

Nanoparticulate Drug Delivery Systems for *Pseudomonas aeruginosa* Infected Lungs in Cystic Fibrosis



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Nazende Günday Türeli

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Dekan:

Prof. Dr. G. Kickelbick

Berichterstatter:

Prof. Dr. M. Schneider

Prof. Dr. R.W. Hartmann

Vorsitzender:

Prof. Dr. C.-M. Lehr

Akad. Beisitzer:

Dr. S. Keßler

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SUMMARY

Current pulmonary treatments against *Pseudomonas aeruginosa* infections in cystic fibrosis (CF) lung suffer from deactivation and immobilization of the drug in thick and viscous biofilm/mucus blend, along with the general antibiotic resistance.

The present work suggests pulmonary antibiotic delivery with high load, capable of penetrating the tight mesh of biofilm/mucus as a solution to existing treatment bottlenecks. The potential use of nanoparticulate drug delivery systems to improve the treatment efficiency of lung infections in CF lungs is investigated.

First chapter describes counter-ion complexes as a strategy to enhance drug load and demonstrates its applicability to different antibiotic classes, as well as counter-ions. The second chapter focuses on the drug delivery system development and its optimization via design-of-experiments approach. For the proof-of-concept studies, biodegradable and biocompatible poly (lactic-co-glycolic acid) was suggested and ciprofloxacin was used as model drug substance. MicroJet Reactor (MJR) technology, a precise preparation technique performed under controlled conditions, was employed. Effect of each process parameter was evaluated to ensure quality-by-design. Final chapter is dedicated to physico-chemical and *in vitro* characterization of the optimized nanoparticles.

Overall, the new established approach offers counter-ion complex loaded PLGA NPs as promising pulmonary nano drug delivery system against *P. aeruginosa* infections in CF lung

ZUSAMMENFASSUNG

Behandlungen von *Pseudomonas aeruginosa* Infektionen der Lunge von zystischer Fibrose (CF) Patienten, leiden unter Deaktivierung und Immobilisierung des Medikaments in dicken und viskosen Biofilm / Schleim Mischungen, zusammen mit der allgemeinen Antibiotika-Resistenz.

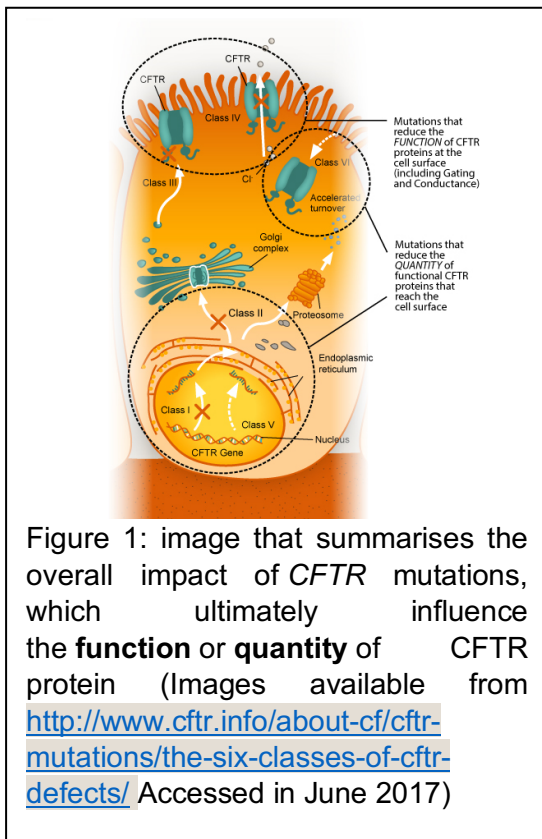
Die vorliegende Arbeit schlägt eine pulmonale Antibiotika Verabreichung mittels Nanopartikeln mit hoher Wirkstoffbeladung vor, die in der Lage sind, das enge Maschenwerk von Biofilm / Schleim zu durchdringen, als eine Lösung für bestehende Behandlungsengpässe. Die potenzielle Verwendung von nanopartikulären *Drug Delivery*-Systemen, zur Verbesserung der Behandlungseffizienz von Lungeninfektionen bei Patienten mit CF, wird untersucht.

Das erste Kapitel beschreibt Gegenion-Komplexe als Strategie zur Verbesserung der Wirkstoffbeladung und zeigt ihre Anwendbarkeit auf verschiedene Antibiotika-Klassen sowie Gegenionen. Das zweite Kapitel konzentriert sich auf die Entwicklung der *Drug Delivery*-Systeme sowie deren Optimierung durch einen *Design-of-Experiments* Ansatz. Für die Machbarkeitsstudien wurde das biologisch abbaubare und biokompatible Poly (lactid-co-glycolid) eingesetzt und Ciprofloxacin als Modell-Wirksamsubstanz verwendet. Die Mikrojetreaktor (MJR) Technologie wurde dafür eingesetzt, da sie eine präzise Herstellung unter kontrollierten Bedingungen ermöglicht. Die Auswirkung jedes Prozessparameters wurde evaluiert, um *quality-by-design* sicherzustellen. Das abschließende Kapitel widmet sich der physikalisch-chemischen und *in vitro*-Charakterisierung der optimierten Nanopartikel.

Insgesamt bietet der neu etablierte Ansatz, von Gegenion-Komplex beladenen PLGA-Nanopartikeln, vielversprechende pulmonale Nano-*Drug Delivery*-Systeme gegen *P. aeruginosa*-Infektionen in Lungen von CF-Patienten.

I. OBJECTIVES

Recent analysis shows that there are already 1400 types of nanoparticles (NPs) available in the world market today¹. Current developments in nanotechnologies applied to medicine (Nanomedicine) have shown that engineered drug delivery systems with the size order of nanometers are intriguing tools, which are capable of solving unmet problems in healthcare.



Cystic fibrosis (CF) is a rare, genetic chronic disease, with its onset occurring in early childhood. The life-span of patients with CF is reported as only 30-40 years [1].

Chronic pulmonary infections, among which *Pseudomonas aeruginosa* is known to be the major pathogen, are reported to be the main cause of mortality among CF patients [2]. Current available CF therapy targets the disease symptoms and not its causation; mainly due to six recognized classes of mutations of cystic fibrosis transmembrane regulator gene (*CFTR*), which

result in an abnormal production of mucus in the lungs (Figure 1). In addition to airway clearance therapies, 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 mucoidic bacteria [3-7]. However, once infection is established in the airways, it is almost impossible to eradicate it. The established biofilm anchors the bacteria to

¹ <http://www.researchandmarkets.com/research/kv6mm5/nanotechnology>

OBJECTIVES

their environment, protects the bacteria and prevents drugs reaching minimum inhibition concentration at the site of action to kill bacteria [8].

Pulmonary drug delivery offers solutions to above mentioned problems, such as avoiding first pass effect, reduced systemic side effects, delivering higher doses at the site of action thus increased local concentration [9], higher bioavailability partially because of relatively low enzymatic activity [10], increasing patient compliance and being a non-invasive drug delivery method [11]. And may be the most important of all, for respiratory diseases such as CF, pulmonary drug delivery ensures local delivery. 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 is intended for the treatment of chronic *P. aeruginosa* infections in CF lungs based on a systematic scientific rationale. Main focus of this dissertation is the development and production of the nanoparticles with the microjet reactor technology to be later introduced into the final dry powder inhalation formulation with an adequate mass median aerodynamic diameter (MMAD) to ensure drug deposition at the upper airways, where the bacteria reside, as shown in Figure 2.

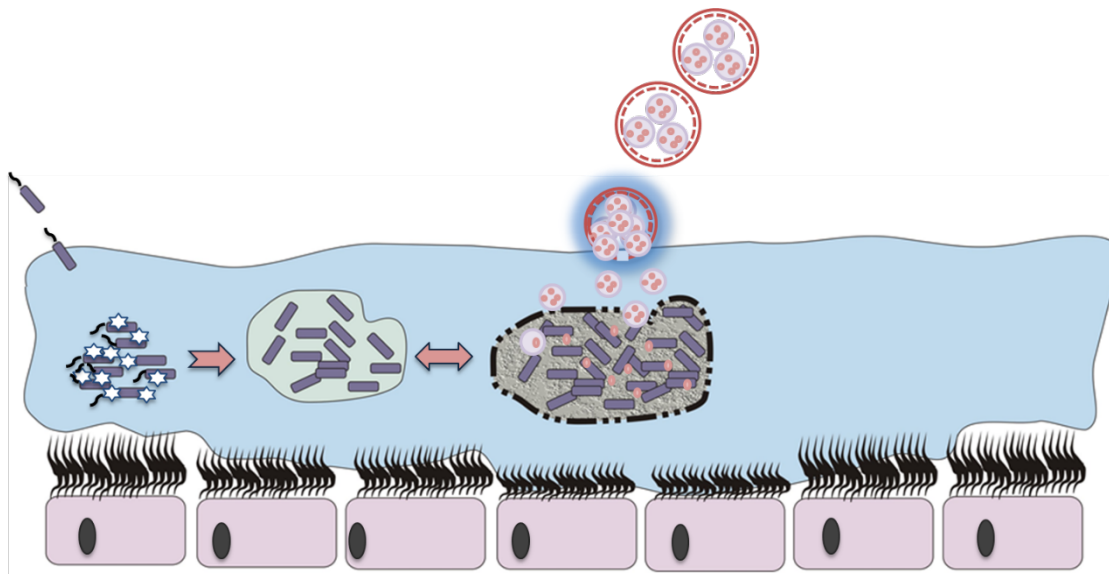


Figure 2: Graphical depiction of the suggested pulmonary drug delivery system for the treatment of chronic *P. aeruginosa* infections in CF

OBJECTIVES

In order to improve the effectiveness of the antibiotics, nanoparticulate dosage forms are to be used in this thesis. The main goal, therefore, is to encapsulate antibiotics with the aid of nanotechnology for the treatment of *P. aeruginosa* infection in patients with CF. These nanocarriers can be further formulated to be inhaled directly into the lungs. Nebulizers or powder inhalers suitable for this purpose are already established on the market in various forms which allow the production of aqueous or solid aerosol particles in the optimum range (MMAD approx. 1-5 μm) for a deposition in the peripheral lung. Nanoparticles can interfere with the pathophysiologically thickened mucus layer and the tailored nanoparticles in a favored size may enable penetration to the thick mucus/biofilm network. Therefore, particles should be engineered to possess particle size smaller than this 3-dimensional complex CF mucus/biofilm meshes, and to prevent adsorption only onto the biofilm and entrapment in the biofilm via surface properties. Thus, higher concentrations of the antibiotics to be used (e.g., gentamycin, tobramycin or ciprofloxacin) can be achieved for a longer period of time at the actual site of action.

For preparation of nanoparticles with such complexity, conventional preparation techniques might suffer from serious disadvantages, such as lack of control on nanoparticle preparation and batch-to-batch reproducibility problem [12, 13]. Although the precipitation represents the universal and inexpensive method for the production of small particles, with the available precipitation methods, particles with pre-set size could not be produced reliably in a reproducible manner.

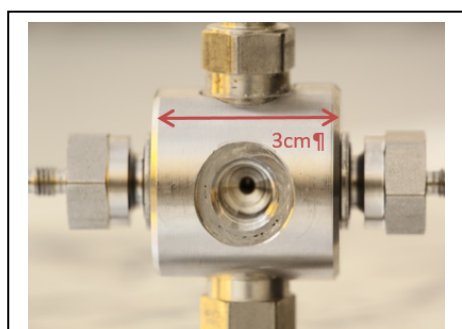


Figure 3: A closer look into the microjet reactor

Therefore, the microjet reactor technology offering advantages, such as continuous manufacturing under fully controlled conditions, to overcome the bottlenecks of conventional methods was employed for preparation of this highly sophisticated drug delivery system. This means a considerable advantage in terms of a controllable particle size, a particle size distribution as

narrow as possible, as well as an easy scaling-up possibility of the process.

OBJECTIVES

Microjet reactor with confined impinging jet principle, enables very high-quality of homogeneously distributed particle production. Combined with statistical experimental planning, efficient development of highly specialized, customized particles is realized. In general, microjet reactor produced nanoparticles possess narrow particle size distribution in comparison to traditional methods, since shorter mixing times (up to < 0.1 ms) and micro mixing can be achieved by microjet reactor. Thus, homogenous dissipation of the entire energy in micro volume is facilitated. Reaction times faster than crystal growth time result in smaller particle sizes and homogenous particle size distribution under controlled conditions. Additionally, in microjet reactor, with the high flow velocity, impinging jets leading to turbulence provide further efficient micro-mixing and shorter nanoparticle formation times. Thus, fast equilibrium establishment conditions and large surface to volume ratio are generated, and the need for additional stabilization is minimized.

In order to be able to meet the requirements listed above, the overall project is divided into three sections:

First section focuses on development of a strategy to encapsulate antibiotics in a suitable carrier at sufficient concentrations. An intensive literature survey was performed to understand the CF disease conditions, treatment bottlenecks and challenges, as well as innovative approaches that have been investigated by other researchers [14]. It was concluded that the NPs should possess surface properties that might prevent adsorption onto the biofilm surface and entrapment in the biofilm and avoid fast clearance. And a controlled release mechanism to ensure drug release at the site of action was preferred. All those expectations could have been realized by poly(lactic-co-glycolic) acid (PLGA), an FDA approved, biocompatible and biodegradable polymer. Considering the current therapy guidelines and clinical studies on-going, fluoroquinolone antibiotic ciprofloxacin was assigned as model drug.

Encapsulation of antibiotics showing pH dependent solubility characteristics along with limited organic solvent solubility was a challenge regardless of the applied preparation techniques.

OBJECTIVES

The drug can neither be dissolved in a common organic solvent due to low solubility nor in pH-adjusted aqueous solvents due to the *in vitro* degradation of the PLGA by acidic and alkaline environment, which is well-known to be accelerated under such conditions [15, 16]. Thus, drug loading was very low. To enhance drug load in nanocarriers, counter-ion complexes was developed and its applicability to different antibiotic classes, as well as counter-ions was demonstrated [17].

The second chapter focuses on the drug delivery system development and its optimization via design-of-experiments approach. In addition to reaching the site of absorption and enhancing the deposition, especially in case of lung diseases like CF, where structural abnormalities are observed, particles should be engineered for an improved interaction. Considering these ultimate goals we can foresee that nanoparticles, owing to their small size, with custom made surfaces may pass the thick mucus layer easily through the gaps within the mesh structured biofilms and reach the bacteria without being trapped in the thick mucus and biofilm upon pulmonary drug delivery [14]. For the proof-of-concept studies, PLGA was employed and ciprofloxacin-SDS complex was used as model drug substance. Effect of each MJR process parameter was evaluated to ensure quality-by-design.

Final chapter is dedicated to physico-chemical and *in vitro* characterization of the optimized nanoparticles. NPs` 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, stability have been evaluated to ensure sustainability of the developed particles.

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II. SCIENTIFIC BACKGROUND

Inhalable Antibiotic Nanoformulations for the Treatment of *Pseudomonas aeruginosa* Infection in Cystic Fibrosis

Drug Delivery Letters, 2014, 4, 193-207

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Inhalable Antibiotic Nanoformulations for the Treatment of *Pseudomonas Aeruginosa* Infection in Cystic Fibrosis – A Review

Nazende Günday Türeli^{1,2}, Akif Emre Türeli¹ and Marc Schneider^{2*}

¹MJR PharmJet GmbH, Unikliniken des Saarlandes, Starterzentrum (Geb.7), D-66424 Homburg, Germany;

²Pharmaceutics and Biopharmacy, Philipps University Marburg, D-35032 Marburg, Germany

Keywords: Antibiotic loaded nanoparticles, bacterial infection, nanoparticles, *Pseudomonas aeruginosa*, pulmonary nanoparticulate, drug delivery.

Online available at:

<http://www.eurekaselect.com/120558/article>

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ABSTRACT

Cystic fibrosis (CF), a genetic lethal chronic disease, causes the body to produce abnormally thick and sticky mucus. As a consequence following bacterial infections threaten ten thousands of people around the world every year. Available tests enable early diagnosis of CF. Nevertheless, current treatments can only serve to improve patient's quality of life. Despite the fact that life span of CF patients is dramatically increased with comprehensive treatments during the last decades, there is no ultimate prevention or cure for CF. Chronic respiratory infections are known to be the major cause of morbidity and mortality. Since the airways provide direct access to these bacteria, it is an attractive target for drug delivery against bacterial infections in CF lung. Current pulmonary treatments are, however, limited since reaching the site of action is highly inhibited by the biofilm, which establishes an efficient obstacle for drug diffusion. Recent developments in nanotechnology have led many researchers to study different types of nanoparticles and nanoformulations for pulmonary drug delivery in the scope of providing a solution to current treatment bottlenecks. This review focuses on the development of nanoparticulate antibiotic pulmonary drug delivery systems for the treatment of *Pseudomonas aeruginosa* infected CF lungs.

III. SCIENTIFIC OUTCOME

CHAPTER 1: Counter-ion Complexes

Günday Türeli N., Türeli A.E., Schneider M., Counter-ion Complexes for Enhanced Drug Loading in Nanocarriers: Proof-of-Concept and Beyond, Int J Pharm, 2016, 511, 994-1001

Online available at: <https://doi.org/10.1016/j.ijpharm.2016.08.004>

CHAPTER 2: Process Optimization

Günday Türeli N., Türeli A.E., Schneider M., Optimization of Ciprofloxacin Complex Loaded PLGA Nanoparticles for Pulmonary Treatment of Cystic Fibrosis Infections: Design of Experiments Approach, Int J Pharm, 2016, 515, 343–351.

Online available at: <https://doi.org/10.1016/j.ijpharm.2016.10.025>

CHAPTER 3: Characterization of Ciprofloxacin Complex Loaded PLGA Nanoparticles

Günday Türeli N., Torge A., Juntke J., Schwarz B.C., Schneider-Daum N., Türeli A.E., Lehr CM., Schneider M., Ciprofloxacin-loaded PLGA nanoparticles against Cystic Fibrosis P. aeruginosa Lung Infections, Eur. J. Pharm. Biopharm.117, 2017, 363-371

Online available at: <https://doi.org/10.1016/j.ejpb.2017.04.032>

CHAPTER 1: COUNTER-ION COMPLEXES

International Journal of Pharmaceutics 511 (2016) 994–1001



Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm



Counter-ion complexes for enhanced drug loading in nanocarriers: Proof-of-concept and beyond



Nazende Günday Türel^{a,b}, Akif E. Türel^a, Marc Schneider^{b,*}

^aMJR PharmJet GmbH, Industriestr. 1B, 66802 Überherrn, Germany

^bDepartment of Pharmacy, Biopharmaceutics and Pharmaceutical Technology, Saarland University, 66123 Saarbrücken, Germany

Keywords: Counter-ion complex, Enhanced encapsulation, Nanomedicine

Online available at:

<https://doi.org/10.1016/j.ijpharm.2016.08.004>

Chemical compounds studied in this article: Acetonitrile (PubChem CID: 6342); Ciprofloxacin (PubChem CID: 2764); Dimethyl sulfoxide (PubChem CID: 679); 1,2-Dipalmitoyl-sn-glycero-3-phosphatidic acid, sodium salt (PubChem CID: 643979); Gentamicin (PubChem CID: 3467); Pluronic® F68 (PubChem CID: 24751); Poly(DL-lactide-co-glycolide) (PubChem CID: 71391); sodium dodecyl sulfate (PubChem CID: 3423265); Tobramycin (PubChem CID: 36294)

²Abbreviations

ACN, Acetonitrile; DMSO, Dimethyl sulfoxide; DPPA, 1,2-Dipalmitoyl-sn-glycero-3-phosphatidic acid, sodium salt; DSC, Differential scanning calorimetry; EE, Encapsulation efficiency; FTIR, Fourier transform infrared spectroscopy; HPLC, High pressure liquid chromatography; MJR, Microjet reactor; NP, Nanoparticle, PDI, Polydispersity index; PLGA, Poly(lactic-co-glycolic) acid; SDS, sodium dodecyl sulfate; XRD, X-Ray diffractometry.

ABSTRACT

Enhanced drug loading is important prerequisite of nanomedicines, to reach administration dose while reducing the amount of excipient. Considering biocompatible and biodegradable polymers such as PLGA, pH dependent solubility characteristics along with limited organic solvent solubility of the drug hampers nanoparticle (NP) preparation. To improve loading of such molecules, a method based on using counter ions for complex formation is proposed. Formed complex alters the intrinsic solubility of active substance via electrostatic interaction without chemical modification. A proof-of-concept study was conducted with sodium dodecyl sulfate as counter-ion to fluoroquinolone antibiotic ciprofloxacin. Complex formation resulted in suppressed pH dependent solubility over pH 1.2 to 9.0 and an additional -80 fold increase in organic solubility was achieved. In consequence, NPs prepared by microjet reactor technology have shown enhanced drug loading efficiencies (- 78%) and drug loading of 14%. Moreover, the counter-ion concept was also demonstrated with another class of antibiotics, water soluble aminoglycosides gentamycin and tobramycin. In addition, the counter ion was substituted by degradable excipients such as phosphatidic acid derivatives. Successful implementation has proven the counter-ion concept to be a platform concept that can be successfully implemented for a variety of active substances and counter-ions to enhance drug loading in nanocarriers.

CHAPTER 2: PROCESS OPTIMIZATION

International Journal of Pharmaceutics 515 (2016) 343–351



Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm



Optimization of ciprofloxacin complex loaded PLGA nanoparticles for pulmonary treatment of cystic fibrosis infections: Design of experiments approach



Nazende Günday Türel^{a,b,*}, Akif Emre Türel^a, Marc Schneider^b

^a MJR Pharmjet GmbH, Industriestr. 1B, 66802 Überherrn, Germany

^b Biopharmaceutics and Pharmaceutical Technology, Department of Pharmacy, Saarland University, 66123 Saarbrücken, Germany

Keywords: Antibiotic-loaded nanoparticles, Design-of-experiments, Process optimization, Quality-by-design

Online available at:

<https://doi.org/10.1016/j.ijpharm.2016.10.025>

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)

ABSTRACT

Design of Experiments (DoE) is a powerful tool for systematic evaluation of process parameters` effect on nanoparticle (NP) quality with minimum number of experiments. DoE was employed for optimization of ciprofloxacin loaded PLGA NPs for pulmonary delivery against *Pseudomonas aeruginosa* infections in cystic fibrosis (CF) lungs. Since the biofilm produced by bacteria was shown to be a complicated 3D barrier with heterogeneous meshes ranging from 100 nm to 500 nm, nanoformulations small enough to travel through those channels were assigned as target quality. Nanoprecipitation was realized utilizing MicroJet Reactor (MJR) technology based on impinging jets principle. Effect of MJR parameters flow rate, temperature and gas pressure on particle size and PDI was investigated using Box-Behnken design. The relationship between process parameters and particle quality was demonstrated by constructed fit functions ($R^2=0.9934$ $p < 0.0001$ and $R^2=0.9983$ $p < 0.0001$, for particle size and PDI, respectively). Prepared nanoformulations varied between 145.2 and 979.8 nm with PDI ranging from 0.050 to 1.00 and showed encapsulation efficiencies $> 65\%$. Response surface plots provided experimental data-based understanding of MJR parameters` effect, thus NP quality. Presented work enables ciprofloxacin loaded PLGA nanoparticle preparations with pre-defined quality to fulfill the requirements of local drug delivery under CF disease conditions.

CHAPTER 3: CHARACTERIZATION OF COMPLEX LOADED PLGA NANOPARTICLES

European Journal of Pharmaceutics and Biopharmaceutics 117 (2017) 363–371



Contents lists available at ScienceDirect

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Research paper

Ciprofloxacin-loaded PLGA nanoparticles against cystic fibrosis *P. aeruginosa* lung infections



Nazende Günday Türeli^{a,b}, Afra Torge^b, Jenny Juntke^c, Bianca C. Schwarz^d, Nicole Schneider-Daum^c,
Akif Emre Türeli^a, Claus-Michael Lehr^{b,c}, Marc Schneider^{b,*}

^a MJR PharmJet GmbH, Industriestr. 1B, 66802 Überherrn, Germany

^b Department of Pharmacy, Biopharmaceutics and Pharmaceutical Technology, Saarland University, 66123 Saarbrücken, Germany

^c Helmholtz Institute for Pharmaceutical Research Saarland (HIPS), Helmholtz Center for Infection Research (HZI), Saarland University, Saarbrücken, Germany

^d Pferdewirtschaft Altforweiler, Raiffeisenstraße 100, 66802 Überherrn, Germany

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

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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)

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%. Release 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 \gg 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.

IV. CONCLUSION

Chronic lung infections are reported to be the main cause of mortality among cystic fibrosis (CF) patients. During the last decades, in addition to airway clearance techniques, high doses of nebulized and inhaled antibiotics have been applied extensively for treatment of early infections as a preventive action against the mucoidic bacteria. However, once the infection is established in the airways, it is almost impossible to eradicate it. CF onset occurs in early childhood and the life-span of patients with CF is reported as only 30-40 years.

The aim of the study was the development of a drug delivery system for the treatment of chronic *P. aeruginosa* infections in CF based on a systematic scientific rational. Under such disease conditions, a local nanoparticle drug delivery system which is capable of penetrating the thick mucus and biofilm, releasing the antibiotic in a controlled manner at the site of action was considered as a scientific basis for the design of a (nano-)particulate drug delivery system for antibiotic drugs.

Main focus of this dissertation was the development and production of the necessary nanoparticles with the microjet reactor technology to be later introduced into a final drug delivery system. The tailor made nanoparticles in a favored size might play a decisive role for the effect at certain target sites in the body, since they may enable penetration to the thick mucus/biofilm network.

In this thesis, a novel approach, counter-ion method, was developed for enhanced drug loading in nanocarriers by altering the solubility of the small molecules, and efficiency of the approach was proven for different classes of antibiotics and counter-ions. Relying on that approach, antibiotic-complex loaded poly (lactic-co-glycolic acid) (PLGA) nanoparticles were prepared by utilizing microjet reactor technology, which allows full control over the manufacturing process. Optimization of those nanoparticles for

- i. *particle size* to minimize size exclusion in meshes of cystic fibrosis lung mucus/biofilm blend,

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- ii. *narrow PDI* to ensure homogeneity

were achieved via design of experiments by optimization the process parameters, whereas,

- iii. *mobility in mucus/biofilm blend* to deliver the drug to the site of action
- iv. *high drug load and controlled release kinetics* to ensure sustained antibiotic concentration at the site of action

were achieved by screening different formulation components and compositions. Finally, performance of optimized antibiotic-complex loaded PLGA nanoparticles were proven in in - vivo simulating environment, as well as horse lung mucus.

In the first section, a strategy to enhance the drug load in nanocarriers was aimed, since early development stages revealed that drug loading into PLGA nanocarrier was hampered, when the drug shows pH dependent solubility characteristics. Neither use of a common organic solvent nor use of buffered solutions were possible due to solubility and stability limitations of both ciprofloxacin and PLGA. In order to overcome those bottlenecks, counter-ion method that relies on electrostatic interaction between oppositely charged small molecule and counter-ion was suggested as solubility modulator to suppress the pH dependent solubility characteristics, thus enhance the drug loading. For the proof-of-concept studies, ciprofloxacin-sodium dodecyl sulfate complex was prepared and fully characterized. Complex formation was confirmed via HPLC, spectral and thermal analysis, as well as X-Ray diffraction. In addition to suppressing pH depending solubility of ciprofloxacin, solubility studies revealed a very pronounced effect on organic solubility and led to 80-fold increase. By this way, a common solvent for both ciprofloxacin and the nanocarrier could be identified without a negative effect on PLGA stability. Occurrence of the complex formation without a chemical modification was proven via similarity analysis of the ciprofloxacin and ciprofloxacin-complex UV-spectra after dissolving in aqueous solutions of wide pH range, proving dissociation of complex to its components upon dissolving. This finding was confirmed by HPLC purity analysis, where no partition difference was observed (same retention time for both complex and ciprofloxacin) and 3D-comparison (in

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dimensions of time-wavelength-absorbance) of the spectra was indicating only ciprofloxacin detection in aqueous environment. Additionally, antibacterial activity of ciprofloxacin complex against lab strains of *Pseudomonas aeruginosa* was significantly enhanced in comparison to free ciprofloxacin.

Applicability of the counter-ion complexes to different antibiotic classes, as well as counter-ions were demonstrated in two case studies. In the first case study, ciprofloxacin complex was prepared by another counter ion, dipalmitoyl phosphatidic acid (DPPA), a precursor of de novo synthesized lung surfactant phosphatidyl choline. It was shown that ciprofloxacin-DPPA complex could be formed by slight modification of the counter-ion approach, allowing solubilization of counter-ion DPPA in an organic solvent. Ciprofloxacin-DPPA complex formation was proven via X-Ray diffraction, thermal and spectral analysis and solubility of the formed complex was characterized in organic solvents. Results showed altered and enhanced organic solvent solubility in accordance with the output of original approach. Antibacterial activity of both ciprofloxacin complexes gathered using SDS and DPPA against lab strains of *P. aeruginosa* were significantly enhanced in comparison to free ciprofloxacin. In the second case study, tobramycin and gentamicin, antibiotics belonging to aminoglycoside classes, complexes were prepared with sodium dodecyl sulfate as counter-ion without any modification of the original approach and successful complex formation was proven via thermal analysis. At the end of first section it was proven that counter-ion complexes enabled altered solubility characteristics without chemical or activity modification and are applicable to a variety of substances/antibiotic classes and counter-ions. Considering that the unlimited combinations of substances with a variety of counter-ion complexes, it was concluded that this approach was not only beneficial for pharma industry but also offering a great potential for nutraceuticals and cosmetics. It also can be employed for tailoring the complexes depending on the administration route (e.g. complex with deoxycholic acid for oral administration etc.).

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The second aim was the drug delivery system development and its optimization. Quality target attributes of the particles were defined as;

- i. particle size; small enough to penetrate through the thick mucus and bacterial biofilm blend, the biobarrier of CF, without being immobilized
- ii. PDI; narrow particle size distributions to ensure that the whole nanoparticle population has uniform sizes
- iii. drug load; high enough to reach the application dose, in other words, therapeutic concentration range at the site of action
- iv. surface stabilization; appropriate stabilizer properties to minimize undesired interactions with the cystic fibrosis biobarrier, hence facilitate penetration

Microjet reactor technology, a precise preparation technique performed under controlled conditions, was employed for continuous particle production. Microjet reactor offers unlimited scale-up since same process parameters can be used for both lab scale and industrial scale manufacturing of nanoparticles. Microjet reactor enables control over the whole process parameters and environment, and turbulent-like micromixing in a gas filled chamber caused by impinging jets provides efficient production for high quality nanoparticles. Nanoparticle quality can be theoretically defined as a function of parameters that govern the whole process. Thus, effect of each process parameter (flow rate, temperature, applied gas pressure) was evaluated to ensure quality-by-design via design of experiments. Particle size and PDI were assigned as target quality prerequisites to facilitate mobility in CF lung local microenvironment. Model fit function, correlating the process parameters and the particle size and PDI, were constructed. Evaluation of the response surface graphs elucidated that all process parameters significantly influenced the particle size and PDI. When two jet streams imping, a disc is formed at the impinging point, where micromixing is achieved. It was concluded that if the mixing time is faster than the nucleation induction time, further improvement in the mixing efficiency via higher flow rates wouldn't affect the particle size and PDI. It was shown that the hydro-dynamics in the impingement disc, where too high flow rates were employed, led to non-optimal mixing conditions, resulted in a reduced residence time in the microjet reactor, thus

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uncontrolled nucleation and eventually higher particle sizes. Temperature was also shown to be significantly affecting the nanoparticle quality and to be within an appropriate range for the exothermic nanoprecipitation process. An increase in temperature caused decrease in particle size due to reduced viscosity of both phases, contributing positively to mixing efficiency and the faster longitudinal movements of both solvent and solute at the mixing interface. On the other hand, an increase in applied gas pressure resulted in higher particle sizes and PDI. This observation was attributed to the distorted impinging disc upon application of high pressures of gas, thus uncontrolled nucleation and particle size growth. The process parameters yielding nanoparticle preparation with the desired quality attributes were extracted from the fit functions. The ability of the model fit function to predict those quality attributes was validated with normal probability plot. Successful validation of the constructed fit functions enabled defining the microjet reactor parameters within the predefined design space to fine-tune quality of nanoparticles.

The final aim was physico-chemical and in-vitro characterization of the optimized nanoparticles for prediction of their in-vivo performance. Since encapsulation was designed for protecting the antibiotic from hostile microenvironment in CF lungs leading to deactivation before it can reach the bacteria, mucus interaction of the particles was evaluated by employing horse lung mucus. Horse lung mucus was chosen as an alternative model, due to very limited access to human lung mucus. Spectral analysis showed that upon interaction with the nanoparticles the mucus network was undergoing a change, optical density of the mucus network was decreasing immediately after mixing and the dense structure was becoming leakier. And this effect was concentration dependent: as the nanoparticle concentration in the mucus increased the disaggregation grade of the mucus increased, as well. These findings were supported by cryo-SEM analysis: mucus incubated with NP showed structural differences in comparison to control mucus. However, it is worth mentioning that sample preparations and sample handling in cryo-SEM requires very intensive method development and due to limited availability of mucus, it was not possible to evaluate the effect in detail. Particle size and zeta potential of

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the nanoparticles did not show any significant change after mucus incubation without an indication of agglomeration. Particles were shown to be stable in mucus environment. Evaluation of the nanoparticle-mucus interaction with those two in-vitro methods suggested the mobility of ciprofloxacin complex loaded PLGA nanoparticles in mucus and capability of penetrating through the mucus owing to their surface properties, size and ZETA potential. If the disaggregation of the network is caused by the nanoparticles repelling the mucus components due to surface properties or due to reduced number of obstacles in the penetration path as a result of corona effect is worth investigating, since this might provide an important know-how for next generations of drug delivery systems developed for treatment of bacterial infections in CF lungs. Finally, the antibacterial activity of complex loaded PLGA nanoparticles were tested against lab strains of *P. aeruginosa*. It was found out that the ciprofloxacin complex loaded PLGA nanoparticles were more effective against bacteria than the free ciprofloxacin and this enhanced antibacterial activity was not related to any synergic effect from other nanoparticle components probably due to better penetration of nanoparticles through agar plates. Furthermore, a controlled drug release reaching to maximum 80% in 8 hours was shown in in-vivo simulating media with dissolution studies and it was found out that the release kinetics were driven by the polymer, indicating successful encapsulation of the active substance in polymer preventing burst effect.

In this thesis, a local nano drug delivery system capable of penetrating the thick mucus and biofilm, showing stability in this microenvironment and releasing antibiotic in a controlled manner at the site of action for treatment of cystic fibrosis bacterial lung infections was presented. High drug loading of the nanoparticles was achieved by innovative counter-ion method, which enabled drug loading up to 14%. On the other hand, it is known that antibiotics are delivered at high dosis is due to low drug concentration at the site of action which is limited by the disease condition. Considering the enhanced antibacterial activity of the complex and complex loaded PLGA nanoparticles, taken together with the ability of the particles to carry the complete drug load directly to the infection area might reduce the application dosis. However,

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this is an expectation that requires further formulation of nanoparticles for possessing right mass median aerodynamic diameter to ensure deposition at the upper airways, where the bacteria reside, in-vivo profiling and testing. Regardless of how high drug loading was achieved, early studies on formulation of those nanoparticles via lyophilization with mannitol was not very promising due to very low antibiotic loaded PLGA nanoparticle: mannitol ratio. Mannitol was assigned as formulation excipient since it offers a very broad range of improvements to the formulation as an excipient. It is already approved as mucolytic and an integrated part of CF therapy in the form of capsules for inhalation. Additionally, it enhances penetration and uptake [1], and serves as C-source to mucoid bacteria at depleted nutrition conditions, thus eventually might be a trigger for favoring the conditions for planktonic bacteria [2]. Once optimum formulation conditions are realized, pulmonary administration of nanoformulations with higher drug loading is a promising tool for treatment of infections.

Thanks to translational research and clinicians actively working on advance in the treatment, today life span of a CF patient is dramatically increased over the last decades, however there is no cure to the disease. Considering that the pulmonary infections are the major cause of morbidity in CF, a very intensive and personalized care is required to keep the airways clear and prevent chronic infections. Pulmonary nano antibiotic delivery systems, as the one developed and presented in this thesis, with high load, capable of penetrating thick mucus and sustaining the local concentrations may offer many advantages to CF bacterial infection treatment, thus increase life quality of the patients and might even prolong the life span.

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NAZENDE GÜNDAY TÜRELI

Date of birth: 10.11.1981 **Place of birth:** Ordu/Turkey

EDUCATION

2012-Present **Saarland University**, Saarbrücken, Germany

PhD candidate in Pharmacy

2010-2012 **Saarland University**, Saarbrücken, Germany

M.Sc. in Biotechnology

1999-2004 **Bogazici University**, Istanbul, Turkey

B.Sc. in Chemistry

EXPERIENCE

11/2014 - Present **MJR PharmJet GmbH**, Überherrn, Germany

R&D Team Leader

10/2010 – 11/2014 **MJR PharmJet GmbH**, Homburg, Germany

Scientist

08/2009 – 09/2010 **Across Barriers GmbH**, Saarbrücken, Germany

Project Leader

08/2008 – 09/2009 **Across Barriers GmbH**, Saarbrücken, Germany

Scientist

07/2004 – 07/2008 **Abdi Ibrahim Pharmaceuticals A.S**, Istanbul, Turkey

R&D Specialist

SCIENTIFIC CONTRIBUTIONS

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