neat azithromycin showed no inhibition at the same concentration (Fig. 10B). Equal inhibitory effects of the MPs and azithromycin were observed when the concentration of azithromycin exceeded the minimum inhibitory concentration (MIC) (Fig. 10B and Table 1 for the antibacterial activity). This might be due to the strong antibacterial effect not profiting from the presence of NAC. Both ciprofloxacin and ciprofloxacin-NAC MPs showed about 65% inhibition of biofilm at 0.1 µg/mL and nearly full inhibition at 0.3 µg/mL, whereas NAC alone showed no inhibition at the equivalent concentrations in both MP formulations (4.9 and 15 µg/mL, respectively) (Fig. 11A).

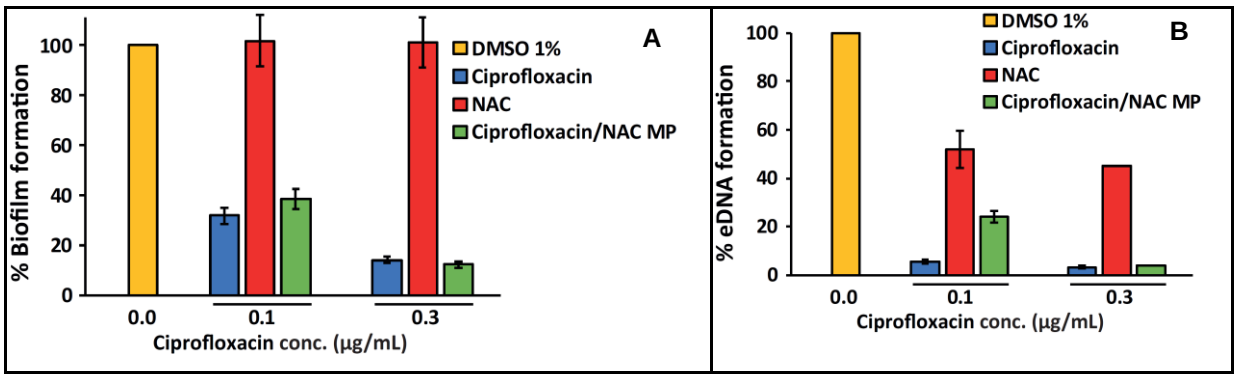

Fig. 11: *Effects of ciprofloxacin, NAC, and ciprofloxacin-NAC MP at concentrations of 0.1 and 0.3 µg/mL on biofilm (A) and eDNA formation (B) in P. aeruginosa PA14*

The equal potency of ciprofloxacin and ciprofloxacin-NAC MPs can be attributed to the antibacterial effect of ciprofloxacin at concentrations of 0.1 and 0.3 µg/mL (see Table 3 for the antibacterial activity), which may mask partially or completely the effect of combination with NAC. Results of eDNA assay are in line with the biofilm assay. Notably, the inhibitory effects of ciprofloxacin-NAC MPs and their individual components on eDNA formation are more pronounced (Fig. 11B). At concentration of 0.1 µg/mL, no significant inhibition of biofilm formation was observed for tobramycin, NAC, and the corresponding MPs (Fig. 12A). Interestingly, tobramycin-NAC MPs showed a synergistic effect on biofilm inhibition at concentration of 0.3 µg/mL compared to each ingredient alone (Fig. 12A).

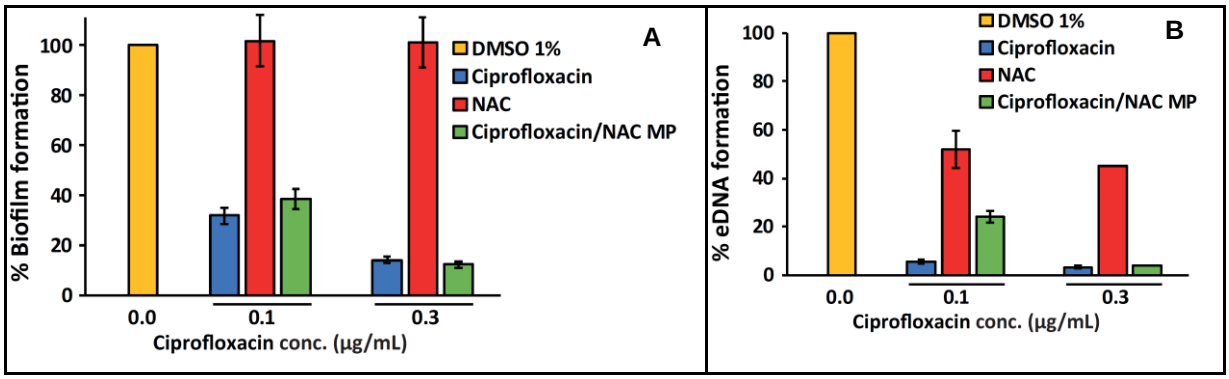

Fig. 12: *Effects of tobramycin, NAC, and tobramycin-NAC MP at concentrations of 0.1, 0.3, and 10.0 µg/mL on biofilm (A) and eDNA formation (B) in P. aeruginosa PA14*

At a concentration of 10 µg/mL, both tobramycin and tobramycin-NAC MPs exhibited almost total inhibition of the biofilm (Fig. 13A). As mentioned above, interference with the antibiofilm effects occurred at high concentration of tobramycin due to inhibition of the bacterial growth of the drug (see Table 3 for antibacterial activity). Effects of the tobramycin-NAC MP formulations on eDNA showed the same profile as their biofilm activities (Fig. 13B). Worth mentioning that in all MP formulations, concentrations of NAC used are much lower than that was required to affect the biofilm of *P. aeruginosa* PAO1 (500 – 2500 µg/mL) [107]. This was reflected in the antibiofilm activity, where the NAC alone did not show biofilm inhibition (Figure 11A, 12A, and 13A), and only a moderate effect on eDNA formation (Figure 11B, 12B, and 13B) at all tested concentrations.

In contrast, synergistic effects were observed when NAC was combined to azithromycin or tobramycin at a concentration of 0.3 µg/mL (Figure 11A and 13A). Taken together, these results demonstrate that the new combinations in the MP formulations especially those of azithromycin and tobramycin have a beneficial effect on biofilm elimination.

4.4.7 *Effect on antibiotic* susceptibility

The antibacterial activity against the clinical strain *P. aeruginosa* PA14 was determined for the antibiotic-containing MPs and the antibiotics alone at the same concentration as in the MPs (Table 3). Susceptibility to the macrolide antibiotic azithromycin has slightly increased by the MPs formulation. For the fluoroquinolone antibiotic ciprofloxacin, no significant difference between the minimum inhibitory concentration (MIC) value of the MPs and the neat antibiotic was observed (MIC values: 0.05 µg/mL vs. 0.06 µg/mL, respectively). Interestingly, the NAC concentration used in this formulation did not negatively affect the MIC value of ciprofloxacin against *P. aeruginosa* PA14 in contrast to a previous finding [124]. Similarly, the growth inhibitory activity of the aminoglycoside tobramycin was not affected by the MPs ingredients

Table 3: *Antibacterial Activities of the Antibiotics with (MP +) and without (MP -) Microparticles.*

Antibiotic	Concentration	*P. aeruginosa* PA14 % growth inhibition or MIC (µg/mL)	
		MP -	MP +
Azithromycin	0.3 µg/mL	25%	36%
Ciprofloxacin	0.1 µg/mL	0.06	0.05
Tobramycin	0.1 µg/mL	n.i.[a]	n.i.
Tobramycin	0.3 µg/mL	10%	10%

an.i. no inhibition

4.5 Conclusion

Respirable multifunctional microparticles for inhalation composed of different antibiotics and N-Acetylcysteine as the matrix was successfully developed. The formation of counter ion complexes between antibiotics belonging to different classes and NAC allowed for improving solubility of the antibiotics. This allowed for the successful formation of a powder formulation using spray drying. In addition, an effect on the redispersibility of the particles at 100% relative humidity was also observed. The shelf-stability of these particles was investigated with respect to their morphology and agglomeration behaviour revealing stable particles for Azithromycin/NAC and Tobramycin/NAC over a range of 12 weeks. Obviously, the formation of the counter ion also reduced the hygroscopicity of the system contributed by NAC. In addition, the obtained spray-dried powders of antibiotic/NAC have shown good aerodynamic properties with fine particle fractions above 60%. An application to the lung is possible since the combined effect of NAC plus the antibiotic has reduced mucus viscosity and allows a direct treatment of residing bacteria. Moreover, the combination of antibiotics and NAC has allowed us to present a novel dry powder formulation for the used route of administration (inhalation) for NAC. By bringing more insight into the mechanism of salification, this might offer help to other drugs to be formulated for inhalation. Beyond the innovative matrix, there is a possibility for further milestone to be developed by loading the MP with a different kind of drug which will be loaded into NPs where a different approach for treatment would benefit from the combination therapy. Moreover, the antibiotic-NAC as MP formulations showed synergistic inhibition of biofilm and eDNA formation for azithromycin and tobramycin at 0.3 µg/mL. The new MP formulations showed a slight enhancement of the azithromycin susceptibility against P. aeruginosa PA14, and they retained the antibacterial activities for all tested antibiotics.

*Multifunctional inhalable microparticles combining antibiotics and a mucolytic with anti-inflammatory drug-loaded PLGA nanoparticles**

*** This chapter is prepared for publication as a journal article:**

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5. Multifunctional inhalable microparticles combining antibiotics and a mucolytic with anti-inflammatory drug-loaded PLGA nanoparticles

5.1 Abstract

Nowadays, the resistance of bacterial biofilms towards the current antibiotics is a serious problem. Therefore, so many efforts were devoted for developing new formulations using nanotechnology. Here, we have innovated 3 novel multifunctional formulations using spray drying technique made of multiple drugs namely Antibiotics, NAC, and Curcumin-loaded PLGA NPs. The microparticles were characterized in terms of size, morphology, and flying properties. Moreover, the microparticle formulations have been assessed for its cell viability with macrophage-like cells. The cells were viable after 20h interaction in a concentration range of 1-135 µg/mL of the antibiotics. From this range, the antibacterial activity of free drugs versus drug in the formulations was evaluated on *P. aeruginosa* and its killing was achieved within 30 µg/mL (MIC50) for azithromycin and less than 1 µg/mL for ciprofloxacin (MIC90), without differences in relation to the spray dry formulation. However, Tobra/NAC microparticles have increased the sensitivity of Tobramycin by 3.5-fold compared to the free drug. Bacteria-derived inflammation was not inhibited by free antibiotics, except on the inhibition of TNF-α. However, NAC and the addition of Curcumin-loaded PLGA NPs increased the potential of those formulations to inhibit TNF-α, IL-8 and IL-1β release.

5.2 Introduction

From individual cells to dense communities, bacteria have stages of adaptation onto biotic or abiotic, static or moving surfaces. The community phase is called biofilm, in which the aggregation of bacteria in combination with extracellular polymeric substances (from extracellular DNA, proteins to alginate) forming its surrounding matrix [125]. Formation of biofilm is ubiquitous and is also of huge relevance for patients influencing the development of diseases. [126]. In Cystic Fibrosis (CF), the protection characteristics of the lung maintained by macrophage clearance, mucociliary escalator, and composition of surface lining antimicrobial fluid [127-129] are altered due to the highly viscous mucus as a result of the defective CFTR gene. This contributes to increased bacterial adherence that translates to biofilm formation, causing host response to be strong accompanied by a local increase of cytokines production and excessive recruitment of neutrophils [130]. Overall, a comprised immune system, the increased bacterial virulence, and the development of drug resistance which is connected to the hampered penetration through mucus and biofilms lead to reduced quality of life for CF patients [126]. Infections are derived from biofilm-forming bacteria like *Pseudomonas aeruginosa* and *Staphylococcus aureus*. In Europe, about 10-60% of CF patients are associated with chronic *P. aeruginosa*. Furthermore, 64% of fatalities were caused due to the bacterial infection in the respiratory tract [131, 132]. Effects against biofilms can be observed by the amount of biofilm formed (biofilm inhibition) or treatment of mature biofilms (eradication) [133]. Biofilms are a kind of hydrogel offering a certain pore size for penetration [27]. In addition to this, the biofilm might be incorporated in mucus also being a gel-like system restricting penetration by interaction filtering and size filtering [18, 134]. However, using nanotechnology is assumed to facilitate drug delivery due to the small size and the adjustable surface chemistry, allowing for the avoidance of the interaction with for example the high viscous mucus in cystic fibrosis [27, 135]. Nanoparticles (NP) will protect the drug and their small sizes will enable to deliver it in the mucus and biofilm and thus to the microorganisms [17, 135].

This will potentially decrease the side effects while increasing the direct interaction with the pathogenic microorganisms [17, 136]. Still, the existence of the defense mechanism against particles such as size-filtering and interaction-filtering effects will limit the efficiency of the therapeutic drug. Nevertheless, nanoparticles in a range from 1 to 500 nm are considered too small in order to be applied efficiently into the lungs as an inhalable powder [29, 137]. Microparticles in the range of 1µm up to 5µm are considered optimal to ensure their deposition into the lower part of the respiratory system without being exhaled [138-140]. The combination of the necessary aspects for inhalation and mucus/biofilm penetration can be achieved by spray drying of nanoparticles using a suitable matrix [114, 141, 142]. The most challenging aspect for producing inhalable formulations is choosing an appropriate matrix to embed the particles which later on could be dispersible within the lung. [143, 144]. Up to now, the conventional way to formulate inhalable formulation out of the nanoparticulate is to use materials such as lactose or mannitol as a matrix to embed this nanoparticulate within it [145]. Previously, we have developed an innovative matrix for inhalation as a dry powder, by combining (antibiotics and N- acetylcysteine) as multifunctional drug delivery system [146]. The idea was to make use of ionic interactions between the acid group in NAC and the free basic amino group(s) in antibiotics, which has enabled us for spray drying the NAC in the presence of antibiotics. At the same time the antibiotics solubility has increased. To address simultaneously the persistent inflammation in CF patients we have integrated PLGA NPs loaded with Curcumin as a natural anti-inflammatory into this innovative matrix [147, 148]. Such a multifunctional formulation, combining a co-mixture of antibiotics, the mucolytic N-acetylcysteine and Curcumin as a natural anti-inflammatory might be a promising approach for the future of cystic fibrosis treatment. The combination would potentially increase drug delivery due to the actions of NAC (fluidize mucus/ better penetration through biofilm).

Also, using the multifunctional formulation would allow benefiting from local effects of the applied drugs instead of the systemic application which will contribute to lower undesirable side effects due to using a low dose [149].

To address the aspects raised before, the inhalable multifunctional formulation was characterized in terms of aerodynamic properties and the geometric sizes and shape using SEM. Furthermore, the properties of the nanocarriers, as well as their recovery after being embedded into the microparticles (MPs) were investigated. Finally, the permeability of Curcumin-loaded PLGA NPs (Cu-PLGA NP) through human pulmonary mucus was confirmed using confocal laser scanning microscopy (CLSM). The biological activity of the formulation was evaluated using acute toxicity (MTT), the antibacterial activity (MIC) as well as the anti-inflammatory potential monitoring cytokines such as IL-8 and TNFα.

5.3 Material and methods

5.3.1 Materials

Polymer (PLGA) (Resomer RG 503H) was obtained from Evonik Industries (Darmstadt, Germany). Rhodamine B (as fluorescence label), acetonitrile (ACN), N-Acetylcysteine (NAC) (MW= 163.19 g/mol), L-Leucine (MW= 131.18 g/mol), Curcumin (MW= 368.39 g/mol), Tobramycin (MW= 467.515 g/mol), Azithromycin (MW= 748.98 g/mol), and Ciprofloxacin (MW= 331.346 g/mol) were purchased from Sigma Aldrich (Steinheim, Germany). *P. aeruginosa* strain PAO1 (DSMZ 22644) and THP-1 monocytes (ACC-16) were purchased from DSMZ (Braunschweig, Germany) and PAO1-GFP from ATCC (Virginia, USA), while ampicillin for GFP expression was bought from Carl Roth (Karlsruhe, Germany). RPMI cell medium was obtained from Gibco (Germany), while FCS (fetal calf serum), LB Broth and phorbol 12-myristate 13-acetate (PMA) from Sigma-Aldrich (Germany). Assays were performed using 96-well plates from Greiner (USA), while ELISA Ready-SET-Go kits from Invitrogen (Vienna, Austria) were assayed on Nunc Maxisorp® 96-well plates from ThermoFisher (Germany).

5.3.2 Curcumin-PLGA NP preparation and characterization

Nanoprecipitation was chosen to prepare polymeric PLGA-Cu NPs, using a microfluidic system (Labsmith, Livermore, USA). PLGA/Curcumin at a ratio of 9:1 was dissolved in acetonitrile (ACN) as organic phase. Then, the organic phase containing PLGA/Curcumin and ACN was injected into the middle channel of a microreactor using a syringe pump (Harvard Apparatus PHD 2000 Syringe, Holliston, USA) to grant a steady flow. The aqueous phase was composed of Pluronic F-68 (0.1 %) dissolved in water. Before injection into the side channels of the microreactor, the aqueous phase was filtered using a membrane of 0.45 mm pore diameter (Millipore, Billerica, USA) to remove undissolved material. To guarantee a steady flow, the aqueous phase was injected into the microfluidic chip using a syringe pump (Multi Programmable Syringe Pump, wpiinc, Sarasota, USA).

The flow ratio was fixed at 0.1 for all preparations, to obtain PLGA-Cu NPs of similar physicochemical properties and to reach encapsulation efficiency similar to previous work. After formation of PLGA-Cu NPs, NPs were collected from the outlet. The organic solvent was removed under stirring (1000 rpm) in a fume hood at RT overnight. Consecutively, the excess amount of stabilizer was removed by washing the nanoparticles 3 times with purified Milli-Q water using centrifugation at 10,000 g for 30 min (Multifuge X1R, Thermo Scientific, Langenselbold, Germany).

5.3.3 PLGA NP characterization

The shape of the manufactured PLGA NPs was confirmed using EVO HD15 electron microscope (SEM) from (Zeiss, Jena, Germany). The accelerating voltage was set to 5 kV. For imaging the sample, ~10 µL suspension was dried under ambient conditions on a silicon wafer by evaporation. Then the samples were sputtered with a thin gold layer (~10 nm) by using a sputter coater Quorum Q150R ES (Quorum Technologies Ltd, East Grinstead, UK).

5.3.4 Manufacturing of multifunctional inhalable microparticles

The multifunctional inhalable microparticles (MP) were engendered by utilizing a BÜCHI B-290 Spray Dryer from Büchi (Flawil, Switzerland). For matrix preparation, N-Acetylcysteine (NAC) was dissolved in Milli-Q together with an antibiotic (Azithromycin, Tobramycin, and Ciprofloxacin) in equimolar amounts. The co-mixed solution was left for 10 min until the solution became transparent. In addition to the (antibiotics and NAC), L-Leucine was integrated. The optimal amount of L-Leucine was determined in our previous study, table 2. Furthermore, Curcumin-loaded PLGA NPs were added making 20% of the final amount. The co-mixed solution for spray drying was adjusted to have 1 wt. % of solid substances. Spray drying was done with constant settings for all formulations. Particularly, the parameter for gas rotameter was set to 50 mm which diverts to current air volume flow of roughly 1050 L/h. The peristaltic feeding pump was tuned to achieve 10% which is approximately equal to (~ 3 mL/min of spray drying solution feed) and the spray rate was adjusted to 100%, as stands for an actual gas flow rate of 35 m^3/h.

All the formulations were sprayed using compressed air. Ultimately, after spray drying the multifunctional formulation, the collected particles were stored in a desiccator at room temperature until usage. For each 100 mg of multifunctional formulation, an approximately 100 µL of a 5 mg/mL of Rhodamine B dissolved in ethanol was added within the feeding solution of the spray dryer to allow for quantification and visualization of the formulation. All experiments were accomplished at least in triplicate under the same conditions.

5.3.5 Morphology of multifunctional inhalable microparticles: SEM and particles size distribution

For visualization the shape of multifunctional microparticles, a scanning electron microscope EVO HD15 from (Zeiss, Jena, Germany) was employed. The multifunctional formulation was immobilized on top of an individual sticky carbon disc. To enable the SEM for visualization, the sample was sputter-coated with a thin layer of gold (roughly around 10 nm) prior to visualization (Quorum Q15R ES, Laughton, UK). The accelerating voltage was 5 kV for all formulations. Images were analyzed in terms of geometric particle sizes utilizing ZEN 2012 (blue edition) software (Zeiss, Jena, Germany). In addition to SEM, particle size distribution was executed using static laser light diffraction with a HORIBA LA-950 (HORIBA Europe GmbH, Darmstadt, Germany). The experiment was conducted using a multifunctional powder formulation under dry condition directly without any solvent. The sample was exposed to vibration and air suction allowing the powder to disperse before crossing the laser beam for analysis.

5.3.6 Examination the aerodynamic properties of multifunctional microparticles

Aerodynamic properties of all multifunctional inhalable formulations were investigated utilizing a Next Generation Impactor (NGI) (Copley Scientific, Nottingham, UK). Prior to each experiment, the impactor pans were plated with a Brij-coating made up of 4 parts, containing 15% Brij 35 in ethanol in six parts of glycerol; 10mL of purified Milli-Q water was filled in the pre-separator. In each experiment, a gelatin capsule size 3 was filled with approximately 20 mg of powder formulation.

Then the capsule was placed in a HandiHaler (Boehringer Ingelheim, Ingelheim, Germany) and then it was punctured. In more detail, the air flow parameter was adjusted to 60 L/min realized by a M1A flowmeter (Copley Scientific, Nottingham, UK). 4 seconds were set for the active time of the vacuum pump (Erweka, Heusenstamm, Germany) and the critical flow controller (Erweka, Heusenstamm, Germany) as well. At the end of the experiment, the multifunctional inhalable powder formulation within the NGI pans was dissolved in a defined quantity of water and analyzed with a Tecan reader infinite 200 (Tecan, Männedorf, Switzerland) to detect the fluorescence signal of Rhodamine B (λ_{ex} = 565 nm and λ_{em} = 625 nm). An individual calibration curve was performed separately for each formulation. All multifunctional inhalable formulations were accomplished in triplicates under constant conditions.

5.3.7 Distribution of PLGA NPs in microparticles

The internal composition of the multifunctional inhalable microparticles was observed using a confocal laser scanning microscope (Zeiss LSM710, Zeiss, Jena, Germany). The multifunctional formulations were spread on top of a nitrocellulose membrane using a Penn-Century device Model DP-4M (Wyndmoor, USA) to guarantee a good distribution of the formulations and then covered by a coverslip. For visualization a 40x (air) objective (Zeiss, Jena, Germany) was used. The excitation wavelengths were set with a bandpass filter between 458 and 514 nm and detection was accomplished with other bandpass filters ranging from 470-551 nm and 551-703 nm respectively to detect Curcumin and rhodamin B.

5.3.8 Recovery of PLGA-Cu NPs out of microparticles

The redispersability of PLGA NPs after being integrated into the microparticle matrix was measured utilizing Zetasizer Nano ZS90 (Malvern Instruments, Worcestershire, UK), to evaluate the influence of spray drying process on the nanoparticles size. For each formulation, 100 mg of the formulation was dissolved in 1 ml of Milli-Q water, after 10 s vortexing. Both, the PLGA NPs size and the PDI of released NPs from the microparticles were measured in DLS with a scattering angle of 173°. Every formulation was tested at least 3 times while keeping the conditions constant.

5.3.9 The permeability of PLGA-Cu NPs through pulmonary human mucus

The ability of PLGA-Cu NPs to penetrate the mucus barrier and potentially deliver their payload was tested using a confocal laser scanning microscope (CLSM) (Zeiss LSM710, Jena, Germany). 5 µg of each multifunctional inhalable microparticle formulation was placed on top of 40 µL of human pulmonary mucus using a Penn-Century device Model DP-4M (Wyndmoor, USA) to ensure homogenous distribution of multifunctional microparticles on the mucus layer. Each 40 µL of mucus was stained unspecifically with 1µL of AlexaFluor488-labelled wheat germ agglutinin [150] and the PLGA-Cu NPs were covalently labelled with rhodamine. For assessing the permeability of the PLGA-Cu NPs through the human pulmonary mucus, the fluorescence signal of labelled PLGA-Cu NPs was tracked using 3D time laps imaging z-stacks. This was after the NPs were released from the disintegrated microparticle matrix. Labelled human pulmonary mucus was detected while using λ_{ex} = 488 nm and a detection range between 467−554 nm. The PLGA-Cu NPs were excited at λ_{ex} = 561 nm and the signal was collected in a wavelengths range of 624−707 nm. Directly after spray drying the formulation, the permeability of PLGA-Cu NPs within human pulmonary mucus was conducted at time points from 0 min up to 1 h for all formulations. All experiment was accomplished in triplicate under the same conditions.

5.3.10 Cultivation of Pseudomonas aeruginosa (PAO1-GFP) and infection of THP1 macrophages

P. aeruginosa strain PAO1 (DSMZ 22644) and the fluorescently labeled strain ATCC® 10145GFP™ were grown in LB broth (Sigma-Aldrich, Darmstadt, Germany) which was supplemented with necessary antibiotics (300 µg/mL ampicillin for GFP expressing strain; Carl Roth, Karlsruhe, Germany). Overnight cultures were grown by inoculating 15-20 mL of LB broth with a single colony of bacteria and incubating the broth for 16-18 hours at 37°C and 180 rpm. For infection, an overnight culture of PAO1 in exponential growth phase was centrifuged (10min; 2800g) washed with PBS, and diluted to a final OD600 of 0.2 (~ $2x10^7$ CFU/mL) in RPMI medium. THP-1 cells were infected with PAO1 for 1 hour, and treated for 6 hours with the above mentioned formulations as well as free drugs.

Infected cultures, treated or not, were incubated for 6 hours under static conditions at 37°C then centrifuged at 1792 g for 5 minutes. The supernatant was separated for analysis of cytokines and filtered MilliQ water was added to the well for cell lysis and freezing at -20°C for later use in CFU assay.

5.3.11 Minimal Inhibitory Concentrations (MIC)

Overnight culture of PAO1 in exponential growth phase was PBS-washed and diluted to a final OD600 of 0.2 (~$2x10^7$ CFU/mL) in LB broth. Free drugs, material, and formulations were weighed and stock solutions were prepared either in DMSO or filtered MilliQ water. One hundred microliters of bacterial suspension was mixed with 100 µL of the formulations on a 96-well plate. Treated and non-treated bacteria were allowed to grow under static conditions for 24 h at 37°C. Optical density (OD 600) was measured with a spectrophotometer (Multiskan GO, Thermo Fisher Scientific, Vantaa, Finland).

5.3.12 Measurements of IL-8, TNF-α and IL-1β by ELISA from infected macrophages

Cytokines ELISA Ready-SET-Go kits (Invitrogen, Vienna, Austria) were used with supernatant of infected cultures, and the samples processed and analysed following the manufacture instructions. Briefly, Nunc Maxisorp® 96-well plates were coated with capture antibody of the respective cytokine and incubated overnight at 4 °C. A series of washing steps were performed with Wash Buffer (PBS/Tween20 0.05%). Then the wells were blocked by incubation with ELISA/ELISPOT diluent for 1 h. Samples were applied (or previously diluted, in the case of IL-8 plates) together with cytokine standards and plates were incubated overnight at 4 °C. Plates were washed, then received detection antibody of the respective cytokine for 1 h incubation at room temperature. Another series of washing steps was performed and plates received Avidin-HRP for detection of biotinylated antibodies for 30 minutes. The last series of washing steps was performed and 3,3',5,5'-Tetramethylbenzidine (TMB) solution was applied as enzymatic/colour inductor from the kit. Reaction was stopped with 1M H_3PO_4 solution and absorbance was measured at 450 nm for detection and 570 nm as background. Values were paired with an internal calibration curve from the cytokine standards.

5.4 Results and discussion

5.4.1 PLGA nanoparticles loaded with Curcumin

PLGA nanoparticles loaded with the hydrophobic drug Curcumin (as a natural anti-inflammatory) were prepared using nanoprecipitation in a microfluidic system. The PLGA-Cu-NP exhibited a small mean hydrodynamic diameter of around 105 nm with a narrow monomodal size distribution (PDI < 0.1) (Table 1). The small sizes and narrow size distribution can be attributed to the usage of short mixing time. This approach enabled us to control sizes of physiochemical identical PLGA-Cu NPs, yielding also, a high drug encapsulation efficiency of 68% (for Curcumin). The zeta potential of PLGA-Cu NPs was a slightly negative charge (-10 mV).

Table 1: *Physicochemical characterization of curcumin loaded into PLGA nanoparticles. Values are indicated as mean ± SD (n = 3).*

Size (nm)	PDI	Zeta potential (mV)	Encapsulation efficiency
105 ± 1.5	0.063 ± 0.019	− 9.1 ± 4.6	68 % ± 7.6

5.4.2 Manufacturing and characterization of multifunctional inhalable microparticles

Previously, we had demonstrated that we could very successfully spray dried microparticles composed of an antibiotic and a mucolytic agent (NAC) as dry powder matrix. Now, the spray dried powder microparticles were taken to the next stage of innovation and were additionally loaded with PLGA NPs as a carrier for a drug of different polarity. For this purpose, Curcumin was loaded into PLGA NP which was stabilized by the muco-inert stabilizer (Pluronic F-68). Curcumin was selected as an additional drug due to its anti-inflammatory properties. This ensures that the multifunctional formulation can cover the whole spectrum of recommended drugs in CF co-therapy namely antibiotics, mucolytic agents and anti-inflammatory compounds [151]. The spray drying feed solution was prepared with 20% NPs content of the total weight as it was reported to be an optimal fraction of PLGA NP for redispersion [114].

The obtained multifunctional formulation after embedding the NPs has shown no change in handling (very easy to collect) as the nanoparticle-free counterparts. Notably, the incorporation of PLGA-Cu NPs showed only a minute effect on the morphology of the microparticles compared to the spray-dried multifunctional formulation without NPs (Fig 1).

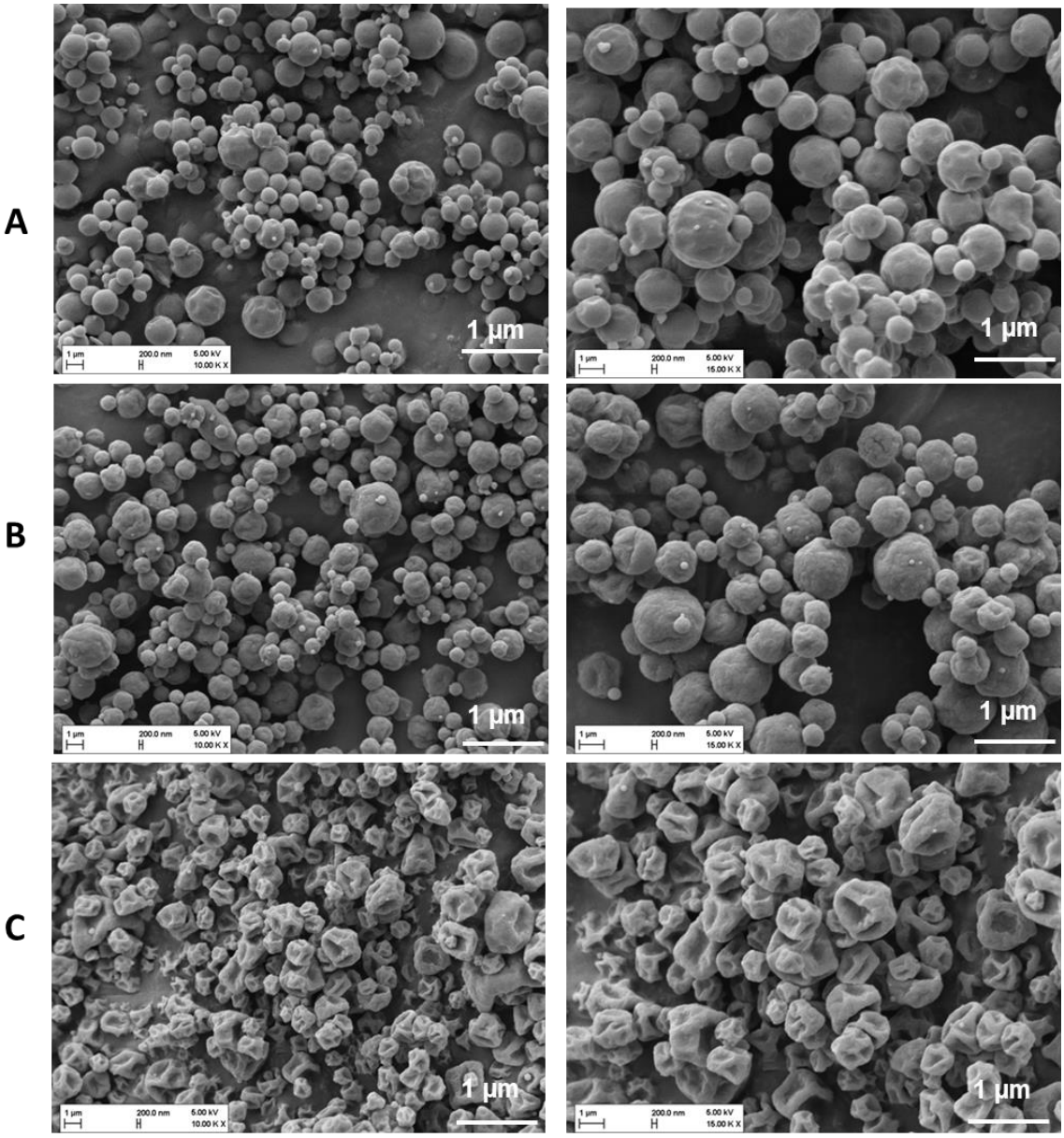


Fig. 1: *SEM micrographs of spray-dried antibiotics /NAC microparticles. Azithromycin/NAC (A) and Ciprofloxacin/NAC (B), Tobramycin/NAC at, 10.000x and 15.000x magnification respectively.*

A very distinct structure of multifunctional microparticle (MP) was obtained. Moreover, investigation the composition of multifunctional formulation from CLSM micrographs (Fig. 2) revealed from the merged images, showing colocalized NP fluorescence and fluorescence from the matrix that the nanoparticles were homogeneously embedded into microparticles.

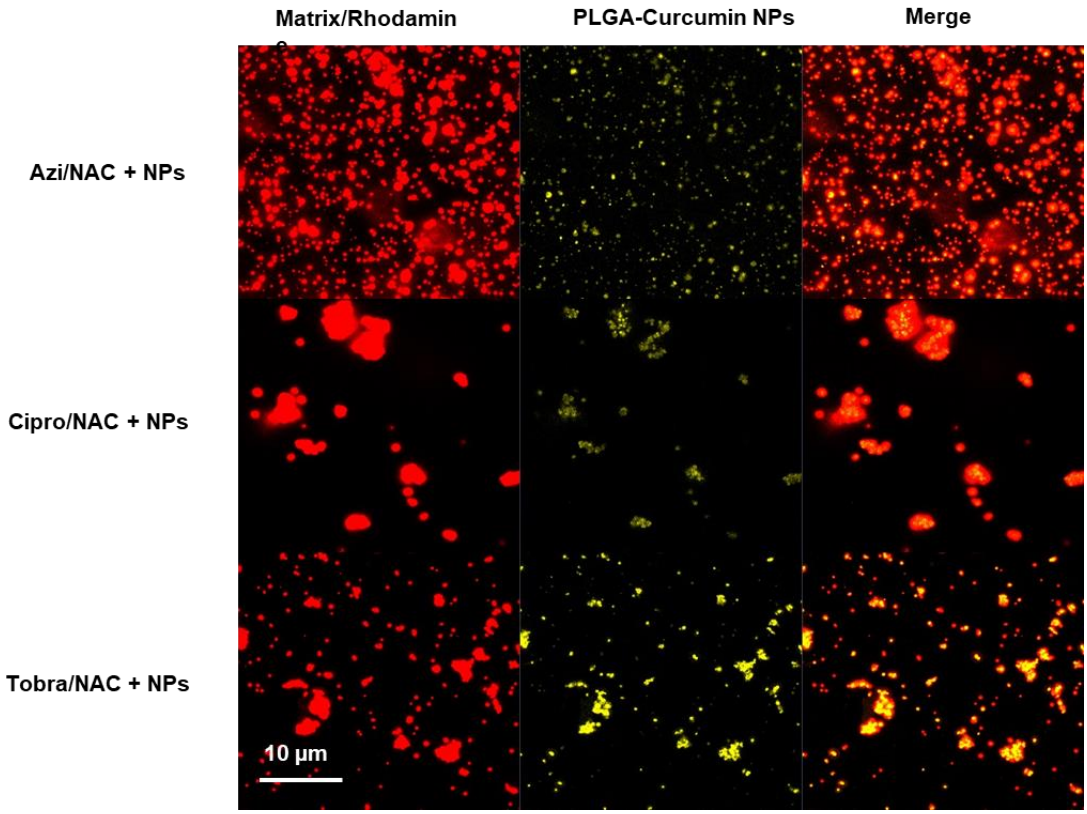


Fig. 2: *Morphology of co-spray-dried microparticles visualized by CLSM; left row shows the Matrix/Rhodamine B fluorescence, the second represent the fluorescence of the curcumin (nanoparticles) and in the right row the merge of both shown. Scale bar represents 10 µm.*

After ensuring the successful preparation of microparticles containing nanoparticles, the next step was to estimate whether the PLGA-Cu NPs will be able to recover after the dissolution of the multifunctional formulation in water. DLS measurements showed that the PLGA-Cu NPs were unchanged (110 nm in diameter, Table. 3).

Table 3: *Nanoparticle recovery out of microparticle matrix after the spray drying.*

	NPs size before spray drying	**NPs after Spray drying (Azith/NAC) formulation**	**NPs after Spray drying (Cipro/NAC) formulation**	**NPs after Spray drying (Tobra/NAC) formulation**
Fresh preparation	105 nm (± 0.07)	109 nm (± 0.02)	110 nm (± 0.01)	109 nm (± 0.07)
After 3 month	----	110 nm (± 0.01)	110 nm (± 0.04)	110 nm (± 0.05)
After 6 month	----	110 nm (± 0.06)	108 nm (± 0.09)	109 nm (± 0.03)
After 9 month	----	109 nm (± 0.03)	110 nm (± 0.02)	112 nm (± 0.08)

This is an important factor as parameters such as temperature and high pressure in the spraying nozzle might contribute to destroying the NPs or result in aggregation of particles. The size distribution of the redispersed embedded nanoparticles is still the same as before spraying drying with a PDI < 0.1, indicating still a monodisperse distribution and the absence of agglomeration. The evaluation was done for freshly prepared samples and after nine months of storage. The data underlined the stability of these PLGA-Cu NPs within the multifunctional microparticles matrix.

5.4.3 Aerodynamic behavior of the microparticles

To obtain particles suited for inhalation therapy, an appropriate aerodynamic behavior is necessary. Generally, it is agreed on, that particulates intended for inhalation should offer aerodynamic diameters between 0.5 and 5 µm [152]. Consequently, a flying study for all multifunctional formulations was conducted using NGI (Next Generation Impactor). The formulations have displayed virtually a congruous FPF > 60% for (NAC/Cipro), (Azi/NAC) and above 90% for (Tobra/NAC) (Table 4). Moreover, the mass median aerodynamic diameter (MMAD) values as an indicator for the size were ranging between 2.16 and 2.63 µm which should be well suited for inhalation use. No difference in aerodynamic properties of Azi/NAC/NPs formulation FPF (68.83 %) in comparison to nanoparticle-free counterpart Azi/NAC (67.40 %), it was the same case for the Cipro/NAC/NPs and Tobra/NAC/NPs (Table 4). This implies that adding nanoparticles as an additional carrier did not affect the aerodynamic behavior of the formulation.

Table 4: *Results of the aerodynamic properties of spray-dried formulation with NPs and without NPs as determined by NGI.*

Formulation	MMAD [µm]	GSD	FPF [%]
Cipro/NAC	2.40 (± 0.02)	1.88 (± 0.008)	61.60 (± 5.23)
Cipro/NAC/NPs	2.56 (± 0.04)	1.62 (± 0.02)	64.48 (± 3.22)
Tobra/NAC	2.16 (± 0.33)	1.53 (± 0.06)	97.52 (± 15.36)
Tobra/NAC/NPs	2.43 (± 0.15)	1.47 (± 0.24)	87.21(± 8.63)
Azi/NAC	2.63 (± 0.03)	1.64 (± 0.13)	67.40 (± 9.41)
Azi/NAC/NPs	2.51 (± 0.06)	1.58 (± 0.04)	68.83 (± 6.11)

5.4.4 In vitro the permeability of NPs through mucus

To profit from the nanoparticle technology to deliver their payload without interaction with the surrounding biological barrier such as the viscous mucus in CF, size and surface chemistry of NPs need to be controlled [153]. Then, the nanoparticles can play a decisive role in overcoming the mucus barrier without being immobilized. Therefore, we have assessed the directed diffusion of the PLGA-Cu-NPs through pulmonary human mucus. As shown in figure 3, the embedded PLGA-Cu- NPs inside the innovative matrix (antibiotic, mucolytic agent) has started to show penetration after 15 min. It was the case for all formulations. After 1 h there was an increase in signal (red) as an indication for more particles which were able to penetrate. It was more or less the same for all formulations as NPs exhibit the same size in all formulations.

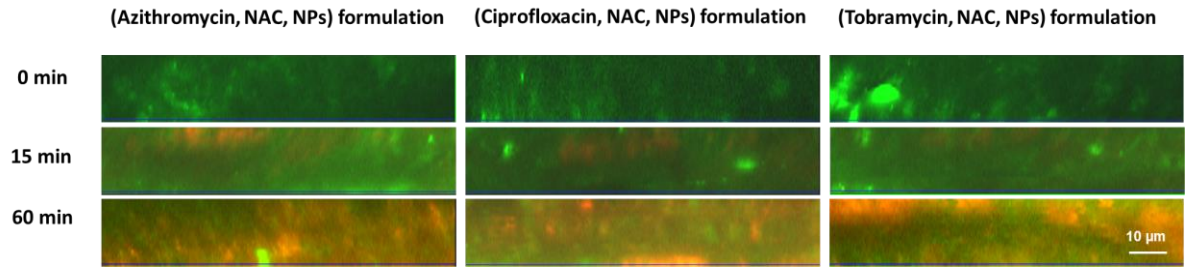


Fig. 3: *Confocal laser scanning microscopy study of the penetration of PLGA loaded curcumin NPs (red fluorescence) through pulmonary human mucus at predetermined time interval. Mucus was stained with wheat germ agglutinin (Green fluorescence).*

5.4.5 Cell viability via the MTT-Formazan assay

When the formulations take a role as muco-penetrant, its fate will directly affect the host lung. So as primary assessment, we used MTT assay to check if the particles and its components could bring cell toxicity. THP-1 macrophages were incubated with antibiotic concentrations (1-135 µg/mL), either free or incorporated into the formulations. After incubaction for 20 h, all formulations with concentrations from 1-30 µg/mL, we observed to show around 70% of cell viability. Even so, our proposed formulations include NAC as component that alters the pH towards an acidic environment [154], our data shows no increase on cell toxicity when NAC is combined with the antibiotics (Figure 4A).

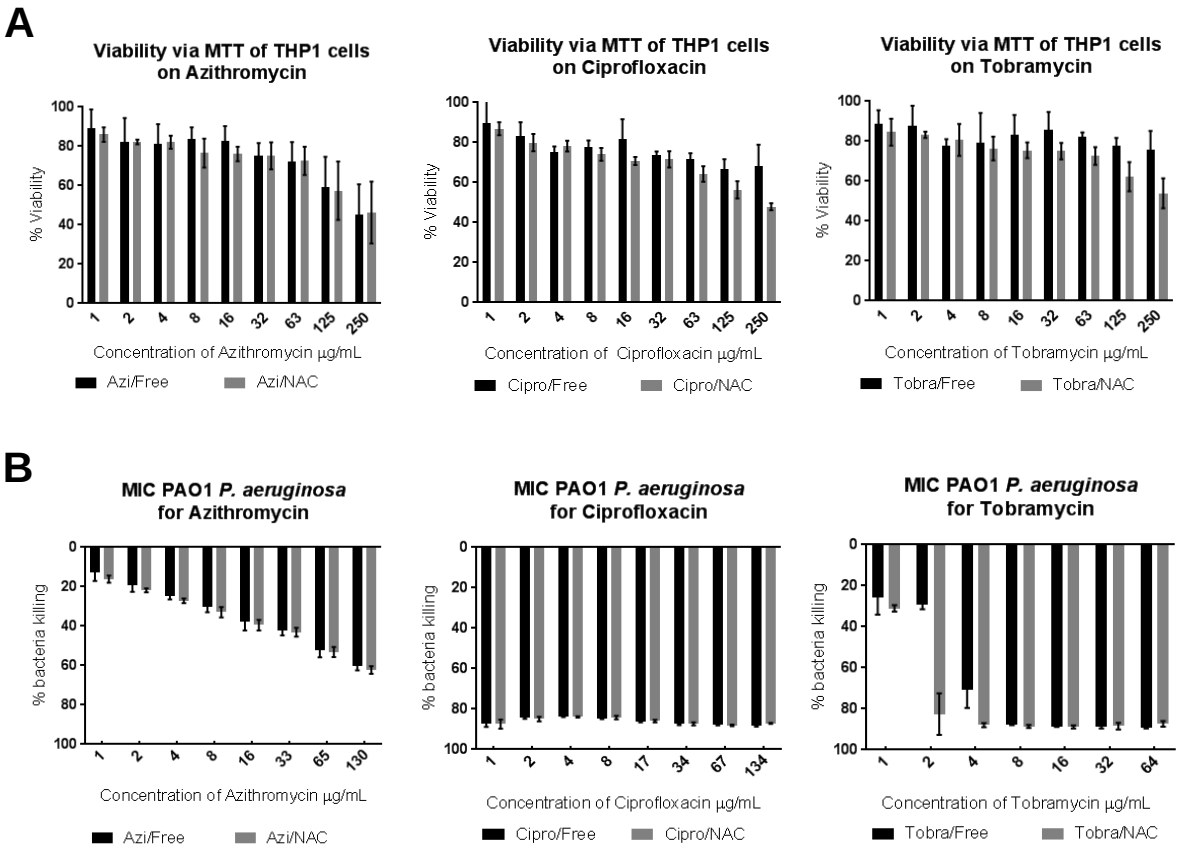


Fig. 4: *MTT Viability of THP1-macrophages (A) and Minimal Inhibitory Concentration (MIC) of P. aeruginosa (B) for free and microparticle formulation of Azithromycin, Ciprofloxacin and Tobramycin. The values needs for bacteria inhibition were found as safe to the macrophages according to the MTT assay.*

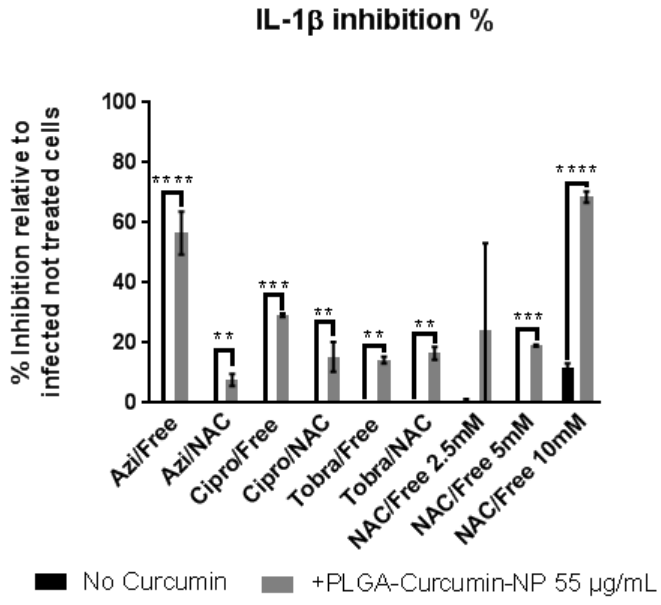
5.4.6 Minimal inhibitory concentration of PAO1 P. aeruginosa exposed to formulation

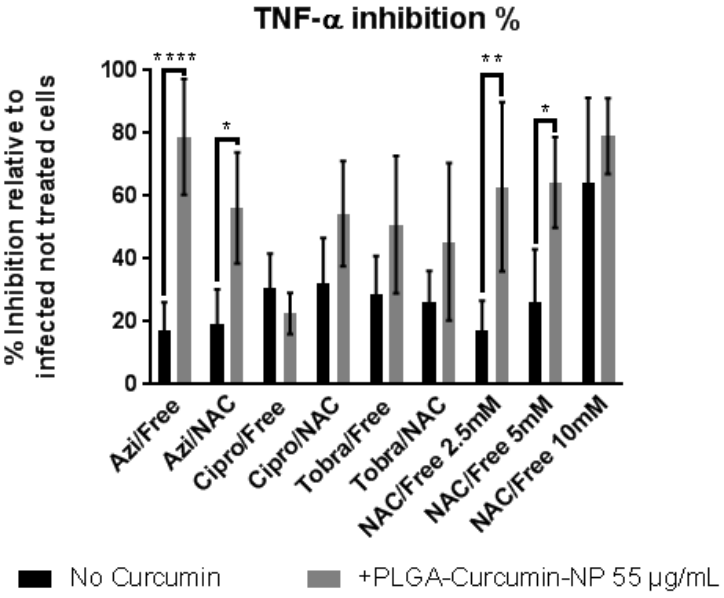
Tobramycin, Azithromycin and Ciprofloxacin are known antibiotics and its use has brought increased chances on Cystic Fibrosis therapy [155], but the challenge of drug dissolution and integration/encapsulation into a targeted formulation is still a challenge in pharmaceutical technology. Our formulation placed these antibiotics in combination with NAC as mucolytic agent potentially facilitating drug penetration. In all cases, we emphasize NAC as co-drug and our data showed no decrease of antibiotic activity, but maintenance of the same levels as the free drug. For Azithromycin, MIC50 is reported as 30 µg/mL and for Ciprofloxacin concentrations lower than 1 µg/mL achieve *P. aeruginosa* killing of 80%. Tobramycin results point to differences on free drug versus formulation. Our proposed formulation increased the sensitivity of *P. aeruginosa* towards Tobramycin resulting in an effective dose 50% (ED50) of 3.4 µg/mL for free Tobramycin and 1 µg/mL for Tobra/NAC spray-dried microparticles (Figure 4B). Considering the development of inhalation therapy, only tobramycin and aztreonam are approved by the US Food and Drug Administration in treating respiratory tract infections [156]. Now, we report the opportunity of 3 new ready-to-inhale formulations. NAC here is acknowledged as an excipient-drug that is not easy to produce with the spray-dryer, muco-penetrating and additive to antimicrobial activity. This in advantage against other mucolytics, as mannitol [157], that do not enhance bacteria killing on CF infection models and would not help to formulate the microparticles in ionized form.

5.4.7 Inhibition of inflammation measured via IL-8, IL-1β and TNF-α

After establishing that anti-pseudomonal activity is present, the next step is to address the excessive inflammation caused by *P. aeruginosa*-host interaction. For that, we modelled inflammation using *P. aeruginosa* grown on top of THP-1 macrophages provoking the increase by inflammatory markers, here represented by interleukin 8 (IL-8), interleukin 1β (IL-1β) and the tumour necrosis factor α (TNF-α).

Those levels from the infected samples were used as controls to normalize the percentage of inhibition obtained by the combination therapy. The treatment with antibiotics alone did not reduce IL-1β nor IL-8, as depicted in the figure 5.





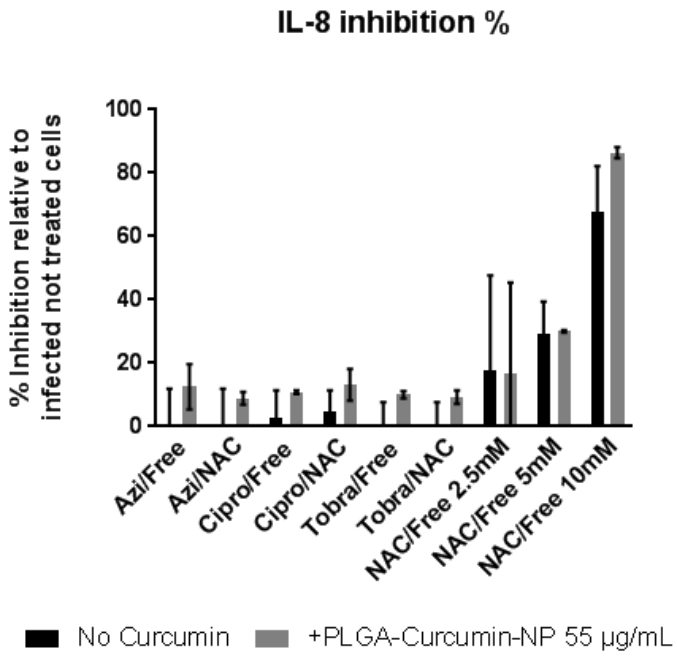


Fig. 5: *Inhibition of inflammation modelled by THP1-macrophages infected with P. aeruginosa treated with free N-acetylcysteine (NAC) or free and microparticle formulation of Azithromycin, Ciprofloxacin and Tobramycin; with and without co-delivery of Curcumin PLGA nanoparticles. An inherent anti-inflammatory activity is observed using NAC alone, while the use of Curcumin NP is additive to the inhibition of IL-8 and TNF-α as trends and statistically significant for IL-1β. Statistics comparing presence of absence of Curcumin NP is shown as * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.*

Only TNF-α was reduced applying free Azithromycin, Ciprofloxacin and Tobramycin. This effect is described in literature and is due to its inhibitory effect on the MAP kinase pathway induced by inflammation-inducers as bacterial lipopolysaccharide onto macrophages [158]. To increase the anti-inflammatory effect, we integrated PLGA-Curcumin nanoparticles (NPs) in the formulation. In Fig 5, the results of Curcumin-NPs co-delivery are observed on the grey bars of inhibition on all 3 inflammation markers analysed. The supplementation with Curcumin-NPs does not strongly inhibit IL-8 (only around 10%), a main recruiter of neutrophils on the inflammation scenario.

Nonetheless, with supplementation, the main signs of pro-inflammatory signalling (IL-1β and TNF-α) can be statistically inhibited by all three formulations. When comparing free drug versus formulation, the advantage of adding PLGA Cu NPs is observed on the reduction of IL-1β (20-60%), TNF-α (20-80%), while, for instance, free drugs without Curcumin NP supplementation could not inhibit IL-1β.

Analysing the effect of the applied drugs, the impact of NAC needs to be considered. As NAC has been described only recently as anti-inflammatory molecule, but also as corrector of chloride efflux on cystic fibrosis cell models [159-161], we used free NAC in three concentrations (2.5, 5 and 10 mM) with and without Curcumin-NPs. Inhibition of inflammation by free NAC is achieved in a dose-dependent manner: 20-60% inhibition of TNF-α and IL-8, and 15% inhibition of IL-1β secretion. These indicate that NAC has more benefit than just ionizing the antibiotics and improving the manufacturing process. Yet, this inhibition can be increased by the addition of PLGA-Cu NPs into the multifunctional formulation, establishing a crucial role for a combination antibiotic-mucopenetrant-anti-inflammatory treatment.

5.5 Conclusion

We achieved the incorporation of Curcumin-loaded nanoparticles into a microparticle formulation via spray drying technique. The matrix of the microparticles combined the mucolytic agent, N-acetylcysteine in combination with different antibiotics like Tobramycin, Ciprofloxacin and Azithromycin. The formulation was done from purely aqueous environment and thus a hydrophobic drug could be incorporated using the nanoparticulates. The redispersability of the particles PLGA-Cu NPs could be ensured also enabling the system to profit from the particles penetration abilities into mucus. Additionally, the inhalable microparticles have exhibited a suitable diameter for inhalation FPF above 60 %. Moreover, the integration of NAC with relevant antibiotics for CF therapy into a spray-dried microparticle was proven to be biocompatible, according to the MTT viability assay, while the formulation did not negatively interfere with antibiotics' activity, but it improved, in the case of Tobramycin. The anti-inflammatory effect was more pronounced on the inhibition of TNF-α. Combining NAC and the Curcumin-loaded particles could results in a synergistic effect showing stronger inhibitory potential as monitored for all investigated cytokines (TNF-α, IL-8 and IL1β); however it requires further investigation on the key factors interaction on this combination.

The proposed multifunctional microparticles combine different beneficial aspects for CF treatment such as muco-penetration, anti-pseudomonal activity, and anti-inflammatory into one inhalable formulation. With such an approach the anti-inflammatory potential could be improved while offering a way to use different types of antibiotics for potential inhalation treatment

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6. Summary and outlook

Cystic fibrosis is an incurable disease. Therefore, the main treatment relies on symptomatic therapy not on the cause of the disease. Currently, the available pharmacotherapy treatments are usually a mucolytic agent, antibiotics, anti-inflammatory and osmotic material. However, the viscous pulmonary mucus remains a challenging barrier for successful treatment of CF as it hinders the permeability of the drugs to reach their site of action. Checking the guideline for the treatment of CF, the need of the combination in co-therapy has inspired us to bring all these functions together and to formulate all of these drugs in one, instead of using every drug alone. Therefore, the aim of this thesis was to focus on the development of multifunctional formulations for the purpose of inhalation. The multifunctional formulations composed out of antibiotics, a mucolytic (N-Acetylcysteine) and an inflammatory compound (Curcumin), three kind of formulations were developed namely (Cipro, N-Acetylcysteine, PLGA NPs loaded Curcumin), (Tobra, N-Acetylcysteine, PLGA NPs loaded Curcumin), (Azith, N-Acetylcysteine, PLGA NPs loaded Curcumin). The muco-penetrating PLGA-NPs loaded with curcumin were manufactured using a microfluidic technique. A variety of different preparation factors were adjusted in order to study their impact on the size of nanoparticles and the encapsulation efficiency of the loaded drug. Using the microfluidic technique for preparing the PLGA-NPs has shown to be the most successful approach for manufacturing monodisperse small NPs. The NPs were less than 100 nm with a high encapsulation efficiency of Curcumin EE of ~68% in comparison to the conventional nanoprecipitation method. Then, a multifunctional microparticle matrix for delivering the PLGA-NPs into the lung was further developed. Based on a salification process, the multifunctional matrix (antibiotics, N-Acetylcysteine) was achieved. Benefiting from the salification approach, a water-soluble matrix was formulated ensuring fast dissolution. Basically, Ciprofloxacin and Azithromycin were not well soluble before salification. Salification has converted it from an insoluble drug into a water-soluble drug.

Aerodynamic properties of the formulation were tested using NGI to evaluate the deposition of dosed formulation inside the lung. Data has shown guarantor that approximately 70 % of multifunctional microparticles are deposited inside the lungs with FPF (69 %). The formulations were also in a range between 2.16 up to 2.63 µm which is suitable for lung application. The obtained results indicate that the multifunctional microparticles have the ability to reach the lungs. A simplified *in vitro* model to assess the disintegration behavior of the multifunctional matrix of microparticles under conditions similar to the lungs was developed. The multifunctional microparticles were deposited on a surface exposed to a high degree of relative humidity. It was observed that the multifunctional microparticles containing (NAC/antibiotics) have already displayed a fast disintegration starting from 1 min, as an indication that adding NAC to those antibiotics has resulted in a water-soluble matrix. As the multifunctional formulation contains NAC as a mucolytic agent, its influence on the macro-rheological of mucus was assessed. The rheometer was used to measure the viscosity of the mucus before adding the formulation and after adding it (treated and an untreated mucus). It was noticed that the multifunctional formulation containing (NAC/antibiotics) resulted in a reduction of mucus viscosity similar to free NAC, indicating that NAC was fully active and that it broke the mucin structure of mucus. The next step was to evaluate the safety of the multifunctional microparticle in cell models, to exclude toxic effects of the particles on human cells. All multifunctional microparticles have exhibited a safe profile. Additionally, the effect of the multifunctional microparticles against biofilm in *P. aeruginosa* was tested. The multifunctional microparticles containing (NAC/antibiotics) has shown a synergetic effect in comparison to free antibiotics, revealing that the presence of the NAC has increased the sensitivity of antibiotics towards bacteria, particularly, this was observed in tobramycin formulation. During the investigation of the state of the drug after spray drying, the multifunctional formulation has displayed an amorphous state without any indication for crystallization.

In this thesis, an innovative multifunctional formulation as a dry powder for inhalation composed out of multi-drug (antibiotics, NAC, Curcumin) was developed for the purpose of treating the bacterial infections of cystic fibrosis. The multifunctional formulation is a milestone for future treatment in cystic fibrosis, taking the benefit from the presence of the NAC to break the mucus network and facilitate the diffusion of the antibiotics and anti-inflammatory drugs through the mucus.

The next milestone of following this approach could be embedding this multifunctional microparticle matrix containing (NAC/antibiotics) with another kind of NPs with a different drug which might be a drug for genetic correction such as Ivacaftor. Moreover, this approach could be used with another kind of antibiotics to test whether there is a possibility to formulate it in salt form. One other option is to use this multifunctional matrix (NAC/antibiotics) to incorporate it into a hydrophilic carrier system such as gelatin NPs.

Finally, *in vivo* data would be needed to give more validation about the benefit of the combination of multiple drugs in one formulation.

In this work, the basis for such an advanced new drug delivery system was developed.

7. ANNEX

7.1 List of abbreviations

DPI	Dry powder inhaler
DLS	dynamic light scattering
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
ASL	airway surface liquid
CLSM	confocal laser scanning microscopy
Cipro	ciprofloxacin
Tobra	tobramycin
Azith	azithromycin
FDA	Food and Drug Administration
NAC	N-Acetylcysteine
P. aeruginosa	*Pseudomonas aeruginosa*
EE%	encapsulation efficiency
FPF	fine particle fraction
GSD	geometric standard deviation
MP	microparticles
AM	artificial mucus
MMAD	mass median aerodynamic diameter
NGI	next generation impactor
NP	nanoparticles
PEG	polyethylene glycol / polyoxyethylene
PLGA	poly(lactic-co-glycolic acid)
SEM	scanning electron microscopy
λem	emission wavelength
λex	excitation wavelength

8. Curriculum vitae

Nashrawan Lababidi

*** Feb 18, 1985 in Hama, Syria**	**Scientific assistant in Biopharmacy and Pharmaceutical Technology** Preparation of Multifunctional Formulations Intended For Pulmonary Delivery Saarland University, Biopharmaceutics and Pharmaceutical Technology (April 2015 – Jun. 2518)
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Jun. 2013 – Nov 2013	**Internships** Department of Clinical Pharmacy-Saarland University "Investigating the impact of gastric pH on the bioavailability of drugs"
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Jun. 2009 – Mar 2010	**Pharmaceutical Sales Representative** Al-Shahba Pharmaceutical Company, Syria
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9. Scientific output

Publications

Nashrawan Lababidi[1], Valentin Sigal[1], Konrad Schwarzkopf[2], Andreas Manz[3], Marc Schneider[1], **Preparation of size-tunable PLGA nanoparticles by microfluidics intended for mucus penetration** (submitted)

Nashrawan Lababidi[1], Valentin Sigal[1], Walid A. M. Elgaher[2], Jörg Haupenthal[2], Bianca C. Schwarz[3], Anna K. H. Hirsch[2],[4], Marc Schneider[1], **Spray-drying for generation of inhalable, multifunctional matrix for the treatment of biofilms as formed in cystic fibrosis** (submitted)

Nashrawan Lababidi[1], Carlos Montefusco[2], Cristiane Wodarz-Carvalho[2], Claus-Michael Lehr [2], Marc Schneider[1], **Multifunctional inhalable microparticles combining antibiotics and a mucolytic with anti-inflammatory drug-loaded PLGA nanoparticles** (submitted)

Poster presentations

Preparation of PLGA Nanoparticles by Microfluidics Intended for Mucus Penetration.
Lababidi, Nashrawan; Manz, Andreas; Schneider, Marc.
CRS- Local Chapter. Marburg, Germany. 2017.

Microfluidics Platform for Synthesis of Polymer Nanoparticles for Bio-Gel Penetration.
Lababidi, Nashrawan; Manz, Andreas; Schneider, Marc.
Solvay workshop on chemical reactions and separation in flows. Brussels, Belgium. 2017.

Synthesis of coated Mucopenetrating PLGA Nanoparticles by Microfluidics for Cystic Fibrosis.
Lababidi, Nashrawan; Manz, Andreas; Schneider, Marc.
7 HIPS Symposium on Pharmaceutical Sciences Devoted to Infection Research. Saarbrücken, Germany. 2017.

Spray Drying of PLGA-based Nanoparticles for Pulmonary Delivery.

Lababidi, Nashrawan; Manz, Andreas; Schneider, Marc.

PhD summer school: Drug delivery – using nano and micro technologies 2017. Copenhagen, Denmark. 2017.

Microfluidics Platform for a Design of Size-tunable PLGA NPs for Enhanced Penetration through Non-cellular Barriers.

Lababidi, Nashrawan; Sigal, V; Manz, Andreas; Schneider, Marc.

Doktorandentag der Naturwissenschaftlich Technischen FakultätIII. Saarbrücken, Germany. 2017.

A novel Approach for Nanoparticle-loaded, Inhalable Microparticles using Smart Excipients as a Matrix.

Lababidi, Nashrawan; Sigal, V; Schneider, Marc.

DPhG Annual Meeting 2017. Saarbrücken, Germany. 2017.

Multifunctional microparticles intended for treatment of cystic fibrosis diseases

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10. Acknowledgement

First of all, I would like to thanks my doctor-father **Prof. Dr. Marc Schneider** for accepting me in his research group and also for his kind supervision during my PhD and for the excellent support and for the exciting and versatile PhD thesis. Also, for giving me the freedom for implementing my own ideas and at the same time providing the necessary support at all times. I have learned a lot from you. Many thanks for your trust and your never-ending encouragement especially during the hard time and moral support.

I would like to thank Prof. Dr. Christian Ducho for his contribution which he has made as my scientific supervisor and expert in this work. Likewise, my thanks go to the other members of the Examination Board. I would like to thank all former and current students of the Schneider Lab group for worthy scientific discussions as well as for spending a nice time. Thanks to Carolin Thum for being always there when I need your help and spending a great time during sharing the office and to still keep our friendship, Baseer, Aljoscha, Marcel, Afra, Agnes, Karola, Thorben, Enkeleda, Alexandra and Nesma. Special thanks to Sarah Barthod (for all supportive time), Nazende, thanks also for introducing me to the project at the beginning and special thanks as well to Valentin Sigal! Isabelle, Peter and Marijas, thanks for your kind help and supports. Thanks to Hips group especially Hanzey Yasar (for the great time and great support, your motivation and passion in the science, always inspire and encourage me), Sara Menina and Robert Richter.

Also many thanks to my best friend **Aysenur Bilgin** (Kuzu) who supported me during my journey in Germany without you I couldn't do it on my own, I can't even put it into words what you have done for me. Of course, I would like to thank my best friend Alice Simpson (Katkoti) for all support and the great days which we have spent it together. Thanks to Ghamdan Beshr for being there always when I need your help and for the great time.

To my dear father and mother, my whole family in Syria many thanks for all your love, kind support, faith and assistance, missing you made me work harder.

To my Cousins, although you passed away, you are always a part of me. I wish we could celebrate this moment together but I hope that you are proud of me.