Ciprofloxacin-loaded PLGA nanoparticles against Cystic Fibrosis *P. aeruginosa* Lung Infections

Nazende Günday Türeli[1,2], Afra Torge[2], Jenny Juntke[3], Bianca C. Schwarz[4], Nicole Schneider-Daum[3], Akif Emre Türeli[1], Claus-Michael Lehr[2,3], Marc Schneider[2*]

[1] MJR PharmJet GmbH, Industriestr. 1B, 66802 Überherrn, Germany

[2] Department of Pharmacy, Biopharmaceutics and Pharmaceutical Technology, Saarland University, 66123 Saarbrücken, Germany

[3] Helmholtz Institute for Pharmaceutical Research Saarland (HIPS), Helmholtz Center for Infection Research (HZI), Saarland University, Saarbrücken, Germany

[4] Pferdeklinik Altforweiler, Raiffeisenstraße 100, 66802 Überherrn, Germany

*** Corresponding author:**

Prof. Dr. Marc Schneider,

Department of Pharmacy

Biopharmaceutics and Pharmaceutical Technology

Saarland University

Campus A4 1

D-66123 Saarbrücken, Germany

phone: 0049 681 302 2438, fax: 0049 681 302 2028,

eMail: Marc.Schneider@mx.uni-saarland.de

ABSTRACT

Current pulmonary treatments against *Pseudomonas aeruginosa* infections in cystic fibrosis (CF) lung suffer from deactivation of the drug and immobilization in thick and viscous biofilm/mucus blend, along with the general antibiotic resistance. Administration of nanoparticles (NPs) with high antibiotic load capable of penetrating the tight mesh of biofilm/mucus can be an advent to overcome the treatment bottlenecks. Biodegradable and biocompatible polymer nanoparticles efficiently loaded with ciprofloxacin complex offer a solution for emerging treatment strategies. NPs were prepared under controlled conditions by utilizing MicroJet Reactor (MJR) to yield a particle size of 190.4 ± 28.6 nm with 0.089 PDI. Encapsulation efficiency of the drug was 79% resulting in a loading of 14%. *R*elease was determined to be controlled and medium-independent in PBS, PBS + 0.2% Tween 80 and simulated lung fluid. Cytotoxicity assays with Calu3 cells and CF bronchial epithelial cells (CFBE41o$^-$) indicated that complex loaded PLGA NPs were non-toxic at concentrations >> MIC_{cipro} against lab strains of the bacteria. Antibacterial activity tests revealed enhanced activity when applied as nanoparticles. NPs' colloidal stability in mucus was proven. Notably, a decrease in mucus turbidity was observed upon incubation with NPs. Herewith, ciprofloxacin complex loaded PLGA NPs are introduced as promising pulmonary nano drug delivery systems against *P. aeruginosa* infections in CF lung.

Keywords: Antibiotic-loaded nanoparticles, biofilm, nanomedicines, pulmonary nanoparticulate drug delivery

Chemical compounds studied in this article

Ciprofloxacin (PubChem CID: 2764); Pluronic® F68 (PubChem CID: 24751); sodium dodecyl sulfate (PubChem CID: 3423265), Poly(DL-lactide-co-glycolide) (PubChem SID 24866924)

INTRODUCTION

Chronic pulmonary infections, among which *Pseudomonas aeruginosa* is known to be the major pathogen, are reported to be the main cause of mortality among cystic fibrosis (CF) patients [1]. In addition to intravenous (i.v.) or oral administration [2, 3], during the last decades, repeated courses of high doses of nebulized and inhaled antibiotics have been applied extensively for treatment of early infections as a preventive action against the mucoidic bacteria [2-6]. However, once infection is established in the airways, it is almost impossible to eradicate it. The established biofilm anchors the bacteria to their environment, protects the bacteria and prevents drugs reaching minimum inhibition concentration (MIC) at the site of action to kill bacteria [7]. Biofilm-forming mucoid strains of *P. aeruginosa* are less susceptible to antibiotics than their planktonic counterparts as a result of different resistance mechanisms. Bacteria show slow growth or proliferation patterns or switch to anaerobic state at certain regions of the biofilm [8], thus bacterial biofilms show heterogonous composition [9]. In addition, diffusion rates of antibiotics in the biofilm are lower in comparison to water, they are prone to inactivation by the biofilm components, and penetration of the antibiotics is strongly affected by the properties of both antibiotics and the heterogeneous biofilm matrix [10-13]. Consequently, antibiotic resistance is enhanced as a result of long term exposure. Under such disease conditions, a local nano drug delivery system capable of penetrating the thick mucus and biofilm, releasing antibiotic in a controlled manner at the site of action can be an intriguing approach to overcome treatment bottlenecks [14-16]. Particles should be engineered to possess particle size smaller than the CF mucus/biofilm meshes [17-19], and to prevent adsorption only onto the biofilm and entrapment in the biofilm via surface

properties [20-22]. With this perspective, the development of pulmonary drug delivery systems for improved interactions with local environment is needed [23].

Pulmonary drug delivery offers many advantages including avoiding first pass effect, reduced systemic side effects, delivering higher doses at the site of action thus increased local concentration [24], higher bioavailability [25], increasing patient compliance and being a non-invasive drug delivery method [26]. Controlled particle size is one of the prerequisites of pulmonary drug delivery and lung deposition.

Engineering nanoparticles at particle size level requires full control over the preparation method. Traditional nanoparticle precipitation techniques lack control over nucleation and growth process, thus particle size and polydispersity index (PDI). Reproducibility problems at lab scale and scale-up problems at industrial level are faced during early and later stages of the development phase. Since particle size and distribution is a critical quality attribute for development of pulmonary nanomedicine against *P. aeruginosa* infections in CF, MicroJet Reactor (MJR) technology, a precise preparation technique performed under controlled conditions, was employed. MJR enables control over the whole process parameters and environment, and turbulent like mixing in micron-volume caused by impinging jets provides high nanoparticle quality. NP quality can be theoretically defined as a function of parameters that govern the whole process [27].

Biocompatibility and biodegradability of the nano-carrier play an important role for pulmonary nanomedicines, especially for the disease cases like CF, where mucociliary clearance (MCC) is impaired [28, 29]. Thus, possible accumulation of the carrier is more likely. Poly(lactic-co-glycolic) acid (PLGA) applications have attracted attention following the first FDA approved product and have been successfully used since decades for

micro-formulated drug delivery systems [30-34]. In addition to its biocompatibility hence low toxicity, it also offers predictable biodegradation kinetics [33]. Even though PLGA is not approved for pulmonary applications, yet, these characteristics make it a promising excipient for a clinically applicable formulation. Ciprofloxacin, a fluoroquinolone antibiotic, which is widely used for treatment of *P. aeruginosa* lung infections in CF patients, was chosen as model drug to be encapsulated with high drug loading in PLGA nano-carriers. Encapsulation of active pharmaceutical ingredients such as ciprofloxacin, showing pH dependent solubility and limited organic solvent solubility, compromise high drug loading regardless of preparation technique. In order to achieve potentially clinically effective drug loading, counter-ion complex of ciprofloxacin was employed [35]. Nanoformulations' success also depends on their potential to reach the bacteria. Thus interaction with mucus, dissolution profile to sustain the local concentration at the site of action and release kinetics to understand the underlying physical and chemical phenomena were characterized. Additionally, safety and efficacy of the nanoformulation have been evaluated to ensure sustainability of the developed nanomedicine.

MATERIALS and METHODS

Materials

Ciprofloxacin base, Pluronic® F-68 and sodium dodecyl sulfate (SDS) were purchased from Sigma Aldrich, Munich, Germany. PLGA (Expansorb ® 10P017, with a lactic to glycolic acid ratio of 50:50) was purchased from PCAS, Longjumeau Cedex, France. All solvents used were of analytical grade and were supplied by VWR, Darmstadt, Germany. All other chemicals used were of pharmaceutical or analytical grade.

Ciprofloxacin-SDS complex preparation

Ciprofloxacin-SDS complex was prepared as previously reported [35]. In brief, equimoles of Ciprofloxacin and SDS were dissolved in 0.1 N HCl solutions of equal volumes. Ciprofloxacin solution was added onto the SDS solution under continuous mixing. Precipitated complex was vacuum-filtered, washed twice with distilled water and dried at 30°C under vacuum.

Nanoparticle preparation

Nanoparticles were prepared according to an optimized nanoprecipitation method [27] by utilizing MJR technology. 2% Ciprofloxacin-SDS complex (1% ciprofloxacin equivalent) dissolved in 0.5% PLGA in DMSO solution (solvent system) and 0.25% Pluronic F68 in water solution (non-solvent system) were delivered to the MJR at 1:10 flow rate ratio at 180° angle by using Smartline S100 pumps (Knauer, Munich, Germany) (Figure 1). Since nitrogen gas was not employed, fourth opening was sealed. Solvent delivering capillaries and the MJR were immersed into a water bath in order to control

the system temperature at 32.5°C. Purification of the nanoparticles was achieved with Visking dialysis membrane with a MWCO 10 kDa (VWR, Darmstadt, Germany).

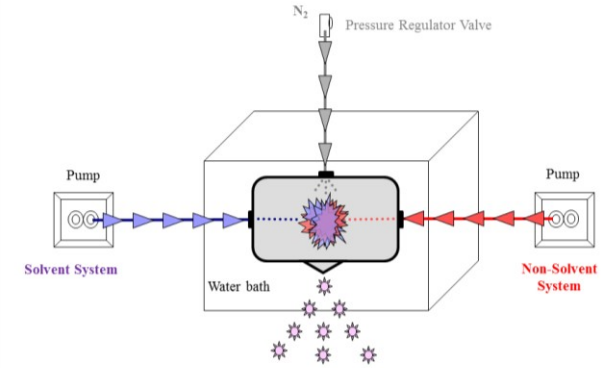

Figure 1 Schematic representation of MJR experimental set-up: Solvent and non-solvent were delivered to the MJR at equal flow rates at 180° angle. NPs were collected from the bottom opening of MJR. The system was immersed in a water bath in order to control the overall temperature.

Physicochemical characterization of the nanoformulation

Particle size, polydispersity index (PDI) and zeta potential of the nanoparticles were determined by Malvern Instruments Nano-ZS90 Zetasizer (Malvern Instruments, Worcestershire, England).

Entrapment efficiency (EE) was calculated with direct method, where incorporated drug substance is determined by extracting the drug from the carrier and comparing with the initial mass of the drug substance employed. EE was determined by HPLC analysis (provided in supplementary materials) and reported as the percentage of ciprofloxacin

incorporated into PLGA nanoparticles relative to the initial total amount of drug in solvent system (n=3).

Drug loading was calculated by HPLC analysis (provided in supplementary materials) of the freeze-dried nanoparticles dissolved in DMSO (n=3). Drug loading was reported as the amount of ciprofloxacin in relation to a certain mass of nanoformulation in percent. Drug based yield was calculated by comparing the theoretical ciprofloxacin amount in the nanoparticle formulations and comparing this with the experimental amount. Yield was reported as percentage. The freeze drying method involved in determining the mass of the nanoformulation is provided in supplementary materials.

Morphology of the particles was determined using atomic force microscopy (AFM). Tapping mode measurements were performed in air using a BioScope BS3-Z2 with a Nanoscope IV controller (Bruker Corporation, Billerica, USA) and silicon cantilevers with tetrahydral tips (OMCL-AC160TS - Olympus, Shinjuku, Japan) with a nominative force constant of 42 Nm^{-1} and a resonance frequency of around 300 kHz. For sample preparation, undiluted nanosuspensions were dropped onto a freshly cleaved mica surface (Plano GmbH, Wetzlar, Germany). After 5 minutes, the liquid was removed with a tissue and the samples were completely dried before imaging. Particles were analyzed with NanoScope Analysis 1.40 software (Bruker Corporation, Billerica, USA).

In vitro evaluation of the nanoformulation

The *in vitro* drug release behavior of the nanoparticles loaded with 100 µM ciprofloxacin equivalent complex in different buffer systems was determined by using dialysis

membrane technique. Release studies were performed at 37.0 ± 0.5°C in triplicate under sink conditions. Refer to supplementary materials for experimental design.

Mucus interaction of the NPs was evaluated by two *in vitro* methods (see supplementary materials for further details). First method was based on turbidimetric measurement of horse lung mucus interaction with NPs over 2h evaluating the absorbance at 650 nm at pre-defined time points. During the turbidimetric measurements, cuvettes were kept steady in the measurement position. Absorbance of horse lung mucus incubated on top with the same amount water as used for NP suspensions were evaluated as control solutions. Zeta potential and size of the NPs after incubation with mucus were used as second *in vitro* method to assess NP and mucus interaction. NP suspension and mucus were mixed at 1:1 volume ratio and incubated at 37°C for 2 h. Zeta potential and size measurement of the NP before and after incubation with mucus was realized by Malvern Instruments Nano-ZS90 Zetasizer. At the end of mucus incubation, NPs were recovered from the mixture via centrifugation for 5 minutes at 5000 g prior to analysis.

The antibacterial activities were compared by the disk diffusion method which was performed in accordance with the recommendations of NCCLS (formerly known as the National Committee for Clinical Laboratory Standards, responsible for establishing guidelines for antimicrobial susceptibility testing) document M2-A9 [36]. Test substances were evaluated simultaneously against *P. aeruginosa* ATCC® 27853 strains. Disk diffusion test was performed in triplicate for a total of 18 h in an incubator at 37°C after inoculation with organisms and placement of disks. Zones of inhibition were measured at 18 h. Detailed description of the set-up is provided in supplementary materials.

Calu-3 HTB-55 and CFBE41o⁻ cells were seeded in 96-well tissue culture plates with a density of 125,000 cells/cm² and 62,500 cells/cm², respectively. Three days after seeding the cells were incubated with the ciprofloxacin-SDS complex loaded NPs or the single components (Ciprofloxacin as free drug, pure SDS, drug-free NPs, free ciprofloxacin-SDS complex) dissolved in Krebs-Ringer modified Buffer (KRB) supplemented with 10% FCS with increasing concentrations from 50-500 µM. Cell viability was assessed with the ToxiLight™ bioassay kit 4, 8 and 24 hours after incubation with the test compounds (for further details see supplementary materials).

Data Analysis

The results presented were expressed as mean ± standard deviation (SD) obtained from three independent experiments (n=3). Comparisons among groups were evaluated by ANOVA analysis, paired Student's t-test, and a P-value of < 0.05 was considered significant.

RESULTS and DISCUSSION

Physicochemical characterization of nanoformulation

NPs prepared with MJR under optimized and controlled conditions are in general expected to have small PDI values, since homogenous dissipation of the entire energy in a micro volume is facilitated. Optimization studies revealed that MJR process parameters (flow rate, temperature and pressure) had a significant effect on particle size of complex loaded PLGA NPs. Regardless of drug incorporation, prepared NPs were

shown to have homogenous particle size distributions [27]. Incorporation of complex in PLGA NPs resulted in 3-4-fold increase in the particle size. Optimized blank NP were shown to be as small as 57.8 ± 17.5 nm with a PDI of <0.090, whereas optimized complex-loaded PLGA NPs showed a particle size of 190.4 ± 28.6 nm with a PDI < 0.090. AFM images of the complex-loaded NPs have confirmed that they are spherical with a smooth surface. Nanoparticle size was, ranging from 51.7 to 124.2 nm in the shown image found to be smaller than measured by photon correlation spectroscopy (PCS). This is commonly observed and represents the difference between geometric radiuses in ambient conditions in contrast to the hydrodynamic radius determined with PCS [37], [38].

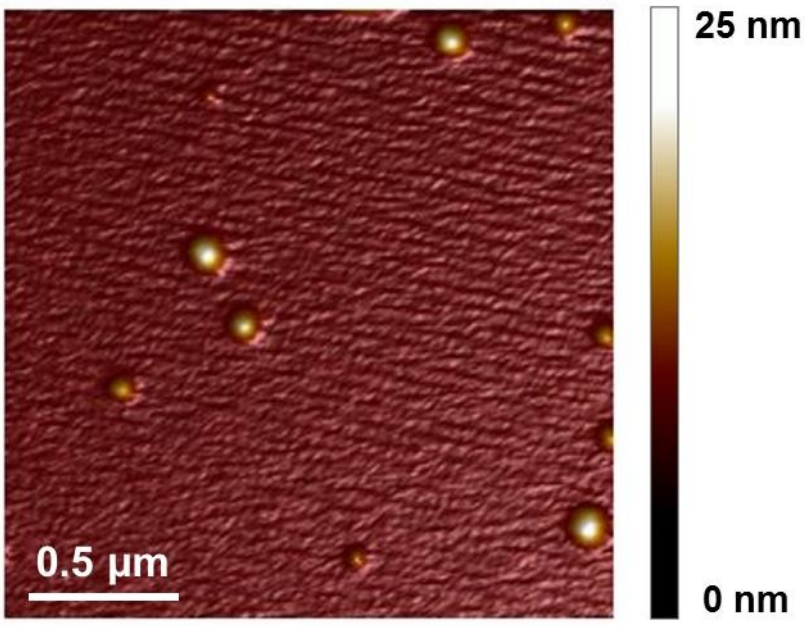

Figure 2: **Complex-loaded nanoparticle morphology:** Atomic force microscopy revealed spherical shape with smooth surface

Both blank and complex loaded PLGA NPs had a negative surface potential of -25.9 ± 7.7 mV and -22.5 ± 5.4 mV, respectively. Ciprofloxacin-based yield of the NP preparation was 101.9 ± 2.3% and EE of 79.3 ± 0.9% was achieved. Drug loading was determined as 13.32 ± 0.52 %.

Lyophilization process was shown to have no negative effect on the quality of the NPs. Particle size, PDI, yield, and EE were compared before and after freeze-drying following redispersion (Table 1).

Table 1: Effect of freeze-drying on NP quality

	Before Lyophilization	**After Lyophilization**
Particle size [nm]	190.4 ± 28.6	236.7 ± 22.6
PDI	<0.090	<0.120
Drug yield [%]	101.9 ± 2.3	101.9 ± 13.0
EE [%]	79.3 ± 0.9	57.5 ± 1.3

However, following redispersion of the lyophilized product, a decrease in EE was observed. This situation was related to loss of the drug load located close to the NP surface upon redispersion and subsequent evaluation of NP. Considering the short diffusion path of those drug molecules, it can be concluded that wetting the particles promoted drug dissolution from the sites close to the surface of the particles. The importance of determination of release kinetics is emphasized by the release of the drug load upon wetting, as it will also be the case when the particles reach the lung lining fluid upon pulmonary application.

In vitro evaluation of the nanoformulation

In vitro release behavior of the NPs was evaluated in three different media shown to be *in vivo* relevant: Phosphate Buffered Saline (PBS) pH 7.4 with or without 0.2% Tween 80 (T80) [39], and simulated lung fluid (SLF) [40]. Additionally, drug leakage testing was performed at 37°C in distilled water. For composition of SLF, please refer to Table S1. Among a variety of different recipes used to simulate the extracellular lung fluids, SLF 3 modeling the interstitial fluid was chosen (see supplementary material for the composition). Results provided as cumulative released amount of ciprofloxacin as percentage over time are depicted in Figure 3.

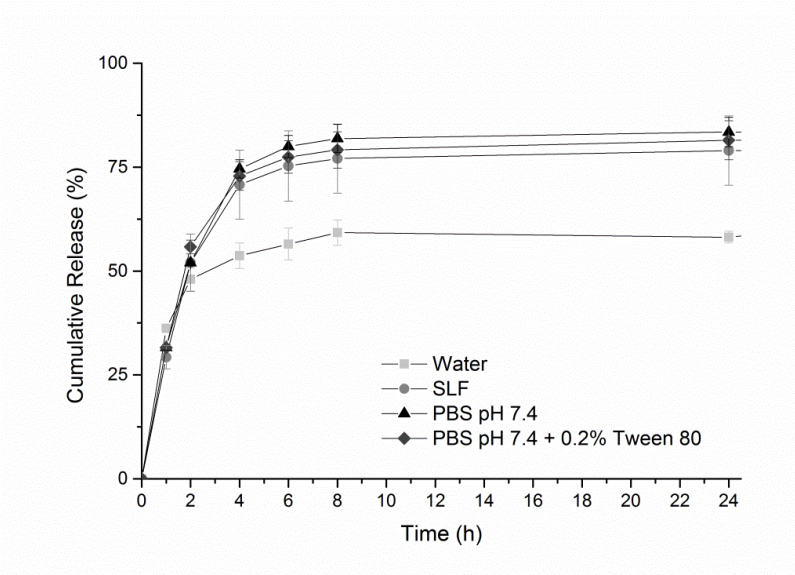

Figure 3: ***In vitro*** **release kinetics of ciprofloxacin in three different *in vivo* representative media and water:** Release kinetics in the *in vivo* relevant media did not show differences.

It was observed that controlled release of ciprofloxacin was achieved within 8 h without burst effect [41] in the very first sampling hours (<30% after 1 h. Please refer to Table S5 for raw data). In water after 8 h, ciprofloxacin release amounted to 59.2% ± 3.0%. Meanwhile, for all the *in vivo* representing media employed, the amount of released ciprofloxacin reached around 80% after 8 h, followed by a very slow release till day 14 to yield ca 90.5% drug release (data provided in Table S5 in supplementary material.). PLGA degradation and release kinetics have been well explored over years [33, 42]. In the initial phase dissolution of the load close to the surface is observed (following Fickian diffusion) and this release is followed by an erosion/degradation and diffusion dominated phase [33, 43].

In order to further investigate the dominating drug release mechanism, Hixson-Crowell, Higuchi, Korsmeyer-Peppas, first order and zero order functions were computed and coefficients of determination (R^2) were evaluated. A summary of the mathematical models and the correlation factors is provided in supplementary material (Table S2 and S3, respectively). It was observed that in all release media release kinetic was best described by Korsmeyer-Peppas model which is used to analyze release of pharmaceutical polymeric dosage forms, where more than one release phenomena could be involved [44]. Used kinetic models and calculated correlation factors for each medium is provided in supplementary materials. Based on calculated release exponent (n), release in all media was defined as anomalous transport (n = 0.6 for all media) which is a combination of diffusion and polymer degradation/erosion dominated release phenomena. This finding was in accordance with the polymeric matrix used as discussed above. The drug release kinetic in water was shown to be the only one being driven by Fick`s diffusion law (n = 0.2); following the model that the diffusional exponent

depends on the size distribution and the general shape of the distribution for drug release in spherical polymer particles [45]. Considering that the diffusion is dominated by the concentration gradient, this result can be attributed to the limited solubility of complex in water (surrounding fluid) at the stagnant layer. Water running into the polymer matrix as a result of osmotic effects reaches saturation much faster than other surrounding fluids tested (PBS, PBS + 0.2% T80 or SLF), thus not experiencing a sharp concentration gradient to forge the release [46].

Mucus interaction properties of the NPs were evaluated by monitoring the turbidity at 650 nm over a period of 2h after mixing NPs with horse lung mucus. NPs and lung mucus samples used as control samples showed significant turbidity difference ($p<<0.001$) and NPs were shown to have a turbidity almost close to zero owing to the applied nanosuspension concentration (0.5 mg NP content in 1 mL). Following incubation of the mucus with NPs, immediately after 15 minutes mucus turbidity decreased and after 2h turbidity was significantly lower than mucus absorption at t=0 ($p<<0.001$) indicating potential disaggregation of the mucus (Figure 4). This phenomenon might be explained by the repulsive forces exerted by the nanoparticles to negatively charged mucus following diffusion thoroughly into the mucus and disassembling the network [47, 48].

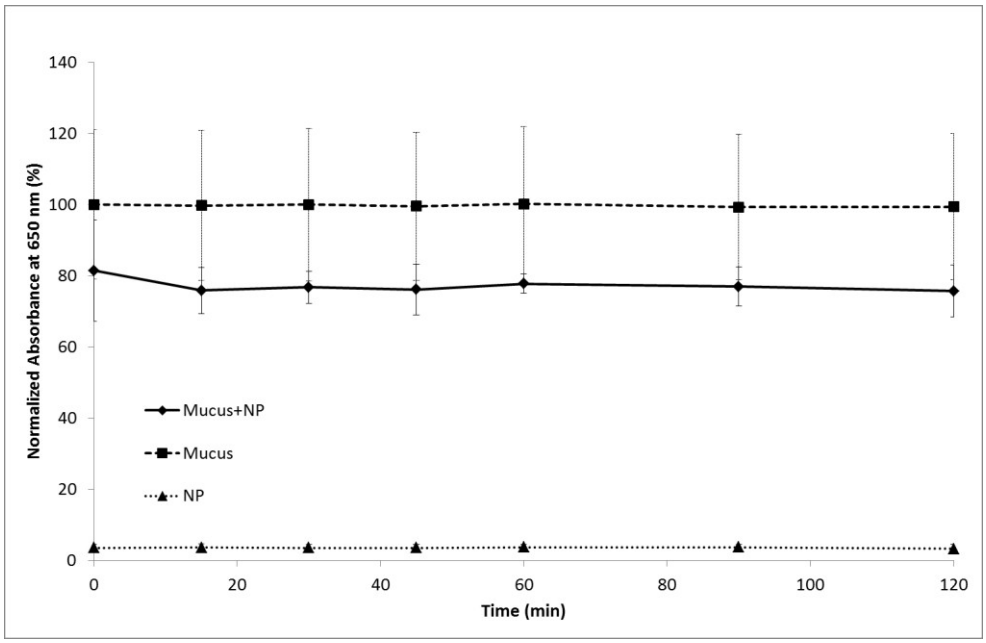

Figure 4: **Mucus and NP interaction evaluation with turbidity analysis at 650 nm:** Following incubation of the mucus with NPs, mucus turbidity decreased significantly ($p<<0.001$) immediately after 15 minutes and stayed constant over 2 h.

Effect of applied NP concentration on disaggregation of the mucus was investigated by incubating the mucus with increasing amounts of NP without changing the applied nanosuspension volume. Results (Figure 5) indicated that mucus disaggregation was directly correlated with the NP concentration: as the applied NP concentration increased, the turbidity decreased, which is related to the disaggregation of the mucus network. These findings might be supported by the cryo-SEM images (see Figure S1), where mucus incubated with NP showed structural differences in comparison to control mucus. Further investigation of this phenomenon is on-going.

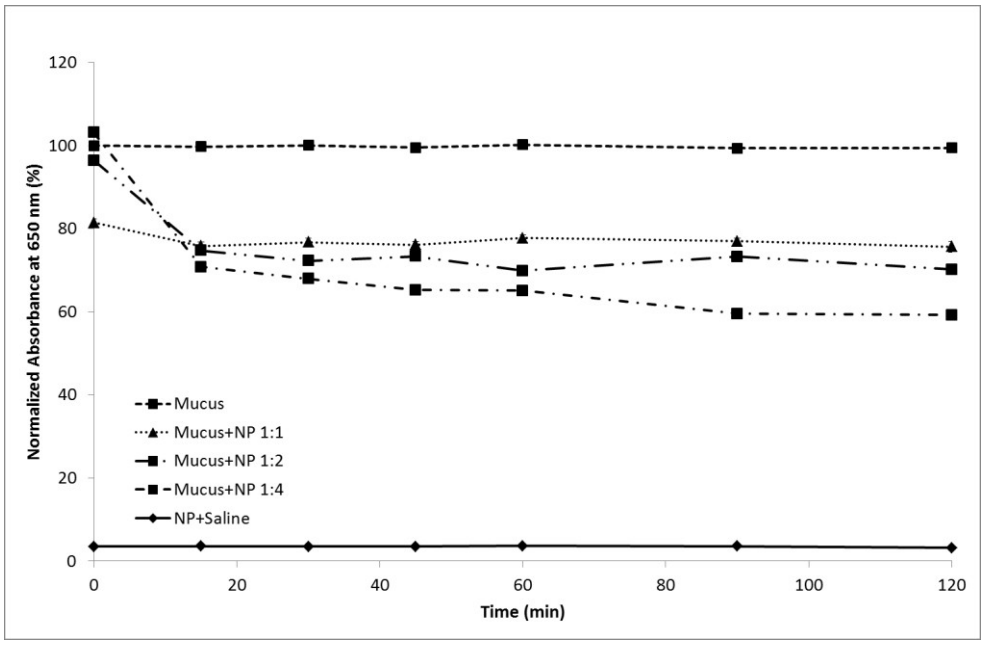

Figure 5: **Concentration dependent mucus and NP interaction evaluation with turbidity analysis at 650 nm:** Following incubation of the mucus with increasing NP concentrations, mucus turbidity decreased already after 15 minutes in direct relation to concentration significantly ($p<<0.001$ for each applied concentration) and stayed constant over 2 h.

Additionally, finding on effect of NP on mucus degradation was supported by particle size and zeta potential of the nanoparticles before and after incubation with mucus. Summarized results of native and incubated NPs are provided in Table 2, where zeta potential of the mucus before incubation was taken as control. Particle size of the nanoparticles was shown to increase only minimally, therefore excluding uncontrolled aggregation and indicating stability of NP in mucus. The shift towards bigger size might be due to corona effect. The formation of a corona is also supported by the fact, that zeta potential of the NPs was shifted slightly (p-value: 0.1524). Before and after each mucus interaction investigation, the count rates of the particle size measurements were

taken as indicator of NP recovery. In all cases the count rate was staying approximately constant.

Table 2: Characterization of NPs before and after incubation with mucus

	Mucus	Particle Size [nm] / PDI	Zeta Potential [mV]
Mucus	+	n.a.*	-14.5 ± 0.00
NP	-	235.2 ± 38.5 / 0.163	-21.0 ± 4.31
	+	284.8 ± 44.8 / 0.302	-27.9 ± 0.00

* n.a. not applicable

Evaluation of the NP-mucus interaction with those two *in vitro* methods fosters the conclusion that presented nanoformulations might show mobility in mucus and be capable of penetrating through the mucus owing to their surface properties, size and potential. Complex mechanism of particle penetration through mucus/biofilm and particle properties effecting the mucus penetration have been subject to many studies. It has been already reported that a size below 200 nm is advantageous for mucus penetration of negatively charged particles [47, 49], and polyoxyethylene-polyoxypropylene block copolymer-stabilized particles, such as Pluronic F68, have been described to enhance mobility in mucus [17, 50]. This might be related to the polyethylene glycol chains involved in the block copolymer, as improved permeability has been also shown for NP with low molecular weight PEGylated surface coverage properties [18, 51, 52]. In the light of those studies, potential mucus penetration capability and mobility of the nanoformulations might be related to a cumulative effect of all those.

Besides reaching the respective bacterial location in the mucus, the activity of the encapsulated and complexed drug is essential. Antibacterial activity of the ciprofloxacin was evaluated by disk diffusion test. According to this test the zone inhibition diameter break point of free ciprofloxacin (5 µg) against susceptible strains of *P. aeruginosa* has been reported as ≥ 25 mm [53]. Figure 6 shows the antibacterial activities of free ciprofloxacin, the used excipient (SDS), complex and complex-loaded PLGA nanoparticles, respectively, against a laboratory strain of *P. aeruginosa*. Inhibition zones obtained from disk diffusion test are provided in table 3. It has been previously reported that ciprofloxacin-SDS complex shows significant difference in antibacterial activity compared to ciprofloxacin at all applied concentrations (p-values 0.01, 0.01 and 0.04 for 0.5 µg, 2.5 µg and 5 µg, respectively) [35].

Table 3: Concentration dependent zone diameters of ciprofloxacin against *P. aeruginosa* ATCC® 27853 strains measured by disk diffusion test

Applied Ciprofloxacin Amount (mg)	**Zone Diameter (mm)**			
	SDS	**Cipro**	**Complex**	**Complex-loaded PLGA**
0.5	0.0	23.0 ± 0.9	26.0 ± 0.5	25.0 ± 0.8
2.5	0.0	27.0 ± 0.9	31.0 ± 0.9	31.0 ± 0.9
5.0	0.0	32.0 ± 0.5	35.0 ± 0.9	36.0 ± 0.8

Since SDS, Pluronic F68 or blank NPs did not show any antibacterial activity at any of the applied concentrations (supplementary data table S6), enhanced antibacterial activity might be related to steric protection of the functional group on position 7 of ciprofloxacin following complex formation. It has been reported that the bulkiness of the substitute at position 7, which is known to directly interact with DNA-gyrase protects quinolones from efflux transporters [54]. After 18 h of incubation, complex loaded PLGA NPs have shown zone inhibitions not significantly differing from complex ($p>0.05$ at all concentrations) as shown in Figure 6 (A).

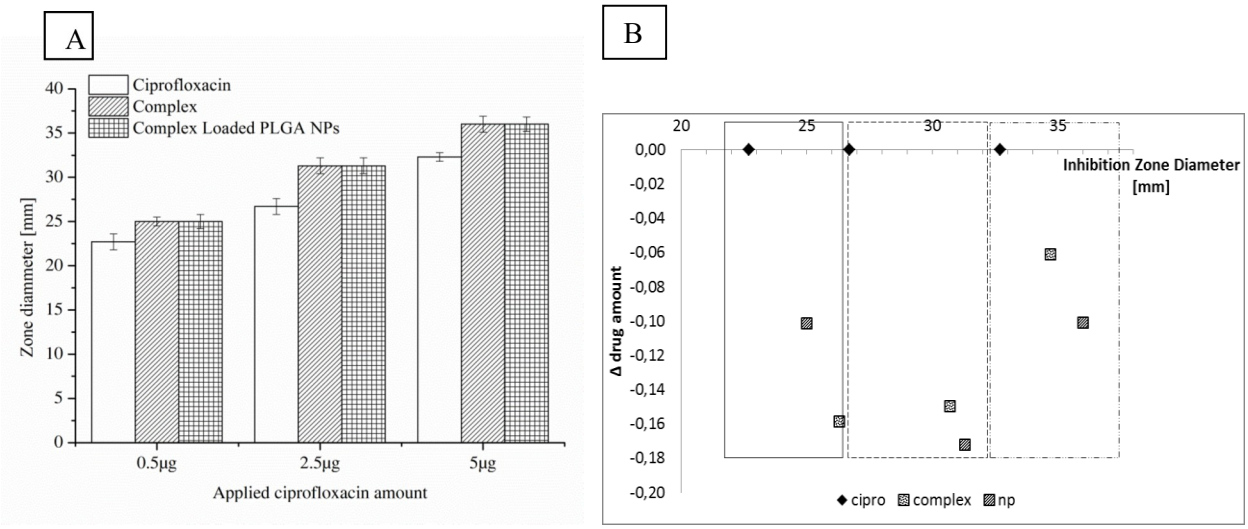

Figure 6: **Inhibition zones of ciprofloxacin against *P. aeruginosa* detected after 18 h of incubation at 37°C (A) and representative results following normalization to released amount of ciprofloxacin (B) as comparison to free ciprofloxacin*:** It was shown that at all applied concentrations complex formation enhances antibacterial activity of ciprofloxacin against *P. aeruginosa* as free complex and complex loaded PLGA NPs, respectively. (*Δ drug amount= free ciprofloxacin amount – released amount of ciprofloxacin)

However, after 18 h only 60% of the drug was released from the polymeric carrier to the water-based environment of agar plates (Figure 3). Taking these released amounts of ciprofloxacin into consideration the enhanced activity of the complex in the NPs in comparison to free complex can be clearly seen (Figure 6 (B)). These data suggests that complex formation enhances antibacterial activity of ciprofloxacin against *P. aeruginosa* for free complex and complex loaded PLGA nanoparticles.

Cytotoxicity of the nanoformulation was assessed over 24 h with Calu-3 bronchial epithelial cell line as a model for healthy lung and CFBE41o- a CF-derived bronchial epithelial cell line as a model for diseased lung. Selected test concentrations were well above the reported *in vivo* maximum ciprofloxacin concentrations (Table 4) and minimum inhibitory concentration (MIC) of ciprofloxacin against susceptible and resistant strains of *P. aeruginosa*, reported between 0.75-3.0 µM [50].

Table 4: Summary of reported *in vivo* ciprofloxacin concentrations

Administration	**Detection site (adult CF patients)**	**Highest detected concentration after 2 h**
i.v. ciprofloxacin 400 mg [55]	Serum	2.37 ± 1.40 µM
	Lung tissue	3.84 ± 1.87 mg/kg
oral ciprofloxacin 500 mg three times daily [56]	induced sputum	3 µM
ciprofloxacin DPI 32.5 mg [56]	induced sputum	99.6 µM

As shown in Figure 7 toxicity of the NPs was dose dependent and no additional cytotoxicity was observed with Calu-3 or CFBE41o- CF bronchial epithelial cell lines as a result of drug-complex loading in PLGA NPs when compared to free ciprofloxacin and SDS. Considering that blank NPs did not show any toxic effect at any applied concentration, it was concluded that observed viability reductions were directly related to drug-complex.

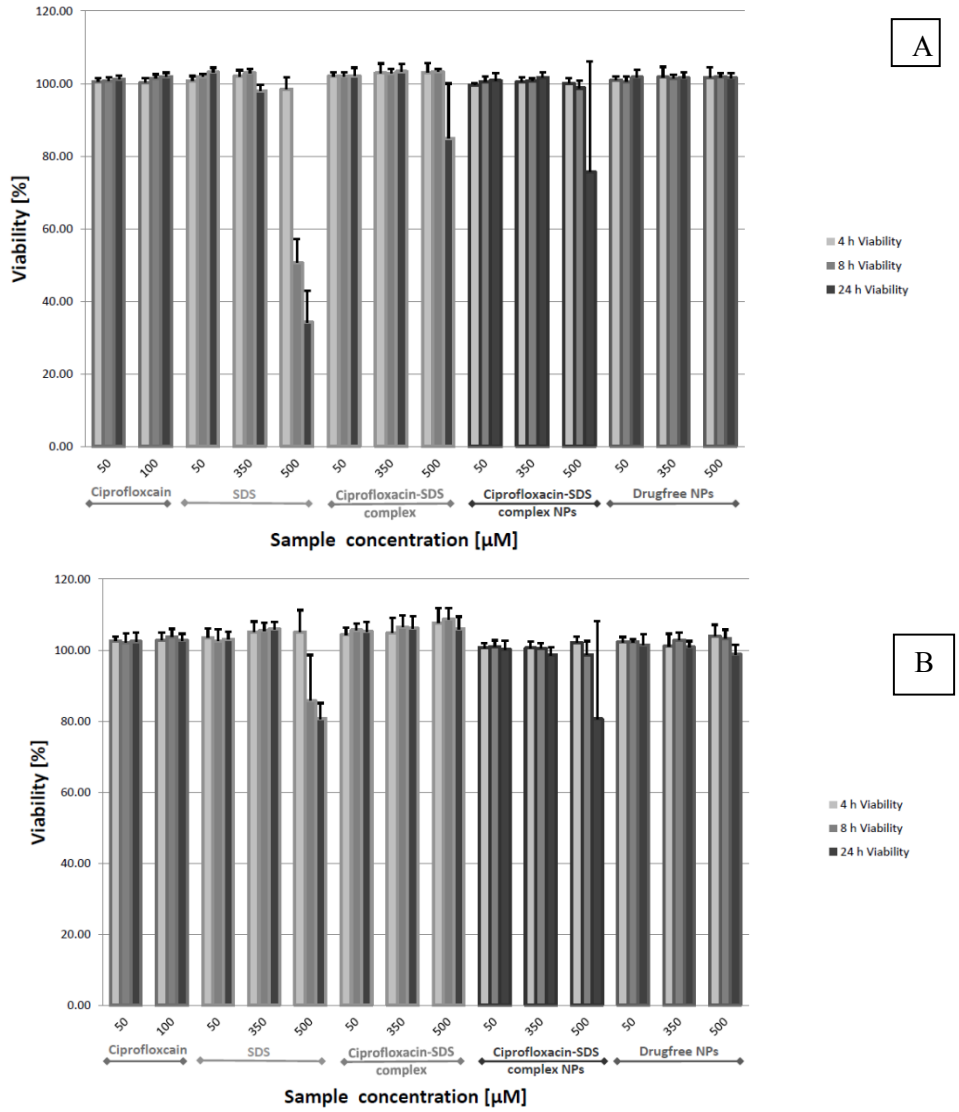

Figure 7 Cytotoxicity assessment with Calu3 (A) and CFBE41o- CF (B) bronchial epithelial cell lines: Cytotoxicity assay details are described in supplementary materials

After 4 hours of incubation none of the tested compounds shows a toxic effect, whereas after 8 hours the highest concentration of SDS (500 µM) decreases the viability of both cell lines. After 24 hours also the highest concentration of the ciprofloxacin-SDS complex loaded NPs (500 µM) decrease the viability to 75%. It can be concluded that the NPs are well tolerated by the epithelial cells and show a low cytotoxic potential.

CONCLUSION

Pulmonary administration of nanoformulations with higher drug loading is a promising tool for treatment of CF infections by overcoming low local antibiotic concentrations at the site of action. Ciprofloxacin complex loaded PLGA NPs presented here not only show high drug loading but also size and surface properties that promise penetration into the negatively charged, thick CF mucus, where the bacteria reside. Controlled release of the antibiotic from particles within 8 h is expected to serve for high and sustained local drug concentration. Additionally, enhanced antibacterial activity of nano-formulated ciprofloxacin against *P. aeruginosa* promises reduced dose, thus reduced side effects. Although a local therapy is aimed in the present work; NPs were shown to be safe against healthy and CF bronchial epithelial cells *in vitro* even at concentrations higher than reported *in vivo* ciprofloxacin concentrations in the lung. Under the light of the presented results, ciprofloxacin complex loaded PLGA NPs are introduced as promising pulmonary nano drug delivery system against *P. aeruginosa* infections in CF lung.

Evaluation of the further effect of those drug delivery systems on *P. aeruginosa,* and also release properties in mucus/biofilm secreted by the bacteria are valuable information on performance of this nano drug delivery system. These properties are under investigation and studies are on-going.

ACKNOWLEDGEMENT

Work reported here is a part of the project “Fibrose Delivery (FiDel)” funded by the German Federal Ministry of Education and Research (BMBF) [grant No: 13N12530]. Authors would like to thank Mr. Michael Hellweg and Dr. Andreas Schaper for their help with cryo-SEM measurements.

REFERENCES

[1] H. Heijerman, Infection and inflammation in cystic fibrosis: a short review, J Cyst Fibros, 4 Suppl 2 (2005) 3-5.

[2] N. Hoiby, Recent advances in the treatment of Pseudomonas aeruginosa infections in cystic fibrosis, BMC medicine, 9 (2011).

[3] N. Hoiby, New antimicrobials in the management of cystic fibrosis, Journal of antimicrobial chemotherapy, 49 (2002) 235 -238.

[4] N. Hoiby, Understanding bacterial biofilms in patients with cystic fibrosis: current and innovative approaches to potential therapies, Journal of Cystic Fibrosis, 1 (2002) 249-254.

[5] G. Döring, N. Hoiby, Early intervention and prevention of lung disease in cystic fibrosis: a European consensus, Journal of Cystic Fibrosis, 3 (2004) 67-91.

[6] H. Heijerman, E. Westerman, S. Conway, D. Touw, G. Doring, Inhaled medication and inhalation devices for lung disease in patients with cystic fibrosis: A European consensus, J Cyst Fibros, 8 (2009) 295-315.

[7] N. Hoiby, Understanding bacterial biofilms in patients with cystic fibrosis: current and innovative approaches to potential therapies, J Cyst Fibros, 1 (2002) 249-254.

[8] A.S. Prince, Biofilms, Antimicrobial Resistance and Airway Infections, The New England journal of medicine, 347 (2002) 1110-1111

[9] A.W. Smith, Biofilms and antibiotic therapy: is there a role for combating bacterial resistance by the use of novel drug delivery systems?, Adv Drug Deliv Rev, 57 (2005) 1539-1550.

[10] J.S. Suk, S.K. Lai, Y.-Y. Wang, L.M. Ensign, P.L. Zeitlin, M.P. Boyle, J. Hanes, The penetration of fresh undiluted sputum expectorated by cystic fibrosis patients by non-adhesive polymer nanoparticles, Biomaterials, 30 (2009) 2591-2597.

[11] J.W. Costerton, P.S. Stewart, E.P. Greenberg, Bacterial Biofilms: A Common Cause of Persistent Infections Science, Science, 284 (1999) 1318-1322.

[12] P.G. Bhat, D.R. Flanagan, M.D. Donovan, Drug diffusion through cystic fibrotic mucus: steady-state permeation, rheologic properties, and glycoprotein morphology, J Pharm Sci, 85 (1996) 624-630.

[13] J. Levy, Antibiotic activity in sputum, The Journal of pediatrics, 108 (1986) 841-846.

[14] S. Azarmi, W.H. Roa, R. Lobenberg, Targeted delivery of nanoparticles for the treatment of lung diseases, Adv Drug Deliv Rev, 60 (2008) 863-875.

[15] W. Yang, J.I. Peters, R.O. Williams, 3rd, Inhaled nanoparticles--a current review, Int J Pharm, 356 (2008) 239-247.

[16] L. Zhang, D. Pornpattananangku, C.M. Hu, C.M. Huang, Development of nanoparticles for antimicrobial drug delivery, Curr Med Chem, 17 585-594.
[17] N. Nafee, A. Husari, C.K. Maurer, C. Lu, C. de Rossi, A. Steinbach, R.W. Hartmann, C.M. Lehr, M. Schneider, Antibiotic-free nanotherapeutics: Ultra-small, mucus-penetrating solid lipid nanoparticles enhance the pulmonary delivery and anti-virulence efficacy of novel quorum sensing inhibitors, J Control Release, 192C (2014) 131-140.
[18] J.S. Suk, S.K. Lai, Y.Y. Wang, L.M. Ensign, P.L. Zeitlin, M.P. Boyle, J. Hanes, The penetration of fresh undiluted sputum expectorated by cystic fibrosis patients by non-adhesive polymer nanoparticles, Biomaterials, 30 (2009) 2591-2597.
[19] K. Forier, K. Raemdonck, S.C. De Smedt, J. Demeester, T. Coenye, K. Braeckmans, Lipid and polymer nanoparticles for drug delivery to bacterial biofilms, J Control Release, 190C (2014) 607-623.
[20] M. Bur, A. Henning, S. Hein, M. Schneider, C.M. Lehr, Inhalative nanomedicine--opportunities and challenges, Inhal Toxicol, 21 Suppl 1 (2009) 137-143.
[21] B.C. Tang, M. Dawson, S.K. Lai, Y.Y. Wang, J.S. Suk, M. Yang, P. Zeitlin, M.P. Boyle, J. Fu, J. Hanes, Biodegradable polymer nanoparticles that rapidly penetrate the human mucus barrier, Proc Natl Acad Sci U S A, 106 (2009) 19268-19273.
[22] T. Yu, Y.Y. Wang, M. Yang, C. Schneider, W. Zhong, S. Pulicare, W.J. Choi, O. Mert, J. Fu, S.K. Lai, J. Hanes, Biodegradable mucus-penetrating nanoparticles composed of diblock copolymers of polyethylene glycol and poly(lactic--glycolic acid), Drug delivery and translational research, 2 (2012).
[23] N. Günday Türeli, A.E. Türeli, M. Schneider, Inhalable Antibiotic Nanoformulations for the Treatment of Pseudomonas aeruginosa Infection in Cystic Fibrosis – A Review, Drug Delivery Letters, 4 (2014).
[24] J.-O.H. Sham, Y. Zhang, W.H. Finlay, W.H. Roa, R. Löbenberg, Formulation and characterization of spray-dried powders containing nanoparticles for aerosol delivery to lung, Int J Pharm, 269 (2004) 457-467.
[25] J. Zhang, L. Wu, H. Chan, W. Watanabe, Formation, characterizatio and fate of inhaled drug nanoparticles, Advanced Drug Delivery Reviews, 63 (2011) 441-455.
[26] S. Gill, R. Lobenberg, T. Ku, S. Azarmi, W. Roa, E.J. Prenner, Nanoparticles: characteristics, mechanims of action, and toxicity in pulmonary drug delivery - a review, Journal of biomedical nanotechnology, 3 (2007) 107-119

[27] N. Günday Türeli, A.E. Türeli, M. Schneider, Optimization of ciprofloxacin complex loaded PLGA nanoparticles for pulmonary treatment of cystic fibrosis infections: Design of experiments approach, International Journal of Pharmaceutics, 515 (2016) 343-351.
[28] J.C. Davies, Pseudomonas aeruginosa in cystic fibrosis: pathogenesis and persistence, Paediatric Respiratory Reviews, 3 (2002) 128-134.
[29] J.J. Smith, S.M. Travis, E.P. Greenberg, M.J. Welsh, Cystic fibrosis airway epithelia fail to kill bacteria because of abnormal airway surface fluid, Cell 85 (1996) 229 - 236
[30] J.M. Barichello, M. Morishita, K. Takayama, T. Nagai, Encapsulation of hydrophilic and lipophilic drugs in PLGA nanoparticles by the nanoprecipitation method, Drug Dev Ind Pharm, 25 (1999) 471-476.
[31] F. Danhier, E. Ansorena, J.M. Silva, R. Coco, A. Le Breton, V. Preat, PLGA-based nanoparticles: an overview of biomedical applications, J Control Release, 161 (2012) 505-522.
[32] T. Govender, S. Stolnik, M.C. Garnett, L. Illum, S.S. Davis, PLGA nanoparticles prepared by nanoprecipitation: drug loading and release studies of a water soluble drug, J Control Release, 57 (1999) 171-185.
[33] H.K. Makadia, S.J. Siegel, Poly Lactic-co-Glycolic Acid (PLGA) as Biodegradable Controlled Drug Delivery Carrier, Polymers, 3 (2011) 1377-1397.
[34] H. Sah, L.A. Thoma, H.R. Desu, E. Sah, G.C. Wood, Concepts and practices used to develop functional PLGA-based nanoparticulate systems, Int J Nanomedicine, 8 (2013) 747-765.
[35] N. Günday Türeli, A.E. Türeli, M. Schneider, Counter-ion Complexes for Enhanced Drug Loading in Nanocarriers: Proof-of-Concept and Beyond, International Journal of Pharmaceutics, (2016) 994-1001.
[36] Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard—Ninth Edition, Clinical and Laboratory Standards Institute, Clinical and Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, 2006.
[37] F.G.R. Jonasz M., Light Scattering by Particles in Water – Theoretical and Experimental Foundations, Elsevier, Amsterdam, 2007.
[38] R. Rietscher, C. Thum, C.M. Lehr, M. Schneider, Semi-automated nanoprecipitation-system--an option for operator independent, scalable and size adjustable nanoparticle synthesis, Pharm Res, 32 (2015) 1859-1863.
[39] Y. Son, M. Horng, M. Copley, J.T. Mcconville, Optimization of an In Vitro Dissolution Test Method for Inhalation Formulations, Dissolution Technologies (2010) 6-13.
[40] M.R.C. Marques, R. Loebenberg, M. Almukainzi, Simulated Biological Fluids with Possible Application in Dissolution Testing, Dissolution Technologies (2011) 15-28.

[41] X. Huang, C.S. Brazel, On the importance and mechanisms of burst release in matrix-controlled drug delivery systems, Journal of Controlled Release, 73 (2001) 121-136.
[42] T.G. Park, Degradation of poly(lactic-co-glycolic acid) microspheres: effect of copolymer composition, Biomaterials, 16 (1995) 1123-1130.
[43] R. Rietscher, J.A. Czaplewska, T.C. Majdanski, M. Gottschaldt, U.S. Schubert, M. Schneider, C.-M. Lehr, Impact of PEG and PEG-b-PAGE modified PLGA on nanoparticle formation, protein loading and release, International Journal of Pharmaceutics, 500 (2016) 187-195.
[44] P. Costa, J.M. Sousa Lobo, Modeling and comparison of dissolution profiles, Eur J Pharm Sci, 13 (2001) 123-133.
[45] P.L. Ritger, N.A. Peppas, A simple equation for description of solute release I. Fickian and non-fickian release from non-swellable devices in the form of slabs, spheres, cylinders or discs, Journal of Controlled Release, 5 (1987) 23-36.
[46] D.R. Paul, Elaborations on the Higuchi model for drug delivery, Int J Pharm, 418 (2011) 13-17.
[47] E. Frohlich, E. Roblegg, Mucus as barrier for drug delivery by nanoparticles, Journal of nanoscience and nanotechnology, 14 (2014) 126-136.
[48] S.K. Lai, Y.Y. Wang, J. Hanes, Mucus-penetrating nanoparticles for drug and gene delivery to mucosal tissues, Adv Drug Deliv Rev, 61 (2009) 158-171.
[49] N.N. Sanders, S.C. De Smedt, E. Van Rompaey, P. Simoens, F. De Baets, J. Demeester, Cystic fibrosis sputum: a barrier to the transport of nanospheres, Am J Respir Crit Care Med, 162 (2000) 1905-1911.
[50] E. Fattal, N. Grabowski, S. Mura, J. Vergnaud, N. Tsapis, H. Hillaireau, Lung toxicity of biodegradable nanoparticles, Journal of biomedical nanotechnology, 10 (2014) 2852-2864.
[51] O. Mert, S.K. Lai, L. Ensign, M. Yang, Y.Y. Wang, J. Wood, J. Hanes, A poly(ethylene glycol)-based surfactant for formulation of drug-loaded mucus penetrating particles, J Control Release, 157 (2012) 455-460.
[52] B.S. Schuster, J.S. Suk, G.F. Woodworth, J. Hanes, Nanoparticle diffusion in respiratory mucus from humans without lung disease, Biomaterials, 34 (2013) 3439-3446.
[53] Breakpoint tables for interpretation of MICs and zone diameters, in, The European Committee on Antimicrobial Susceptibility Testing, 2014.
[54] L.R. Peterson, Quinolone molecular structure-activity relationships: what we have learned about improving antimicrobial activity, Clinical infectious diseases : an official publication of the Infectious Diseases Society of America, 33 Suppl 3 (2001) S180-186.

[55] M.C. Birmingham, R. Guarino, A. Heller, J.H. Wilton, A. Shah, L. Hejmanowski, D.E. Nix, J.J. Schentag, Ciprofloxacin concentrations in lung tissue following a single 400 mg intravenous dose, Journal of Antimicrobial Chemotherapy, 43 (1999) 43-48.
[56] H. Stass, J. Nagelschmitz, S. Willmann, H. Delesen, A. Gupta, S. Baumann, Inhalation of a dry powder ciprofloxacin formulation in healthy subjects: a phase I study, Clinical drug investigation, 33 (2013) 419-427.