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Electrosprayed poly-butylene-succinate microparticles for sustained release of ciprofloxacin as antimicrobial delivery system

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Electrosprayed Poly-butyl-succinate microparticles for sustained release of Ciprofloxacin as an antimicrobial delivery system

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Abstract

In this study polymeric microparticles by electrospraying using polybutylene succinate (PBS), a biodegradable and biocompatible polyester, were produced. This versatile and easy-to-use technique enabled the incorporation of the poorly water-soluble ciprofloxacin into the polymer matrix, a fluoroquinolone antibiotic, inhibiting bacterial replication and effectively treating various infections. The microparticles were characterized by means of different techniques (SEM-EDX, XRD, ATR-FTIR, DSC), and their degradation rate was tested in DPBS and human plasma. Moreover, the as-produced DDS enabled the sustained release of CPX for several days, which proved effective against S. aureus and P. aeruginosa and also against a reference group of bacteria of skin microbiota. MIC and MBC assays were conducted using different culture media. Effective antibacterial activity was observed, along with inhibition of P. aeruginosa biofilm formation at sub-MIC concentrations. An ex

vivo permeation study on porcine skin, evaluated the drug permeation to assess potential enhancement in drug permeation.

Keywords

Electrospraying; polybutylsuccinate; polymeric microparticles; ciprofloxacin; sustained release; antibacterial treatment

1. Introduction

Given the widespread criticality of bacterial infections on wounds to the healthcare system and patient quality of life, improving efficacy of the antibacterial treatments is crucial. (Mustoe et al., 2006; Tricco et al., 2015) Moreover, chronic infections can play a role during tissue reparation, dysregulating the wound healing process and leading to its chronicity over time.

Clinical protocols for treating infected wounds may vary depending on the severity and type of infection. These protocols typically involve a combination of debridement, systemic antibiotics, and topical antimicrobial agents. Antiseptics are used in conjunction with antibiotics in clinical practice to treat infected wounds to reduce the presence of bacteria in the wound, accelerate the resolution of inflammation, and promote tissue healing. (Caldwell, 2020).

The use of topical antibiotics offers several advantages over systemic administration. It helps minimize systemic side effects and toxicity by delivering a high concentration of the antibacterial agent directly to the site of infection. This localized approach also reduces the likelihood of developing resistant microorganisms, as the antibiotic is primarily targeting the specific wound area. Topical antibacterial products provide flexibility in their application, allowing for both prevention and treatment of bacterial infections. (Lio and Kaye, 2004)

Moreover, the development of antibiotic systems with slow release holds great promise as a strategy to address antibiotic resistance. These systems aim to avoid sub-minimum inhibitory concentration (sub-MIC) doses, which may provide selective pressure, allowing bacteria to adapt and alter their phenotype, leading to resistance. By delivering antibiotics at controlled and sustained rates, the proposed systems have the potential to maintain therapeutic levels while minimizing the risk of resistance development (Gao et al., 2011; Larsson and Flach, 2022).

Polymeric microparticles are excellent drug delivery systems for topical antibacterial administration. (Xiong et al., 2014) They can be produced by electrospraying, which is an easy-to-use and versatile technique that can be tuned suitably to incorporate drugs in a polymer matrix. (Gaskell, 1997; Jaworek, 2007)(Nath et al., 2013). The electrospray technique derives from electrospinning. Following the application of a high electric field, when the electrostatic force applied is high enough, the solution at the tip of the needle elongates and forms a conical jet which eventually breaks into tiny droplets. As the droplets travel toward the electrically charged collector, they repel each other, remaining dispersed, the solvent evaporates, and the solid particles are collected. (Cam et al., 2019; Hsu et al., 2018a) This technique allows poor water-soluble drugs to be incorporated into a polymeric matrix as microparticles. De Pieri et al. exploited electrospraying in order to delivery successfully resveratrol, which otherwise undergoes to easily degradation or depletion. The microparticles as produced were able to microencapsulate resveratrol, preserving its antioxidant properties, and tested them for the local treatment of chronic non-healing wounds (De Pieri et al., 2022). Electrospraying was also employed to load several types of drugs, such as lidocaine/vancomycin/ceftazidime, into the microparticles produced by Hsu et al. (Hsu et al., 2018b) with analgesic and antimicrobial activity.

Ciprofloxacin (CPX) is a broad-spectrum poorly soluble fluoroquinolone antibiotic that was used as a model antibiotic in this study, despite the fact that it is not the drug of choice for topical infections, which are more often treated with mupirocin, fusidic acid, or retapamulin. (Kwak et al., 2017) The rationale behind selecting CPX as the model antibiotic was its low solubility and the potential for

local administration in a slow-release manner, which could serve as an optimal model for DDS. By exploring the feasibility of using CPX in this context, we aim to contribute to the development of innovative therapeutic approaches.

CPX possesses several qualities that make it an attractive candidate for our research. Its broad-spectrum activity allows for effective targeting of various bacterial pathogens commonly associated with chronic wounds. Moreover, ciprofloxacin has a well-established pharmacological profile and is widely used as a systemic antibiotic in wound healing (Tsou et al., 2005; Uhljar et al., 2021; Yu et al., 2006). Although our study does not focus on systemic antibiotics, the extensive use of ciprofloxacin in wound healing supports its consideration as a suitable antibiotic model. The low cost, strong bactericidal effect, and high antibacterial activity are all characteristics of CPX that make it the optimal drug for a slow-release polymeric delivery system for the resolution of chronic wounds. (Federico et al., 2021; Fiorica et al., 2021)

Future research should explore the feasibility of employing this drug delivery system for drugs of choice, such as fusidic acid and mupirocin, which are commonly recommended for topical infections. Further investigation is needed to assess the applicability of this DDS to optimize the delivery of drugs specifically recommended for topical applications.

For biomedical uses, such as implant devices, tissue scaffolds, and wound dressings, polymers such as poly(lactic acid) (PLA), polycaprolactone (PCL), poly(glycolic acid) (PGA), and poly-(butylene succinate) (PBS) are in high demand. Due to their biodegradability, biocompatibility, and substantial capacity for drug loading, these polymers are perfect for these applications (Cooper et al., 2018; Zong et al., 2002). Among the several types of polymers for biomedical applications, Polybutylene-1,4-butylene succinate (PBS) offers unique advantages as it combines high biodegradability, biocompatibility, substantial drug-loading capacity, and good mechanical strength. That is also proved by the growing number of publications about its use. (Gigli et al., 2016) PBS is a well-known water-insoluble polymer with tuneable chemical-physical properties obtained from the

polycondensation reaction between succinic acid and butanediol. It is biocompatible and biodegrades at different rates depending on the application site and therefore is well suited to tissue regeneration or wound healing applications. Miceli et al. used PBS as an electrospun polymeric tubular scaffold for tissue engineering applications as biocompatible and biodegradable material. They proved that nanofibers made of PBS provide structure and function over time and support host cell remodelling. (Miceli et al., 2022) While Di Prima et al. (Di Prima et al., 2019) produced hydrophobic microfibrillar scaffolds employing an electrospinning technique for ocular insert with features like wettability, mucoadhesion, and cytocompatibility. They could load triamcinolone acetonide, a lipophilic drug with a prolonged release for up to 30 days. Moreover, smooth microspheres of PBS were synthesized by Mohanray et al. for administering levodopa, a drug with poor bioavailability, short half-life, and side effects in treating Parkinson's disease. (Mohanraj et al., 2013) Finally, Cicero et al. demonstrated that PBS scaffolds play an essential role in peripheral nerve regeneration through X-ray microcomputed tomography and magnetic resonance imaging in vivo. (Cicero et al., 2022)

In the present study, we aimed to use the electrospraying technique to develop biodegradable PBS microparticles in a single solvent of chloroform ($CHCl_3$) system loaded with CPX for a sustained antimicrobial release for wound healing applications. The system thus developed showed an excellent ability to capture the drug and release it constantly for an extended time. Furthermore, as previously reported, the biocompatible polymer used to produce the microparticles degrades slowly, facilitating tissue regeneration and wound healing (Cicero et al., 2022; Miceli et al., 2022; Vigni et al., 2022). Microbiological tests showed that the microparticles exhibit antibacterial action against *S. aureus* and *P. aeruginosa*. These strains are particularly dangerous because they are considered respectively of high or critical priority category based on their resistance as stated on the global priority list of antibiotic-resistant pathogens published by the World Health Organization (WHO). ("WHO publishes list of bacteria for which new antibiotics are urgently needed," n.d.) In addition, tested pathogens often cause chronic infections and delay wound healing (DeLeon et al., 2014). Moreover,

microparticles were also tested against a reference group of bacteria of skin microbiota which can play a pathogenic role in the nonhealing wound (Tomic-Canic et al., 2020).

2. Materials and Methods

2.1.Materials

Poly(1,4-butylene succinate), extended with 1,6-diisocyanatohexane (PBS), Ciprofloxacin (CPX), n-octanol, Dulbecco's phosphate buffered saline (DPBS), Mueller−Hinton broth (MHB), cation-adjusted Mueller−Hinton broth (MHB2), tryptic soy agar (TSA) and crystal-violet were purchased from Sigma, Italia. Chloroform was purchased from VWR chemicals. Human plasma was extracted from volunteers upon informed consent at the University of Palermo, Palermo, Italy. For the antibacterial tests in vitro, *Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 15442, reference strains in official tests (UNI EN European Standard), and reference skin bacteria *Staphylococcus hominis* ATCC 27844, *Staphylococcus epidermidis* ATCC 12228, *Cutibacterium acnes* ATCC 11827 and *Streptococcus agalactiae* ATCC 13813 were purchased from PBI-VWR (Italy).

2.2. *Methods*

2.2.1. Microparticles Preparation by electrospraying

To prepare microparticles, CPX (5% w/w) was dissolved in chloroform with PBS at a concentration of 15% w/v by vortexing and sonicating multiple times at room temperature. Then, microparticles were obtained by electrospraying the dispersion using an electrospinning NF-103 (MECC, Japan) at room temperature and loading the dispersion into a 5 ml syringe. The flow passed through a PTFE tube connected to a 20G flat-tip needle. The electric field applied was 17.5 kV, and the feed rate was varied from 1.5 ml/h to 1.9 ml/h. The microparticles were collected on a 10 mm diameter stainless-steel rotator collector placed approximately 19 cm from the needle. The rotation speed of the collector was set at 40 rpm, and the needle moved across a transversal distance of 5 cm at a rate of 8 mm/s. Once the deposition process was complete, the microparticles were detached from the collector using

a scalpel. Prior to the batch here described, several tests were conducted using various drug and polymer concentrations to enhance the incorporation efficiency and overall process yield. Additionally, the electrospraying parameters, including feed rate, applied electric field, and other variables, were also optimized. This process facilitated the replication of the batch of microparticles described above, which exhibits the processability and reproducibility compared to other batches prepared.

2.2.2. Microparticles Characterization

In order to evaluate the morphology of the as-prepared microparticles, they were observed by SEM imaging technique, using Phenom ProXSEM (Alfatest, Italy) equipped with Energy Dispersive X-ray (EDX). The SEM analysis was performed with an electron-accelerating voltage of 10kV. Samples were prepared by dispersing the microparticles on a double-sided adhesive tape previously applied on a stainless-steel stub and dried under vacuum (0.1 Torr) before analysis. All the SEM analyses were performed at 25.0 °C ± 0.1 °C. The average diameter (d) ± standard deviation (SD) (mean ± SD, n = 3) of the microparticles was determined from the mean value of 100 measurements using the software ImageJ (Madison, WI, USA, version 1.46 v).

X-ray powder diffraction (XRD) patterns of MPs-PBS, pure CPX, and MPs-PBS-CPX were recorded with a Bruker D8 Advance diffractometer in the Bragg-Brentano geometry using a Ni-filtered Cu Kα radiation (λ = 1.54 Å) in the 2Θ range 5°-35° (setup conditions: tube voltage of 40 kV, current 40 mA, 0.05°/s). XRD patterns and the degree of crystallinity (DC) of each sample were analyzed and calculated using Match! 3 (Crystal Impact GbR) software.

Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (ATR-FTIR) spectra were acquired using an FTIR Bruker Lumos model ALPHA. Spectra result from 64 scans in the wavenumber range 4000–400 cm^{-1}, with a resolution of 2 cm^{-1}. After each measurement, the ATR diamond crystal was cleaned with a 70% ethanol/water mixture. To obtain optimum contact between

the material and the ATR crystal, the microparticles were pressed against the diamond. The baseline correction has been performed by using the OPUS® software.

Differential scanning calorimetry (DSC) studies were carried out using a Setaram DSC131 EVO at a heating rate of 20 °C/min in the range of 20-300°C, using 120 µl aluminum non-hermetic crucibles, with samples of 8-10 mg in the range of 20-300°C. The analysis included the MPs with and without CPX and the drug CPX alone.

The degradation tests were carried out in an incubator at a constant temperature of 37°C and 5% CO_2 in two different mediums: a buffer solution of DPBS with the addition of sodium azide 0.05% w/v and in human plasma. The microparticles were weighted (w_0) using a precision balance, weighing about 20 mg for each sample. Then 1 ml of medium (buffer or plasma) was added to the microparticles and incubated. At precise intervals (7 days, 15 days, and 30 days), a sample was sacrificed, washed thoroughly with Milli-Q water, and filtered over a nylon filter. It was then frozen at -80°C and freeze-dried. The microparticles were weighed afterward. The mean recovered weight divided by the standard deviation was used to represent the findings of these three investigations, which were done in triplicate, plotting the data versus time zero.

2.2.3. Determination of CPX loading

To design the dissolution studies experiments, the content of CPX in PBS microparticles was first evaluated. The drug loading (DL%) was quantified using a UV-Vis spectrophotometer (Jasco V-760 spectrophotometer) reading at the maximum wavelength (λ max) of the CPX in chloroform of 282 nm (cuvette 1 mL, optical path 10 mm). A standard calibration curve of CPX was formerly obtained by measuring the absorbance of known concentrations of CPX (0.1– 0.001 $mg \cdot ml^{-1}$) in chloroform at 282 nm (R^2= 0.98).

Then, a known amount of microparticles (about 10 mg) were dissolved in chloroform under magnetic stirring for about 2 hours. The solution was passed through a 0.22 mm membrane filter (Millipore),

and then the CPX content was assayed by measuring the absorbance of the sample at 282 nm after suitable dilution. The drug loading (DL%) was then calculated according to the below formula:

$$DL\ \% = \frac{Mass\ of\ CPX\ in\ microparticles}{Mass\ of\ microparticles} \times 100$$

The encapsulation efficiency (EE%) was determined by comparing the actual content of CPX with the drug content that was present in the theoretical quantity of microparticles according to the following formula:

$$EE\ \% = \frac{Experimentally\ calculated\ amount\ of\ CPX\ in\ the\ microparticles}{Theoretical\ amount\ of\ CPX\ in\ the\ solution} \times 100$$

2.2.4. In Vitro Dissolution Studies

The *in vitro* release of the CPX loaded in the MPs was assessed using a basket that consists of a stainless steel 40 mesh construction. MPs along with an equivalent amount of pure CPX were placed into the metal basket in an empty beaker. A bi-phasic medium consisting of 100 mL of DPBS with Tween 0.05% w/w and 25 mL of n-octanol was then added to the beaker. To ensure solubility and accurate measurement of drug release, the solubility of CPX in the release medium was carefully evaluated prior to the study.

The systems were maintained under stirring at constant temperature (150 rpm, 37 °C); these conditions were held for the entire experiment. One milliliter sample of the upper organic phase (n-octanol) was taken at regular intervals over a period of six day. Each sample was immediately examined with the spectrophotometer, allowing for the detection and quantification of CPX, and poured back into the beaker. The amount of CPX was then calculated from a standard calibration curve of CPX by measuring the absorbance of known concentrations of CPX (0.01– 0.0001 $mg{\cdot}ml^{-1}$) in n-octanol at 284 nm ($R^2 = 0.99$).

It is important to note that although saturation was observed in the cumulative release of pure CPX within the experimental timeframe, an increase in the solubility of loaded CPX is still evident following the electrospray process. While this release study model may not perfectly replicate the dynamic nature of wound exudates observed in real clinical scenarios, it serves as a valuable tool to assess the constant release of drugs from the MPs within the receiving compartment and the difference between the free drug and the one incorporated into the MPs.. We acknowledge the need for future studies that more closely mimic clinical conditions and validate the clinical relevance of our findings.

2.2.5. Ex-Vivo Permeation Studies

Ex-vivo permeation studies were conducted using vertical Franz cells and porcine skin in an orbital shaker at 37°C and 70 rpm. The acceptor compartment contained 5 mL of n-octanol, while the donor compartment contained 0.3 mL of physiological solution with MPs-PBS-CPX or CPX alone. Samples of 200 µL were collected from the acceptor compartment at different time points (0.5, 1, 2, 4, 6, 8, and 24 hours) and immediately diluted with n-octanol before being analyzed with a spectrophotometer (UV Jasco V-760) using a 1 mL cuvette with an optical path of 10 mm. Each time a sample was taken, an equal volume of n-octanol was added to the acceptor compartment to maintain sink conditions. The permeation studies were performed in triplicate, and the results were plotted as graphs of the CPX permeation over time. In addition, at the end of the permeation studies, the amount of drug (CPX) remaining in the porcine skin was quantified after extraction with n-octanol from the skin used for the experiment. The amount of CPX residual in the donor compartment was quantified as well after freeze-drying of the samples and extraction of remaining CPX with n-octanol. The morphology of the skin porcine tissue was evaluated via Dino-Lite digital microscope (Dino-Lite, model AM4115T-CFVW, AnMo Electronics Corp., Hsinchu, Taiwan).

2.2.6. Determination of Minimum Inhibitory Concentrations (MICs) by broth dilution micro-method

Minimum inhibitory concentrations (MICs) of the microparticles were determined toward reference strains of *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 15442, *Staphylococcus hominis* ATCC 27844, *Staphylococcus epidermidis* ATCC 12228, *Cutibacterium acnes* ATCC 11827 and *Streptococcus agalactiae* ATCC 13813, by a serial dilution method by using three different media for comparative purposes, Mueller Hinton broth (MHB), Mueller Hinton Broth II (MHBII) and Tryptic Soy Broth (TSB) as previously described (Girasolo et al., 2006).

Briefly, 0.1 mL of a sterile stock solution of microparticles suspended in water was added into a well of a sterile 96-well plate, and 1:2 dilution series with each broth medium was performed. The inoculum suspension was added (10 µl of a bacterial suspension from a 24h culture containing 10^6 CFU/ml) and incubated at 37 °C for 24 h. After this time, the optical density (OD) at 570 nm was read using a spectrophotometer microplate reader (GloMax Multi Detection System TM 297 Promega, Milan, Italy). The lowest concentrations of samples whose optical density read at 570 nm was comparable with that of the negative control, which contained only inoculated broth, were considered the MICs. The accurate carrying out of the procedure is indicated by bacterial growth in the wells, marked as a positive control.

2.2.7. Determination of Minimum Bactericidal Concentrations (MBCs)

In order to determine the Minimum Bactericidal concentration (MBC), we sub-cultured onto substance-free TS agar plates, 0.1 ml of each negative well, and from the positive control of MIC determinations. Then the plates were incubated at 37 °C for 24 h. The lowest concentration of the substance, which produced subcultures growing no more than three colonies, was defined as the MBC.

2.2.8. Biofilms susceptibility testing by using the crystal violet method

In order to determine the Minimum Bactericidal concentration (MBC), we sub-cultured onto substance-free TS agar plates, 0.1 ml of each negative well, and from the positive control of MIC

263 determinations. Then the plates were incubated at 37 °C for 24 h. The lowest concentration of the
264 substance, which produced subcultures growing no more than three colonies, was defined as the
265 MBC. Sub-MIC concentrations of microparticles ranging from 1 to 0.05 μg/mL were tested for their
266 capability of interfering with the growth as biofilm form of *P. aeruginosa* ATCC 15442, as previously
267 reported (Carbone et al., 2020). The bacterial strain was incubated in tested tubes containing TSB
268 with 2% *v/v* glucose at 37 °C for 24 h, then 2.5 μL of the diluted bacterial suspension (10^6 CFU/ml),
269 was added to each well of a sterile flat-bottom 96 well loaded with 200 μL of TSB with 2% *v/v*
270 glucose (Federico et al., 2022). Aliquots of sub-MIC concentration of microparticles were directly
271 added to the wells and the plates were incubated at 37 °C for 24 h. After biofilm formation, wells
272 were washed twice with sterile NaCl 0.9% and stained with 200 μL of 0.1% *v/v* crystal violet solution
273 for 30 minutes at 37 °C. The surplus solution was removed, and the plates were washed twice using
274 tap water. After each stained well was processed, 200 μL of ethanol were added to solubilize the dye.
275 OD was read with wavelength of 600 nm using a microplate reader (Glomax®-Multi Detection
276 System, Promega s.r.l., Milan, Italy). The experiments were carried out at least in triplicates, and
277 three independent experiments were performed. The percentage of biofilm inhibition formation was
278 determined through the following formula:

279 $$\%\ of\ inhibition = \frac{(mean\ OD\ growth\ control\ -\ mean\ OD\ sample)}{mean\ OD\ growth\ control}$$

280 ***2.2.9. Statistical Analysis***

281 The mean standard deviation (SD) (n = 3) was used to describe the results from numerous samples.
282 The Student's t-Test was used to examine statistical differences. The statistical significance level was
283 established as p-value of 0.05.

284 **3. Results and Discussion**

285 **3.1. Preparation and characterization of MPs-PBS-CPX**

The production of PBS nanofiber-based scaffolds obtained by electrospinning has already been discussed in previous research (Cicero et al., 2022; Di Prima et al., 2019). By changing the solvent used to dissolve the PBS, we observed not the formation of nanofibers but instead the formation of microparticles thus obtained by electrospraying, which can be used as drug delivery system for poor-soluble drugs. For this purpose, the experimental conditions were varied and optimized to incorporate CPX into the as-prepared microparticles. The setup of the instrument is schematized in Figure 1a.

Figure 1.

After production, MPs-PBS were analyzed by means of SEM in order to highlight the microscopic features and porosity structure. Fig. 1 shows representative SEM images of empty MPs (Fig. 1b) and MPs loaded with CPX (Fig. 1c). The morphologies of the microparticles were remarkably similar in both the empty and drug-loaded batches, appearing highly porous. It is possible to observe that the empty microparticles are larger than those loaded with CPX, this may suggest that the presence of CPX may influence the microparticle characteristics. It is hypothesized that factors such as surface tension and viscosity could potentially contribute to this discrepancy, as they are known to affect particle formation during electrospraying processes. (Okutan et al., 2014; Tan et al., 2005) These properties can influence the behaviour of the solution during the electrospraying process, potentially leading to variations in microparticle size. In Fig. 1e and 1f, the size distributions of the microparticles are presented. The distribution of the mean diameter is centred at 43 ± 8 μm for the MPs-PBS and at 17 ± 5 μm for the MPs-PBS-CPX, indicating a more uniform size distribution for the former. These findings suggest that the presence of CPX may contribute to increased heterogeneity and smaller size of the loaded microparticles. Future studies could explore the relationship between surface tension, viscosity, and microparticle size to further elucidate the underlying mechanisms responsible for these observations.

Then, to assess if all the chloroform present in the mixture was effectively removed by evaporation during the electrospraying process, an Energy Dispersive X-ray (EDX) analysis was performed,

311 which provides a quick nondestructive determination of the elemental composition of the
312 sample readily identifying atoms such as chlorine, barium, potassium, and strontium, with a detection
313 limit of about 1-10% wt. (Goldstein et al., 2017) In Fig. 1d it is possible to see the peaks exhibited by
314 the microparticles, which can be attributed to carbon, oxygen, and nitrogen atoms, without any
315 residual chlorine detection, indicating that no chloroform is retained in the final material.

316 **Figure 2.**

317 The X-ray diffraction (XRD) technique was employed in this study to investigate the presence of
318 CPX within the MPs and to assess the structural changes induced by its incorporation which are
319 crucial to understand the properties of the MPs, including solubility, dissolution rates, and drug
320 release kinetics. Fig. 2a shows the XRD pattern conducted on the MPs, this analysis provides
321 evidences supporting the presence of CPX within them. Despite the low intensity, characteristic peaks
322 of the antibiotic are visible indeed, which can be attributed to the limited concentration of CPX into
323 the MPs. These distinct peaks serve as a clear indication of the presence of CPX in the loaded MPs.
324 Furthermore, the calculation of the degree of crystallinity (DC) offers valuable insights into the
325 structural changes induced by the presence of CPX. A comparison of the DC values between empty
326 MPs (59.2%) and MPs loaded with CPX (39.6%), and pure CPX (60.2%) reveals a significant
327 alteration in the crystalline structure of the MPs loaded with CPX. The decrease in DC signifies that
328 the incorporation of CPX results in a more amorphous nature of the MPs. The increased
329 amorphousness observed in the CPX-loaded MPs can be attributed to the interactions between the
330 antibiotic and the polymeric matrix of the MPs. These interactions disrupt the regular packing
331 arrangement of the polymer chains, leading to a decrease in the long-range order and crystallinity.
332 The presence of CPX within the MPs also hinders the growth of well-defined crystal structures,
333 further contributing to the observed decrease in crystallinity. Understanding the changes in
334 crystallinity is of great significance as it can have a profound impact on the functional properties and
335 behavior of the MPs. Amorphous materials typically exhibit altered solubility, dissolution rates, and

drug release kinetics compared to their crystalline counterparts. Therefore, the increased amorphousness induced by the incorporation of CPX in the MPs has the potential to affect the release profile and therapeutic efficacy of the antibiotic.

The ATR-IR analysis was used to evaluate the effective incorporation of CPX within the microparticles, as well as the absence of any degradation to the components of the formulation (Figure 2b). Characteristic peaks of CPX can be identified at 707, 822, and 866 cm^{-1} (Tewes et al., 2016) those peaks are present both in the raw CPX and in the microparticles loaded with CPX. At the same time, they are not found in the unloaded microparticles spectrum. They can be attributed to the typical C-H aromatic bending, as well as δ CH (COO^-), which are related to the aromatic ring of CPX. (Refat et al., 2011). This data confirms the efficient incorporation of ciprofloxacin onto microparticles without any degradation.

In Figure 2c are shown the DSC thermograms of three samples: the MPs made by just PBS, the ones loaded with CPX, and the drug CPX alone. It is possible to observe that although the thermogram of the MPs-PBS-CPX is very similar to that of the PBS-only microparticles sample, the shape of the exothermic peak is changed. This change could be attributed to the incorporation of the drug into the microparticles. However, the peaks in the CPX thermogram are not observable in the CPX-loaded microparticles one; this might mean that the drug is incorporated into the polymer matrix.

Figure 3.

In Figure 3, the residual mass of the microparticle degradation after 7, 15, and 30 days in different mediums such as DPBS and Human Plasma is shown. No significant degradation of microparticles was observed within one month. This observation suggests a potential favourable aspect of the microparticles, as their stability over time may indicate a reduced likelihood of stimulating an inflammatory response and a possibility of promoting tissue regeneration. Further studies and experimental data are needed to confirm these hypotheses.

3.2.Evaluation of dissolution and permeation properties

An in vitro dissolution study was carried out to highlight the differences in the release profiles of CPX encapsulated in the MPs compared to the pure active. In this regard, an in vitro model was used to simulate the distribution of the active molecules between the aqueous phase of the biological fluid and the lipid phase of the biological membranes. The experimental setup previously used (Terracina et al., 2022) has been suitably modified to prevent the microparticles from precipitating and modifying the release. Therefore, metal baskets for the dissolution of tablets were used to evaluate how much drug is released from the microparticles into the lipidic phase.

Figure 4.

From the results obtained in the dissolution studies (Figure 4), it was possible to observe that tested microparticles had dissolution profiles controlled compared to the pure active principle. The release of CPX is controlled and slowed thanks to their incorporation into microparticles and, at the same time, favouring the prolonged release of the drug thanks to the gradual degradation of the polymer.

The permeation of microparticles loaded with CPX through porcine skin was investigated through an ex vivo permeation study. This experimental approach allowed to assess the potential increase in permeation of encapsulated drug through the porcine skin, which served as a model tissue for human skin.

Figure 5.

Franz's vertical diffusion cells were employed as an in vitro ex-vivo permeation model (Figure 5a). Despite not replicating wound conditions, using intact porcine skin as the model served two purposes. Firstly, it established a reference for normal skin permeability, enabling future comparisons with wound permeation. Secondly, it allowed for the evaluation of dermal component effects on permeation. This approach provides valuable insights before studying specific wound conditions and considers the limited availability of wound tissue samples. Overall, intact porcine skin serves as an

informative starting point for evaluating permeation mechanisms and establishing a foundation for further studies.

Figure 5b shows the drug release profile through the skin during 24 hours. It can be stated that MPs increase drug permeation through the skin compared to the free CPX, whose permeation is slower, which can lead to accumulation and precipitation in the membrane itself, preventing effective distribution. The improved drug permeation profile through the skin in the as-produced DDS is therefore favourable, as it confirms that the electrospraying process enhances the properties of the encapsulated drug compared to the free drug, thus paving the way to other biomedical applications. Furthermore, a comparison of the residual drug levels in three different compartments: donor, acceptor, and membrane, is depicted in figure 5c. As a result of the improved permeation in the donor compartment, the microparticles contain a lower amount of CPX than the free drug. This, in turn, leads to a higher concentration of CPX in the acceptor compartment of the microparticles than in the one of the free CPX. within the membrane while facilitating a gradual and extended transdermal crossing.

In conclusion, the encapsulation of the drug within the microparticles results in reduced retention of the drug by the membrane. This is attributed to the controlled drug release by the microparticles, which effectively prevents its accumulation and entrapment within the membrane while facilitating gradual and extended skin permeation. Additionally, the ability of the microparticles to release the drug in a gradual and prolonged manner may decrease the systemic toxicity of the drug and increase its therapeutic efficacy.

3.3. Study of antibacterial and antibiofilm activity

In addition to their potential as a drug delivery system for skin permeation applications, the microparticles studied have also been evaluated for their antibacterial properties. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of drug-loaded microparticles have been determined against various bacterial strains, including those found on the

skin. In particular, different culture media were used in antibacterial studies to evaluate the effectiveness of the drug-loaded microparticles against various bacterial strains under a range of growth conditions. The antibacterial activity in terms of MIC was found to be more effective in the Mueller Hinton-2 culture medium against the two pathogens, *S.aureus* and *P.aeruginosa* (Table 1). Furthermore, in TSB, the antibacterial activity values against the strains mentioned above are almost similar to those obtained with the MH culture medium in terms of MIC. Therefore, it is assumed that the presence of a richest medium (TSB) that promotes better microbial growth did not affect the susceptibility of the tested strains to microparticles containing ciprofloxacin than a conventional medium (MH or MHII) used in susceptibility testing. Regarding dermatological strains, no significant difference in susceptibility was observed compared to the tested medium (see Table 1).

Table 1.

In Table 2 are reported MBC values; a better activity for this second assay was observed, starting from the MHII medium in relation to the two pathogens, *S.aureus* and *P.aeruginosa* (Table 2). On the other hand, the MIC and MBC for the *P. aeruginosa* strain in the MH medium have the same value. In this case, the different composition of the TSB medium has influenced the antibacterial activity in terms of MBC of the microparticles (especially for *S. aureus* and skin strains).

Table 2.

Furthermore, the ability of the microparticles to inhibit biofilm formation of the *P. aeruginosa* strain was tested. Sub-MIC concentrations ranging from 1 to 0.05 μg/ml were tested. The inhibition activity, expressed as a percentage of inhibition with respect to the growth control, at the screening concentration of 1 μg/ml (maximum concentration tested) is equal to 52.5%. The data was obtained from the average of three independent experiments performed individually. By comparing this result to similar studies, we found that our reported inhibition activity of 52.5% aligns with the range of values reported (Jensen et al., 2013; Magalhães et al., 2017). These findings further validate the potential effectiveness of MPs-PBS-CPX as a promising approach to also treat P. aeruginosa biofilms.

4. Conclusions

In the present study, PBS microparticles encapsulating a poorly water-soluble drug, ciprofloxacin, were successfully prepared by electrospraying to develop an effective drug delivery system for topical antibacterial therapy. Electrospraying was carried out in a single solvent system, chloroform, which evaporates and does not remain in the sample, as confirmed by elemental analysis. The system produced was fully characterized and showed good drug encapsulation properties and sustained drug release, associated with a decrease in the degree of crystallinity of CPX-loaded MPs, as confirmed by XRD analysis. The polymer used, PBS, was selected for its slow degradation, so the drug delivery system thus produced allows, on the one hand, to promote wound regeneration as it is highly biocompatible and, on the other hand, to release the drug over a long period of time, interfering with bacterial growth. In addition, results from skin permeation studies have shown that microparticles possess the ability to increase drug permeation through the skin of pigs, thus facilitating more efficient drug delivery. CPX-loaded microparticles have also been shown to counteract biofilm formation of the pathogen P. aeruginosa, which often colonizes chronic wounds. The drug delivery system investigated in this study holds great promise for further in vivo investigation in tissue regeneration of chronic wounds. In addition, CPX-loaded microparticles showed excellent antibacterial activity against a group of commensal skin bacteria that can engage in pathogenic behaviour in diabetic foot and venous leg ulcers and unhealed surgical wounds (Swaney and Kalan, 2021). In addition, electrospraying has proved to be a very interesting technique that can be applied for the production of polymeric microparticles, as, by appropriately changing the parameters, different drugs can be loaded from time to time and the dosing system can be exploited for other future applications, or even multiple drugs can be combined for synergistic therapeutic action. The developed and characterized DDS is presented as a promising delivery model that can be subjected to further testing in future studies. This includes evaluating its efficacy in loading different drugs and exploring its potential applications within the field of antibacterial therapy.

Credit authorship contribution statement

Giorgia Puleo: Formal analysis, Writing – original draft, Investigation. Francesca Terracina: Methodology, Writing—Review and Editing. Valentina Catania: Methodology, Data curation, Writing – review & editing. Domenico Schillaci: Investigation, Methodology, Data curation, Writing – review & editing. Mariano Licciardi: Conceptualization, Methodology, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known financial or interpersonal conflicts that might have noticed to have influenced the research presented in this article.

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Tables

Table 1. Values of MIC (µg/ml) determined in vitro by using different culture media growth (MH, MHII, TSB).

	MIC (µg/mL)		
Strains	**MH medium**	**MHII medium**	**TSB medium**

***S. aureus* ATCC 25923**	12.5	1.5	6.2
***P. aeruginosa* ATCC15442**	12.5	0.75	12.5
***S. hominis* ATCC 27844**	1.5	1.5	3.1
***S. epidermidis* ATCC 12228**	6.2	6.2	3.1
***C. acnes* ATCC 11827**	12.5	12.5	12.5
***S. agalactiae* ATCC 13813**	3.1	1.5	3.1

Table 2. Values of MBC (µg/ml) determined in vitro starting from MICs obtained in different culture media growth (MH, MHII, TSB).

	MBC (µg/mL)		
Strains	**MH medium**	**MHII medium**	**TSB medium**
***S. aureus* ATCC 25923**	50	50	>100
***P. aeruginosa* ATCC15442**	12.5	3.1	25
***S. hominis* ATCC 27844**	50	>100	100
***S. epidermidis* ATCC 12228**	50	12.5	50
***C. acnes* ATCC 11827**	100	50	100
***S. agalactiae* ATCC 13813**	50	25	50

Figure Captions

Figure 1. a) Electrospraying setup: the arrows indicate the movements along which the needle travels during the deposition and rotation of the collector. A power supply is connected to the needle, which charges it with a positive voltage, while the collector is grounded. b) Scanning Electron Microscopy of PBS microparticles with 2050 X magnification and (c) PBS microparticles loaded with CPX with 2400 X magnification. d) EDX spectrum of microparticles of PBS made by electrospraying, after production, and relative weight percentage abundance. Size distribution of PBS microparticles (e) and PBS-CPX microparticles (f).

Figure 2. a) XRD patterns of MPs-PBS (black), MPs-PBS-CPX (blue), and CPX (red). Typical peaks of CPX within the MPs-PBS-CPX sample are highlighted with a green star. b) FTIR-ATR spectra from 675 cm^{-1} to 875 cm^{-1} of MPs-PBS (black), MPs-PBS-CPX (blue), and CPX (red). c) DSC thermograms of MPs-PBS (black), MPs-PBS-CPX (blue), and CPX (red).

Figure 3. Residual mass of MPs after degradation in DPBS and Plasma after 7, 15, and 30 days.

Figure 4. a) schematic illustration of the experimental setup: a bi-phasic system composed of buffer DPBS with Tween and n-octanol. The microparticles are loaded into the dissolution grid, and at certain time intervals, the drug concentration in the n-octanol phase is measured by a spectrophotometer. b) Graph with the percentage of CPX released as a function of time: in black pristine CPX alone, in red the CPX released from the loaded microparticles. The inset graph shows the release profile of the systems through 8 hours. $p < 0.05$ (*****) for the MPs versus pure CPX after 8 hours.

Figure 5. a) Photo of the experimental setup for evaluating drug permeation through porcine skin using a vertical Franz cell and a close-up of the skin sample. The magnified view allows for a detailed examination of the skin's texture. b) cumulative amount (µg) of CPX permeated through the porcine skin as a function of time until 24 hours. $p < 0.05$ (*) for the tested microparticles versus pure drug after 24 h c) amount of residual CPX levels in different compartments: donor, acceptor, and membrane, $p < 0.05$ of microparticles versus pure drug at 24 hours.

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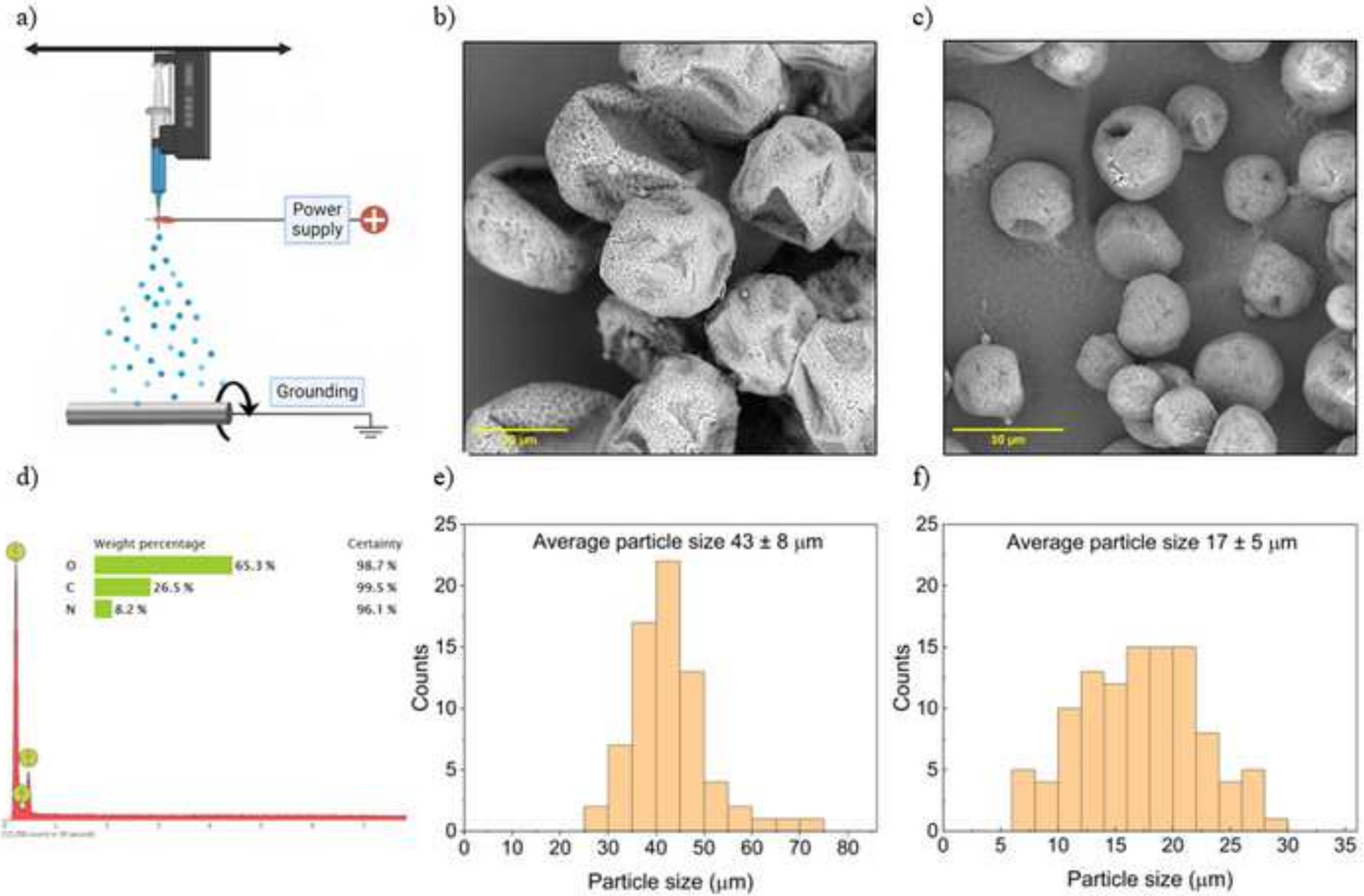

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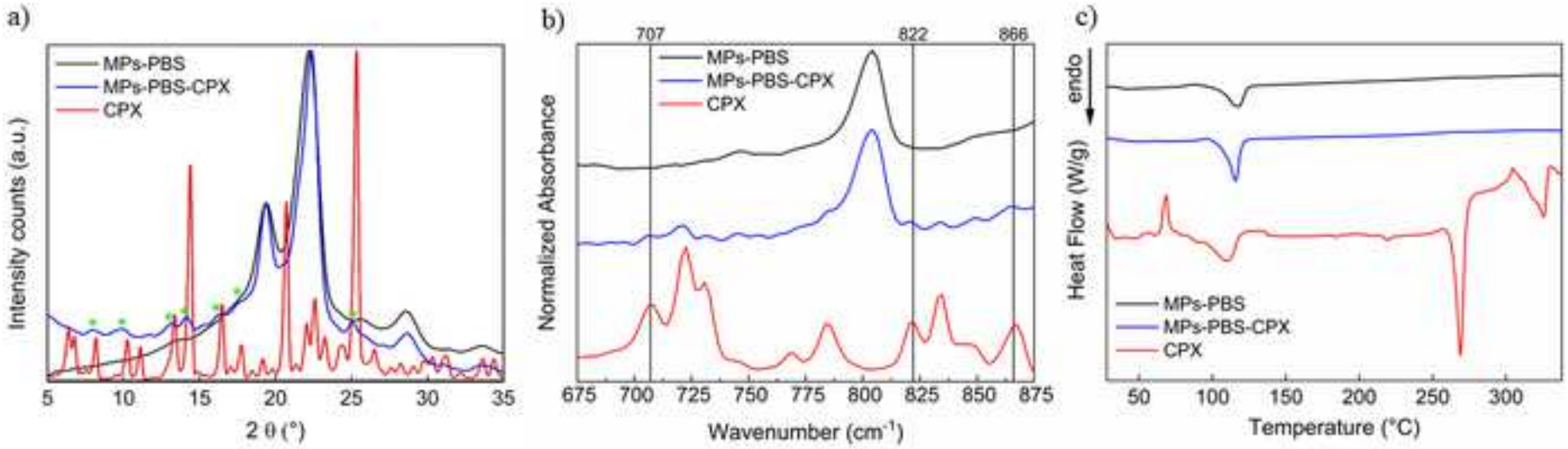

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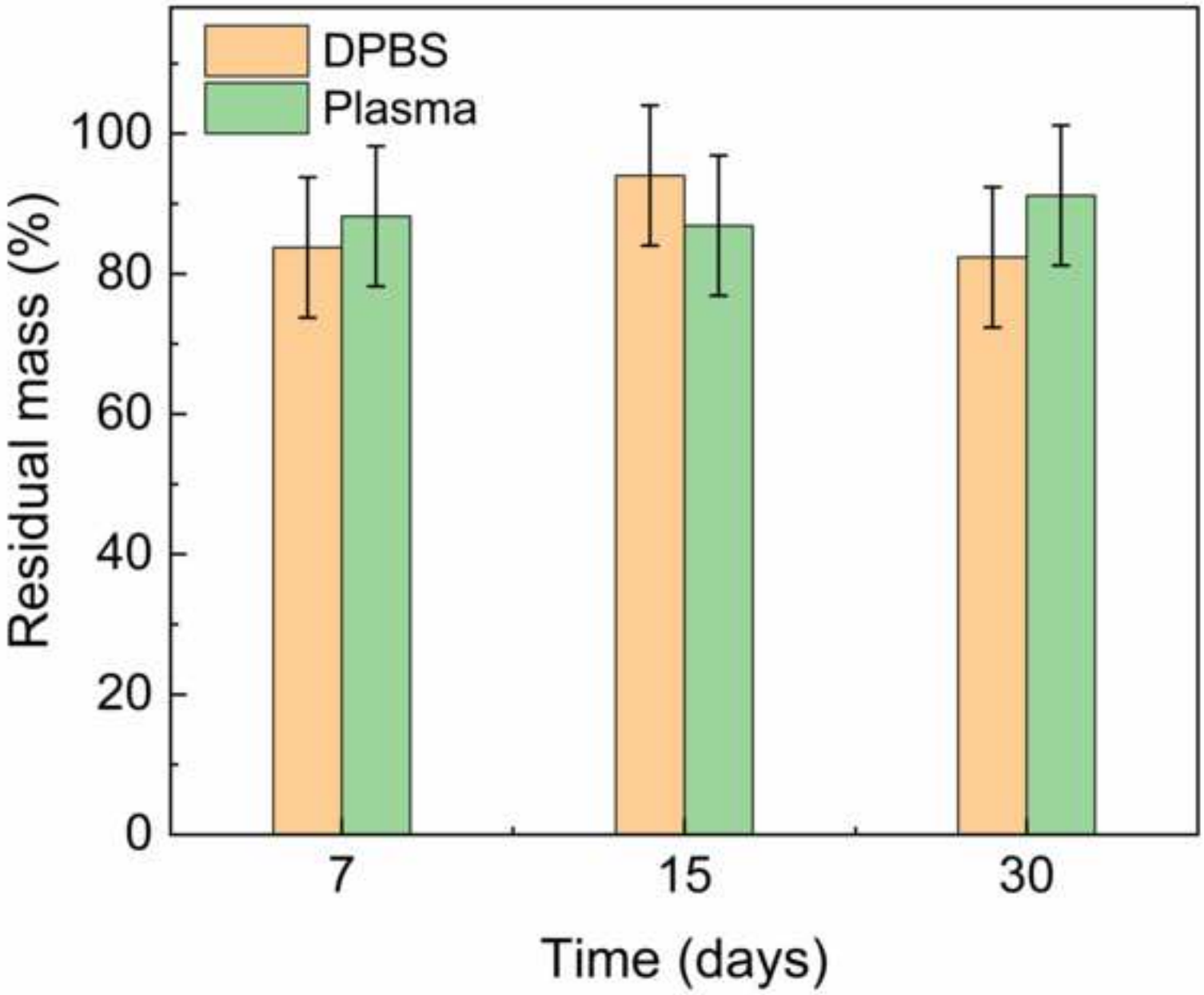

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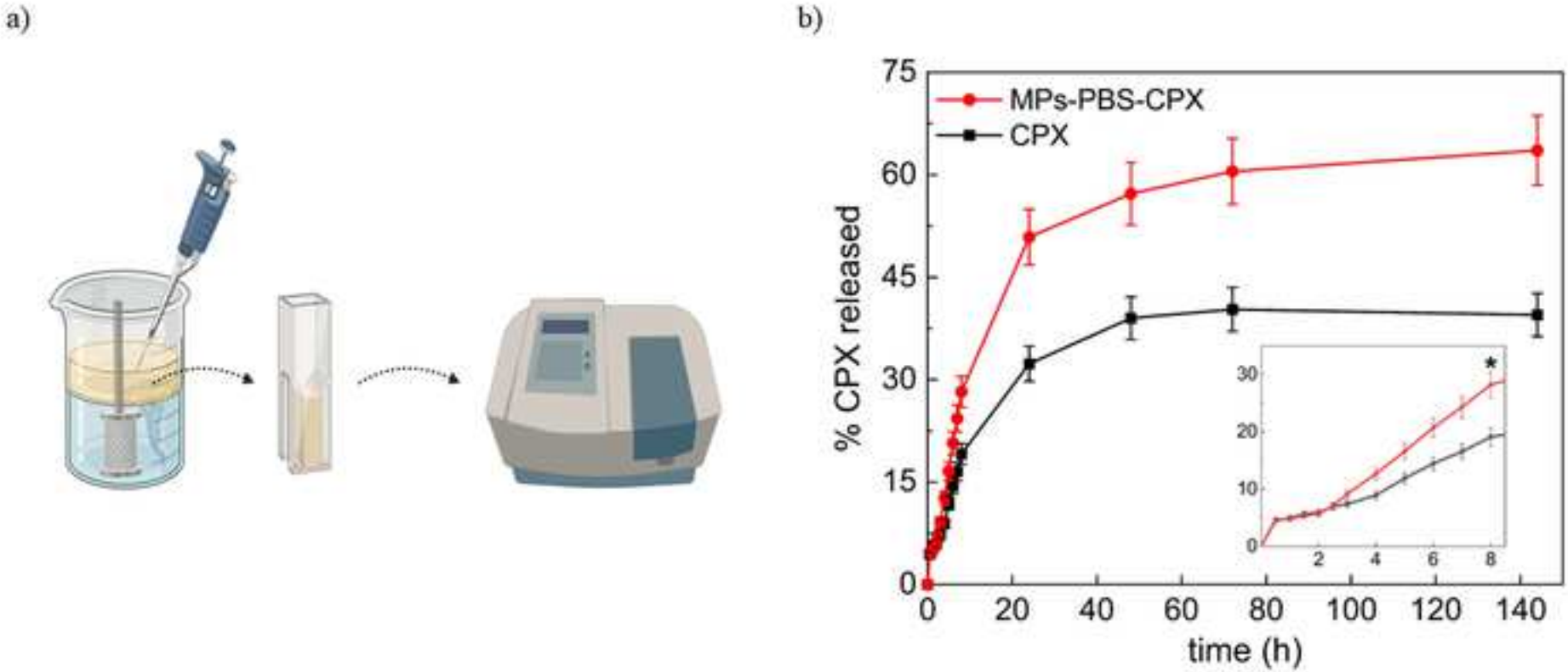

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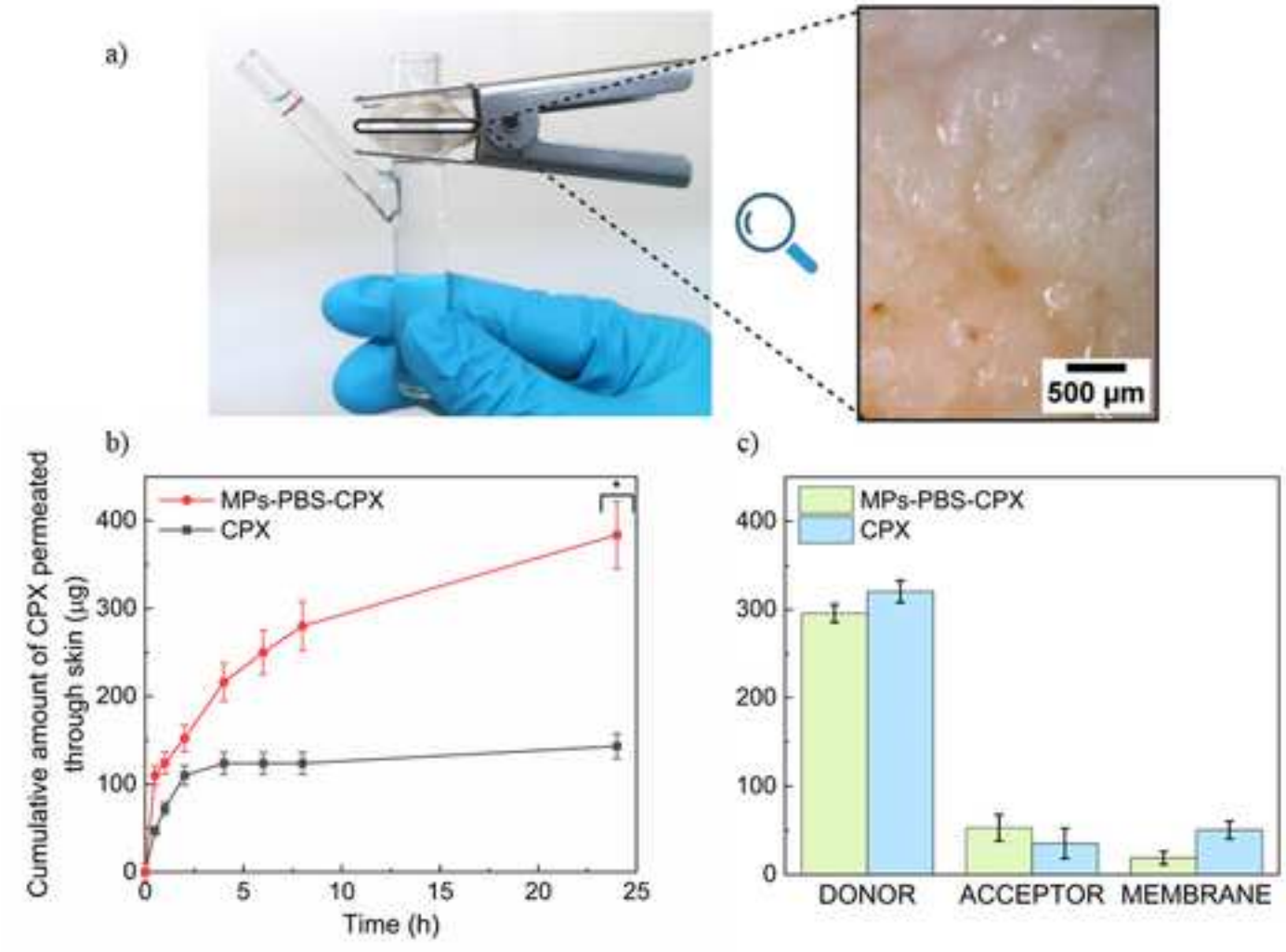

To the EDITORIAL OFFICE:

Editor-in-Chief

Dear Editor,

Here enclosed you will find the online submission of the manuscript entitled "**Electrosprayed Poly-butyl-succinate microparticles for sustained release of Ciprofloxacin as an antimicrobial delivery system** by Giorgia Puleo, Francesca Terracina, Valentina Catania, Sergio Scirè, Domenico Schillaci and Mariano Licciardi to be considered for publication as an original article in Powder Technology.

The above article was transferred to Powder Technology from International Journal of Pharmaceutics, upon a first reviewer's evaluation in the latter journal. Before submission to Powder Technology the manuscript was properly revised and improved, according with reviewer's comment. At the end of this letter, you'll find a detailed list of changes we made in the manuscript according to the reviewer's suggestions. It is the author's opinion that the revised manuscript in now appropriate for publication in Powder Technology and strongly fulfills the scope of the journal. The manuscript provides a proof of the potential utility of new polymeric microparticles produced by electrospraying technique, loading the antibacterial molecule Ciprofloxacin (CPX) and arising to important advantages: microparticles improve the release of CPX and show a sustained release profile of CPX over long time; the electrospray process modifies the crystalline state, improving performances; effectiveness against S. aureus, P. aeruginosa and pathogenic skin bacteria was demonstrated.

All authors are convinced that our contribution is of high innovation and scientific impact that justify the publication as an original article in Powder Tchnology. We believe that these studies prove useful for a large community of scientists in different research fields covering microparticles production and characterization as well as Drug Delivery and Devices.

Please consider for correspondence the following address: Mariano Licciardi, e-mail: mariano.licciardi@unipa.it.

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Below are reported point-by-point answers to reviewer.

Reviewer #1

1. *In this study, Polybutylsuccinate (PBS) is not widely reported polymer. Other materials and techniques used in the manuscript are not new, e.g. electrospray technique, ciprofloxacin, sustained release microparticles, and techniques used for characterization and evaluation of the microparticles. Although the authors developed the microparticles for a wound healing purpose, there were no wound healing evaluation included in the study. The effectiveness of the microparticles against S. aureus, P. aeruginosa and pathogenic skin bacteria is also expected. In the view of the reviewer, this study is lack of novelty.*

Given that this is a paper describing the procedure for obtaining polymeric microparticles to create a model drug delivery system capable of loading different types of drugs, we recognize the versatility of the electrospraying technique in fabrication. In our study, we selected Polybutylsuccinate (PBS) as the polymer due to its unique properties, such as biocompatibility and biodegradability, which make it suitable for various biomedical applications that have already been extensively investigated by our research group. Our objective was to explore the potential of PBS as a material for sustained-release microparticles intended for antibacterial therapies.

While we acknowledge that the electrospray technique, ciprofloxacin, sustained release microparticles, and characterization techniques are not novel, we believe that our study offers a fresh approach by combining these elements within the context of developing drug delivery systems (DDSs) using polymeric materials for sustained release of drugs in biomedical applications. Our primary focus was to develop a formulation utilizing PBS-based microparticles and assess their physicochemical properties, drug release kinetics, and antibacterial activity. Our aim was to establish a comprehensive foundation for the development of a versatile DDS that could be further utilized in various applications.

We understand your observation regarding the absence of wound healing evaluation in our study. We apologize for not including this aspect and acknowledge that its inclusion would have provided valuable insights into the research. Consequently, we have thoroughly revised the article in order to address this limitation. The revised version of the manuscript now provides a better-defined aim for this paper.

2. *The title of the manuscript is overstated as there is no assessment of wound healing although the microparticles exhibited sustained-release profile and antimicrobial effect.*

   Following the instruction of the reviewer the title has been changed.

3. *There is only one electrosprayed microparticle formulation was reported. There is no formulation and process optimization in the manuscript.*

   We would like to address this concern and provide clarification. While it is true that only one electrosprayed microparticle formulation is reported in the manuscript, it is important to note that this formulation was the result of a comprehensive optimization process involving multiple trials and experiments with different parameters.

   In order to achieve the desired formulation, we produced several batches that were not explicitly included in the manuscript. These additional batches were primarily focused on optimizing the concentrations of the polymer and drug, as well as fine-tuning the electrospraying parameters. The purpose of these preliminary batches was to identify and refine the optimal conditions for the electrospraying process.

   Due to space limitations and the focus of the manuscript, we made the decision to present the final formulation and its corresponding characterization and performance analysis. However, proceeded to provide a summary of the optimization process in the manuscript, emphasizing the various concentrations tested, the different parameters explored, and the rationale behind the selection of the final formulation.

4. *The solid state of ciprofloxacin in the microparticles is not clear, which is one of the key information required to understand the release profiles and storage stability of the microparticles.*

   Thank you to the reviewer for this observation. In the revised version of the manuscript, we have improved the description of the solid-state property of ciprofloxacin encapsulated in microparticles. We have performed XRD analysis, which confirms that the crystalline state of the MPs-PBS-CPX produced via electrospraying has a lower degree of crystallinity. This information has been appropriately included in the manuscript, and Figure 2 has been updated to include the corresponding graph.

5. *It is not clear how the in vitro release conditions for the microparticles and ciprofloxacin were decided if the microparticles were developed to against bacteria in skin wounds.*

   We thank the reviewer to have highlighted this aspect. The experimental setup for the in vitro release investigation was chosen to assess the differences in release between the pure drug and the microparticles containing Ciprofloxacin. Although the skin wounds may not precisely replicate the release conditions, when considering the combined results of the release study and the permeation through pig skin, we obtained

preliminary data that confirms a notable enhancement in the release of the drug from the microparticles compared to the unencapsulated form. This favorable outcome is attributed to the production method employed, which alters the solid-state structure of the drug delivery system.

6. *The permeation study on the microparticles using skin is confusing given the fact that the microparticles were developed to against bacteria in skin wounds rather than transdermal delivery.*

   We have included in the manuscript the following explanations to better address the valuable comment from the reviewer, whom we would like to thank. We have provided a more detailed rationale for conducting the permeation study using Franz's cells, despite not replicating wound conditions. This choice allowed us to utilize porcine skin as the model, serving two important purposes. Firstly, it served as a reference for assessing the permeability of normal skin, enabling future comparisons with wound permeation. Secondly, it facilitated the examination of dermal component effects on permeation. By adopting this approach, we gain valuable insights before studying specific wound conditions and take into consideration the limited availability of wound tissue samples. In summary, intact porcine skin serves as an informative starting point for evaluating the dynamics of permeation and establishing a solid foundation for further investigations.

7. *The interpretation and reflection on the data in the section of the R&D are superficial.*
   E.g. the different sizes between loaded and empty microparticles, there is no date proving the difference in surface tension.
   why the dissolution rates of ciprofloxacin were slower than the release rate of the microspheres?
   what is the detection limit of EDX? Have you validated whether this analysis is capable of detecting residual chlorine?

   As suggested by the reviewer the R&D section was revised and improved, and the points highlighted by the comments have been improved in order to give a more thorough and effective description.

Reviewer #2

1. *According to the general editor rules, authors for whom English is not their native language are encouraged to have their paper checked before submission for grammar and clarity. This manuscript needs to be revised (e.g., making the wound not heal, porose, etc.).*

   Following the reviewer's suggestion, the manuscript was thoroughly examined and re-read, and any English errors found were corrected.

2. *I do not think a slow releasing antibiotic system would prevent antibiotic-resistance. The key issue is to prevent doses below the MIC which could allow bacteria to change its phenotype and adapt. Please consider in the introduction section how your proposed system could prevent resistance because it is not clear.*

   In the introduction manuscript, we have included the following explanations to better address the comment made by the reviewer regarding antibiotic resistance, and we would like to express our gratitude for this comment.
   According to (Lio and Kaye, 2004) , topical antibacterial products offer flexibility in their application, allowing for both the prevention and treatment of bacterial infections.
   Furthermore, the development of antibiotic systems that release drugs slowly shows promise as a strategy to potentially prevent antibiotic resistance. These systems aim to avoid administering doses below the minimum inhibitory concentration (sub-MIC), which can exert selective pressure on bacteria, leading to their adaptation and the development of resistance. By delivering antibiotics at controlled and sustained rates, the proposed systems have the potential to maintain therapeutic levels while minimizing the risk of resistance development, as suggested by (Gao et al., 2011; Larsson and Flach, 2022)

3. *The author say: "wounds are often treated with systemic and topical antibiotics" Can they support that with clinical protocols? Usually infected wounds are treated with antiseptics to avoid resistance. Please address how infected wounds are pharmacologically treated.*

   Following the reviewer's advice, the topical clinical protocols have been further elaborated in the introduction in order to obtain a more comprehensive view of the current clinical treatment.

4. *The antibiotic choice of the study does not correlate with the most used topical antibiotics (Mupirocin and fusidic acid) although as I mentioned before topical antibiotics are not recommended, antiseptics do. They say: "Ciprofloxacin… is one of the most often used antibiotics in wound healing" Is that correct? They also say "the optimal drug" Are they sure about that? Can they correlate with records of the most prescribed clinical topical antibiotics?*

   In response to the reviewer's comment, we have provided a more detailed explanation of our choice to use Ciprofloxacin (CPX) in this study. We have included the following explanations in the manuscript:
   Although Ciprofloxacin (CPX) is not the first-line drug for topical infections, which are typically treated with mupirocin, fusidic acid, or retapamulin (Kwak et al., 2017), we selected CPX as the model antibiotic for several reasons. Firstly, CPX is a poorly soluble fluoroquinolone antibiotic with broad-spectrum activity, making it suitable for targeting various bacterial pathogens commonly associated with chronic wounds. Secondly, its low solubility and potential for local administration in a slow-release manner make it an ideal candidate for studying drug delivery systems (DDS). By exploring the feasibility of using CPX in this context, we aim to contribute to the development of innovative therapeutic approaches.
   CPX possesses several qualities that make it an attractive candidate for our research. Ciprofloxacin has a well-established pharmacological profile and is widely used as a systemic antibiotic in wound healing (Tsou et al., 2005; Uhljar et al., 2021; Yu et al., 2006). Although our study does not focus on systemic antibiotics, the extensive use of ciprofloxacin in wound healing supports its consideration as a suitable antibiotic model. Furthermore, CPX exhibits a strong bactericidal effect, high antibacterial activity, and is cost-effective, all of which are important characteristics for a slow-release polymeric delivery system aimed at resolving chronic wounds (Federico et al., 2021; Fiorica et al., 2021).

5. *They say: "It is becoming more popular due to its ease of use and capacity to generate particles with average diameters ranging from micrometers to nanometers" More popular than what?*

   We thank the reviewer for the comment. Our intention is to highlight the versatility and ease of use of the electrospraying technique compared to other methods for producing microparticles. To this end, we have revised the introduction to better emphasize this aspect.

6. *Instead of using an example of antibiotic loaded electrosprayed microparticles for pulmonary delivery (Arauzo et al.) it would be better to focus on examples of topical delivery because this is the topic of the paper.*

   We thank the reviewer for their valuable feedback. Following their comment, we have replaced the bibliographic reference on pulmonary delivery with that of a study on topical treatment using resveratrol-based microparticles (De Pieri et al., 2022).

7. *The advantages of their used polymer compared to others (PCL, PGA, PLA) are not clear.*

   We thank the reviewer for their comment, we have further emphasized this aspect in the manuscript. While considering PCL, PLGA, and PLA as some of the most well-known and widely used polymers for biomedical applications, PBS, the subject of this study, is not to be overlooked. In fact, our group has extensively studied PBS for various applications, such as peripheral nerve and bone regeneration. Knowing

its versatility and biocompatibility, we chose to use it for this study, especially considering the increasing number of publications on PBS in recent years (Gigli et al., 2016)

8. *They say: "The fibers were collected on a 10 mm diameter stainless-steel rotator collector placed approximately 19 cm from the needle." Fibers? Are they particles?*

   We thank the reviewer and apologize for the mistake. The sentence has been appropriately reviewed and corrected.

9. *They say: "It is possible to observe that the empty microparticles are larger than those loaded with CPX; this can be due to a different surface tension of the mixture with CPX." They should evaluate surface tension and viscosity of both samples to corroborate their hypothesis.*

   Following the comment of the reviewer whom we thank, we have modified our statements regarding the difference in size between the microparticles, better explaining that this could probably be due to a difference in surface tension, and that we intend to investigate this phenomenon further in future studies, which we know from the literature affects a lot the size of the microparticles made by electrospraying.

10. *Chemical bond identification should be performed in the FTIR analysis. They use a reference (Tewes et al.) but it would be better to describe which bonds are those that they use to identify the presence of the antibiotic.*

    As suggested by the reviewer, whom we thank, the results and discussion section pertaining to FTIR analysis has been revised, and the assignment of bonds to the peaks observed in the analysis has been further explored.

11. *They say: "this might mean that the drug is incorporated into the polymer matrix in an amorphous state." XRD is needed to corroborate their hypothesis. XRD performed on the empty microparticles, on the antibiotic and on the antibiotic-loaded microparticles.*

    As suggested by the reviewer, to provide stronger support for our hypotheses, we performed XRD analysis to better evaluate the solid-state of the microparticles and thus assess the degree of crystallinity. This analysis was conducted on empty microparticles, as well as on pure CPX antibiotic and CPX-loaded microparticles.

12. *The authors say: "This is favourable because the material within the tissues does not stimulate any inflammatory response but promotes tissue regeneration,…" That is not demonstrated in the current manuscript. I do not think that the authors have included experimental data to support such statement.*

    This statement arises from the previously use of this material both in vitro and in vivo in other studies conducted by the group (Cicero et al., 2022; Miceli et al., 2022; Vigni et al., 2022) but we are aware that we have not tested this aspect in the current manuscript. Therefore, we have carefully reviewed and revised this section as suggested by the reviewer.

13. *Saturation is observed for pure Ciprofloxacin in the release studies. "One ml sample of the upper organic phase (n-octanol) was taken at regular intervals (for six days), immediately read with the spectrophotometer, and poured back into the beaker" Do they need to work under sink conditions to avoid saturation? It is not clear why the cumulative release saturates. In a real scenario saturation could not be reached because wound exudates would continuously washed away the drug. It is not clear.*

    In order to better explain this phenomenon rightly pointed out by the reviewer and we have modified the paragraph to better explain our statements. Here is the revised and corrected paragraph:

The systems were maintained under stirring at constant temperature (150 rpm, 37 °C); these conditions were held for the entire experiment. One milliliter sample of the upper organic phase (n-octanol) was taken at regular intervals over a period of six day. Each sample was immediately examinated with the spectrophotometer, allowing for the detection and quantification of CPX, and poured back into the beaker. The amount of CPX was then calculated from a standard calibration curve of CPX by measuring the absorbance of known concentrations of CPX (0.01– 0.0001 $mg \cdot ml^{-1}$) in n-octanol at 284 nm ($R^2 = 0.99$). It is important to note that although saturation was observed in the cumulative release of pure CPX within the experimental timeframe, an increase in the solubility of loaded CPX is still evident following the electrospray process. While this release study model may not perfectly replicate the dynamic nature of wound exudates observed in real clinical scenarios, it serves as a valuable tool to assess the constant release of drugs from the MPs within the receiving compartment and the difference between the free drug and the one incorporated into the MPs.. We acknowledge the need for future studies that more closely mimic clinical conditions and validate the clinical relevance of our findings.

14. *In figure 5b if the release is cumulative how is it possible that the amount decreases from 4 to 7h? also, how that release can be explained. It does not make sense that the release increases, then decreases and then increases again.*

    We gratefully acknowledge the reviewer's insightful comments on Figure 5b in our work. We modified our study and thoroughly examined the issue on the basis of his comments. Following further experiments and analysis, we identified a mistake in the initial data analysis and interpretation. We sincerely apologize for the confusion created by our initial findings. We revised the section of the manuscript after addressing the issue.

15. *Also the rationale behind the skin permeation experiment does not make sense. When wounding no epidermal tissue is present. They should have performed the permeation experiment on a wound (only dermis).*

    As previously mentioned to Reviewer #1, we would like to reiterate our response to Reviewer #2, whom we also extend our gratitude for his valuable feedback. Both reviewers raised a similar observation, and in our response, we explained that we have chosen to utilize this study on permeation through the skin despite it not precisely replicating wound conditions. The reason for this choice is that it still provides essential insights, serving as a reference for evaluating normal skin permeability and investigating the impact of dermal components on drug permeation from the Drug Delivery System produced.
    In our future investigations, we plan to use the information obtained from intact porcine skin as a foundation for our research, with a specific focus on studying infected wound tissue.

    The antibiofilm activity should also be compared with the literature. How does that 52,5% compare with other studies?

    We have incorporated this explanation into the manuscript, where we state that our reported inhibition activity of 52.5% falls within the range of values reported in previous studies (Jensen et al., 2013; Magalhães et al., 2017). These findings provide additional support for the potential efficacy of MPs-PBS-CPX as a promising treatment approach for P. aeruginosa biofilms.

Kind Regards

Mariano Licciardi

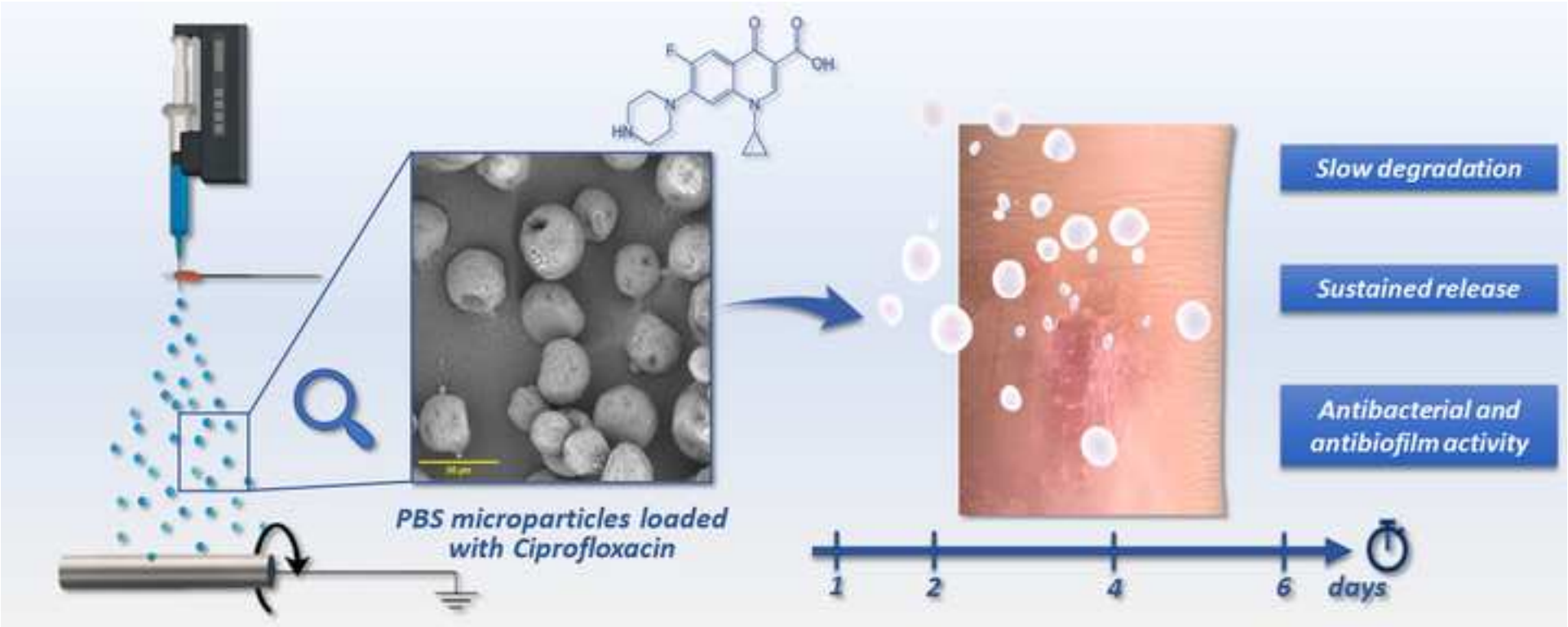
Abstract

In this study polymeric microparticles by electrospraying using polybutylene succinate (PBS), a biodegradable and biocompatible polyester, were produced. This versatile and easy-to-use technique enabled the incorporation of the poorly water-soluble ciprofloxacin into the polymer matrix, a fluoroquinolone antibiotic, inhibiting bacterial replication and effectively treating various infections. The microparticles were characterized by means of different techniques (SEM-EDX, XRD, ATR-FTIR, DSC), and their degradation rate was tested in DPBS and human plasma. Moreover, the as-produced DDS enabled the sustained release of CPX for several days, which proved effective against S. aureus and P. aeruginosa and also against a reference group of bacteria of skin microbiota. MIC and MBC assays were conducted using different culture media. Effective antibacterial activity was observed, along with inhibition of P. aeruginosa biofilm formation at sub-MIC concentrations. An ex vivo permeation study on porcine skin, evaluated the drug permeation to assess potential enhancement in drug permeation.

Highlights

- Electrospraying enables production of polymeric microparticles (MPs) loading drugs.
- MPs improve release of Ciprofloxacin (CPX), a poorly water-soluble antibiotic.
- Polybutylsuccinate (PBS) aids wound healing, degrades slowly, and holds promise as a biodegradable polymer.
- Electrosprayed MPs show a long release profile of CPX over long periods of time.
- MPs-PBS-CPX showed effectiveness against S. aureus, P. aeruginosa and pathogenic skin bacteria.

Graphical Abstract (for review)



To the Editor of Powder Technology

Declaration of Competing Interest

Of the manuscript entitled "Electrosprayed poly-butylene-succinate microparticles for sustained release of ciprofloxacin as antimicrobial delivery system" by Giorgia Puleo, Francesca Terracina, Valentina Catania, Sergio Scirè, Domenico Schillaci and Mariano Licciardi.

The authors declare that they have no known financial or interpersonal conflicts that might have noticed to have influenced the research presented in this article.Sincerely

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