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Microcontainer Delivery of Antibiotic Improves Treatment of *Pseudomonas aeruginosa* Biofilms

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Keywords

Bacterial biofilm, microdevices, drug delivery, polymeric coatings, ciprofloxacin

Abstract

Biofilm-associated infections are difficult to treat effectively with antibiotics despite repeated treatments. Polymeric microdevices (microcontainers) have previously been shown to engulf in mucus layers and to provide tunable release. Such devices may overcome the challenge of delivering antibiotics into the biofilm, increasing the local drug concentration and hence improve local bacterial killing. In this work, microcontainers are loaded with the antibiotic, ciprofloxacin hydrochloride, and functionalized with polymeric lids of polyethylene glycol (PEG), chitosan or Eudragit[®] S100. The PEG lid gives rise to a drug release comparable to uncoated microcontainers showing complete release after 8 h, whereas Chitosan and Eudragit[®] S100 lids result in continuous release during the course of 24 h. All antibiotic-containing microcontainers inhibit planktonic growth of *Pseudomonas aeruginosa* (PAO1) cells, but the degree of inhibition depends on the coating. Microcontainers with ciprofloxacin hydrochloride kill about three times more biofilm-associated PAO1 cells compared with a single standard bolus. Moreover, the use of microcontainers in biofilm result in bacterial killing equal to a constant flow of a three times higher concentration of solubilized antibiotics. These studies suggest that microcontainers can be useful for antibiotic delivery in treatment of biofilm-associated infections, resulting in more effective treatment and reduced use of antibiotics.

45 Graphical abstract 326 **1. Introduction**

327 A biofilm is a consortium of bacteria living together in a self-produced extracellular polymeric
 328 substances (EPS) matrix consisting of various polysaccharides, proteins and DNA.^[1,2] The bacteria
 329 adhere to each other and to surfaces, aggregates develop, and the biofilm promotes bacterial survival
 330 in otherwise hostile environments.^[3,4] Biofilm-associated infections are difficult to treat, and they are
 331 often associated with chronic infections, resulting in recurrent inflammation and exacerbations
 332 despite intense treatment with antibiotics. In contrast, bacteria living as planktonic, single-cell
 333 populations in suspension show decreased tolerance towards antibiotics, and hence are easier to
 334 treat.^[1,2,5]

335 *Pseudomonas aeruginosa* is a Gram-negative pathogenic bacterial species forming biofilms and
 336 causing devastating chronic infections in immuno-compromised individuals.^[3,6] *P. aeruginosa* can
 337 cause serious intestinal infections, where it for example has been isolated from patients suffering from
 338 inflammatory bowel disease and cancer patients.^[7,8] The gastrointestinal tract is often considered to
 339 be an important reservoir for *P. aeruginosa* and the presence in the intestines is responsible for
 340 increased mortality in gut-derived sepsis and bacteremia and facilitates hematogenous spread of

341 infections to other organs.^[8] *P. aeruginosa* often causes chronic lung infections in patients suffering
342 from cystic fibrosis due to the accumulation of thick, sticky mucus and to the consequentially
343 impaired mucociliary clearance.^[4,5]

344 Antibiotic treatment is the standard therapy for chronic infections with *P. aeruginosa*, but it is difficult
345 to fully combat *P. aeruginosa* infections.^[9] One of the most often used antibiotics is ciprofloxacin.
346 This is a broad spectrum second generation fluoroquinolone with a reported low frequency of
347 spontaneous bacterial resistance.^[10,11] Delivery of several antibiotics to biofilm-associated infections
348 is, however, obstructed by the EPS matrix. This viscous structure creates diffusional barriers that may
349 deactivate the antibiotics in the outer layers of the biofilm faster than they diffuse, leading to
350 insufficient antimicrobial effects.^[4,12,13] Also, the EPS allows development of phenotypically
351 different subpopulations with reduced metabolic activities and growth properties, making the bacteria
352 more tolerant to antibiotic treatment.^[2]

353 Standard delivery of antibiotics is usually untargeted, and will result in distribution of the drugs to all
354 parts of the body. Consequently, the efficacy of the antibiotics may decrease, since reduced
355 concentrations of the antibiotics may reach the actual site of the infection.^[14] The recalcitrance of
356 infectious biofilms towards antibiotics may be overcome by the use of a local antibiotic delivery
357 system creating a high local concentration, and hence a more effective treatment. In recent years,
358 nanotechnology-based drug delivery systems such as mesoporous silica nanoparticles, polymeric
359 nanoparticles and liposomes have been broadly studied as antibiotic carriers facilitating penetration
360 and ultimately killing of infectious biofilms.^[13] However, many of these formulations suffer from
361 poor storage stability^[13], versatility may be limited and usage depends on the properties of the active
362 pharmaceutical drug i.e. charge, size and hydrophilicity. Microfabricated drug delivery devices have
363 shown promise towards increasing local drug concentration^[15,16], and might as well be a suitable
364 system for delivering antibiotics to specific sites of infection. One of such devices is a microcontainer,
365 which is a micrometer sized polymeric device mainly developed for oral delivery of therapeutics.^[17]

366 Microcontainers have proven to be able to improve oral bioavailability of small drugs, most likely
367 due to the fact that microcontainers have mucoadhesive properties and are engulfed within the
368 intestinal mucus.^[18,19] In addition, microcontainers have the benefit of being very versatile and can
369 be used for delivery of any active pharmaceutical ingredient no matter size, charge or hydrophilicity.
370 The microcontainers can be functionalized by applying a polymeric lid onto the cavity of drug-loaded
371 microcontainers.^[20] Polymers with different properties can be used to e.g. control the drug release
372 profiles, to achieve additional mucoadhesive or mucus penetrating effects, and/or to provide
373 additional antibacterial activity. Polyethylene glycol (PEG) is an uncharged hydrophilic polymer that
374 has been shown to possess either mucoadhesive or mucus penetrating properties, depending on the
375 surface density and the molecular weight.^[21,22] Wang *et al.* documented improved mucus penetration
376 of polystyrene nanoparticles coated with low molecular weight (MW) PEG, whereas higher MW PEG
377 improved mucoadhesion.^[23] Chitosan is a cationic polysaccharide that has been widely explored as a
378 mucoadhesive polymer. In addition, it provides controlled drug release, prolonging the therapeutic
379 effect of the drug^[24,25], and has also been reported to possess antimicrobial properties.^[26] Previously,
380 the inhibitory effect of chitosan solutions and nanoparticles without antibiotics against *P. aeruginosa*
381 clinical isolates in planktonic and biofilm conditions was investigated, and it was shown that
382 planktonic bacteria were more effectively eradicated than biofilm-associated bacteria.^[27] Eudragit®
383 S100 is an anionic co-polymer widely used for pH-responsive formulations as it dissolves at pH
384 values above 7.^[28,29] It has previously been reported that using Eudragit microparticles for antibiotic
385 delivery caused a significant reduction in adherent methicillin-resistant *Staphylococcus aureus*
386 compared to treatment with free antibiotics.^[30]

387 The aim of this study is to investigate the potential impact of using microcontainers in the treatment
388 of *P. aeruginosa* biofilms. The microcontainers are suggested to adhere to the biofilm and release the
389 loaded ciprofloxacin in a controlled and localized manner. Ciprofloxacin-loaded microcontainers
390 were coated with polymeric lids of PEG, chitosan and Eudragit® S100. Together with investigating

the drug release, the resulting antimicrobial activity obtained from the functionalized microcontainers were tested on planktonic bacterial cells as well as on biofilm consortia of *P. aeruginosa* and compared to delivery of unconfined antibiotic.

2. Results & Discussion

2.1. Loading of antibiotic into microcontainers and lid depositing

Microcontainers (**Figure 1A** and Sup. Info, **Figure S1**) with an inner diameter of $232 \pm 1 \mu\text{m}$ and an inner height of $214 \pm 3 \mu\text{m}$ (mean $\pm$ SD, $n=3$) were produced and successfully loaded with $2.75 \pm 0.48 \text{ mg}$ ($n=44$ chips, mean $\pm$ SD) ciprofloxacin hydrochloride (CIP) per chip corresponding to $4.39 \pm 0.77 \mu\text{g}$ in each microcontainer. The loaded microcontainers were visualized using scanning electron microscopy (SEM), which confirmed efficient loading (**Figure 1B**). Following the drug loading, the microcontainers were coated on the cavity with either PEG, chitosan or Eudragit[®] S100, and the height and morphology of the spray coated lids were characterized by profilometry and SEM. Coating with PEG resulted in a uniform lid (**Figure 1C**), which was also confirmed by profilometry measurements revealing a low surface roughness and a coating thickness of $32.4 \pm 1.9 \mu\text{m}$ (mean $\pm$ SD, $n=3$). Mazzoni *et al.* reported a coating thickness of $17.0 \pm 5.6 \mu\text{m}$ when coating microcontainers using 0.7 % w/v PEG dissolved in dichloromethane.^[20] In this study, 2.66 % w/v of PEG has been utilized with water as solvent, and it was observed that these changes increased the coating thickness with a factor two. The chitosan coating (**Figure 1D**) resulted in the thinnest lid among the three polymers, since the polymer concentration was lower and the spray coating parameters different. The chitosan lid did not cover the open cavity of the microcontainer completely, and structures of the CIP crystals were still visible. However, we aimed for a thin layer as chitosan is hygroscopic and quickly swells when in contact with water, hence creating a hydrogel lid affecting the release of CIP.^[31,32] The thickness of the chitosan coating was $8.9 \pm 0.7 \mu\text{m}$ (mean $\pm$ SD, $n=6$), in accordance with previously reported heights.^[20] Eudragit[®] S100 coating was shown to cover the entire opening of the

415 microcontainer and to be homogenously distributed (**Figure 1E**), and the height was $25.0 \pm 5.3 \mu\text{m}$
 416 (mean $\pm$ SD, n= 6).

417
 418 **Figure 1.** Scanning electron microscopy (SEM) images of a microcontainer (A) empty, (B) loaded with ciprofloxacin hydrochloride
 419 (CIP), (C) coated with polyethylene glycol (PEG), (D) coated with chitosan and (E) coated with Eudragit® S100.

420 2.2. *In vitro* release of ciprofloxacin hydrochloride from microcontainers

421 The release of CIP from uncoated and coated microcontainers was evaluated in a modified minimal
 422 medium, FAB medium^[33]. When no coating was applied onto the microcontainers, it appears that two
 423 types of release kinetics apply. Within the first min an initial burst release of $42.1 \pm 4.5 \%$ of CIP
 424 occurred, followed by 7 h sustained release before reaching a plateau (**Figure 2A**). Applying a PEG
 425 lid on the microcontainers resulted in a lower initial release ($31.5 \pm 1.9 \%$ CIP released from PEG-
 426 coated microcontainers after 10 min compared to $44.7 \pm 4.9 \%$ CIP released from uncoated
 427 microcontainers). However, after 90 min no significant differences in the release profiles were
 428 observed (**Figure 2A**). This is consistent with PEG being a water-soluble polymer^[34], which quickly
 429 solubilizes in the aqueous FAB medium. Chitosan coating gave rise to an initial release of 25.9 ± 5.6
 430 % CIP after 90 min, followed by a subsequent sustained release with $99.5 \pm 9.1 \%$ being released after
 431 28 h (**Figure 2B**). In accordance, it has previously been shown that chitosan nanoparticles also
 432 provide a burst release of CIP followed by a sustained release behavior.^[35] SEM images confirmed
 433 swelling and after 28 h the hydrogel was still present on the microcontainers (data not shown),
 434 presumably due to the limited solubility of chitosan at physiological pH.^[32] The sustained release
 435 profiles are likely caused by a slow but constant diffusion of entrapped CIP through the chitosan
 436 hydrogel. Eudragit® S100 was included as a coating to provide protection of CIP until the
 437 microcontainers had reached the biofilm growing bacteria in the flow cell system, and fast release

438 could be induced by increasing the pH of the FAB medium. As can be seen from **Figure 2C**, the
439 coating was kept intact for up to 15 min, and release was triggered when increasing the pH from 6.8
440 to 7.4. This is in accordance with Eudragit[®] S100 being solubilized at pH values above 7.^[29] To
441 facilitate inoculation of the microcontainers into a flow cell system, they were shortly embedded in a
442 gelatin hydrogel at pH 6.8. The release data support that the lid was kept intact during the time in the
443 hydrogel, and that release was triggered in the growth medium with a pH of 7.4. However, the release
444 of CIP from Eudragit[®] S100-coated microcontainers was sustained, with only 46.1 ± 3.0 % being
445 released after 4 h and 90.2 ± 2.8 % after 18 h. This complies with previous findings in the literature,
446 observing that albumin-containing microspheres coated with Eudragit[®] S100 showed a sustained
447 release in intestinal pH reaching complete release after 16 h.^[36]

Figure 2. *In vitro* cumulative release of ciprofloxacin hydrochloride (CIP) from microcontainers in FAB medium as a function of time. **A)** Uncoated microcontainers compared to microcontainers coated with polyethylene glycol (PEG), chitosan or Eudragit® S100. Release differed significantly from uncoated microcontainers until $t=90$ min (PEG), $t=11$ h (Eudragit® S100) and $t=13$ h (chitosan). **B)** Zoom-out depicting the release of CIP during 40 h with chitosan coating. **C)** Magnified view showing the initial release of CIP with Eudragit® S100 coating. In experiments with Eudragit® S100 the chips were placed in FAB medium at pH 6.8 for 15 min followed by immersion in FAB medium at pH 7.4. Data are presented as mean $\pm$ SD ($n=3-6$) and an unpaired t-test was applied for determination of significant difference (with statistical significance defined as $p < 0.05$).

2.3. Impact of microcontainers on planktonic growth of PAO1 cells

The impacts of empty, CIP-loaded and polymer-coated microcontainers on bacterial growth inhibition were determined from addition of these to planktonic PAO1 cultures. Bacterial growth inhibition observed for CIP-loaded uncoated and PEG-coated microcontainers, respectively, differed

462 significantly after 3 h from the PAO1 control growth ($p\text{-value}\leq 0.0017$), whereas CIP mediated
463 bacterial growth inhibition with chitosan and Eudragit[®] S100, respectively, differed significantly
464 from control after 4 h ($p\text{-values}\leq 0.0013$) (**Figure 3A**). These results align with the release data (cf.
465 **Figure 2**), in which only limited differences in release profiles were observed for uncoated and PEG-
466 coated microcontainers, whereas delayed releases of CIP were observed for both chitosan and
467 Eudragit[®] S100. Addition of all loaded and polymer-coated microcontainers led to reductions in
468 viable counts of PAO1, reaching the lowest bacterial viability after 8 h of treatment with viable counts
469 reduced from app. 10^9 colony forming units (CFU) mL^{-1} (control) to between 10^4 - 10^6 CFU mL^{-1}
470 (**Figure 3B**). The increase of OD_{600} during the first 3h after addition of the microcontainers seemingly
471 conflicts with the decrease in CFU during this period. We suggest that this ‘conflict’ is a reflection
472 of a reduced plating efficiency of the treated bacteria, which shows that although the antibiotic exerts
473 its inhibitory effect of reducing viability of the bacteria, it takes time to stop increase of the optical
474 density. This increase most likely reflects that the cells may continue to grow in size without dividing
475 and quickly loose the capacity to form colonies on plates. The delayed cell lysis is observed later as
476 a decrease in OD_{600} . After 24 h, re-growth of viable cells was observed in all treated cultures, reaching
477 an average of 1.73×10^8 CFU mL^{-1} (**Figure 3B**). Regrowth may be due to a subpopulation of resistant
478 cells not affected by the applied CIP concentrations. The empty microcontainers had no effect on
479 growth of PAO1 cells (**Figure 3A and B**). Treating the planktonic bacterial suspension with 20 μL
480 of a 0.22 mg/ml CIP solution (same amount as confined in one microcontainer) resulted in identical
481 growth inhibition (**Figure 3A and B**), proving that administration of CIP in microcontainers did not
482 decrease the efficacy of the antibiotic towards planktonic suspensions of PAO1.

Figure 3. Impact of the microcontainers on planktonic growth inhibition of PAO1 cells. **A)** Measurement of the optical density (LogOD₆₀₀) as a function of time. Significant difference after $t=3$ h (CIP solution; uncoated; PEG) and $t=4$ h (chitosan, Eudragit® S100) when compared to PAO1 control growth. **B)** Viable counts measured as CFU mL⁻¹ as a function of time. Color codes represent microcontainers loaded with ciprofloxacin hydrochloride (CIP) and coated with polyethylene glycol (PEG), chitosan or Eudragit® S100. Controls include PAO1 growth control without microcontainer addition, empty microcontainers, uncoated microcontainers as well as CIP in solution. Data are depicted as mean $\pm$ SD (n=3-4) and one-way ANOVA with Tukey's multiple comparison was applied for determination of significant difference (with statistical significance defined as $p < 0.05$).

2.4. Impact of microcontainers on biofilm growth of PAO1 cells

Antibiotics, which normally inhibit growth and kill bacteria in suspended cultures, have been shown to be much less efficient in bacterial biofilms.^[1] This specific behavior of biofilm bacteria is a serious medical problem, since many infections are characterized by biofilm growth in the human body. Microcontainers potentially may deliver a large dose of antibiotics into close contact with a localized infection, increasing the local concentration of antibiotics, which may improve the chance of eradicating the bacteria in the biofilm. Controlling the release rate by the use of different coatings makes it possible to expose the biofilm to specific concentrations of CIP over time. PAO1 biofilms were allowed to mature for 96 h in flow cell chambers, reaching a biomass of $5.35 \pm 3.74 \mu\text{m}^3 \mu\text{m}^{-2}$ (Sup. Info, **Figure S2**). Subsequently, microcontainers were successfully introduced into the flow cell biofilm. A bright field image of the top of a PEG-coated microcontainer together with a corresponding confocal scanning laser microscopy (CSLM) image is shown in **Figure 4**.

Figure 4. Example of a bright field (BF) image of a microcontainer (located below the dotted line and marked with an arrow) and the corresponding confocal scanning laser microscopy (CLSM) image of a 96 h old biofilm after 7 h of treatment with CIP-loaded and polyethylene glycol (PEG)-coated microcontainers. Green represents live GFP fluorescent bacteria. Red represents dead bacteria after PI staining. Scale bar: 30 μ m.

To determine efficacies of PEG-, chitosan- or Eudragit[®] S100-coated microcontainers on killing of biofilm-associated PAO1 cells, confocal images were acquired at locations in close proximity to the microcontainers (**Figure 5**).

Figure 5. PAO1 biofilm grown for 96 h under flow to a mature state, and thereafter exposed to app. 120 μg ciprofloxacin hydrochloride (CIP) confined in microcontainers coated with either polyethylene glycol (PEG), chitosan or Eudragit® S100. **A-D**) Confocal scanning laser microscopy (CSLM) images of mature biofilm before treatment and 2, 7 and 24 h after treatment with polymer-coated microcontainers as well as a 24 h untreated control. Scale bar: 30 μm. **E**) Quantitative analysis of the biomass (μm³ μm⁻²) converted to the fraction of live/dead (%). Green represents live bacteria. Red represents dead bacteria. Data are depicted as mean+SD (n=4-24).

After 2 h of treatment with CIP-loaded microcontainers, containing app. 120 μg CIP in total, limited bacterial killing occurred, with an average dead biomass of 11.1 ± 14.2 %, 3.3 ± 1.5 % and 8.5 ± 12.2 % for PEG, chitosan and Eudragit® S100 coatings, respectively. At this time point, PEG coating led to the greatest amount of CIP released (61.3 %) compared to chitosan and Eudragit® S100 (28.1 % and 23.6 %, respectively) (cf. **Figure 2A**). The differences in amount of released CIP were paralleled by the mean killing of biofilm-associated PAO1 cells after 2 h. Note, however, that utilizing a PEG coating with a faster release did not significantly improve the killing compared to the sustained release achieved with chitosan and Eudragit® S100. After 7 h, the dead bacterial fraction increased to

527 19.7±16.2 %, 23.5±19.6 % and 13.5±10.2 % for PEG, chitosan and Eudragit® S100, respectively. All
528 coatings reduced bacterial motility after 7 h compared to 2 h. In addition, bacterial morphology
529 changed as the bacteria appeared coccoid, a sign of slow growth and starvation.^[37] At the same time
530 many bacteria appeared extremely elongated indicating that they are stressed and still metabolically
531 active, but cell division is inhibited.^[37] This observation correlates with the reported mechanism of
532 ciprofloxacin, which interferes with DNA replication and transcription by inhibiting of topoisomerase
533 II (also known as DNA gyrase) and topoisomerase IV, thereby inhibiting cell division.^[11] When
534 planktonic PAO1 cells were treated with coated microcontainers, a significant killing was observed
535 after 8 h (cf. **Figure 3**). Microcontainer therefore had a faster and more effective impact on planktonic
536 cell inhibition, which correlates with previous reported decreased antibiotic tolerance of planktonic
537 bacteria compared to the biofilm-associated cells.^[1] After treating for 24 h, in which almost all
538 antibiotic had been released from the microcontainers, the live population of PAO1 were significantly
539 reduced and the dead biomass increased to 75.7±16.2 %, 88.2±5.3 % and 73.6±14.8 % for PEG,
540 chitosan and Eudragit® S100, respectively.

541 Altogether, the uncharged PEG coating did not appear to improve the antimicrobial effect compared
542 to the anionic, non-mucoadhesive Eudragit® S100 coating, whereas the cationic chitosan coating
543 appeared to be the most promising coating (**Figure 5**). Interestingly, the charge of the polymers did
544 not appear to have any prominent effect on the eradication of the biofilm. Du *et al.* reported a 3.2-
545 fold increase in elimination of an established *P. aeruginosa* biofilm when treating with PEG-
546 conjugated tobramycin compared to the effect of a solution of the antibiotic.^[38] Moreover, Suk *et al.*
547 showed that low MW PEG-coated nanoparticles moved through undiluted cystic fibrosis sputum up
548 to 90-fold faster than uncoated particles, but the penetration of identically coated larger nanoparticles
549 were strongly hindered.^[39] For this reason, larger particles or devices like the microcontainers coated
550 with PEG are likely to be mostly mucoadhesive, however, this did not improve the bacterial killing
551 as no difference between PEG and Eudragit® S100 coating was observed. Chitosan has previously

552 been shown to be mucoadhesive presumably due to hydrogen bonding and ionic interactions with the
 553 negatively charged mucins.^[26] The same mechanism may be relevant in the biofilm, in which the
 554 positively charged chitosan can interact with the negatively charged components of the EPS (such as
 555 alginate)^[40], hence increasing adhesion. In addition, Machul *et al.* reported an inhibitory effect of
 556 chitosan on both planktonic as well as biofilm-associated *P. aeruginosa*.^[27] The chitosan may interact
 557 with the negatively charged microbial cell membranes causing leakage of the intracellular
 558 components hence cell disintegration.^[24,26] Both the additional antimicrobial effect as well as
 559 improved adhesion might have contributed to the enhanced killing observed when using chitosan
 560 coating in this study.

561 2.4.1. *Impact of one bolus dose or constant perfusion of ciprofloxacin hydrochloride on PAO1* 562 *biofilm*

563 To determine whether CIP confined in microcontainers would provide improved killing compared to
 564 the same dose of unconfined antibiotic given as one bolus injection, a 96 h old PAO1 biofilm was
 565 treated with one bolus dose of 120 µg CIP and images were acquired 2, 7 and 24 h after exposure.
 566 After 2 h, 26.7±25.4 % was found dead and no significant change occurred the following 24 h (**Figure**
 567 **6**). In absence of antibiotics, the biofilm biomass showed minimal killing after 24 h as expected.
 568 These findings indicate that using the same dose of CIP but confining it in microcontainers improved
 569 the *in vitro* antibacterial properties of CIP significantly.

Figure 6. PAO1 biofilm grown for 96 h under flow to a mature state and thereafter exposed to one bolus injection of $60 \mu\text{g mL}^{-1}$ ciprofloxacin hydrochloride (CIP) with a total dose of $120 \mu\text{g}$. **A-B**) Confocal scanning laser microscopy images of the biofilm before treatment and 2, 7 and 24 h after treatment as well as a 24 h control biofilm not treated. Scale bar: $30 \mu\text{m}$. **C**) Quantitative analysis of the biomass ($\mu\text{m}^3 \mu\text{m}^{-2}$) converted to the fraction of live/dead (%). Green represents live bacteria. Red represents dead bacteria. Data are depicted as mean+SD (n=3).

Moreover, a 96 h old PAO1 biofilm was perfused with CIP in a concentration of $4 \mu\text{g mL}^{-1}$ for 24 h, corresponding to a total amount of $330 \mu\text{g}$ CIP delivered to the biofilm. The perfusion resulted in 82.0 ± 6.2 % killing of the total amount of biomass after 24 h (**Figure 7**). In control chambers, PAO1 cells were still alive and the biomass increased over 24 h as expected. Comparing these results to biofilm treatment with CIP confined in microcontainers (cf. **Figure 5E**), it was found that microcontainers provided bacterial killing similar to a 2.75 times higher concentration of solubilized antibiotic constantly perfused. Therefore, it appears that confining CIP in microcontainers is a promising strategy, providing the same antibacterial effect with a significantly reduced amount of antibiotic.

Figure 7. PAO1 biofilm grown for 96 h under flow to a mature state and thereafter exposed to perfusion of $4 \mu\text{g mL}^{-1}$ ciprofloxacin hydrochloride (CIP) for 24 h corresponding to a total amount of $330 \mu\text{g}$ CIP delivered to the biofilm. **A)** Confocal scanning laser microscopy images of the biofilm before treatment, after 24 h with no treatment and after 24 h treatment with CIP. Scale bar: $30 \mu\text{m}$. **B)** Quantitative analysis of the biomass ($\mu\text{m}^3 \mu\text{m}^{-2}$) converted to the fraction of live/dead (%). Green represents live bacteria. Red represents dead bacteria. Data are depicted as mean+SD (n=5).

No complete eradication of PAO1 biofilm was observed in any of the treatments tested, since 10-20% of the biomass remained alive despite treatment with CIP confined in microcontainers or as constant perfusion. The remaining fraction might be a subpopulation of antibiotic-tolerant persister cells in a temporary dormant state in which cell division is minimized.^[41,42] Many antibiotics (including ciprofloxacin) require a certain degree of metabolic activity in the cells to be effective as the mechanism of action often involves disruption of the cellular processes.^[4] To achieve a full eradication therapy on the biofilm cells with microcontainers, it is possible that delivery of a nutrition-rich compound prior to antibiotic therapy may stimulate bacterial growth, and thus improve the antibiotic efficacy.

Using microcontainers to achieve a site-specific delivery of antibiotics could potentially reduce the required dose, and thereby reduce the risk of resistance development. The local delivery would also prevent antibiotic exposure in the gastrointestinal tract and its microbiota, thereby decreasing the risk of gastrointestinal side effects often seen for patients needing antibiotics for extended time periods.^[9] The microcontainers could potentially be used to prevent recurring infections and to treat initial and chronic infections. An additional benefit of the microcontainers is the possibility of personalizing the

606 treatment, by dosing the amount of microcontainers needed for treatment of a certain infection in
607 different patients.

608 3. Conclusion

609 Microcontainers were successfully loaded with CIP and functionalized with lids of PEG, chitosan or
610 Eudragit® S100. Release profiles from coated microcontainers showed that PEG facilitated a faster
611 release of CIP, whereas Eudragit® S100 and chitosan gave rise to a sustained release. All antibiotic
612 containing microcontainers inhibit planktonic growth of PAO1 cells, but the degree of inhibition
613 depends on the choice of coating. Treating a mature PAO1 biofilm with a bolus dose of CIP only
614 resulted in killing of a 26.1 ± 16.9 % of the biofilm cells after 24 h. The same dose confined in coated
615 microcontainers killed 75.7 ± 16.2 %, 88.2 ± 5.3 % and 73.6 ± 14.8 % of the biomass in close proximity
616 to the microcontainer, when using PEG, chitosan and Eudragit® S100 coatings, respectively.
617 Microcontainers provided bacterial killing similar to a 2.75 times higher concentration of solubilized
618 antibiotic constantly perfused. This proves that using microcontainers as a delivery system for
619 antibiotic treatment of biofilm-associated infections could be a promising new strategy.

620 4. Experimental Section

621 4.1. Fabrication of microcontainers

622 Microcontainers were fabricated on silicon wafers as previously described with two-steps of
623 photolithography using the negative epoxy-based photoresist SU-8.^[43] Silicon wafers (4-in. b100N
624 n-type) were supplied by Okmetic, Vantaa, Finland, whereas SU-8 2075 and SU-8 developer were
625 purchased from Microresist Technology GmbH (Berlin, Germany). For biofilm assays, the
626 microcontainers were fabricated on top of a fluorocarbon coated silicon wafer enabling detachment
627 of single microcontainers from the wafer,^[44] whereas for the other studies, the microcontainers were
628 fabricated directly on the silicon wafer.^[43] The wafers with the fabricated microcontainers were cut
629 into chips of 12.8×12.8 mm², containing 625 microcontainers on each chip, using a laser cutter

630 (microSTRUCT vario, 3D Microac AG, Chemnitz, Germany). The dimensions of the
631 microcontainers were measured on an Alpha-Step IQ Stylus Profilometer (KLA-Tencor Corporation,
632 Milpitas, USA).

633 **4.2. Loading ciprofloxacin hydrochloride in microcontainers**

634 A mask was used to cover the gaps between the microcontainers to allow only drug loading in the
635 cavity of the microcontainers.^[45] The microcontainers were then manually loaded with CIP (Fagron,
636 Uitgeest, The Netherlands) by distributing powder over a chip with microcontainers using a small
637 brush. The mask was gently removed after loading and any excess of drug in between the
638 microcontainers was removed with pressurized air. The chips with microcontainers were weighed
639 before and after loading to determine the amount of drug loaded into the microcontainers

640 **4.3. Deposition of a lid on ciprofloxacin hydrochloride-loaded microcontainers**

641 The microcontainers were coated with three different lids of either PEG, chitosan or Eudragit® S100.
642 A 2.66 % w/v solution of low molecular weight 11-15 kDa PEG (Sigma Aldrich, St. Louis, USA) in
643 water was made. The chitosan solution was prepared in a 0.5 % w/v concentration by dissolving low
644 molecular weight chitosan (12 kDa, 75-85 % deacetylated, Sigma Aldrich, St. Louis, USA) in 0.1 M
645 acetic acid (Sigma Aldrich, St. Louis, USA). After dissolving, the chitosan solution was filtered using
646 a 5-13 µm filter with vacuum suction. A solution of 1 % w/v Eudragit® S100 (Evonik Industries,
647 Darmstadt, Germany) and 5 % w/w in relation to the polymer of dibutyl sebacate (Sigma Aldrich, St.
648 Louis, USA) was dissolved in isopropanol (Honeywell, Muskegon, USA). The solutions were
649 sprayed on top of the CIP-loaded microcontainers using a spray coating system equipped with an
650 ultrasonic nozzle actuated at 120 kHz (ExactaCoat, Sono Tek, USA). The nozzle of the spray coater
651 moved across the microcontainer chip in a x-y path covering the area of the chip. One loop
652 corresponded to the nozzle moving across the sample twice, with an offset of 2 mm in the x direction

the second time as the beam diameter was 4 mm. Z-distance represents the distance between the spray nozzle and the sample (**Table 1**).

Table 1. Parameters used for ultrasonic spray coating with either 2.66 % w/v polyethylene glycol (PEG), 0.5 % w/v chitosan or 1 % w/v Eudragit® S100 applied on ciprofloxacin hydrochloride (CIP)-loaded microcontainers.

Coating parameters	PEG	Chitosan	Eudragit® S100
Path speed (mm s ⁻¹)	20	25	10
Infusion rate (mL min ⁻¹)	0.1	0.1	0.1
Generator power (W)	1.3	1.3	2.2
Shaping air pressure (kPa)	0.02	0.03	0.02
Heat plate temperature (°C)	40	50	40
Z-distance from nozzle to sample (cm)	6.0	5.5	3.0
No. of loops	150	120	30

The thickness of the coatings was evaluated using a contact Profilometer (Alpha-Step IQ Stylus Profilometer, KLA-Tencor, Corporation, Milpitas, USA). Each of the polymers was sprayed on top of a flat silicon chip covered with a layer of SU-8. Half of the chip was covered by a glass cover slip hence, enabling coating of only half a chip suitable for height evaluation. The height profiles were measured using an 8 mg tip force with a scan speed of 50 µm s⁻¹ and a sampling rate of 50 Hz. Measurements were conducted at three different locations on each coated chip and presented as mean±SD.

4.4. Scanning electron microscopy of the microcontainers

The quality of the loading and the coating was evaluated using a Tabletop SEM (Hitachi High-Technologies Europe GmbH, Krefeld, Germany). The chips with microcontainers 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 for highest quality images.

4.5. *In vitro* release of ciprofloxacin hydrochloride from microcontainers

The *in vitro* release of CIP from the microcontainers was measured using a μ Diss Profiler (Pion Inc. Woburn, MA, USA) at a constant temperature of 37°C. Stirring rate was kept at 100 RPM and the absorbance was measured *in situ* at 350 nm. The path length of the UV probes was 5 mm. Each probe of the μ Diss was calibrated prior to each release study. Each chip with CIP-loaded microcontainers was attached to cylindrical magnetic stirrers with carbon tape and placed in a glass vial. Modified FAB medium was prepared as described in Sup. Info, **Table S1** with a pH of 6.8 and pH 7.4. The chips were covered with 20 mL of FAB medium pH 6.8 and CIP release from microcontainers was measured for 18-40 h depending on the polymer coating. For Eudragit[®] S100, the chips were placed in FAB medium at pH 6.8 for 15 min and subsequently, in FAB medium adjusted to pH 7.4. The percentage of drug release to each time point was calculated from the known amount of drug loaded per chip and presented as mean $\pm$ SD. The release studies were in 3-6 replicates for uncoated microcontainers and microcontainers coated with either PEG, chitosan or Eudragit[®] S100.

4.6. Bacterial strain and media preparation

P. aeruginosa PAO1 was genetically modified to express green fluorescent protein (GFP) constitutively by insertion into a neutral intergenic region in the genome with no impact on the cell growth physiology.^[6,46,47] An overnight culture of PAO1 was prepared from a -80°C stock by inoculating a small amount into 5 mL LB and allowing growth until stationary phase for 12-16 h at 37 °C at 150 RPM. Plate assays were conducted in LB medium. Biofilms were grown in modified FAB minimal medium with trace metals suitable for PAO1 growth and with glucose as the only carbon source (Sup. Info, **Table S1**).^[33]

4.7. Planktonic growth of PAO1 and growth inhibition testing

To study the ability of microcontainers to inhibit bacterial growth, a PAO1 overnight culture was diluted to an optical density (OD₆₀₀) of 0.05 in 20 mL of LB medium adjusted to pH 6.8, except when

694 used with Eudragit® S100 where pH was pH 7.4. One microcontainer either being empty or loaded
695 with CIP or loaded with CIP and coated with PEG, chitosan or Eudragit® S100 was added to each
696 flask. For the control with CIP in solution, 20 µL of a 0.22 mg/ml CIP solution was added to the flask.
697 The flasks were placed in a 37°C incubator at 150 RPM. Samples were taken at selected time points
698 between 20 min and 24 h after introduction of the microcontainer. Bacterial density was monitored
699 using OD measurements at 600 nm on an automated plate reader (Synergy H1, BioTek, USA). Viable
700 counts were obtained after 3, 8 and 24 h by serial dilution followed by spot plating on LB agar plates.
701 The plates were left to incubate overnight at 37°C and CFU were counted the following day.

702 **4.8. Setup of flow system to grow biofilms**

703 Biofilms of PAO1 were grown at 37°C under laminar flow in flow chambers at 58.4 µL/min with a
704 16-channel Watson Marlow pump as described by Tolker-Nielsen *et al.*^[48] However, the flow cells
705 were slightly modified implementing an inlet channel on the side of the flow chamber to allow
706 inoculation of the microcontainers. Sterilization of the flow system was performed by pumping 1 L
707 of 0.5 % v/v hypochlorite through the system over a period of 4 h followed by a cleaning procedure
708 with filling and emptying the system 3 times with 2 L of autoclaved MilliQ water. Thereafter, the
709 system was filled with FAB medium at pH 6.8 and left with a flow of 58.4 µL/min overnight to allow
710 saturation of the tubings with the medium. The medium flow in the system was stopped and inlet was
711 clamped to avoid backflow. The inlet silicone tube was wiped with 70 % v/v ethanol and 250 µL of
712 a diluted overnight culture (with 0.9 % NaCl to OD₆₀₀ of 0.05) was inoculated carefully into the flow
713 chamber. The inlet needle hole was wiped with ethanol and resealed with a thin layer of silicone glue.
714 To allow the bacteria to attach to the glass surface the flow chambers were left for 1 h without flow
715 before medium flow was resumed. Bacterial biofilms were allowed to develop for 96 h before
716 antibiotic treatment.

4.8.1. *Treating PAO1 biofilm with ciprofloxacin hydrochloride confined in coated microcontainers*

For inoculation of microcontainers, a solution of 2.5 % w/v gelatin (Sigma Aldrich Chemie GmbH, Steinheim, Germany) was used to allow the microcontainer to be dispersed in the syringe instead of sticking to the walls. The gelatin was weighed into sterile tubes and dissolved in 100 mM phosphate-buffered saline (adjusted to pH 6.8) in a water bath at 37 °C and 100 RPM. For sterilization purposes, chloroform (Sigma Aldrich, St. Louis, USA) was added to the gelatin solution with a final concentration of 0.5 % v/v. The tubes were stored at room temperature. Chloroform evaporation was allowed and the microcontainers coated with either PEG, Chitosan or Eudragit® S100 were added to the gelatin just prior to the experiment. Microcontainers loaded with app. 120 µg CIP in total were introduced into the flow cell and the system was left without flow for 15 min before starting the flow again. In Eudragit® S100 channels the pH of the medium was increased to 7.4. Images used for quantification purposes were acquired at a distance of 140-320 µm from the open side of the microcontainer, as the presence of the microcontainer in the image would interfere with the quantitative COMSTAT analysis (see Section 4.9).

4.8.2. *Treating PAO1 biofilm with unconfined ciprofloxacin hydrochloride as bolus dose or as constant perfusion*

A 96 h old PAO1 biofilm was treated with unconfined CIP in two ways: 1) as a single bolus dose or 2) as a constant perfusion. When treating with one bolus dose, the tubing after the bubble traps were clamped off and 2 mL of a 60 µg mL⁻¹ CIP solution were introduced into the system, corresponding to a total dose of 120 µg of CIP. Tubings were afterwards sealed with silicone. For the perfusion study, the medium bottle was changed with a medium bottle containing 4 µg mL⁻¹ of CIP and medium in bubble traps was exchanged with antibiotic-containing medium. The biofilm was treated for 24 h corresponding to a total delivered dose of 330 µg.

4.9. Microscopy parameters, image acquisition and analysis

Microscopic observations of bacterial biofilms and microcontainers were completed using an inverted Leica TCS SP8 CLSM (Mannheim, Germany) equipped with an argon/krypton laser and detectors and filter sets for simultaneous monitoring of GFP (excitation: 488 nm, emission: 493-558 nm) for live cell imaging and propidium iodide (excitation: 543 nm, emission: 558-700 nm) for dead cell staining. Sequential line scanning was used to avoid cross talk. Images were obtained using an HC PL Apo CS2 63x oil objective (numerical aperture 1.4). Propidium iodide was injected into the top of the bubble traps and allowed to flow into the flow chambers for 10 min prior to image acquisition. Images were acquired with z-intervals of 1 μ m. As control, six biological experiments were performed acquiring two images (technical replicates) of non-treated biofilm in each experiment at random positions within the flow cell to account for any heterogeneity within the biofilm. Stacked images were generated using Imaris software (Version 7.7.1, Bitplane AG, Zürich, Switzerland). Volume of biomass was calculated using the image-analysis COMSTAT version 2.1 software.^[33,49] Graphs depicting the fraction of live/dead biomass were generated by calculating the percentage of live/dead in relation to the total biomass measured either before treatment (0 h), after 24 h without treatment as control or 2, 7 and 24 h after treatment.

4.10. Statistical analysis

Data are expressed as the mean $\pm$ SD unless otherwise noted. 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 5 % ($p < 0.05$).

763 Supporting information

764 Supporting Information is available from the Wiley Online Library or from the author.

765

766 Conflict of interest

767 The authors declare no conflict of interest.

768

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Supporting Information**Microcontainer Delivery of Antibiotic Improves Treatment of *Pseudomonas aeruginosa*****Biofilms***Stine Egebro Birk*, Janus Anders Juul Haagensen, Helle Krogh Johansen, Søren Molin, Line Hagner**Nielsen and Anja Boisen*

Figure S1. A) Photograph of a silicon wafer with 32 individual chips (12.8 x 12.8 mm) each containing 625 SU-8 microcontainers. **B)** Scanning electron microscopy (SEM) image of empty microcontainers on a chip.

Figure S2. Graphs depicting the biomasses of PAO1 biofilm grown under flow for 96 h to a mature state and thereafter exposed to ciprofloxacin hydrochloride (CIP)-loaded microcontainers coated with either polyethylene glycol (PEG), chitosan or Eudragit[®] S100 (A) or a bolus dose of CIP (B) or constant perfusion of CIP (C). Green represents live bacteria. Red represents dead bacteria. Data are depicted as mean+SD (n=4-24).

Table S1. The composition of modified FAB medium. A10 buffer, FB minimal medium with trace metals and the carbon source were autoclaved separately and mixed afterwards. Concentrations are given as final concentrations.

A10 buffer	FB minimal medium with trace metals		Carbon source
33.7 mM Na ₂ HPO ₄ ·2H ₂ O	1 mM MgCl ₂	2 μg L ⁻¹ CuSO ₄ ·5H ₂ O	0.3 mM glucose
22.0 mM KH ₂ PO ₄	0.1 mM CaCl ₂	2 μg L ⁻¹ ZnSO ₄ ·7H ₂ O	
15.1 mM (NH ₄) ₂ SO ₄	20 μg L ⁻¹ CaSO ₄ ·2H ₂ O	1 μg L ⁻¹ CoSO ₄ ·7H ₂ O	
51 mM NaCl	20 μg L ⁻¹ FeSO ₄ ·7H ₂ O	1 μg L ⁻¹ NaMoO ₄ ·H ₂ O	
	2 μg L ⁻¹ MnSO ₄ ·H ₂ O	0.5 μg L ⁻¹ H ₃ BO ₃	