Co-delivery of ciprofloxacin and colistin using microcontainers for bacterial biofilm treatment

Birk, Stine Egebro; Mazzoni, Chiara; Mobasharah Javed, Madeeha; Borre Hansen, Morten; Krogh Johansen, Helle; Anders Juul Haagenzen, Janus; Molin, Søren; Hagner Nielsen, Line; Boisen, Anja

Published in:
International Journal of Pharmaceutics

Link to article, DOI:
[10.1016/j.ijpharm.2021.120420](https://doi.org/10.1016/j.ijpharm.2021.120420)

Publication date:
2021

Document Version
Peer reviewed version

[Link back to DTU Orbit](#)

Citation (APA):
Birk, S. E., Mazzoni, C., Mobasharah Javed, M., Borre Hansen, M., Krogh Johansen, H., Anders Juul Haagenzen, J., Molin, S., Hagner Nielsen, L., & Boisen, A. (2021). Co-delivery of ciprofloxacin and colistin using microcontainers for bacterial biofilm treatment. *International Journal of Pharmaceutics*, 599, [120420]. <https://doi.org/10.1016/j.ijpharm.2021.120420>

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Co-delivery of ciprofloxacin and colistin using microcontainers for bacterial biofilm treatment

Stine Egebro Birk^{a}, Chiara Mazzoni^a, Madeeha Mobasharah Javed^a, Morten Borre Hansen^b, Helle Krogh Johansen^{d,e}, Janus Anders Juul Haagensen^c, Søren Molin^c, Line Hagner Nielsen^a and Anja Boisen^a*

^aThe Danish National Research Foundation and Villum Foundation's Center for Intelligent Drug Delivery and Sensing Using Microcontainers and Nanomechanics (IDUN), Department of Health Technology, Technical University of Denmark, Ørstedes Plads 345C, 2800 Kongens Lyngby, Denmark

^bNovo Nordisk Foundation Center for Intestinal Absorption and Transport of Biopharmaceuticals, Department of Health Technology, Technical University of Denmark, Produktionstorvet 423, 2800 Kongens Lyngby, Denmark

^cNovo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, Kemitorvet 220, 2800 Kongens Lyngby, Denmark

^dDepartment of Clinical Microbiology, Section 9301 Copenhagen University Hospital Rigshospitalet, Henrik Harpestrengs Vej 4A, Copenhagen Ø 2100, Denmark

^eDepartment of Clinical Medicine Faculty of Health and Medical Sciences University of Copenhagen, Blegdamsvej 3B, Copenhagen N 2200, Denmark

*Corresponding author

Stine Egebro Birk
Ørstedes Plads 345C
2800 Lyngby
stegha@dtu.dk
+45 28 45 01 97

Abstract

In many infected patients, bacterial biofilms represent a mode of growth that significantly enhances the tolerance to antimicrobials, leaving the patients with difficult-to-cure infections. Therefore, there is a growing need for effective treatment strategies to combat biofilm infections. In this work, reservoir-based microdevices, also known as microcontainers (MCs), are co-loaded with two antibiotics: ciprofloxacin hydrochloride (CIP) and colistin sulfate (COL), targeting both metabolically active and dormant subpopulations of the biofilm. We assess the synergistic effect of the two drugs in a time-kill study of planktonic *P. aeruginosa* and find that co-loaded MCs are superior to monotherapy, resulting in complete killing of the entire population. Biofilm consortia of *P. aeruginosa* grown in flow chambers were not fully eradicated. However, antibiotics in MCs work significantly faster than simple perfusion of antibiotics (62.5 ± 8.3 % versus 10.6 ± 10.1 % after 5 h) in biofilm consortia, showing the potential of the MC-based treatment to minimize the use of antimicrobials in future therapies.

Graphical abstract

1. Introduction

Antibiotic resistance is one of the greatest challenges facing the global healthcare system (Davies and Davies, 2010). Microbes are developing resistance at an alarming rate, while the ‘golden era’ of antibiotic discovery is long over (O’Neill, 2016). This leaves us with a clear demand for novel strategies to combat multi-drug resistant (MDR) bacterial infections (Parish, 2019).

Besides from MDR, it has become evident that the ability of bacteria to organize themselves into matrix-enclosed aggregates, also known as biofilm, is responsible for most chronic bacterial infections (Hall-Stoodley et al., 2004). In fact, it has been shown that the biofilm-lifestyle renders the bacteria with a 10-1,000-fold higher tolerance towards antibiotics than their planktonic counterparts (Mah and O’Toole, 2001; Stewart, 2002). Gradients of nutrients and oxygen exist from the top to the bottom of the biofilm, with oxygen levels being high at the surface and low in the center of the biofilm. Similarly, metabolic activity is stratified with high activity at the surface and low or no growth in the center (Ciofu and Tolker-Nielsen, 2019; Pamp et al., 2008). As the antimicrobial effect of antibiotics often depends on the metabolic activity of the bacteria, this dormancy-phenomenon is one of the explanations for the reduced susceptibility of biofilm-associated bacteria towards antibiotics (Høiby et al., 2010). Consequently, monotherapy with antibiotics, which are only active against growing cells, leads to a reduction of bacteria without complete eradication of the biofilm (Anwar and Costerton, 1990).

Combining antibiotics with different cellular targets is widely recognized as a useful strategy to increase the likelihood of achieving early adequate bacterial eradication of infections, where monotherapy has failed, and at the same time minimizing the risk of resistance development (Tamma et al., 2012; Traugott et al., 2011). Ciprofloxacin is a fluoroquinolone antibiotic showing broad activity mainly against Gram-negative bacteria, such as *Pseudomonas aeruginosa* that are associated with debilitating infections in the airways (Bjarnsholt, 2011; Schwerdt et al., 2018) and wounds (Pastar et al., 2013). Ciprofloxacin exhibits bacteriostatic and bactericidal effects by inhibiting the DNA gyrase and topoisomerase IV enzymes essential for DNA replication (Aldred et al., 2014; Silva et al., 2011), thus targeting mainly the actively dividing cells found in the outer edges of the biofilm (Høiby et al., 2010). The cyclic cationic lipopeptide, colistin, belonging to the family of polymyxins, has recently received raising attention because of its significant effect on MDR Gram-negative bacteria (Li et al., 2006; Nation et al., 2015). Colistin binds to the lipid A component in lipopolysaccharides disordering the cell membrane structure, making it more permeable (a detergent-like effect), which ultimately results in cellular death (Bialvaei and Samadi Kafil, 2015). This means that colistin kills dormant bacterial subpopulations found in the core of the biofilm (Haagensen et al., 2007; Pamp et al., 2008). The use of colistin was originally hampered due to its serious nephrotoxicity, but the limited therapeutic options have driven its revival as a last line of defense against severe *P. aeruginosa* infections (Li et al., 2006). Colistin has shown attractive synergistic antimicrobial activity against *P. aeruginosa* when combined with antibiotics such as azithromycin (Lin et al., 2015), rifampicin (Giamarellos-Bourboulis et al., 2003), and ciprofloxacin (Buyck et al., 2015).

83 Recently it was documented that liposomes co-loaded with ciprofloxacin and colistin showed an increase
84 in of the antimicrobial efficacy against *P. aeruginosa* compared to monotherapies, while, at the same
85 time, showing no toxicity towards the pulmonary epithelial cells (Chai et al., 2019; Wang et al., 2018; Yu
86 et al., 2020). Liposomes are one example of a particulated drug delivery system, which are gaining
87 considerable attention to improve the therapeutic efficacy of antibiotic treatments (Gao et al., 2018).
88 By using particulates to deliver antibiotics, a sufficiently high drug concentration can be achieved at the
89 site of infection, while reducing the administered dose, keeping systemic toxicity at a minimum as well
90 as limiting the risk of development of resistance (Liu et al., 2019). However, many of these formulations
91 suffer from poor drug loading capabilities as well as from restrictions concerning which drugs that can
92 be encapsulated into the particles.

93
94 Recent and promising approaches have focused on reservoir-based polymeric microdevices serving as
95 drug carriers for oral drug delivery (Nielsen et al., 2018). The microdevices have shown a promising
96 potential towards prolonging the retention of drugs at the site of absorption as well as providing a higher
97 local drug concentration (Ainslie et al., 2009; Chirra et al., 2014; Nielsen et al., 2016). Previously, we
98 have proposed microcontainers (MCs), which are polymeric cylindrical structures, as a means to deliver
99 high loads of antibiotics to the site of infection (Birk et al., 2020). Compared to other drug delivery
100 systems, such as liposomes, the MCs possess the unique capability of allowing delivery of any antibiotic
101 no matter the size, charge or lipo-/hydrophilicity. Therefore, the ratio between co-loaded antibiotics can
102 easily be adjusted as desired. Moreover, MCs have demonstrated mucoadhesive properties as they can
103 embed and engulf in mucus (a habitat which biofilms reside in) (Dalskov Mosgaard et al., 2019; Mazzoni
104 et al., 2017; Nielsen et al., 2016). Moreover, adhesion was shown to be further promoted by coating
105 the MCs with a lid of chitosan (Mazzoni et al., 2019). Previously we have shown that local delivery of
106 ciprofloxacin hydrochloride (CIP) confined in MCs resulted in eradication of *P. aeruginosa* biofilm equal
107 to a constant flow of an about three times higher concentration of the solubilized antibiotic. However,
108 10-20 % of the biomass remained alive despite treatment with CIP (Birk et al., 2020).

109
110 The aim of the present study was to exploit the potential of using MCs for co-delivery of synergistic
111 antibiotics as a more efficient treatment of *P. aeruginosa* biofilms compared to monotherapies. CIP and
112 colistin sulfate (COL) were co-loaded in MCs and coated with a mucoadhesive coating of chitosan. *In*
113 *vitro* release kinetics were characterized together with time-kill investigations of planktonic *P.*
114 *aeruginosa* and compared to antibiotic monotherapy. Lastly, the impacts of co-loaded MCs were
115 investigated on biofilm consortia of *P. aeruginosa* and compared to single-loaded MCs and simple
116 antibiotic perfusion.

2. Materials and Methods

2.1. Materials

Silicon (Si) wafers (4" (100) n-type) were acquired from Okmetic (Vantaa, Finland), while the SU-8 constituents (SU-8 2075 and SU-8 Developer) were purchased from Micro Resist Technology (Berlin, Germany). Ciprofloxacin HCl (CIP) was from Fagron (Uitgeest, The Netherlands), while colistin sulfate (COL), chitosan (low MW 50-190 kDa, 75-85 % deacetylation), acetic acid, Luria Bertani (LB) medium, potassium phosphate, disodium phosphate, sodium chloride, calcium chloride, ammonium sulfate, magnesium chloride, trace metals, glucose, propidium iodide and glutaraldehyde (25 % solution) were all bought from Sigma-Aldrich (St. Louis, MO USA). Osmium tetroxide (4 % in water) was acquired from ACROS Organics. Ultrapure water was obtained from a Q-POD® dispenser (Merck Millipore, Burlington, MA, USA).

2.2. Fabrication of MCs

MCs were fabricated on silicon wafers using a mask-based two-step photolithography process, an approach originally introduced for drug delivery devices by Tao *et al.* and later modified by Nielsen *et al.* (Nielsen *et al.*, 2012; Tao *et al.*, 2007). Starting out with clean Si substrates, a release layer consisting of 5 nm titanium (Ti) and 20 nm gold (Au) was deposited (Temescal FC-2000, Ferrotec Corporation, Santa Clara, CA, USA) using electron beam evaporation. The release layer ensured adequate adhesion of the MCs to the chip during loading and coating, while simultaneously enabling the detachment of MCs from the chip without damaging them (Nielsen *et al.*, 2016). Subsequently, the Ti/Au-Si wafers were covered with a layer of the negative epoxy photoresist SU-8 and exposed to a number of baking steps allowing the formation of the bottom and the side walls of the MCs. After fabrication, the wafers were diced (Automatic Dicing Saw DAD 321, DISCO, Tokyo, Japan) into squared chips (12.8 × 12.8 mm²), with each chip containing 625 individual MCs. The inner and outer diameters of the individual MCs were determined with an Eclipse L200 bright-field optical microscope (Nikon, Tokyo, Japan), whereas the inner and outer heights were evaluated by vertical scanning interferometry using a PLu Neox 3D Optical Profiler (Sensofar, Terrassa, Spain).

2.3. Loading of CIP and COL into MCs and coating with chitosan

Prior to drug loading, a shadow mask was aligned on top of the chip containing MCs in order to minimize the amount of drug powder being distributed into the gaps between the MCs, as previously described (Abid *et al.*, 2017).

For the co-loading, CIP and COL powders were mixed in a 1:8 w/w ratio. CIP:COL or pure COL powder was loaded into MCs using a centrifuge method previously described (Jørgensen *et al.*, 2019). In brief, the powder was distributed on top of the mask and the chip was subsequently centrifuged in a flat-bottomed Falcon tube using a Heraeus Megafuge 16R Centrifuge (Thermo Fisher Scientific, Waltham, MA, USA) at 3720 g for 120 s at room temperature. Following this, the chip was removed from the Falcon tube and additional powder was embossed into the cavity of the MCs by applying a pressure of 0.49·10⁻¹ Torr for 15 s with a compact digital pressure controlled electric crimper-MSK-160E (MTI Corporation,

Richmond, CA, USA). The centrifugation and compression steps were conducted twice to ensure adequate drug loading. Afterwards, the shadow mask was removed and any excess drug around the MCs was gently removed using pressurized air. CIP was loaded into the MCs using only compression. The chips were weighed before and after loading to quantify the amount of loaded drug. Drug loading capacity was calculated as the amount of loaded drug relative to the weight of a drug-loaded MC. CIP:COL- and COL-loaded chips were stored in the freezer at -18 °C until usage to ensure drug stability, whereas CIP-loaded chips were stored at room temperature.

Functionalization of the drug-loaded MCs was achieved by spray coating a lid of a chitosan solution over the chip using an Exacta Coat Ultrasonic Spray System (Sonotek, USA) with an accumist nozzle operating at 120 kHz. The chitosan solution was prepared by dissolving 0.5 % w/v chitosan in 0.1 M acetic acid, heated overnight at 50°C and subsequently, filtered using a 5-13 µm filter with vacuum suction. Each chip was coated with two alternating spray paths having an offset of 2 mm, resulting in a total of 90 passages for CIP:COL and COL, and 120 passages for CIP. To facilitate solvent evaporation, the plate underneath the chip was heated to 50°C. Generator power was kept at 1.3 W, path speed at 25 mm/s and infusion rate at 0.1 mL/min. The applied shaping air pressure was 0.020 bar for CIP:COL and for COL MCs, and 0.028 bar for CIP-loaded MCs. The distance between the spray nozzle and the sample was set to 5.5 cm.

The quality of the loading and the coating of the MCs was evaluated using a tabletop scanning electron microscope (SEM) (Hitachi High-Technologies Europe GmbH, Krefeld, Germany). The MC chips were placed on a 30° tilted holder and SEM images were acquired using the scattered electron (SE) detector and an accelerating voltage of 15 kV.

2.4. Characterization of *in vitro* drug release from MCs using LC-MS

The *in vitro* release from co-loaded MCs was investigated using liquid chromatography-mass spectrometry (LC-MS), and the MCs loaded with either CIP or COL were utilized as controls. A chip containing 625 MCs was diced into mini-chips containing 100-110 MCs using a diamond cutter. The mini-chip was placed in a Falcon™ tube containing 5 mL FAB medium (buffered minimal medium used for bacterial growth; for preparation see **Supp. Materials, Table S1**) at 100 rpm and 37°C. Samples of 200 µL were taken at specified time points over the course of 48 h and replaced with 200 µL blank FAB medium. Samples with drug concentrations above the upper limit of detection for the LC-MS assay were diluted with FAB medium as appropriate. All samples were stored at -18 °C prior to the analysis.

Standards (0-50 µM CIP or COL) and samples were analyzed with a Shimadzu Nexera X2/Prominence HPLC (Shimadzu Europe, Duisburg, Germany) and ESI micrOTOF-Q III (Bruker Daltonics, Bremen, Germany) LC-MS setup. The LC was performed by a 5 µL injection of the analyte on a Poroshell 120 SB-C8 column, 2.7 µm, 2.1x50 mm (Agilent, Santa Clara, CA, USA) followed by elution with a linear gradient of MeCN and 2.5 mM NH₄OH in water with 0.1 % formic acid (from 0 to 100 % over 9 min) at a flow rate of 0.4 mL/min. The chromatographic front (1.75 min) was diverted to waste, while the remaining run was injected into the ESI micrOTOF-Q III mass spectrometer. A calibration solution consisting of 2.5 mM

NaOH, 2.25 mM formic acid in 90 % *i*-PrOH/water was injected into the ion source between 1.75 and 1.85 min at a flowrate of 30 μ L/h for internal calibration of the spectra. MS analysis was performed in positive mode in the range of 50-3,000 m/z at a rate of 2 Hz. For COL, a capillary potential of 5,000 V was employed, the nebulization pressure was 1.5 bar and the drying gas flow was set to 3 L/min at 180°C. For CIP, the parameters were 4,500 V, 0.3 bar and 4 L/min at 200°C. All mass spectra were analyzed with the software QuantAnalysis (Bruker Daltonics, Bremen, Germany) to generate extracted ion chromatograms for 332.16 $\pm$ 0.05 m/z (for CIP) and 1155.7 $\pm$ 0.1 m/z (for COL) and the resulting peaks were integrated.

All experiments were carried out in 3-6 replicates and data were normalized to 100 % in relation to the measured amount of drug release after 48 h.

2.5. Bacterial strains and overnight culturing

For all bacterial studies, a *P. aeruginosa* PAO1 strain (Klockgether et al., 2010) genetically modified to express green fluorescent protein (GFP) constitutively by insertion into a neutral intergenic region in the genome was used (Holloway and Morgan, 1986; Klausen et al., 2003; Stover et al., 2000). Overnight cultures of PAO1 were grown in lysogeny broth (LB) at 150 rpm at 37°C for 18-20 h.

2.6. Time-kill studies of planktonic *P. aeruginosa* using co-loaded MCs

For studying the ability of co-loaded MCs to inhibit bacterial growth, an inoculation of PAO1 overnight culture was diluted to a final concentration of approximately 1x10⁶ cells/mL. As controls, single loaded MCs were used in the same set-up. To each vial containing 10 mL culture, treatment was added as either uncoated MCs, coated MCs or antibiotic solution (for concentrations see **Figure 1**). PAO1 in LB without treatment and pure LB were used as negative control. The number of added MCs varied according to the difference in antibiotic loading per MC (determined per individual chip) and the minimum inhibitory concentration (MIC) values of the antibiotics (aiming for reaching 4xMIC for each antibiotic). Vials were stored at 150 rpm and 37 °C during the course of the experiment. Samples were taken at 0, 3, 8 and 24 h, and viable counts were obtained by serial dilution followed by spot plating on LB agar plates. The plates were left to incubate overnight at 37 °C and colony forming units (CFU) were counted the following day.

Figure 1. Schematic presentation of the production of microcontainers (MCs) and the conducted experiments on *P. aeruginosa* PAO1. **1)** Dimensions of fabricated MCs (mean±SD, n=6-12), co-loading or single-loading of ciprofloxacin hydrochloride (CIP) and/or colistin sulfate (COL) followed by coating with chitosan. **2)** MCs were tested against PAO1 cultures in shaken LB medium. Samples were taken over time for counting the colony forming units (CFU/mL). MCs were tested both with and without chitosan coating and compared to treatment with antibiotic in solution. See table for applied concentrations. Scanning electron microscopy (SEM) was applied as a visual assessment of bacterial killing after 24 h. **3)** MCs were tested against PAO1 in biofilm mode of growth using flow-chambers and with confocal microscopy monitoring. See table for applied concentrations.

2.7. SEM observation of *P. aeruginosa* cells post treatment with MCs

A qualitative study was performed to investigate the ability of the coated CIP:COL, CIP or COL MCs to inhibit biofilm formation on glass slides over 24 h. Microscopic glass slides were cut into smaller rectangles of approximately 3x7 mm size using a diamond cutter. The glass slides were sterilized in ethanol for 20 min, left to air dry and subsequently, mounted onto vials with Pressure Sensitive Adhesive (PSA) tape. Growth and treatment were conducted as described in Section 2.6.

Sample fixation was completed as previously described by Weber *et al.* (Weber et al., 2014). In brief, the samples were covered with 2.5 % glutaraldehyde for 2 h, washed three times in phosphate buffered saline (PBS) and then transferred to wells containing 0.5 mL 1 % osmium tetroxide for 1 h. Thereafter,

the samples were dehydrated in solutions with increasing ethanol percentages (35 %, 50 %, 75 %, 2x90 %, and 2x100 %) for 30 min in each solution. After the last dehydration step, the samples were placed in a critical point dryer (Leica EM CPD300 Critical point dryer, Germany) for 2 h to allow further dehydration of the sample without causing structural changes to the bacteria. Lastly, the samples were placed in a desiccator overnight.

Prior to SEM analysis, the biofilm samples on glass slides were coated with a thin conducting layer of gold (Quorum Q150T ES Coater, Quorum Technologies, Lewes, United Kingdom) to prevent sample charging with the electron beam. Tooling factor was 2.3. Sputter current was kept at 20 mA and a sputter time of 65 s were used, whereas the clean current was 60 mA and clean time of 30 s. The stage was rotating at a speed of 30 rpm. SEM images were acquired using a QFEG 200 Cryo-ESEM (FEI Company, USA) using the Everhart-Thornley (ETD) detector with high vacuum mode and a voltage of 5 kV.

2.8. Growth of *P. aeruginosa* biofilms in flow chambers and treatment with antibiotics

2.8.1. Growth of PAO1 biofilms

The PAO1 biofilm growth was conducted at 37°C under laminar flow in self-fabricated flow chambers. The flow chambers were fabricated by milling in a 6 mm thick sheet of polycarbonate as previously described (Tolker-Nielsen and Sternberg, 2011). Additionally, they were redesigned with an inlet channel on the side of the flow chamber to which a small piece of silicon tubing was glued, allowing for inoculation of MCs (Birk et al., 2020). A sterile system was achieved by pumping 1 L of 0.5 % v/v hypochlorite through the system over a period of 4 h followed by a cleaning procedure with filling and emptying the system three times with 2 L of autoclaved MilliQ water. The system was perfused overnight with modified FAB minimal medium with trace metals (for preparation see **Supp. Materials, Table S1**) at 58.4 µL/min with a 16-channel Watson Marlow pump. The flow was stopped, and the tubings were clamped to avoid backflow and sterilized with 70 % v/v ethanol on the outside. Thereafter, 250 µL of a diluted overnight culture (with 0.9 % NaCl to OD₆₀₀ of 0.05) was inoculated carefully into the flow chamber and resealed with silicone glue. Flow chambers were left without flow for 1 h to allow bacterial attachment to the glass surface, before medium flow was resumed. Bacterial biofilms were allowed to develop for 72 h before antibiotic treatment.

2.8.2. Treatment with antibiotics

Treatment with CIP and COL were conducted in two ways: I) as confined in coated MCs (either as co-loaded or as single-loaded as controls), or II) as a constant perfusion. 70 MCs were added to each flow chamber, and constant perfusion was achieved by adding CIP and/or COL to the medium bottle (see **Figure 1** for concentrations). To allow inoculation of MCs, a solution of 2.5% w/v gelatin (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was used to disperse the microcontainer in the syringe instead of sticking to the walls of the syringe as described previously (Birk et al., 2020). Biofilms were treated for 24 h, after which images were acquired at a distance of 140-320 µm from the open side of the MCs and in case of perfusion at random locations in the flow chamber.

2.8.3. Microscopy parameters, image acquisition and analysis

Biofilms were monitored using an inverted Leica TCS SP8 CLSM (Mannheim, Germany) equipped with an argon/krypton laser and detectors and filter set for sequential monitoring of GFP (excitation wavelength: 488 nm, emission range: 493-558 nm) for live cell imaging and propidium iodide (excitation wavelength: 543 nm, emission range: 558-700 nm) for dead cell staining. Images were obtained in z-intervals of 1 μ m using an HC PL Apo CS2 63x oil objective (numerical aperture 1.4). PAO1 expressed GFP consecutively, and propidium iodide had to be injected into the bubble trap. As control, four biological experiments were performed acquiring two images (technical replicates) of non-treated biofilm in each experiment at random positions within the flow chamber. For each treatment, 3-5 biological replicates with 3-5 technical replicates were acquired. Stacked images and 3D images were generated using Imaris software (Version 7.7.1, Bitplane AG, Zürich, Switzerland). Volume of biomass was calculated using the image analysis software COMSTAT version 2.1 (Heydorn et al., 2000; Vorregaard, 2008). Graphs depicting the fraction of live/dead biomass were generated by calculating the percentage of live/dead in relation to the total biomass measured at the specified time-point.

2.9. Statistical analysis

Data are expressed as the mean $\pm$ SD, unless otherwise stated. For comparison of two individual mean values an unpaired t-test was applied, whereas a one-way ANOVA with Tukey's multiple comparison was used if more than two mean values were compared. Graphs and tests were conducted in GraphPad Prism (Version 8.0.1, GraphPad Software, CA, USA) and p-values were considered statistically significant when below 1 % ($p < 0.01$).

3. Results and Discussion

3.1. Loading of antibiotics into MCs and coating depositing

MCs were produced with good reproducibility in the desired dimensions (see **Figure 1** for dimensions). For the co-loading of CIP:COL, we chose a 1:8 w/w ratio based on the MIC values of the individual antibiotics towards PAO1 cells (0.125 μ g/mL for CIP (Soares et al., 2019); 1 μ g/mL of COL (Bergen et al., 2010)). The loading resulted in 3.85 $\pm$ 0.37 μ g/MC CIP:COL, 5.16 $\pm$ 0.54 μ g/MC CIP or 3.32 $\pm$ 0.57 μ g/MC COL ($n=12-18$, mean $\pm$ SD), corresponding to a drug loading capacity of 14.6-21.0 % w/w. Efficient loading was confirmed with SEM (**Figure 2A** and **Supp. Materials, Figure S1**). After drug loading, the cavity of the MCs was coated with a layer of chitosan, serving to promote bioadhesion. Moreover, chitosan is known to contribute to the killing of *P. aeruginosa* (Perinelli et al., 2018). The coating of CIP:COL- and COL-loaded MCs resulted in a uniform, fully covering lid, whereas the coating of CIP-loaded MCs was less uniform, a tendency also previously observed (Birk et al., 2020) (**Figure 2B** and **Supp. Materials, Figure S1**).

Figure 2. Scanning electron microscope (SEM) images of microcontainers (MCs) co-loaded with ciprofloxacin hydrochloride and colistin sulfate (1:8 w/w) (A) and subsequently coated with chitosan (B). (1-column figure)

3.2. *In vitro* drug release from MCs

The release of CIP and/or COL from chitosan-coated MCs was evaluated at 37 °C in FAB medium at pH 6.8 (Figure 3) (for release from uncoated MCs, see Supp. Materials Figure S2).

The co-loaded chitosan-coated MCs released their entire COL cargo within the first 30 min reaching a release of 98.7 ± 5.7 %. This was similar to what was observed from the single-loaded COL MCs (105.2 ± 8.8 %), and co-loading therefore did not influence the release profile of COL. The release of CIP was significantly accelerated from the co-loaded MCs (79.0 ± 6.4 % within 30 min) as compared to when CIP was single-loaded (13.8 ± 1.2 % within 30 min). This tendency was also observed in a previous study with liposomes, where incorporation of COL significantly accelerated the CIP release compared to liposomes loaded only with CIP (Wang et al., 2018). Sustained release from chitosan particles is a well-known phenomenon (Mohammed et al., 2017) and has previously been shown for CIP-loaded chitosan NPs (Patel et al., 2019) and CIP- or lysozyme loaded MCs (Birk et al., 2020; Mazzoni et al., 2019). The chitosan lid is shown to swell due to the hydrogel properties of chitosan (Ahmadi et al., 2015) creating pores which allows diffusion of CIP and COL. The diffusion rate depends on the hydrophilicity of the drugs, and the different release profiles can therefore be attributed to the higher aqueous solubility of COL (50 mg/ml (Wang et al., 2016)) compared to CIP (varying solubilities reported: 0.17-30 mg/mL (Olivera et al., 2011; Ross and Riley, 1994; Varanda et al., 2006)). Moreover, it is well known that surfactants influence the degree of hydrogel swelling and can provide micellar solubilization of poorly water-soluble drugs (Gunathilake et al., 2018; Wallace et al., 2010). As COL is amphiphilic in nature, we believe that COL served as a surfactant, affecting the swelling of chitosan and the associated drug release. In the current study, COL constituted approximately 90 % of the co-loaded MC (due to the large difference in MIC values of CIP and COL). Seemingly, the water soluble and fast-releasing COL drove the release of the otherwise less soluble CIP.

Figure 3. *In vitro* release of ciprofloxacin hydrochloride (CIP) and colistin sulfate (COL) from co-loaded or single-loaded microcontainers (MCs) coated with chitosan. Release study was performed at 37 °C in FAB medium at pH 6.8 and quantified by LC-MS. Data was normalized to 100 % and presented as mean±SD, n=3-4. (2-column figure)

3.3. Time-kill of planktonic *P. aeruginosa* using co-loaded MCs

The capabilities of the drug-loaded and coated MCs to inhibit and kill planktonic bacteria were investigated by adding MCs to a growing population of *P. aeruginosa* (**Figure 4**) (for effect of uncoated MCs, see **Supp. Materials Figure S3**). Treatment with CIP:COL, CIP or COL significantly reduced bacterial growth compared to the PAO1 growth control ($p \leq 0.0001$).

For co-loaded MCs, growth inhibition was increased compared to the monotherapies. The three types of treatments for the co-loaded MCs (uncoated, coated and solution) resulted in similar viable counts, which is in accordance with the rapid release of CIP:COL from the MCs (**Figure 3** and **Supp. Materials Figure S2**). After 8 h, no growth was observed except for the coated MCs (note: only 1 out of 4 replicates showed growth). At 24 h, no growth was observed for any of the treatments.

When treating with CIP in MCs or in solution, the maximum killing was reached at 8 h and regrowth was observed at 24 h, indicating a possible resistant subpopulation. No differences was observed between CIP in solution or confined in MCs throughout the 24 h period ($p \geq 0.36$). Presumably, the starting bacterial density was sufficiently low to allow a similar inhibition despite the sustained release observed with the chitosan coating (**Figure 3**).

Similar reductions in viable counts were observed when treating with COL confined in MCs or in solution, which correlates with the release data showing that all COL was released within the first 30 min. In contrast to CIP treatment, treating with COL generally did not give rise to a significant difference ($p \geq 0.02$) between the viable counts after 3, 8, and 24 h, thus indicating that the amount of COL was sufficient to prevent further regrowth.

Altogether, the combination CIP:COL showed greater inhibition effect on PAO1 after 24 h compared to single-loaded MCs, a synergistic effect of the two antibiotics reaching levels where all subpopulations were killed with no regrowth. The synergistic mechanism of CIP and COL on planktonic cells is not yet completely understood, but may be attributed to the ability of COL to enhance the uptake of CIP by destabilizing the outer membrane of the Gram-negative bacterium (Buyck et al., 2015). Recently, Yu *et al.* treated two COL-resistant *P. aeruginosa* strains with 8 mg/L CIP and 2 mg/L COL delivered in a liposomal formulations and found an enhanced *in vitro* antimicrobial activity compared to the monotherapies (Yu et al., 2020). However, one strain showed regrowth after 24 h despite treating with the two antibiotics, whereas the other only showed a 2-log reduction. In our study (treating PAO1 with 0.5 mg/L CIP: 4 mg/L COL), we observed a full eradication, and the difference may be attributed to the selection of the strain or the applied ratio of antibiotics.

Figure 4. Inhibition of planktonic growth of *P. aeruginosa* (PAO1) over time when co-delivering ciprofloxacin hydrochloride (CIP) and colistin sulfate (COL) in chitosan-coated microcontainers (MCs) or as antibiotic solution. As controls, treatment with CIP or COL alone (as coated MCs or antibiotic solutions) as well as a positive control for PAO1 growth were included. Data is presented as mean±SD (n=3-6). (2-column figure)

3.4. SEM observation of *P. aeruginosa* cells post treatment with MCs

SEM imaging of *P. aeruginosa* biofilms supported the viable counting results. Non-treated biofilms grew unevenly across the glass slide with certain areas containing huge and thick clusters (**Figure 5A**). These clusters were not found in the MC-treated samples, but instead single bacteria or small clusters were spread almost uniformly across the glass surface. However, the bacteria exposed to co-loaded and coated MCs were clearly affected by the treatment, and morphological changes appeared to correlate

well with the mechanism of action of the individual antibiotics. For the co-loaded CIP:COL MCs, the predominant effect on bacterial morphology originated from COL as cellular debris and deformed bacteria were observed (**Figure 5B**), yet, single bacteria did also appear elongated as evident from the zoom-in.

When the biofilm was treated with CIP-loaded MCs, the bacteria appeared elongated, which is in accordance with the mechanism of CIP, inhibiting DNA replication and ultimately cellular division (**Figure 5C**) (Aldred et al., 2014). Also, the membrane disordering effect of COL (Bialvaei and Samadi Kafil, 2015) was evident in the samples treated with COL-loaded MCs, as extracellular material could be observed between the bacteria (**Figure 5D**). Moreover, the cell length appeared to be diminished and the bacteria to be collapsed. This is in accordance with previous results from the investigation of the effect of COL on the cellular structure of *P. aeruginosa* using atomic force microscopy (Mortensen et al., 2009). They observed that after only 3 h of COL treatment, the cells had almost completely lost their morphological structure. The visual assessment after treatment with CIP:COL MCs revealed that COL had the biggest impact on the bacteria, a tendency which is probably a consequence of two factors. Firstly, that the co-loaded CIP:COL MCs contained significantly more COL than CIP, and secondly, that COL quickly destabilizes the membrane thereby, evading the cellular elongation as otherwise observed after the treatment with only CIP.

Figure 5. Scanning electron microscopy (SEM) images of a *P. aeruginosa* PAO1 biofilm grown for 24 h and **A)** left without antibiotic treatment, **B)** treated with coated co-loaded ciprofloxacin hydrochloride (CIP) and colistin sulfate (COL) MCs, **C)** CIP MCs and **D)** COL MCs. Scale bars on the inserts are 10 μm . (2-column figure)

3.5. Eradication of pre-formed *P. aeruginosa* biofilms

The combination of COL targeting the metabolically inactive subpopulations with CIP being active against the metabolically active subpopulation has been shown to enable eradication of *P. aeruginosa* biofilms *in vitro* (Pamp et al., 2008). As previously reported, MC-based delivery increased the local antibiotic concentration, thus improving eradication (Birk et al., 2020). In this study, we evaluated the effect of co-delivering CIP and COL in MCs on biofilm eradication. Before treatment, biofilms were grown for 72 h resulting in a biomass of $5.39 \pm 3.31 \mu\text{m}^3/\mu\text{m}^2$ ($n=43$).

CIP:COL co-loaded in MCs worked significantly faster than CIP:COL in solution, as a much larger fraction of the biomass was found dead after 5 h ($62.5 \pm 8.3\%$ versus $10.6 \pm 10.1\%$, $p \leq 0.0001$), which is also evident from the confocal images (**Figure 6**). We believe this effect is due to the burst release of the antibiotics from the MCs (**Figure 3**) creating an immediate high local drug concentration and ultimately more dead biomass. After 24 h, $69.6 \pm 13.8\%$ and $74.1 \pm 20.4\%$ of dead biomass was found for CIP:COL in MCs and

for CIP:COL in solution, respectively, showing no significant difference (p-value of 0.4653). Interestingly, the biomass did not regrow after 24 h, showing, that despite all drug being released within 30 min from the MCs, a long-lasting effect still applies.

COL in MCs worked significantly faster than COL in solution (56.3 ± 12.3 % versus 3.7 ± 5.1 % dead biomass after 5 h, $p \leq 0.0001$) (**Supp. Materials, Figure S4**). No regrowth occurred after 24 h, and confocal images revealed dead biomass only in the core of the biofilm cluster. Notably, COL in MCs gave rise to a larger dead biomass than COL in solution (56.8 ± 17.4 % versus 29.2 ± 18.0 %, $p \leq 0.0001$), which may indicate that less COL could be used to achieve the same bacterial killing.

Treatment with CIP monotherapy for 5 h and 24 h resulted in a dead biomass of 14.1 ± 10.8 % and 81.9 ± 9.8 % with CIP in MCs and 26.8 ± 13.9 % and 85.8 ± 10.5 % with CIP in solution, showing no statistical difference ($p \geq 0.05$, **Supp. Materials, Figure S4**) despite the local and sustained CIP release observed from the MCs. As evident from the confocal cross-section (**Supp. Materials, Figure S4**), CIP killed the bacteria in the periphery of the biofilm clusters. Previously, we reported that 88.2 ± 5.3 % of the biomass was killed, when exposing the biofilm to MCs loaded with about one-third of the CIP dose administered in the present study (~ 120 μg versus 336 μg) (Birk et al., 2020). This is very interesting, as no further improvement in the bacterial killing was found despite the increased concentration of CIP. This proves that CIP-loaded MCs are effective even with less antibiotic used. However, they are not able, despite increasing the dose, to eradicate the remaining fraction, which might be due to a dormant or resistant subpopulation not affected by CIP. To address this subpopulation, we aimed at delivering a synergistic CIP and COL therapy, but, as evident from the present study, no full biofilm eradication occurred neither with the solutions nor when confined in MCs.

Only few studies have been published employing drug delivery carriers for co-delivery of CIP and COL, and these all involve liposomes (Chai et al., 2019; Wang et al., 2018; Yu et al., 2020). This focus on liposomes is likely due to their biocompatibility combined with a unique property allowing fusion with bacterial phospholipid bilayers, whereby channels to release their antimicrobial cargo directly into the intracellular space of the bacterium are created (Forier et al., 2014). However, liposomes often suffer from a poor drug loading capacity, which is usually much lower than 10 % w/w (Lee, 2020), and as a result, the quantity of drug is not sufficient to reach therapeutic levels in the body, or the amount of the carrier material required is too high which may cause undesirable side-effects (Couvreur, 2013).

This is the first time that two synergistic antibiotics have been delivered in microfabricated devices such as MCs. The drug loading capacity of the applied MCs varied between 14.6-21.0 % w/w, being considerably higher than loading values reported for liposomes. Our results clearly prove that the antibiotics confined in MCs works significantly faster than the antibiotic solutions. This shows the potential of the MCs in delivery of immediate high local concentrations of multiple antibiotics at the site of infection. Future studies will include a detailed investigation of the influence of varying antibiotic concentrations as well as treatment with antibiotics with other mechanisms of actions. Pamp *et al.* showed that a combined treatment with CIP and COL was able to kill almost all cells in a *P. aeruginosa* biofilm as less than 10 cell/mL survived the combinational treatment compared to 3.80×10^5 - 2.25×10^7

cells/mL on average in separately treated biofilm (Pamp et al., 2008). The concentrations of applied antibiotics were significantly higher than the concentrations applied in this study (60 µg/mL CIP and 25 µg/mL COL versus 4 µg/mL CIP and 2.5 µg/mL COL) and a full eradication may therefore be achieved by increasing the drug concentrations. Moreover, to achieve full biofilm eradication other combinational strategies may be included such as incorporation of agents that degrade the extracellular matrix, promoting disruption of biofilms. Such a mechanism might promote phenotypical alterations making the otherwise tolerant dormant cells more susceptible.

Figure 6. 72 h old *P. aeruginosa* (PAO1) biofilm treated with ciprofloxacin hydrochloride and colistin sulfate (CIP:COL) confined in MCs coated with chitosan or as a solution. Quantitative analysis of the biomass ($\mu\text{m}^3/\mu\text{m}^2$) converted to the fraction of live/dead (%) cells. Data is depicted as mean+SD ($n=4-5$ biological replicates with 3-5 technical replicates for each type of treatment). Representative confocal laser scanning microscopy (CLSM) images of the biofilm before treatment, and 5 and 24 h post treatment with CIP:COL in MCs or as a solution. Green represents live bacteria and red shows dead bacteria. Scale bars: 30 µm. (2-column figure).

4. Conclusion

Previous studies utilizing MCs have primarily focused on delivery of single drug entities. In this study, we exploited the potential of co-delivery of two synergistic antibiotics, namely CIP and COL using MCs. We demonstrated that the activity of the antibiotics was retained while loaded in MCs. Combining CIP:COL in MCs showed greater effects on the killing of planktonic *P. aeruginosa* than the monotherapies, thus

the two antibiotics synergistically killed all subpopulations as no regrowth occurred. In contrast, full eradication could not be achieved when treating a 72 h old biofilm with MCs, yet the MCs worked significantly faster than the solution. These results show that the MCs have a great potential as delivery system for biofilm treatment by reaching immediate high local drug concentration of multiple drugs at the site of infection.

Author contributions

Conceptualization and methodology, S.E.B., C.M., H.K.J., J.A.J.H., S.M., L.H.N. and A.B.; supervision, S.E.B., C.M., J.A.J.H., S.M., L.H.N. and A.B; investigation, S.E.B., C.M., M.M.J., M.B.H.; resources, S.M. and A.B.; writing—original draft preparation, S.E.B.; writing—review and editing, all authors; funding acquisition, A.B. All authors have read and agreed to the published version of the manuscript.

Supplementary material

Supplementary material is available from Elsevier or from the author.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements and funding

The authors would like to acknowledge the Center for Intelligent Drug Delivery and Sensing Using Microcontainers and Nanomechanics (IDUN) whose research is funded by the Danish National Research Foundation (DNRF122) and Villum Foundation (Grant No. 9301). Micro- and nanofabrication specialist Lasse Højlund Eklund Thamdrup, DTU Health Tech is thanked for help with fabrication of the microcontainers. Furthermore, associate Professor Claus Sternberg, DTU Bioengineering is greatly acknowledged for providing access to the confocal scanning laser microscope. Helle Krogh Johansen was supported by The Novo Nordisk Foundation as a clinical research stipend (NNF12OC1015920), by Rigshospitalets Rammebevilling 2015–17 (R88-A3537), by Lundbeckfonden (R167-2013-15229), by Novo Nordisk Fonden (NNF15OC0017444, NNF18OC0052776), by RegionH Rammebevilling (R144-A5287) and by Independent Research Fund Denmark/Medical and Health Sciences (FSS-4183-00051, DFF-9039-00037A).

5. References

- Abid, Z., Gundlach, C., Durucan, O., von Halling Laier, C., Nielsen, L.H., Boisen, A., Keller, S.S., 2017. Powder embossing method for selective loading of polymeric microcontainers with drug formulation. *Microelectron. Eng.* 171, 20–24. <https://doi.org/10.1016/j.mee.2017.01.018>
- Ahmadi, F., Oveisi, Z., Samani, S.M., Amoozgar, Z., 2015. Chitosan based hydrogels: characteristics and pharmaceutical applications. *Res. Pharm. Sci.* 10, 1–16.
- Ainslie, K.M., Lowe, R.D., Beaudette, T.T., Petty, L., Bachelder, E.M., Desai, T.A., 2009. Microfabricated Devices for Enhanced Bioadhesive Drug Delivery: Attachment to and Small-Molecule Release Through a Cell Monolayer Under Flow. *Small* 5, 2857–2863. <https://doi.org/10.1002/sml.200901254>
- Aldred, K.J., Kerns, R.J., Osherooff, N., 2014. Mechanism of quinolone action and resistance. *Biochemistry* 53, 1565–1574. <https://doi.org/10.1021/bi5000564>
- Anwar, H., Costerton, J.W., 1990. Enhanced activity of combination of tobramycin and piperacillin for eradication of sessile biofilm cells of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 34, 1666–1671. <https://doi.org/10.1128/AAC.34.9.1666>
- Bergen, P.J., Bulitta, J.B., Forrest, A., Tsuji, B.T., Li, J., Nation, R.L., 2010. Pharmacokinetic/Pharmacodynamic Investigation of Colistin against *Pseudomonas aeruginosa* Using an In Vitro Model. *Antimicrob. Agents Chemother.* 54, 3783–3789. <https://doi.org/10.1128/AAC.00903-09>
- Bialvaei, A.Z., Samadi Kafil, H., 2015. Colistin, mechanisms and prevalence of resistance. *Curr. Med. Res. Opin.* 31, 707–721. <https://doi.org/10.1185/03007995.2015.1018989>
- Birk, S.E., Haagen, J.A.J., Johansen, H.K., Molin, S., Nielsen, L.H., Boisen, A., 2020. Microcontainer Delivery of Antibiotic Improves Treatment of *Pseudomonas aeruginosa* Biofilms. *Adv. Healthcare Mater.* 9, 1901779. <https://doi.org/10.1002/adhm.201901779>
- Bjarnsholt, T., 2011. *Pseudomonas aeruginosa* Biofilms in the Lungs of Cystic Fibrosis Patients, in: Moser, C., Jensen, P.Ø., Høiby, N. (Eds.), *Biofilm Infections*. Springer, pp. 167–185. <https://doi.org/10.1007/978-1-4419-6084-9>
- Buyck, J.M., Tulkens, P.M., Van Bambeke, F., 2015. Activities of Antibiotic Combinations against Resistant Strains of *Pseudomonas aeruginosa* in a Model of Infected THP-1 Monocytes. *Antimicrob. Agents Chemother.* 59, 258–268. <https://doi.org/10.1128/AAC.04011-14>
- Chai, G., Park, H., Yu, S., Zhou, F., Li, J., Xu, Q., Zhou, Q. (Tony), 2019. Evaluation of co-delivery of colistin and ciprofloxacin in liposomes using an in vitro human lung epithelial cell model. *Int. J. Pharm.* 569, 118616. <https://doi.org/10.1016/j.ijpharm.2019.118616>
- Chirra, H.D., Shao, L., Ciaccio, N., Fox, C.B., Wade, J.M., Ma, A., Desai, T.A., 2014. Planar Microdevices for Enhanced In Vivo Retention and Oral Bioavailability of Poorly Permeable Drugs. *Adv. Healthcare Mater.* 3, 1648–1654. <https://doi.org/10.1002/adhm.201300676>
- Ciofu, O., Tolker-Nielsen, T., 2019. Tolerance and Resistance of *Pseudomonas aeruginosa* Biofilms to Antimicrobial Agents—How *P. aeruginosa* Can Escape Antibiotics. *Front. Microbiol.* 10, 1–15. <https://doi.org/10.3389/fmicb.2019.00913>
- Couvreux, P., 2013. Nanoparticles in drug delivery: Past, present and future. *Adv. Drug Delivery Rev.* 65, 21–23. <https://doi.org/10.1016/j.addr.2012.04.010>
- Dalskov Mosgaard, M., Strindberg, S., Abid, Z., Singh Petersen, R., Højlund Eklund Thamdrup, L., Joukainen Andersen, A., Sylvest Keller, S., Müllertz, A., Hagner Nielsen, L., Boisen, A., 2019. Ex vivo

549 intestinal perfusion model for investigating mucoadhesion of microcontainers. *Int. J. Pharm.* 570,
550 118658. <https://doi.org/10.1016/j.ijpharm.2019.118658>

551 Davies, J., Davies, D., 2010. Origins and Evolution of Antibiotic Resistance. *Microbiol. Mol. Biol. Rev.* 74,
552 417–433. <https://doi.org/10.1128/MMBR.00016-10>

553 Forier, K., Raemdonck, K., De Smedt, S.C., Demeester, J., Coenye, T., Braeckmans, K., 2014. Lipid and
554 polymer nanoparticles for drug delivery to bacterial biofilms. *J. Controlled Release* 190, 607–623.
555 <https://doi.org/10.1016/j.jconrel.2014.03.055>

556 Gao, W., Chen, Y., Zhang, Y., Zhang, Q., Zhang, L., 2018. Nanoparticle-based local antimicrobial drug
557 delivery. *Adv. Drug Delivery Rev.* 127, 46–57. <https://doi.org/10.1016/j.addr.2017.09.015>

558 Giamarellos-Bourboulis, E.J., Sambatakou, H., Galani, I., Giamarellou, H., 2003. In Vitro Interaction of
559 Colistin and Rifampin on Multidrug-Resistant *Pseudomonas aeruginosa*. *J. Chemother.* 15, 235–
560 238. <https://doi.org/10.1179/joc.2003.15.3.235>

561 Gunathilake, T.M.S.U., Ching, Y.C., Chuah, C.H., Ilias, H.A., Ching, K.Y., Singh, R., Nai-Shang, L., 2018.
562 Influence of a nonionic surfactant on curcumin delivery of nanocellulose reinforced chitosan
563 hydrogel. *Int. J. Biol. Macromol.* 118, 1055–1064. <https://doi.org/10.1016/j.ijbiomac.2018.06.147>

564 Haagenensen, J.A.J., Klausen, M., Ernst, R.K., Miller, S.I., Folkesson, A., Tolker-Nielsen, T., Molin, S., 2007.
565 Differentiation and distribution of colistin- and sodium dodecyl sulfate-tolerant cells in
566 *Pseudomonas aeruginosa* biofilms. *J. Bacteriol.* 189, 28–37. <https://doi.org/10.1128/JB.00720-06>

567 Hall-Stoodley, L., Costerton, J.W., Stoodley, P., 2004. Bacterial biofilms: from the Natural environment
568 to infectious diseases. *Nat. Rev. Microbiol.* 2, 95–108. <https://doi.org/10.1038/nrmicro821>

569 Heydorn, A., Nielsen, A.T., Hentzer, M., Sternberg, C., Givskov, M., Ersbøll, B.K., Molin, S., 2000.
570 Quantification of biofilm structures by the novel computer program comstat. *Microbiology* 146,
571 2395–2407. <https://doi.org/10.1099/00221287-146-10-2395>

572 Høiby, N., Bjarnsholt, T., Givskov, M., Molin, S., Ciofu, O., 2010. Antibiotic resistance of bacterial biofilms.
573 *Int. J. Antimicrob. Agents* 35, 322–332. <https://doi.org/10.1016/j.ijantimicag.2009.12.011>

574 Holloway, B.W., Morgan, A.F., 1986. Genome Organization in *Pseudomonas*. *Annu. Rev. Microbiol.* 40,
575 79–105. <https://doi.org/10.1146/annurev.mi.40.100186.000455>

576 Jørgensen, J.R., Jepsen, M.L., Nielsen, L.H., Dufva, M., Nielsen, H.M., Rades, T., Boisen, A., Müllertz, A.,
577 2019. Microcontainers for oral insulin delivery – In vitro studies of permeation enhancement. *Eur.*
578 *J. Pharm. Biopharm.* 143, 98–105. <https://doi.org/10.1016/j.ejpb.2019.08.011>

579 Klausen, M., Heydorn, A., Ragas, P., Lambertsen, L., Aaes-Jørgensen, A., Molin, S., Tolker-Nielsen, T.,
580 2003. Biofilm formation by *Pseudomonas aeruginosa* wild type, flagella and type IV pili mutants.
581 *Mol. Microbiol.* 48, 1511–1524. <https://doi.org/10.1046/j.1365-2958.2003.03525.x>

582 Klockgether, J., Munder, A., Neugebauer, J., Davenport, C.F., Stanke, F., Larbig, K.D., Heeb, S., Schöck,
583 U., Pohl, T.M., Wiehlmann, L., Tümmler, B., 2010. Genome Diversity of *Pseudomonas aeruginosa*
584 PAO1 Laboratory Strains. *J. Bacteriol.* 192, 1113–1121. <https://doi.org/10.1128/JB.01515-09>

585 Lee, M.-K., 2020. Liposomes for Enhanced Bioavailability of Water-Insoluble Drugs: In Vivo Evidence and
586 Recent Approaches. *Pharmaceutics* 12, 264. <https://doi.org/10.3390/pharmaceutics12030264>

587 Li, J., Nation, R., Turnidge, J., Milne, R., Coulthard, K., Rayner, C., Paterson, D., 2006. Colistin: the re-
588 emerging antibiotic for multidrug-resistant Gram-negative bacterial infections. *Lancet Infect Dis* 6,
589 589–601. [https://doi.org/10.1016/S1473-3099\(06\)70580-1](https://doi.org/10.1016/S1473-3099(06)70580-1)

590 Lin, L., Nonejuie, P., Munguia, J., Hollands, A., Olson, J., Dam, Q., Kumaraswamy, M., Rivera, H., Corriden,
591 R., Rohde, M., Hensler, M.E., Burkart, M.D., Pogliano, J., Sakoulas, G., Nizet, V., 2015. Azithromycin

592 Synergizes with Cationic Antimicrobial Peptides to Exert Bactericidal and Therapeutic Activity
 593 Against Highly Multidrug-Resistant Gram-Negative Bacterial Pathogens. *EBioMedicine* 2, 690–698.
 594 <https://doi.org/10.1016/j.ebiom.2015.05.021>

595 Liu, Y., Shi, L., Su, L., van der Mei, H.C., Jutte, P.C., Ren, Y., Busscher, H.J., 2019. Nanotechnology-based
 596 antimicrobials and delivery systems for biofilm-infection control. *Chem. Soc. Rev.* 48, 428–446.
 597 <https://doi.org/10.1039/C7CS00807D>

598 Mah, T.F.C., O'Toole, G.A., 2001. Mechanisms of biofilm resistance to antimicrobial agents. *Trends*
 599 *Microbiol.* 9, 34–39. [https://doi.org/10.1016/S0966-842X\(00\)01913-2](https://doi.org/10.1016/S0966-842X(00)01913-2)

600 Mazzoni, C., Jacobsen, R.D., Mortensen, J., Jørgensen, J.R., Vaut, L., Jacobsen, J., Gundlach, C., Müllertz,
 601 A., Nielsen, L.H., Boisen, A., 2019. Polymeric Lids for Microcontainers for Oral Protein Delivery.
 602 *Macromol. Biosci.* 19, 1900004. <https://doi.org/10.1002/mabi.201900004>

603 Mazzoni, C., Tentor, F., Strindberg, S.A., Nielsen, L.H., Keller, S.S., Alstrøm, T.S., Gundlach, C., Müllertz,
 604 A., Marizza, P., Boisen, A., 2017. From concept to in vivo testing: Microcontainers for oral drug
 605 delivery. *J. Controlled Release* 268, 343–351. <https://doi.org/10.1016/j.jconrel.2017.10.013>

606 Mohammed, M., Syeda, J., Wasan, K., Wasan, E., 2017. An Overview of Chitosan Nanoparticles and Its
 607 Application in Non-Parenteral Drug Delivery. *Pharmaceutics* 9, 53.
 608 <https://doi.org/10.3390/pharmaceutics9040053>

609 Mortensen, N.P., Fowlkes, J.D., Sullivan, C.J., Allison, D.P., Larsen, N.B., Molin, S., Doktycz, M.J., 2009.
 610 Effects of Colistin on Surface Ultrastructure and Nanomechanics of *Pseudomonas aeruginosa* Cells.
 611 *Langmuir* 25, 3728–3733. <https://doi.org/10.1021/la803898g>

612 Nation, R.L., Li, J., Cars, O., Couet, W., Dudley, M.N., Kaye, K.S., Mouton, J.W., Paterson, D.L., Tam, V.H.,
 613 Theuretzbacher, U., Tsuji, B.T., Turnidge, J.D., 2015. Framework for optimisation of the clinical use
 614 of colistin and polymyxin B: the Prato polymyxin consensus. *Lancet Infect. Dis.* 15, 225–234.
 615 [https://doi.org/10.1016/S1473-3099\(14\)70850-3](https://doi.org/10.1016/S1473-3099(14)70850-3)

616 Nielsen, L.H., Keller, S.S., Boisen, A., 2018. Microfabricated devices for oral drug delivery. *Lab Chip* 18,
 617 2348–2358. <https://doi.org/10.1039/C8LC00408K>

618 Nielsen, L.H., Keller, S.S., Gordon, K.C., Boisen, A., Rades, T., Müllertz, A., 2012. Spatial confinement can
 619 lead to increased stability of amorphous indomethacin. *Eur. J. Pharm. Biopharm.* 81, 418–425.
 620 <https://doi.org/10.1016/j.ejpb.2012.03.017>

621 Nielsen, L.H., Melero, A., Keller, S.S., Jacobsen, J., Garrigues, T., Rades, T., Müllertz, A., Boisen, A., 2016.
 622 Polymeric microcontainers improve oral bioavailability of furosemide. *Int. J. Pharm.* 504, 98–109.
 623 <https://doi.org/10.1016/J.IJPHARM.2016.03.050>

624 O'Neill, J., 2016. Tackling drug-resistant infections globally, Review on Antimicrobial Resistance.
 625 <https://doi.org/10.4103/2045-080X.186181>

626 Olivera, M.E., Manzo, R.H., Junginger, H.E., Midha, K.K., Shah, V.P., Stavchansky, S., Dressman, J.B.,
 627 Barends, D.M., 2011. Biowaiver monographs for immediate release solid oral dosage forms:
 628 Ciprofloxacin hydrochloride. *J. Pharm. Sci.* 100, 22–33. <https://doi.org/10.1002/jps.22259>

629 Pamp, S.J., Gjermansen, M., Johansen, H.K., Tolker-Nielsen, T., 2008. Tolerance to the antimicrobial
 630 peptide colistin in *Pseudomonas aeruginosa* biofilms is linked to metabolically active cells, and
 631 depends on the *pmr* and *mexAB-oprM* genes. *Mol. Microbiol.* 68, 223–240.
 632 <https://doi.org/10.1111/j.1365-2958.2008.06152.x>

633 Parish, T., 2019. Steps to address anti-microbial drug resistance in today's drug discovery. *Expert Opin.*
 634 *Drug Discov.* 14, 91–94. <https://doi.org/10.1080/17460441.2019.1550481>

635 Pastar, I., Nusbaum, A.G., Gil, J., Patel, S.B., Chen, J., Valdes, J., Stojadinovic, O., Plano, L.R., Tomic-Canic,
636 M., Davis, S.C., 2013. Interactions of Methicillin Resistant *Staphylococcus aureus* USA300 and
637 *Pseudomonas aeruginosa* in Polymicrobial Wound Infection. *PLoS One* 8, e56846.
638 <https://doi.org/10.1371/journal.pone.0056846>

639 Patel, K.K., Tripathi, M., Pandey, N., Agrawal, A.K., Gade, S., Anjum, M.M., Tilak, R., Singh, S., 2019.
640 Alginate lyase immobilized chitosan nanoparticles of ciprofloxacin for the improved antimicrobial
641 activity against the biofilm associated mucoid *P. aeruginosa* infection in cystic fibrosis. *Int. J. Pharm.*
642 563, 30–42. <https://doi.org/10.1016/j.ijpharm.2019.03.051>

643 Perinelli, D.R., Fagioli, L., Campana, R., Lam, J.K.W., Baffone, W., Palmieri, G.F., Casettari, L., Bonacucina,
644 G., 2018. Chitosan-based nanosystems and their exploited antimicrobial activity. *Eur. J. Pharm. Sci.*
645 117, 8–20. <https://doi.org/10.1016/j.ejps.2018.01.046>

646 Ross, D.L., Riley, C.M., 1994. Dissociation and complexation of the fluoroquinolone antimicrobials - an
647 update. *J. Pharm. Biomed. Anal.* 12, 1325–1331. [https://doi.org/10.1016/0731-7085\(94\)00081-6](https://doi.org/10.1016/0731-7085(94)00081-6)

648 Schwerdt, M., Neumann, C., Schwartbeck, B., Kampmeier, S., Herzog, S., Görlich, D., Dübbers, A., Große-
649 Onnebrink, J., Kessler, C., Küster, P., Schültingkemper, H., Treffon, J., Peters, G., Kahl, B.C., 2018.
650 *Staphylococcus aureus* in the airways of cystic fibrosis patients - A retrospective long-term study.
651 *Int. J. Med. Microbiol.* 308, 631–639. <https://doi.org/10.1016/j.ijmm.2018.02.003>

652 Silva, F., Lourenço, O., Queiroz, J.A., Domingues, F.C., 2011. Bacteriostatic versus bactericidal activity of
653 ciprofloxacin in *Escherichia coli* assessed by flow cytometry using a novel far-red dye. *J. Antibiot.*
654 (Tokyo). 64, 321–325. <https://doi.org/10.1038/ja.2011.5>

655 Soares, A., Alexandre, K., Lamoureux, F., Lemée, L., Caron, F., Pestel-Caron, M., Etienne, M., 2019.
656 Efficacy of a ciprofloxacin/amikacin combination against planktonic and biofilm cultures of
657 susceptible and low-level resistant *Pseudomonas aeruginosa*. *J. Antimicrob. Chemother.* 74, 3252–
658 3259. <https://doi.org/10.1093/jac/dkz355>

659 Stewart, P.S., 2002. Mechanisms of antibiotic resistance in bacterial biofilms. *Int. J. Med. Microbiol.* 292,
660 107–113. <https://doi.org/10.1078/1438-4221-00196>

661 Stover, C.K., Pham, X.Q., Erwin, A.L., Mizoguchi, S.D., Warren, P., Hickey, M.J., Brinkman, F.S.,
662 Hufnagle, W.O., Kowalik, D.J., Lagrou, M., Garber, R.L., Goltry, L., Tolentino, E., Westbrook-
663 Wadman, S., Yuan, Y., Brody, L.L., Coulter, S.N., Folger, K.R., Kas, A., Larbig, K., Lim, R., Smith, K.,
664 Spencer, D., Wong, G.K., Wu, Z., Paulsen, I.T., Reizer, J., Saier, M.H., Hancock, R.E., Lory, S., Olson,
665 M. V., 2000. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic
666 pathogen. *Nature* 406, 959–64. <https://doi.org/10.1038/35023079>

667 Tamma, P.D., Cosgrove, S.E., Maragakis, L.L., 2012. Combination Therapy for Treatment of Infections
668 with Gram-Negative Bacteria. *Clin. Microbiol. Rev.* 25, 450–470.
669 <https://doi.org/10.1128/CMR.05041-11>

670 Tao, S.L., Popat, K., Desai, T.A., 2007. Off-wafer fabrication and surface modification of asymmetric 3D
671 SU-8 microparticles. *Nat. Protoc.* 1, 3153–3158. <https://doi.org/10.1038/nprot.2006.451>

672 Tolker-Nielsen, T., Sternberg, C., 2011. Growing and analyzing biofilms in flow chambers. *Curr. Protoc.*
673 *Microbiol.* 21, 1B.2.1–1B.2.17. <https://doi.org/10.1002/9780471729259.mc01b02s21>

674 Traugott, K.A., Echevarria, K., Maxwell, P., Green, K., Lewis, J.S., 2011. Monotherapy or Combination
675 Therapy? The *Pseudomonas aeruginosa* Conundrum. *Pharmacotherapy* 31, 598–608.
676 <https://doi.org/10.1592/phco.31.6.598>

677 Varanda, F., Pratas De Melo, M.J., Caço, A.I., Dohrn, R., Makrydaki, F.A., Voutsas, E., Tassios, D.,
678 Marrucho, I.M., 2006. Solubility of antibiotics in different solvents. 1. Hydrochloride forms of

679 tetracycline, moxifloxacin, and ciprofloxacin. *Ind. Eng. Chem. Res.* 45, 6368–6374.
680 <https://doi.org/10.1021/ie060055v>

681 Vorregaard, M., 2008. Comstat2 - A Modern 3D Image Analysis Environment for Biofilms. Technical
682 University of Denmark, DTU.

683 Wallace, S.J., Li, J., Nation, R.L., Prankerd, R.J., Velkov, T., Boyd, B.J., 2010. Self-Assembly Behavior of
684 Colistin and Its Prodrug Colistin Methanesulfonate: Implications for Solution Stability and
685 Solubilization. *J. Phys. Chem. B* 114, 4836–4840. <https://doi.org/10.1021/jp100458x>

686 Wang, S., Yu, S., Lin, Y., Zou, P., Chai, G., Yu, H.H., Wickremasinghe, H., Shetty, N., Ling, J., Li, J., Zhou, Q.,
687 2018. Co-Delivery of Ciprofloxacin and Colistin in Liposomal Formulations with Enhanced In Vitro
688 Antimicrobial Activities against Multidrug Resistant *Pseudomonas aeruginosa*. *Pharm. Res.* 35, 187.
689 <https://doi.org/10.1007/s11095-018-2464-8>

690 Wang, W., Zhou, Q.T., Sun, S.P., Denman, J.A., Gengenbach, T.R., Barraud, N., Rice, S.A., Li, J., Yang, M.,
691 Chan, H.K., 2016. Effects of Surface Composition on the Aerosolisation and Dissolution of Inhaled
692 Antibiotic Combination Powders Consisting of Colistin and Rifampicin. *AAPS J.* 18, 372–384.
693 <https://doi.org/10.1208/s12248-015-9848-z>

694 Weber, K., Delben, J., Bromage, T.G., Duarte, S., 2014. Comparison of SEM and VPSEM imaging
695 techniques with respect to *Streptococcus mutans* biofilm topography. *FEMS Microbiol. Lett.* 350,
696 175–179. <https://doi.org/10.1111/1574-6968.12334>

697 Yu, S., Wang, S., Zou, P., Chai, G., Lin, Y.W., Velkov, T., Li, J., Pan, W., Zhou, Q.T., 2020. Inhalable liposomal
698 powder formulations for co-delivery of synergistic ciprofloxacin and colistin against multi-drug
699 resistant gram-negative lung infections. *Int. J. Pharm.* 575, 118915.
700 <https://doi.org/10.1016/j.ijpharm.2019.118915>

701