Sustained release of antimicrobials from double-layer nanofiber mats for local treatment of periodontal disease, evaluated using a new micro flow-through apparatus

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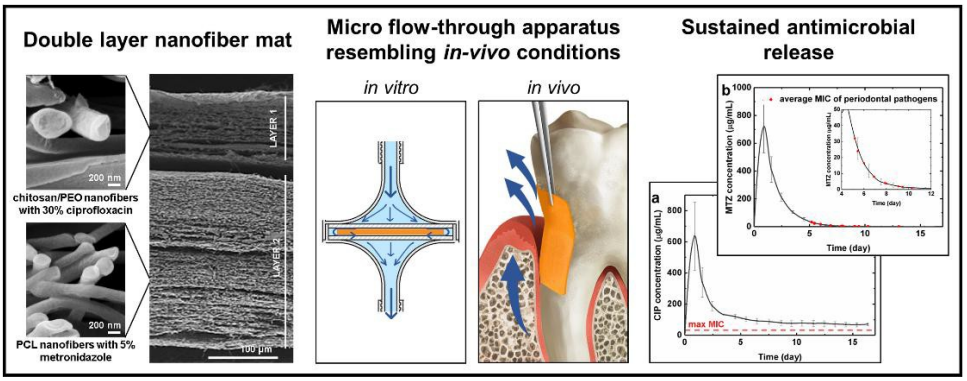
Abstract

Periodontal disease is a widespread chronic condition associated with degradation of periodontal tissues that requires more effective approaches for its treatment. Thus, the aim was to develop a nanodelivery system for local application of antimicrobials, with evaluation *in vitro* using a newly developed micro flow-through apparatus that simulates local *in-vivo* conditions in the periodontal pocket: small resting volume, and low gingival crevicular fluid flow rate. We successfully developed a double-layer nanofiber mat composed of a chitosan/ poly(ethylene) oxide nanofiber layer with 30% ciprofloxacin, and a poly(ε-caprolactone) nanofiber layer with 5% metronidazole. The precisely designed composition enabled sustained *in-vitro* release of the antimicrobials according to their specific drug-release mechanisms. The rate-limiting step of ciprofloxacin release was its own low solubility at pH 7.4, when there was excess of solid drug present in the delivery system. In contrast, sustained release of metronidazole was due to slow penetration of dissolution medium through the hydrophobic poly(ε-caprolactone) nanofiber layer. The double-layer nanofiber mat developed showed antibacterial activity against *Escherichia coli* and *Aggregatibacter actinomycetemcomitans* based on plate antibiogram assays. The antimicrobial concentrations released from the nanofiber mats determined using the developed apparatus were above the MICs against these periodontal pathogens for up to 7 days, which is valuable information for prediction of the efficacy of the nanodelivery system. Although this apparatus was specifically designed for characterization of formulations associated with treatments for periodontal disease, its applicability is much wide, as for development of any delivery system for application at target sites that have similar local conditions.

Keywords: nanofibers, periodontal disease, electrospinning, *in-vitro* method, drug delivery, controlled release



1 INTRODUCTION

Periodontal disease is a chronic inflammatory disease of the periodontium that can cause irreversible degradation of the periodontal ligaments and alveolar bone, which results in deepening of the periodontal pockets, and eventual loosening and loss of the teeth [1]. The overall quality of the life of a patient can be additionally reduced by the higher associated risk of development of other systemic diseases [2]. This represents a huge health problem, as 64.7 million people in the USA suffer from periodontitis, which represents 46% of US adults; 8.9% of these people have an advanced form of periodontitis [3].

The pathology of periodontal disease includes two major causes: a shift in oral microbiota that results in increased numbers of periodontal pathogens in the biofilm, and insufficient immune response of the host [2, 4]. The additional treatment to the mechanical removal of the biofilm, which in the case of advanced periodontitis and deep periodontal pockets does not sufficiently eradicate the periodontal pathogens [2, 5], is antimicrobial therapy [6]. More recent studies have revealed that a combination of antimicrobials is the most effective treatment, which also decreases the development of bacterial resistance [7, 8]. The combination of metronidazole and ciprofloxacin is highly efficient due to their synergistic actions against the major periodontal pathogens, such as *Aggregatibacter actinomycetemcomitans* [9, 10]. On the other hand, beneficial bacteria that have the potential to inhibit periodontal pathogens, such as nonperiodontopathic viridans streptococcal species, are resistant to both of these antimicrobials [10]. Furthermore, ciprofloxacin also shows immunomodulatory effects in human and murine systems [11]. Different induced disease models have shown that ciprofloxacin can reduce the levels of TNFα, interleukin-1β, and interleukin-8 [12], and increase the levels of interleukin-2, interleukin-3, and granulocyte-macrophage colony-stimulating factor [13, 14].

Antimicrobial treatments can be systemic, via oral administration, or local, via application into the periodontal pockets. Local treatments have several advantages, such as higher local drug concentrations, very limited or no systemic side effects, better patient compliance, and reduced risk for development of bacterial resistance [6, 15, 16]. However, a special challenge remains in the development of local delivery system for antimicrobials: they need to be retained in the periodontal pocket and released slowly at therapeutic concentrations over periods of 1 week or more.

For such applications, nanofibers represent a promising drug-delivery system with specific advantages, such as high loading capacity, high encapsulation efficiency, simultaneous delivery of diverse therapeutics, and cost-effectiveness [15, 17, 18]. Depending on the therapeutic application, different controlled-release profiles can be obtained using nanofibers, including biphasic, delayed, sustained, and pulsatile [18, 19]. With respect to the materials used in the preparation of monolithic, blended, or core-shell nanofibers, several drug-release mechanisms have been suggested, such as controlled drug diffusion [20, 21], controlled drug desorption from the polymer matrix [22, 23], controlled air displacement from a superhydrophobic nanofiber mat [24, 25], and controlled swelling, erosion, or degradation of the polymer matrix [21]. Despite significant progress in the development and characterization of nanofibers for drug delivery over recent years, there remain several issues that need to be addressed [18]. One of these is the incorporation of therapeutic doses of hydrophilic drugs that can be released over prolonged periods of time [26]. The challenge is even greater when the delivery of a combination of such drugs is desired, to achieve better therapeutic outcomes.

In our previous study, we incorporated the two hydrophilic antimicrobials metronidazole and ciprofloxacin into poly(ε-caprolactone) (PCL) nanofibers. We achieved sustained metronidazole release for up to 20 days; however, ciprofloxacin alone and the combination of both drugs were released completely in 2 days [9]. Thus, the challenge to develop a delivery system with sustained release of both of these antimicrobials remained unfulfilled.

Moreover, even if sustained drug release can be confirmed in *in-vitro* studies using conventional methods, the prediction of drug concentrations at the site of administration is extremely difficult, as *in-vitro* conditions poorly resemble the *in-vivo* microenvironment. The periodontal pocket is a narrow gingival crevice that is supplied with a slow flow of gingival crevicular fluid that is measured in microliters per hour, with a small resting volume that ranges from 0.4 µL to 1.5 µL, depending on the size of the periodontal pocket [27, 28]. These conditions are far from those used in standard dissolution tests (e.g., United States Pharmacopeia [USP], Apparatus I). The discrepancy between *in-vitro* and *in-vivo* drug-release profiles was also confirmed by Chen *et al.* [29]. Thus, with the latest trends focused on minimizing the use of *in-vivo* testing, there was the need to develop a reliable *in-vitro* method that mimics as closely as possible the true *in-vivo* conditions. Such methods would

provide representative data *in vitro*, and thus decrease the cost and time of development of new dosage forms [30, 31].

The aim was thus to develop a double-layer nanofiber mat that incorporates a combination of selected antimicrobials that would be released in a sustained manner at concentrations higher than the minimal inhibitory concentrations (MICs) against the main periodontal pathogens. Therefore, we first developed a chitosan/ poly(ethylene) oxide (PEO) nanofiber mat with as high a content of ciprofloxacin and chitosan as possible, to increase the bioadhesivity of the delivery system, and then we produced a PCL nanofiber mat with metronidazole. The optimal composition of the nanofibers for ciprofloxacin and metronidazole were selected and electrospun in the form of a double-layer nanofiber mat. Here we hypothesized that we can take advantage of the low ciprofloxacin solubility at pH 7.4 to achieve its sustained local release under conditions with low fluid volumes and flow rates. To prove this concept and to predict the antimicrobial concentrations in the periodontal pocket, we also developed a new *in-vitro* micro flow-through apparatus that mimics the conditions in the periodontal pocket. In addition, the efficacy of the developed nanodelivery systems were tested on *Escherichia coli* and the periodontal pathogen *A. actinomycetemcomitans*.

2 MATERIALS AND METHODS

2.1 Materials

Metronidazole, ciprofloxacin, chitosan (low molecular weight [MW]), PCL (MW, 80 kDa), and PEO (MW, 8 MDa) were from Sigma-Aldrich (China, UK or USA). Glacial acetic acid (100%), formic acid (98%-100%), potassium dihydrogen phosphate, sodium hydroxide, methanol, and acetonitrile were from Merck (Finland or USA). Standard culture media, nutrient broth, and agar-agar were from Roth (Germany), and brain–heart infusion broth was from Biolife (Italy). All of the chemicals were used as received, without further purification or modification. Drug-release experiments were performed using 50 mM phosphate buffer, pH 7.4 as the dissolution medium, and the samples were filtered using 0.2-µm filters (Minisart RC 15; Sartorius StedimBiotech GmbH, Germany).

2.2 Electrospinning of nanofiber mats

The chitosan/PEO solution (92.5:7.5; w/w) was prepared in 70% (w/w) acetic acid, to a total polymer concentration of 4% (w/w). Just prior to electrospinning, different amounts of

ciprofloxacin were added to the polymer solution, as 10% to 40% (w/w) theoretical drug loading of the nanofibers. A 15% (w/w) PCL solution was prepared in a mixture of acetic acid and formic acid (3:1; w/w), which enabled dissolution of metronidazole [9]. One hour before electrospinning, the metronidazole was added to the polymer solution to 5% theoretical drug loading of the nanofibers.

The nanofiber mats were produced using vertical electrospinning technology with a Fluidnatek LE-100 instrument (BioInicia, Spain). The syringe with polymer and drug solution was placed in the pump, which generated a constant flow rate through the needle. Positive high voltage was applied to the needle and negative to the rotating drum collector (diameter, 9.55 cm) that was positioned 15 cm from the nozzle. All of the parameters used in the electrospinning of the chitosan/PEO and PCL solutions are given in Table 1. To produce homogeneously thick nanofiber mats, the drum collector rotation was set to 150 rpm, and the nozzle was simultaneously moving in a range of 3 cm along the collecting drum, at 6.0 mm/s. The electrospinning of the single-layer PCL nanofiber mat with 5% (w/w) metronidazole and the chitosan/PEO nanofiber mats with 10%, 20%, 30%, and 40% (w/w) ciprofloxacin were carried out for 6 h. Drug-free nanofiber mats were prepared under the same conditions as described, without the drugs added to the polymer solutions.

Table 1. Parameters used for the preparation of the electrospun drug-loaded chitosan/PEO and PCL nanofiber mats.

Sample	Composition of polymer solution (%; w/w)	Solvent	Drug content[a] (%; w/w)	Environmental temperature (°C)	Relative humidity (%)	Flow rate (mL/h)	Applied voltage (kV)	
							Nozzle	Collector
Chitosan/PEO	4	70% (w/w) acetic acid	10-40	42 ±1	12 ±1	0.5	+23.6	-5.5
PCL	15	Acetic acid: formic acid, 3:1 (w/w)	5	21 ±1	35 ±1	1	+ 19	-5.5

[a]theoretical drug content in nanofibers

The double-layer nanofiber mats were prepared using two-step electrospinning. First, the chitosan/PEO solution with ciprofloxacin was electrospun for 13 h, and then the PCL solution with metronidazole was electrospun for 8 h, under the conditions shown in Table 1. The theoretical drug loadings in the nanofibers were 30% (w/w) for the chitosan/PEO nanofibers, and 5% (w/w) for the PCL nanofibers.

2.3 Physical characterization of nanofiber mats

2.3.1 Nanofiber morphology and thickness of nanofiber mats

Field emission scanning electron microscopy (SEM; Supra 35 VP; Carl Zeiss, Oberkochen, Germany) was used for evaluation of the morphology and mean diameters of the nanofibers. The nanofiber samples without the coating applied prior to the imaging were fixed on standard pin studs and examined using an acceleration voltage of 1 kV and a secondary detector. The mean fiber diameter was determined based on the SEM images, with measurement of 50 randomly selected nanofibers using the ImageJ 1.44p software (NIH, USA).

To evaluate the morphology and thickness of the single-layer and double-layer nanofiber mats, the samples were frozen in liquid nitrogen and cut perpendicular to the surface of the mats. Then they were pasted to the studs at a 90° angle, in such a way that the nanofiber mat cross-section was facing upwards. Prior to the SEM imaging, the samples were coated with a 5-nm Au layer. The morphology of the nanofiber mat cross-sections was observed under SEM at an acceleration voltage of 7 kV. The images were generated by signal mixing of the secondary and InLens detector at the ratio of 40:60. The nanofiber mat thickness was measured in triplicate, using a stereomicroscope (Olympus SZX12, Japan).

2.3.2 Thermal analysis

Pure ciprofloxacin, ciprofloxacin acetate (prepared by drying its solution in 70 % acetic acid), chitosan, PEO powder, the ciprofloxacin-loaded chitosan/PEO nanofibers were analyzed using differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA). DSC measurements were carried out using ~5 mg samples placed in aluminum pans and scanned in the temperature range from 0 °C to 300 °C at a heating rate of 10 °C/min. The DSC curves were normalized to the sample mass. TGA was performed between 30 °C and 500 °C at a heating rate of 10 °C/min, with the initial sample mass of 5 mg to 10 mg. Both measurements were performed under constant nitrogen flow of 50 mL/min. The solid-state analysis of metronidazole and metronidazole-loaded PCL nanofibers was performed in our previous study [9].

2.4 Drug-release studies using the adjusted conventional dissolution test

Prior drug-release studies ciprofloxacin solubility in the dissolution medium were carried out by addition of excess ciprofloxacin powder to 50 mM phosphate buffer (pH 7.4), in a glass vial. The vial was shaken at 150 rpm at 37 °C, and after 24, 48, and 72 h, 1 mL samples were taken, filtered, diluted, and analyzed using ultra-performance liquid chromatography (UPLC).

Ciprofloxacin release studies were performed under sink and non-sink conditions, whereas metronidazole release was investigated under sink condition. The theoretical maximal drug concentration achieved under the sink conditions was at least a third of that of the drug saturation solubility in the phosphate buffer at 37 °C, whereas under the non-sink conditions, this condition was not fulfilled. Thus, the appropriate mass of chitosan/PEO nanofibers loaded with 30% ciprofloxacin was used in the drug-release study under sink and non-sink conditions. The samples of the ciprofloxacin-loaded nanofiber mats were each immersed in 19 mL phosphate buffer in a glass vial, whereas 18 mg samples of the metronidazole-loaded nanofiber mats were immersed in 10 mL. phosphate buffer. The glass vials were, shaken at 150 rpm and 37 °C throughout the tests. At predetermined times, 1 mL medium was withdrawn, and for the test under the sink conditions, the samples were replenished with fresh medium. The samples were filtered immediately, and then diluted and analyzed using UPLC. To examine the morphology of the ciprofloxacin-loaded nanofiber mats after the drug release study under the non-sink conditions, the samples were washed with distilled water, frozen at -80 °C, lyophilized (Chris Beta 1-8K; Martin Chris, Germany) using a method described previously [32], and then analyzed by SEM without any coating applied prior to imaging (section 2.3.1).

2.5 Set-up and drug release using the newly developed micro flow-through apparatus

To evaluate the drug release under conditions that mimicked the *in-vivo* environment of periodontal pockets, we developed a specific micro flow-through apparatus. The apparatus and the conditions used were based on the available *in-vivo* data of the microenvironment of the periodontal pocket. This apparatus was composed of three main components: (i) pump; (ii) temperature-controlled micro flow-through dissolution chamber; and (iii) collecting system. In more detail, the pump was attached to a reservoir of fresh dissolution medium and provided a constant flow of dissolution medium through the temperature-controlled micro flow-through dissolution chamber. The test samples, namely the nanofiber mats, were placed in the flow-through chamber, which was circular in shape with a surface area of 3 cm^2, and

had a low internal volume, to resemble the dimensions of periodontal pockets. The needle connected the flow-through chamber with the vials, where the dissolution medium was collected and then analyzed using UPLC. To increase the number of simultaneously tested samples, we used 10 parallel set-ups, as described above.

To perform drug release from nanofibers, the ciprofloxacin-loaded chitosan/PEO nanofiber mats (5 ±1 mg), the metronidazole-loaded PCL nanofiber mats (9 ±1 mg), and the double-layer nanofiber mats (27.4 ±0.2 mg) were cut into circles (surface area, 2.6 cm^2) and placed into the chambers of the micro flow-through apparatus. The flow rate of dissolution medium through the chambers that contained the nanofiber mats was set to 15 µL/h and 30 µL/h. All of the nanofibers were tested using 50 mM phosphate buffer (pH 7.4) as the dissolution medium, while for 30% ciprofloxacin-loaded chitosan/PEO nanofiber mats, 0.1 M HCl (pH 1) was also used. The medium with the released drugs was collected in vials, and these samples were filtered. Then the drug concentrations were determined using UPLC analysis. The vials were changed daily, and thus the dissolution medium collected each day contained the average drug concentration being released from the nanofibers during each specific day.

2.6 UPLC analysis of antimicrobials

Ciprofloxacin and metronidazole were analyzed using a UPLC system (Acquity; Waters Corp., USA) equipped with a UV–VIS photodiode array module with a high-sensitivity flow cell and column, and an associated pre-column (Acquity UPLC CSH C18; 1.7 µm 2.1 × 50 mm). The injection volume was 5 µL, the column temperature was maintained at 50 °C, and the mobile phase flow-rate was set to 0.5 mL/min. Gradient elution was used to achieve chromatographic separation of both of the antimicrobials, with mobile phases A (25 mM phosphate buffer, pH 3, 10% methanol) and B (98% acetonitrile). The elution was according to the following gradient: 0.0-1.0 min, 0% B; 1.0-3.0 min, 0%-20% B; 3.0-3.2 min, 20%-50% B; 3.2-3.6 min, 50% B; 3.6-4.4 min, 50%-0% B; 4.4-5.0 min, 0% B. The analytical run-time for each sample was thus 5 min, and the UV detection was set to 271 nm. During the analyses, the autosampler temperature was kept at 4 °C. Calibration curves were prepared for both of the antimicrobials in the concentration range from 0.5 µg/mL to 40 µg/mL (R^2 = 0.9999, for both of the antimicrobials).

2.7 Antimicrobial tests

2.7.1 Antimicrobial activities of antimicrobials released from nanofiber mats

The antimicrobial activities of the antimicrobials incorporated into the single-layer and double-layer nanofiber mats were assessed using plate antibiogram assay. Nutrient agar and brain–heart infusion agar were used with the *E. coli* TOP10 and *A. actinomycetemcomitans* DSM 8324 test strains, respectively. For each test, a total of 100 µL active bacterial liquid culture (OD_{600}, 0.8-1.0) was spread evenly over the surface of the agar medium. Then, 4-mm-diameter discs were cut aseptically from the drug-free and antimicrobial-loaded single-layer and double-layer chitosan/PEO and PCL nanofiber mats, and placed on the agar plates. The plates were incubated for 24 h at 37 °C. Plates with *E. coli* were incubated aerobically, while for *A. actinomycetemcomitans*, a low oxygen environment was provided using the candle jar method of Slots (1982) [33]. The growth of the bacteria on the surface of the agar medium was inhibited due to the diffusion of the antimicrobials into the agar. The diameters of the clear areas around the pockets with nanofibers, defined as the inhibition zones, were measured after 24 h, to indicate the efficacies of the growth inhibition. Drug-free nanofiber mats were used as the negative controls.

2.7.2 Determination of MICs of antimicrobials against E. coli and A. actinomycetemcomitans

The MICs of the antimicrobials released from the nanofiber mats into the buffer were evaluated using the broth microdilution method for *E. coli* TOP10 and *A. actinomycetemcomitans* DSM 8324. Approximately 10 mg antimicrobial-loaded chitosan/PEO and PCL nanofiber mats, and control chitosan/PEO and PCL nanofiber mats without drug, were separately immersed in 10 mL phosphate buffer and shaken at 150 rpm at 37 °C. After 1 week, the samples were filtered and the antimicrobial concentrations in the buffers were analyzed using UPLC. Mats without drugs were used as negative controls to ensure that the polymers themselves did not inhibit the growth of the bacteria. The antimicrobial standards were prepared separately by dissolving the powdered drugs in phosphate buffer, initially at 50 µg/mL. Dilution series of the released antimicrobials or dissolved standards and double-strength growth media were mixed in 1:1 volume ratios. Nutrient broth and brain–heart infusion medium were used for *E. coli* and *A. actinomycetemcomitans*, respectively. The tests were prepared in sterile clear-bottomed, 96-well plates, where each well contained 100 µL suspension of growth medium and the antimicrobial, to which a total of 1 µL of the test bacterial liquid culture of *E. coli* or *A. actinomycetemcomitans* was added (at OD_{600}, 1.0-1.3). Growth medium without the antimicrobials was used as the positive control of growth, and growth medium containing the

antimicrobials without the bacterial inoculants was used as the negative control. The samples were examined for the MICs after 24 h incubation at 37 °C. For *A. actinomycetemcomitans*, a low oxygen environment was provided using the candle jar method of Slots (1982) [33]. OD_{600} values were determined using a plate reader (Synergy H4; Biotek, USA). The MICs were determined as the lowest concentrations of the antimicrobials at which no growth of *E. coli* or *A. actinomycetemcomitans* was detected.

2.8 Statistical analysis

Data are expressed as means ±standard deviation. The drug-release studies were performed in triplicates, and the antimicrobial tests were repeated three times. The data were analyzed statistically using one-way analysis of variance (ANOVA), followed by Tukey's *post-hoc* tests, using the OriginPro 2017 software (OriginLab Corporation, USA). $p < 0.05$ was taken as significant.

3 RESULTS AND DISCUSSION

3.1 Development of single-layer and double-layer nanofiber mats with antimicrobials

3.1.1 Single-layer chitosan/PEO nanofiber mats with ciprofloxacin

In this study, a novel delivery system based on electrospun nanofibers was designed for administration of the antimicrobials in the periodontal pockets, as the target sites for local treatment of periodontal disease. Efficacy of this local therapy can only be expected if the formulation adheres to the pocket surface and is retained there for a prolonged period of time [15]. For development of such delivery systems, the bioadhesive polymers are suggested, among which chitosan presents a very promising candidate [34]. Additional benefit of using chitosan as excipient is its bacteriostatic effect on Gram-negative bacteria [35]. Although the electrospinning of chitosan is known to be challenging [36], our goal was to prepare nanofibers with the maximal amount of chitosan, to achieve stability of the nanofiber morphology in a physiological environment, and to retain the nanofiber bioadhesive properties.

To fulfil this aim, the composition of polymer solution and the electrospinning parameters were systematically varied, including for the ratio of chitosan to PEO, the molecular weight of PEO used, the flow-rate of the polymer solution, and the voltage applied. The formulation with a chitosan/PEO ratio of 92.5:7.5 using high molecular weight PEO (8

MDa) prepared from 20% (w/w) acetic acid was selected as the most promising for further optimization based on the experimental design (see Supplementary Materials, section 2.1 for more details). Statistical analysis revealed that the best nanofiber formulation was obtained with 70% (w/w) acetic acid, where the nanofibers were beadless, with a mean diameter of 255 ±120 nm (Fig. 1a). The nanofiber polymer composition enabled incorporation of up to 40% (w/w) ciprofloxacin without any visible drug crystals on the nanofiber surface (Fig. 1b-e). High drug loading is often a crucial parameter in the preparation of nanodelivery systems with therapeutic doses. Here, this high drug loading was a consequence of good compatibility between the components, as in contrast, ciprofloxacin crystallized on the surface of PCL nanofibers already at 5% drug loading [9]. However, increased ciprofloxacin loading from 10% to 40% significantly influenced the morphology of the electrospun product, as the mean diameter was increased, and the fibrillar morphology was changed to a ribbon structure (Fig. 1b-e). At the highest investigated drug loading, these electrospun ribbons fused into a mesh-like morphology (Fig. 1e). Nanofibers with 30% ciprofloxacin showed the optimum properties in terms of the small nanofiber diameter (Fig. 1d) and the high drug loading, which were used for the development of the double-layer nanofiber mats. The preserved nanofiber morphology of chitosan/PEO nanofiber mats with 30% ciprofloxacin was confirmed also after the completed drug release study under the non-sink conditions (Fig. 1f).

Differential scanning calorimetry was used to determine the solid-state form of the ciprofloxacin incorporated into the nanofibers (Fig. 1h). All of the DSC curves associated with the chitosan/PEO nanofibers showed two endothermic peaks: one at ~100 °C and the other at ~180 °C. The DSC curves of chitosan/PEO nanofibers with 30% and 40% ciprofloxacin had additional endothermic peaks at 257 °C and 264 °C, respectively. Most of components started to degrade above 260 °C (Fig. 1i). The peak at ~100 °C appears to be associated with the evaporation of adsorbed water, as also seen in the DSC curve of pure chitosan (Fig. 1h). This correlates with the weight loss detected by TGA analysis (Fig. 1i). The endothermic peak at ~180 °C can be ascribed to the evaporation of acetic acid, as the polymer solution for electrospinning was prepared in 70% (w/w) acetic acid, and thus ciprofloxacin might form a salt during the solution preparation or the electrospinning process. To support this, the DSC curve of ciprofloxacin dissolved in 70% (w/w) acetic acid and then dried was recorded. This revealed endothermic peaks at 85 °C, 178 °C, and 275 °C, where the first two correlated with the weight reduction, as detected by TGA. It would appear that the first peak represents the evaporation of residual water and the second peak represents the

evaporation of bound acetic acid. The endothermic peak at 275 °C represents the melting of ciprofloxacin, which is also present in the DSC curve of pure ciprofloxacin. The endothermic peak at 74 °C in the DSC curve of pure PEO represents its melting. This was less profound in the chitosan/PEO nanofibers due to the small amount of PEO (i.e. 7.5%) in the polymer mixture and overlap with the endothermic peak that represents water evaporation from chitosan.

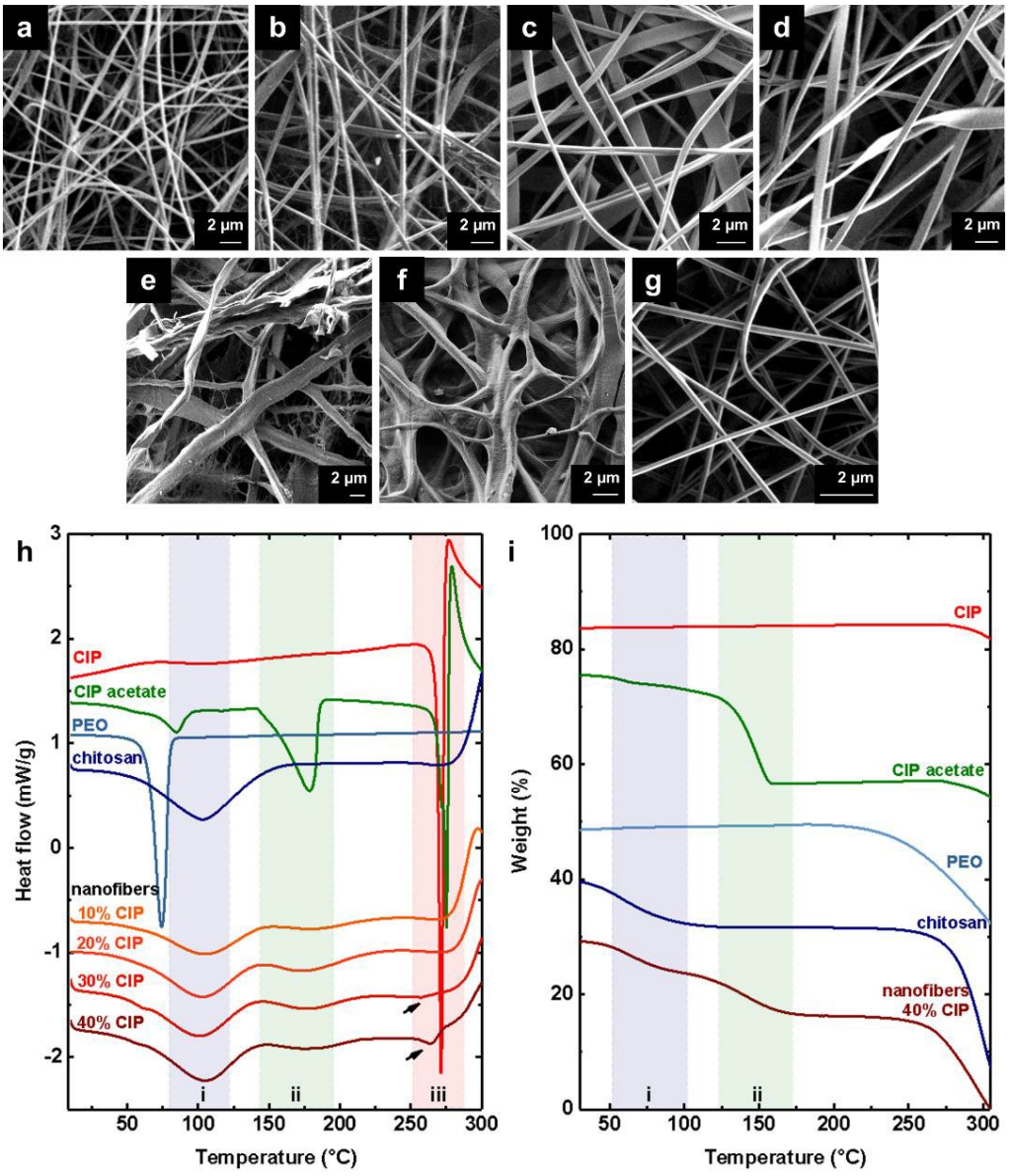


Figure 1. SEM images of chitosan/PEO nanofibers (a) without drug and loaded with (b) 10%, (c) 20%, (d) 30%, and (e) 40% ciprofloxacin, and (f) with 30% ciprofloxacin, after drug-release tests had been carried out under non-sink conditions. (g) SEM image of PCL nanofibers with 5% metronidazole. (h) Differential scanning calorimetry and (i) thermogravimetric analysis curves of pure ciprofloxacin (CIP), ciprofloxacin acetate, pure

PEO, pure chitosan, and chitosan/PEO nanofibers with ciprofloxacin. The temperature ranges of water desorption (i), acetic acid evaporation (ii), and melting of ciprofloxacin (iii) are shaded.

To sum up, DSC and TGA analysis showed that a proportion of the ciprofloxacin in the chitosan/PEO nanofibers was in the form of ciprofloxacin acetate; furthermore, crystalline ciprofloxacin was not detected in the nanofibers with 10% and 20% drug loading, and thus its amorphous form can be assumed. Some crystalline ciprofloxacin was detected in the nanofibers with 30% and 40% drug loading.

3.1.2 Single-layer PCL nanofiber mat with metronidazole

PCL nanofibers with 5% metronidazole loading showed no visible crystals on the surface, which indicated successful incorporation of the metronidazole into the polymer matrix (Fig. 1g). The mean nanofiber diameter was 290 ±75 nm, and the mean thickness of the nanofiber mat was 199 ±34 μm. In our previous study [9], a X-ray diffraction revealed that part of metronidazole crystalized within the nanofibers in its initial crystalline form, and another part was in its amorphous form.

3.1.3 Double-layer nanofiber mat with combination of antimicrobials

The double-layer nanofiber mat was prepared by two-step electrospinning, where the first layer was electrospun from chitosan/PEO solution with 30% ciprofloxacin, and the second layer from PCL solution with 5% metronidazole. The thickness of the chitosan/PEO nanofiber layer was 82 ±16 μm, whereas the PCL nanofiber layer had a thickness of 229 ±17 μm (Table 2, Fig. 2). The mean nanofiber diameter of the individual types of nanofibers in double-layer nanofiber mats did not differ significantly ($p > 0.05$) from the same type of nanofibers prepared as single layers.

Table 2. Characteristics of the double-layer nanofiber mats.

Characteristic	**Layer 1 Chitosan/PEO nanofibers with 30% ciprofloxacin**	**Layer 2 PCL nanofibers with 5% metronidazole**	**Double layer (total)**
Layer thickness (μm)	82 ±16	229 ±17	311 ±33
Mass of nanofiber mat (mg/cm^2)	3.30 ±0.15	7.11 ±0.00	10.42 ±0.15
Mass of antimicrobial in nanofiber mat (μg/cm^2)	991	356	1347
Loading of antimicrobial in nanofiber mat (μg/mg)	300	50	129

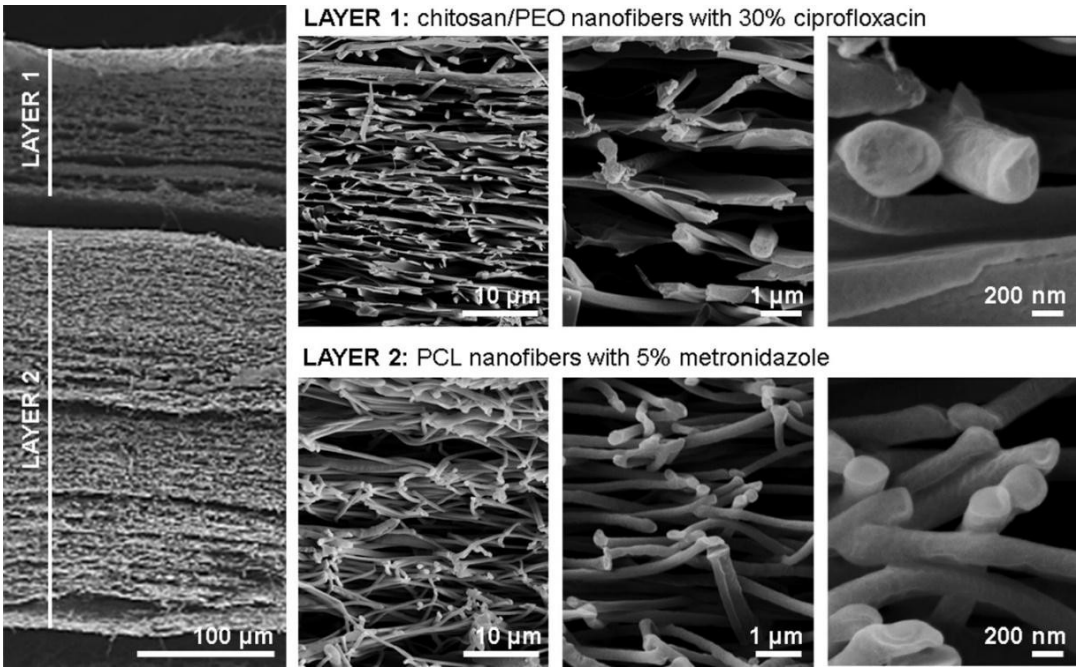


Figure 2. Cross-section of a double-layer nanofiber mat composed of chitosan/PEO nanofibers with 30% ciprofloxacin (layer 1) and PCL nanofibers with 5% metronidazole (layer 2). The cross-section is shown at various magnifications. Scale bars, as indicated.

3.2 Drug release in the adjusted conventional dissolution test

The ciprofloxacin release from the nanofiber mats developed was evaluated first using the adjusted conventional *in-vitro* dissolution test in the dissolution medium at pH 7.4 (Fig. 3d). Here, the volume of the dissolution medium was lower compared to USP Apparatus I, and the mixing of the dissolution differed between the methods. Under sink conditions, ciprofloxacin was completely released from the chitosan/PEO nanofibers in the first 4 h (Fig. 3a), where the PEO dissolved and chitosan swelled and thus allowed rapid ciprofloxacin diffusion out of the polymer matrix. Ciprofloxacin has been studied already in nanofibers prepared from different polymers [9, 23, 37-40]. The general conclusion across all of these studies is that ciprofloxacin release mainly depends on the polarity of the polymers and the drug loading. Ciprofloxacin was released from hydrophilic nanofibers in less than 1 day [39], which is in line with the present study (Fig. 3a). Hydrophobic polymers sustained ciprofloxacin release at 1% drug loading [23, 37, 38], while ciprofloxacin loading of more than 5% resulted in its precipitation on the surface of the PCL or polycarbonate urethane nanofibers [9, 40]. In such cases, the ciprofloxacin was immediately released.

Under non-sink conditions, only 26% of ciprofloxacin was released (Fig. 3a). Ciprofloxacin showed supersaturation in the first few hours, with a maximal concentration of 313 µg/mL. This decreased to 300 µg/mL over the next 4 days (Fig. 3b), and during the experiment it remained significantly higher ($p < 0.05$) compared to the pure ciprofloxacin solubility under these conditions (110 µg/mL; Fig. 3b). This ciprofloxacin supersaturation is the consequence of its partial amorphization in the nanofibers (Fig. 1h), as the polymers alone did not improve its solubility (data not shown). The remaining 74% of the drug, i.e., the excess amount of ciprofloxacin in the nanofiber mat, would only be released if fresh dissolution medium is added.

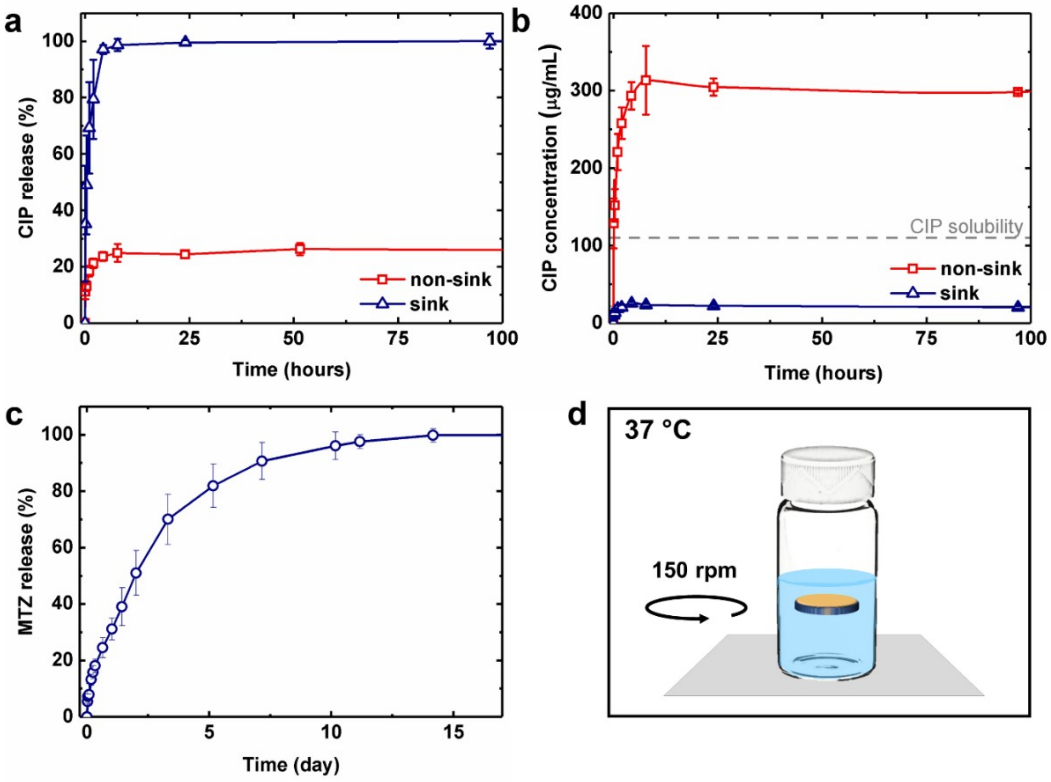


Figure 3. Ciprofloxacin (CIP) release profiles from chitosan/PEO nanofibers with 30% ciprofloxacin under sink and non-sink conditions, (a) as cumulative drug release from nanofiber mats, and (b) as concentration of ciprofloxacin in the release media. (c) Cumulative metronidazole (MTZ) release from PCL nanofibers with 5% metronidazole loading under sink conditions. (d) Illustration of the adjusted conventional dissolution test used in this study.

Metronidazole release from the PCL nanofiber mats was slow, and lasted over 14 days (Fig. 3c); this was in line with our previous study [9]. If metronidazole is incorporated into chitosan/PEO nanofibers, it would be released in <1 day [41].

3.3 The design of the new micro flow-through apparatus

The sustained release of ciprofloxacin was not confirmed *in vitro* using the adjusted conventional drug dissolution test under either sink or non-sink conditions. Consequently, we developed a new micro flow-through apparatus, where the apparatus set-up and the test parameters used were chosen to simulate *in-vivo* conditions in periodontal pockets; namely for temperature, pH, and volume and flow of the gingival crevicular fluid through the periodontal pocket. Periodontal pockets are narrow gingival crevices between plaque bacteria that adhere to the surface of the tooth root and the epithelium lining of the pocket space. The main source of body fluid in these pockets is gingival crevicular fluid, which flows through them and into the mouth at a rate that is measured in microliters per hour; saliva does not generally diffuse from the oral cavity into the pockets [28]. Apart from unidirectional fluid flow, the specificity of the pockets is also their small resting volume, which ranges from 0.4 µL to 1.5 µL, depending on the size of the periodontal pockets [27]. The main characteristics of the gingival crevice were mimicked in our *in-vitro* apparatus by the narrow chamber in the micro flow-through apparatus, which simulated the small resting fluid volume *in vivo.* A comparison between the *in-vivo* conditions and the *in-vitro* apparatus set-up is shown in Figure 4. The flow of the dissolution medium from the upper part of the chamber through and around the delivery system resembles the gingival crevicular fluid flow from the inflamed epithelium through and around the delivery system inserted in a periodontal pocket. Detailed calculations of the theoretical flow rates of gingival crevicular fluid and other *in- vivo* conditions based on available literature data are described in the Supplementary Materials, section 2.2.

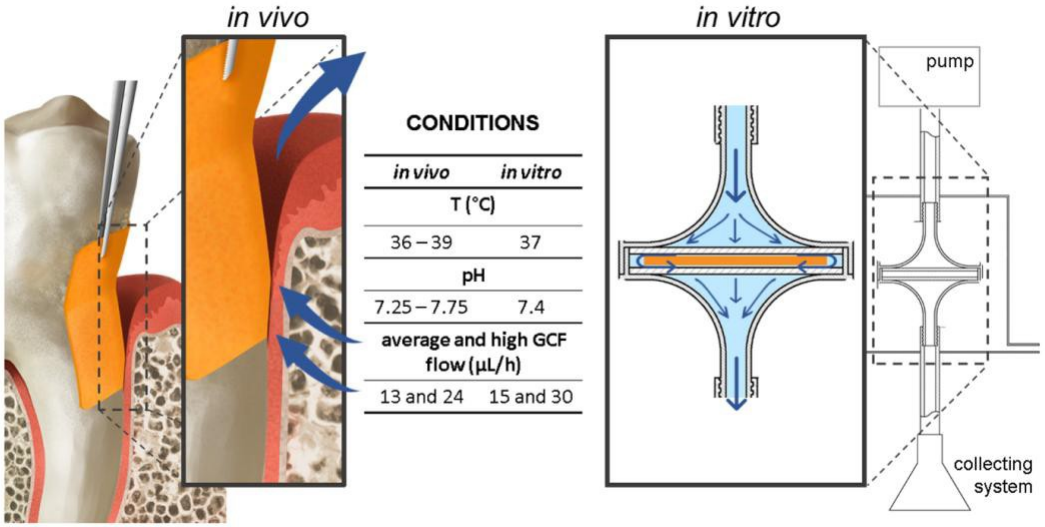


Figure 4. Comparison between the *in-vivo* conditions in a periodontal pocket and the *in-vitro* conditions in the micro flow-through apparatus. GCF, gingival crevicular fluid.

3.4 Drug release in the newly developed micro flow-through apparatus

3.4.1 Antimicrobial release from single-layer nanofiber mats

With the newly developed micro flow-through apparatus, we tested the hypothesis that low ciprofloxacin solubility at pH 7.4 can be used to achieve its sustained local release from nanofibers under conditions with low volumes and flow rates of the dissolution medium. Ciprofloxacin is according to the biopharmaceutics classification system (BCS) classified in Class IV [42]. It is a hydrophilic drug (i.e., logP, –1.7 to 0.94 [42]), and it shows pH-dependent solubility. In acidic medium at 37 °C, its solubility is above 30 mg/mL, whereas at pH 7.4 and 37 °C is only 110 μg/mL. As seen in Figure 5, the dissolution medium pH has a key role in ciprofloxacin release from chitosan/PEO nanofibers at 30% drug loading. Over 90% of ciprofloxacin was released in 2 days at pH 1, and in 21 days at pH 7.4 (Fig. 5a).

Sustained release of ciprofloxacin was confirmed for all of the ciprofloxacin-loaded chitosan/PEO nanofiber mats with 10% to 40% ciprofloxacin loading, over a minimum of 7 days (Fig. 5b). In more detail, the data showed strong correlation between ciprofloxacin loading and ciprofloxacin release, which was more sustained for the nanofiber mats with higher ciprofloxacin loading. The changes in the ciprofloxacin concentrations in the dissolution medium with time are shown in Figure 5b (right panel). Three regions can be identified in the ciprofloxacin-release profiles: (*i*) the region of supersaturation, which occurs at the beginning of the ciprofloxacin release as a consequence of its partial amorphous state in the nanofibers (Fig. 1h); (ii) the region where the ciprofloxacin concentration in the dissolution medium lies at its solubility (~110 μg/mL), which occurs 2-3 days after the beginning of the ciprofloxacin release; and (iii) the region where the ciprofloxacin concentration slowly decreases below its solubility level. The ciprofloxacin concentration after 21 days was still ~6.5 μg/mL and ~87.5 μg/mL for the 30% and 40% ciprofloxacin-loaded nanofiber mats, respectively. In many studies, increased drug loading causes an acceleration of the rate of drug release [21, 43, 44], which is contrary to the present data. The reason for this relates to the different mechanisms of drug release, as usually the polymer matrix represents the barrier that hinders the rate of drug release. Here, the rate-limiting step for ciprofloxacin release was its own solubility, when there was an excess of solid drug present in the nanofibers. As seen from Figure 5b, the higher the drug loading, the more

ciprofloxacin was in the nanofibers, and the region with ciprofloxacin concentration around its solubility was longer.

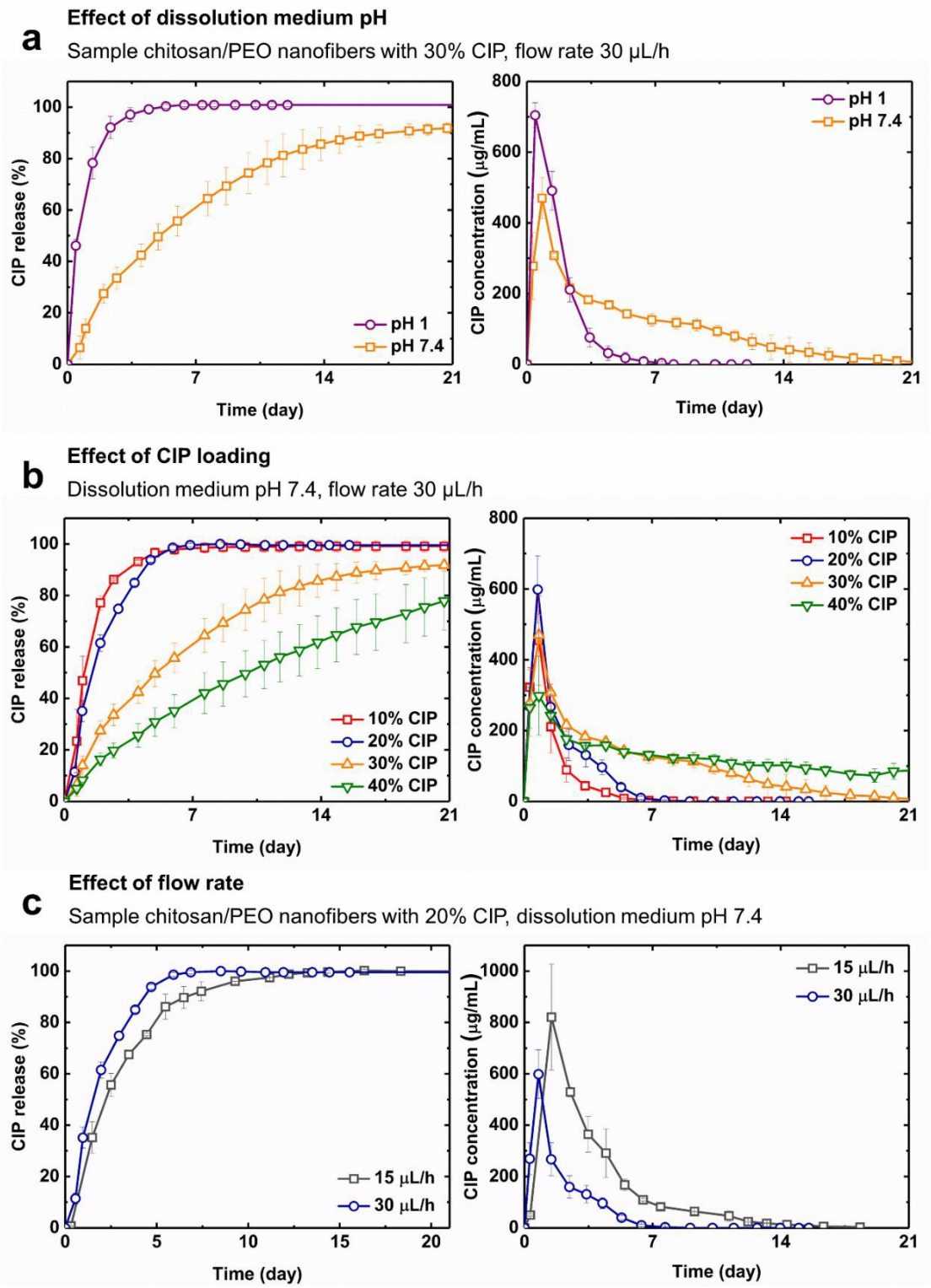


Figure 5. Ciprofloxacin (CIP) release from chitosan/PEO with ciprofloxacin, as determined using the micro flow-through apparatus, while varying: (a) pH of dissolution medium; (b) ciprofloxacin loading; and (c) dissolution medium flow rate. Left panel: Cumulative

ciprofloxacin release; right panel: Ciprofloxacin concentration in the dissolution medium during the release.

The flow rate of gingival crevicular fluid increases with progression of periodontal disease [27], which can also be simulated in the new micro flow-through apparatus. Thus, we investigated ciprofloxacin release at the mean (15 µL/h) and maximum (30 µL/h) flow rates of the dissolution medium from chitosan/PEO nanofibers with 20% ciprofloxacin. The lower flow rate prolonged the ciprofloxacin release, while achieving higher concentrations compared to higher flow rates (Fig. 5c). Thus, at sites where a limited volume of body fluid is available for drug release, low solubility and excess of solid drug are the key factors responsible for the sustained release. Thus, it was shown that ciprofloxacin, as a BCS class IV drug, has a high potential to be slowly released at the sites with low fluid flow and residual volume due to its low solubility at pH 7.4, whereas its low permeability can decrease systemic absorption and consequently reduce undesirable side effects.

Prolonged release of the metronidazole from PCL nanofibers was shown in the adjusted conventional dissolution test (Fig. 3c) as well as in the newly developed micro flow-through apparatus, where the flow rate of the dissolution medium was 30 µL/h and the metronidazole release lasted 11 days (Fig. 6a). We also investigated metronidazole release at the mean (15 µL/h) and maximum (30 µL/h) flow rates of the dissolution medium. These data revealed that the release of metronidazole was faster with the increased flow rate, although the metronidazole concentration in the dissolution medium was lower (Fig. 6b). The same trend of drug release was observed for both drugs (Figs. 5c, 6). The maximal metronidazole concentration in the dissolution medium was reached after ~20 h, and for the lower flow rate the concentration was 2-fold that at the higher flow rate.

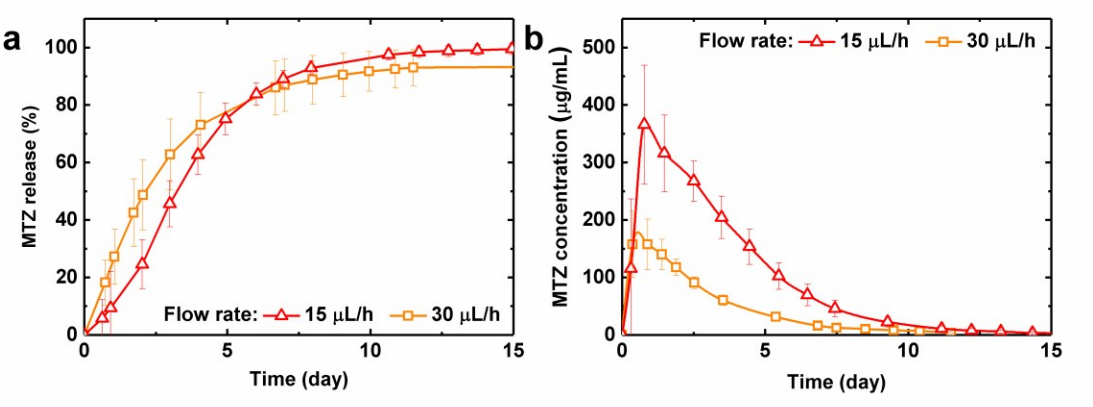


Figure 6. Metronidazole (MTZ) release profiles from PCL nanofiber mats with 5% drug loading at different flow rates, as determined using the micro flow-through apparatus. Left

panel: (a) Cumulative metronidazole release. (b) Concentrations of metronidazole released into the dissolution medium.

The mechanism of metronidazole release from the hydrophobic PCL nanofibers was different from that of ciprofloxacin release from the hydrophilic chitosan/PEO nanofibers. The PCL nanofiber mats loaded with metronidazole showed a hydrophobic character due to the chemical structure of the polymer and the air entrapped in the pores between the nanofibers in the mats. The air hydrophobicity resulted in slow penetration of the dissolution medium into the nanofiber mats. After the air was displaced by the dissolution medium, the nanofibers were wetted, and the metronidazole was able to dissolve and diffuse out of the nanofiber mats [9]. A similar mechanism has been reported for drug release from superhydrophobic fiber mats [24, 25]. Therefore, for a sparingly soluble drug, as is metronidazole (10 mg/mL; pH 2.5-8.0; [45]), it is possible to use a hydrophobic polymer to hinder access of the medium needed for drug dissolution. This mechanism is possible with a sufficiently thick hydrophobic nanofiber mat [9].

The vertical cutting of the nanofiber mat to smaller pieces did not affect the metronidazole release (Supplementary Fig. S2), which is in line with our previous study [9]. This finding is vital for clinical applications, as it means that the antimicrobial doses can be adjusted by simply cutting the appropriate size of nanofiber mat, which would fit into a specific periodontal pocket without adversely affecting the overall drug release profile. Of note, although PCL is biodegradable, the degradation of the nanofiber mat in the periodontal pocket would take longer compared to its function as a drug-delivery system, and thus its removal after 2 weeks by the dentist would be advisable.

3.4.2 Antimicrobial release from double-layer nanofiber mats

For the double-layer nanofiber mats composed of the PCL nanofiber layer with 5% metronidazole and the chitosan/PEO nanofiber layer with 30% ciprofloxacin, the release of the antimicrobials was sustained, as determined using the micro flow-through apparatus (Fig. 7). Metronidazole was completely released in 12 days, while ciprofloxacin release was slower, and only 53% of it was released over the time of the experiment; i.e., 17 days (Fig. 7a). The initial peak concentration of ciprofloxacin was 636 ± 220 µg/mL, and for metronidazole, 720 ±189 µg/mL, and these were both achieved in the first 24 h of the tests (Fig. 7b). The metronidazole concentration gradually decreased over the following days,

while that of ciprofloxacin remained constant, at 80 ±10 µg/mL from the 5th to the 17th day (Fig. 7b). The additional antimicrobial release studies using the micro flow-through apparatus varying the orientation of double-layer nanofiber mats and the impact of chitosan/PEO nanofiber layer on metronidazole-loaded PCL nanofiber layer with calculated similarity factor between samples are described in the Supplementary Materials, section 2.3.

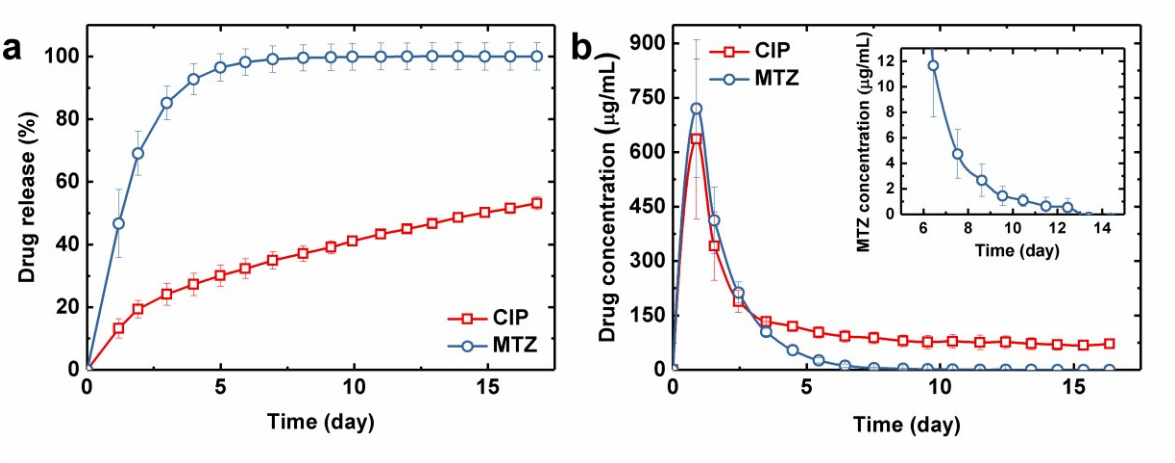


Figure 7. Ciprofloxacin (CIP) and metronidazole (MTZ) release from the double-layer nanofiber mats composed of the PCL nanofiber layer with 5% metronidazole and the chitosan/PEO nanofiber layer with 30% ciprofloxacin, as determined using the micro flow-through apparatus with a dissolution medium flow-rate of 30 µL/h. (a) Cumulative drug release. (b) Concentrations of drugs released into the dissolution medium. Insert: Enlarged part of the metronidazole release profile.

3.5 Antibacterial activities of antimicrobials released from the nanofiber mats

The antimicrobial-loaded nanofiber mats were shown to be effective against the growth of two Gram-negative bacteria inoculated onto the surface of agar plates: *E. coli* and *A. actinomycetemcomitans* (Fig. 8). The ciprofloxacin and metronidazole successfully diffused from nanofiber mats placed onto the surface of the solid agar medium as round disks, and they inhibited the growth of both of these bacteria, with the formation of inhibition zones around the disks (Fig. 8). Comparisons of the effects of orientation of the double-layer mat on the agar plate revealed that the size of the inhibition zones was larger when the ciprofloxacin-loaded layer was facing downwards and was in contact with the agar medium. These data also showed that tight contact must be created between the PCL nanofiber mat and the agar

medium for efficient diffusion of the metronidazole into the agar medium (Supplementary Fig. S4). Drug-free nanofiber mats (i.e., negative controls) had no inhibitory effects on the growth of these bacteria (Fig. 8).

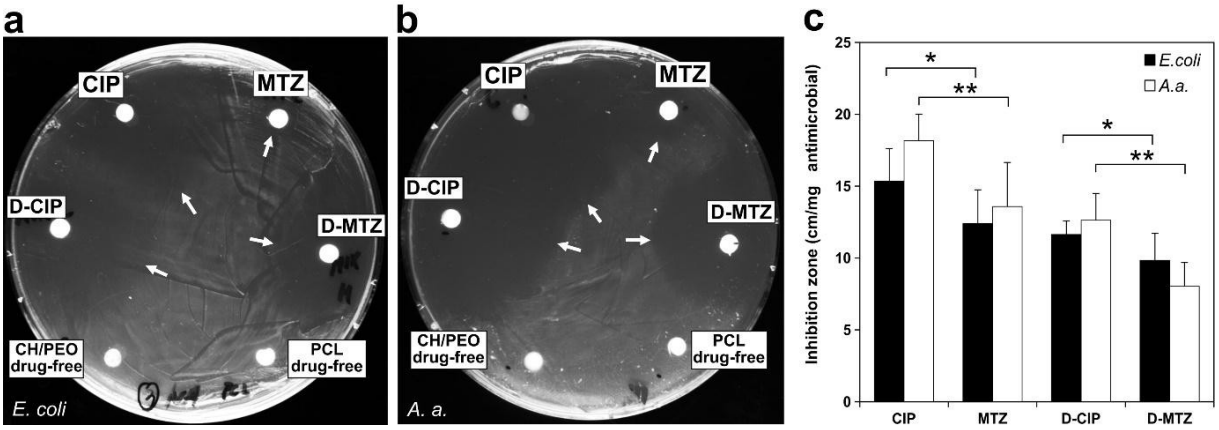


Figure 8. Representative *in-vitro* testing for growth inhibition of Gram-negative bacteria using the antimicrobial loaded nanofiber mats. Antibiogram tests were performed on solid agar medium using test strains of (a) *E. coli* and (b) *A. actinomycetemcomitans* (*A.a.*). The inhibition zones are marked with white arrowheads. (c) Quantification of the inhibition effects with the inhibition zones normalized to the antimicrobials contents. CIP, ciprofloxacin; MTZ, metronidazole, D-CIP, double-layer mat, consisting of PCL layer loaded with metronidazole and the chitosan/PEO (CH/PEO) layer loaded with ciprofloxacin facing the agar medium; D-MTZ, double-layered mat as described above, with the PCL layer loaded with metronidazole facing the agar medium. The chitosan/PEO (CH/PEO drug-free) and PCL drug-free nanofiber mat controls are also shown (i.e., without antimicrobials loaded). * P <0.05, ** P <0.01.

The stability and consequently the activity of the antimicrobials could be hindered by the electrospinning process, the exposure to the polymer solution, and/or the dissolution medium. Thus, here, the antimicrobial activities of the ciprofloxacin and metronidazole released from the nanofiber mats were compared with the antimicrobial activities of the antimicrobial standards (Table 3). Ciprofloxacin was very effective, as its MIC when released from the chitosan/PEO nanofiber mats was 0.6 ng/mL to 1.3 ng/mL against *E. coli*, and 2.5 ng/mL to 5.0 ng/mL against *A. actinomycetemcomitans*. The MICs of the metronidazole released from the PCL nanofiber mats were approximately 1,000-fold those of ciprofloxacin, as they were in the range of 5.0 µg/mL to 10.0 µg/mL against *E. coli*, and 2.5 µg/mL to 5.0

μg/mL against *A. actinomycetemcomitans*. The antibacterial efficacies of these antimicrobials did not change after their incorporation into the nanofiber mats, as their MICs when released into the dissolution medium did not significantly differ from their MICs obtained as the antimicrobial standards. In addition, the drug-free nanofiber mats did not release any compounds that showed inhibition of the growth of these bacteria.

Table 3. Minimal inhibitory concentrations for inhibition of growth of the Gram-negative bacteria by ciprofloxacin and metronidazole. The resolution of the dilution series was two-fold.

Antimicrobial	**Form***	**Minimal inhibitory concentrations**	
		E. coli	***A. actinomycetemcomitans***
Ciprofloxacin	Standard	0.6-1.3	2.5-5.0
(ng/mL)	Released	0.6-1.3	2.5-5.0
Metronidazole	Standard	5.0-10.0	2.5-5.0
(μg/mL)	Released	5.0-10.0	2.5-5.0

*Standard, antimicrobial standards dissolved in phosphate buffer; Released, antimicrobials released from the nanofiber mats.

3.6 Correlation between data obtained from micro flow-through apparatus and the MICs of antimicrobials used against periodontal pathogens

Drug-loaded nanofibers are mainly developed for local treatments of different diseases, where are present low resting volumes and flow rates of body fluids, such as for chronic wounds and periodontal disease. However, the conventional dissolution methods poorly simulate the physiological conditions, and there remains a need for better standardization of *in-vitro* methods to evaluate drug release for such applications. To partially fill this gap, we developed this micro flow-through apparatus with low flow rate and resting volume of the dissolution medium. By changing the flow rates and modifying the chemical composition of the dissolution medium, such as changing pH or potentially the addition of esterases and other proteins that might be encountered *in vivo*, it would be possible to simulate both a specific disease and also the different stages of a disease, if the physical parameters present at a local site are known. Thus, the micro flow-through apparatus represents a very useful tool for accurate analyses of drug release from different formulations, for determination of drug-release mechanism, and for evaluation of drug concentrations under selected conditions. This screening method can contribute to the selection of the most promising formulation for their

evaluation in the complex study systems that more closely simulate the physiological conditions, such as with bacterial biofilms in a microfluidic device [46, 47], for a 'lab-on-a-chip' with human cells [48], or to perform clinical studies to confirm the efficacy and safety of a delivery system.

For the treatments here to be effective, the local antimicrobial concentrations at the target site need to exceed the MICs of each of the targeted periodontal pathogens over a sufficiently long period of time. The concentrations of ciprofloxacin released from the double-layered nanofiber mats was >60 μg/mL over 15 days, which exceeded the MICs determined against the periodontal pathogens by a minimum of 20-fold (Fig. 9, Supplementary Table S3) [22, 23, 30] and thus they could be sufficient to eradicate multispecies bacteria grown in a biofilm. Nevertheless, these concentrations of ciprofloxacin are also sufficient for its immunomodulatory activity in the periodontal pocket [11], and this might represent a risk for human cells in the first 2 days of application. Should this indeed be a problem, it could eventually be solved by modification of the delivery system containing the crystalline ciprofloxacin, or by addition of an antioxidant [43, 44].

For metronidazole, the susceptibility of different periodontal pathogens varies more compared to ciprofloxacin. The MICs of most of the bacterial strains tested are from <1 μg/mL to 8 μg/mL, with only a few strains showing MICs >32 μg/mL (Supplementary Table. S3)[22, 30-32]. Our double-layered nanofiber mats assured metronidazole concentrations of >8 μg/mL for 7 days, and >32 μg/mL for 5 days (Fig. 9). These would ensure sufficient levels to inhibit all of the strains of the bacteria here for up to 5 days, and for most of the strains for up to 7 days. In addition, metronidazole-loaded PCL nanofibers did not show any negative affect on the viability of baby hamster kidney fibroblasts *in vitro* [9].

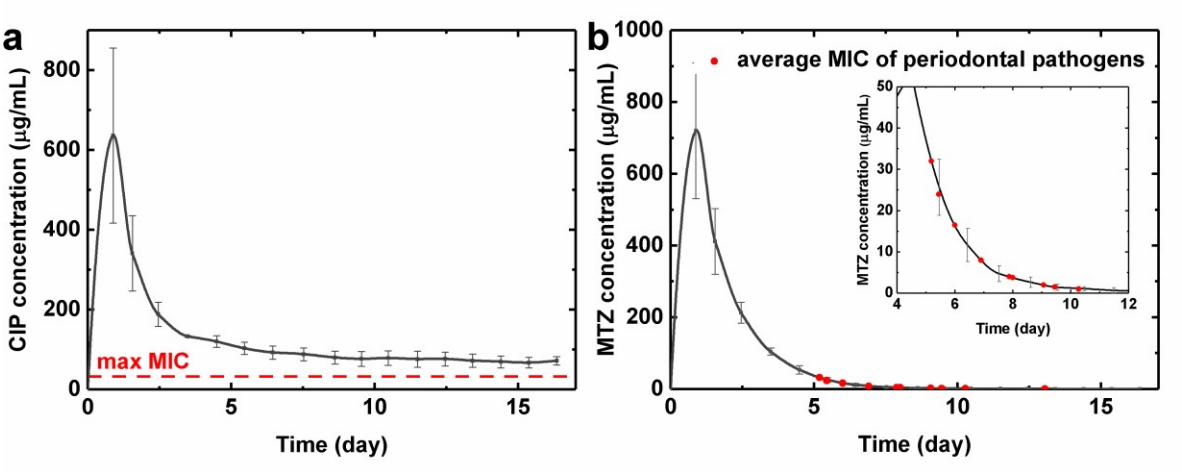


Figure 9. Ciprofloxacin (a; CIP) and metronidazole (b; MTZ) release from the double-layer nanofiber mats composed of the PCL nanofiber layer with 5% metronidazole and the chitosan/PEO nanofiber layer with 30% ciprofloxacin, as tested in the micro flow-through apparatus with dissolution medium flow rate of 30 μL/h. Red dashed line in (a), maximal ciprofloxacin MIC reported; red dots in (b), mean MICs of metronidazole against different periodontal pathogens, where these MICs follow the metronidazole concentration released. The MICs are based on literature data [49, 50].

However, it must be noted that these *in-vitro* tests performed here that involved single periodontal pathogens represent the first step toward determination of the clinically relevant efficacy of this formulation. *In vivo*, the different periodontal pathogens can include *A. actinomycetemcomitans*, *P. gingivalis*, *Tannerella forsythia*, and *Treponema denticola*, together with other microorganisms, whereby they form a complex polymicrobial association in the form of a biofilm attached to biotic or abiotic surfaces [51]. The microorganisms embedded in such an extracellular matrix can also share their genetic information, and communicate and cooperate with each other. As a result, 100-fold to 1,000-fold greater concentrations might be needed to destroy the same strains grown in biofilms compared to those grown planktonically [52]. To overcome this problem, future studies of complex systems are needed to help to formulate the optimal clinical approaches for treatments, which can include the classical mechanical removal and disruption of such biofilms, plus the subsequent local treatment with a combination of antibiotics in patient-friendly delivery system, as developed in the present study. Furthermore, the final stage of treatment would consist of the re-population of the microenvironment with autochthonous probiotics to prevent or slow the recurrence of periodontal disease [53, 54].

4 CONCLUSIONS

A double-layer nanofiber mat composed of chitosan/PEO nanofibers with 30% ciprofloxacin and PCL nanofibers with 5% metronidazole has been developed here using electrospinning. The precisely designed composition of the double-layer nanofiber mat enabled sustained *in-vitro* release of the drugs over 7 days at concentrations above their MICs against the most important periodontal pathogens, as determined using this new micro flow-through apparatus. It closely simulates the conditions of the periodontal pockets *in vivo*, with respect to flow rate, volume, temperature, and pH of the gingival crevicular fluid. The development of this apparatus represents an important step to fill the gap for the need for new methods and possible standardization of release tests that mimic local physiological conditions in the periodontal pocket. This apparatus has also contributed to identification of the ciprofloxacin release mechanism. Ciprofloxacin was released in a sustained manner due to its low solubility at pH 7.4, when there was excess of solid drug present in the delivery system. In contrast, sustained release of metronidazole was caused by the slow air displacement by the dissolution medium in the hydrophobic PCL nanofiber mat. The double-layer nanofiber mat developed here provided antibacterial activities against *E. coli* and *A. actinomycetemcomitans*, and it represents promising potential to be further evaluated in *in-vivo* studies.

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Disclosure

The authors have no conflicts of interest.

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