European Journal of Pharmaceutics and Biopharmaceutics 139 (2019) 59–67

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

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

Research paper

Nano spray dried antibacterial coatings for dental implants

Elias Baghdan[a,1], Michael Raschpichler[a,1], Walaa Lutfi[a], Shashank Reddy Pinnapireddy[a], Marcel Pourasghar[b], Jens Schäfer[a], Marc Schneider[b], Udo Bakowsky[a,*]

[a] Department of Pharmaceutics and Biopharmaceutics, University of Marburg, Robert-Koch-Str. 4, 35037 Marburg, Germany
[b] Department of Pharmacy, Biopharmaceutics and Pharmaceutical Technology, Saarland University, Campus C4 1, 66123 Saarbrücken, Germany

ARTICLE INFO

Keywords:
Antibacterial
Biocompatible
Nanoscale
Coating
Implant
PLGA
Norfloxacin

ABSTRACT

Nanostructured coatings of dental implants have shown great potential in overcoming many challenges responsible for implant failure. In this study, nano spray drying technology was utilized to produce novel biocompatible nanocoatings with antibacterial activity. The experiments were applied on titanium discs, which were used as a model material for dental implants. The produced nanocoatings consisted of poly(lactic-*co*-glycolic acid) as a biodegradable polymer and norfloxacin as a model antibiotic. Scanning electron microscopy results revealed an average particle size ranging between 400 and 600 nm. *In vitro* release studies showed a biphasic drug release profile with a burst release within the first 48 h, followed by a sustained release phase until the end of the experiment. The antibacterial activity of the nanocoatings was evaluated against *Escherichia coli* where the norfloxacin loaded nanocoatings achieved up to 99.83% reduction in the number of viable bacterial colonies. Finally, *in vitro* biocompatibility of the nanocoatings was investigated using mouse fibroblasts (L929) as a standard sensitive cell line for cytotoxicity assessment. Cell proliferation on the surface of the titanium discs was studied using fluorescence microscopy followed by cell counting assay. Both methods confirmed the biocompatibility of the examined nanocoatings. In conclusion, nano spray drying is a promising technique for preparing tailor-made nanocoatings, thereby representing an innovative approach for the surface modification of dental implants.

1. Introduction

The U.S. Food and Drug Administration (FDA) defines medical implants as devices or tissues which are inserted into the human body or placed on its surface in order to replace parts of the body or their functionality (U.S. Food and Drug Administration, 2018). Dental implants are commonly used in restoring either a partly or entirely toothless area [1–3]. They are characterized by screw-like or cylindrical shape and are typically made of inert materials like pure titanium or titanium alloys [3]. Dental implant insertion is a well-established procedure with a success rate of 90–95%. However, several complications can occur leading to implant failure, especially peri-implantitis which is a serious inflammation surrounding the dental implant [1]. The emergence of peri-implantitis can be described as multifactorial. Potential risk factors are, for example, inadequate oral hygiene, genetic predisposition, smoking or bacterial contamination during the surgery [2,4]. Bacterial accumulation on the implant surface induces an inflammation of the surrounding tissues leading to bone degradation and the removal of the infected implant [4].

Even though antibiotic prophylaxis is commonly indicated to avoid such complications, the improper use of antibiotics is often associated with several side effects, e.g. bacterial resistance development, secondary infections or antibiotic toxicity [2]. Moreover, the high antibiotic dosage needed to achieve an adequate bioavailability is linked to

Abbreviations: CFU, colony-forming unit; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; DSMZ, German Collection of Microorganisms and Cell Cultures GmbH; EBSS, Earle's balanced salt solution; NFX, norfloxacin; OD_{600}, optical density at $\lambda = 600$ nm; PBS, phosphate-buffered saline; PLGA, poly(lactic-*co*-glycolic acid); Ra, roughness average; RH, relative humidity; RMS, root mean square; SEM, scanning electron microscopy; *Tg*, glass transition temperature; Ti-disc, titanium disc

* Corresponding author.
E-mail addresses: elias.baghdan@pharmazie.uni-marburg.de (E. Baghdan), michael.raschpichler@pharmazie.uni-marburg.de (M. Raschpichler), walaa.lutfi@pharmazie.uni-marburg.de (W. Lutfi), shashank.pinnapireddy@pharmazie.uni-marburg.de (S.R. Pinnapireddy), marcel.pourasghar@uni-saarland.de (M. Pourasghar), j.schaefer@staff.uni-marburg.de (J. Schäfer), marc.schneider@uni-saarland.de (M. Schneider), ubakowsky@aol.com (U. Bakowsky).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.ejpb.2019.03.003
Received 27 January 2019; Received in revised form 28 February 2019; Accepted 1 March 2019
Available online 02 March 2019
0939-6411/

an increase in the probability of allergic or gastrointestinal reactions [2,5]. Until now, there is still no agreement among experts for standard guidelines concerning peri-implantitis prophylaxis or treatment [1–3]. Therefore, the search for a better alternative continues.

One such promising approach is implant coating and surface modification which has shown promising potential in preventing implant failure. Antimicrobial implant coatings offer several advantages over systemic administration of antibiotics, especially in terms of their local activity against biofilm formation [6,7]. Furthermore, the emergence of nanotechnology took the capabilities of surface coatings to the next level. Several methods have been applied to produce nanostructured dental implants with desired features, e.g. reduced bacterial adhesion and enhanced osseointegration [8,9].

Recently, the nano spray drying technology was utilized as a single-step technique for the coating and surface modification of dental implants [10]. This was achieved using the Nano Spray Dryer B-90 which is equipped with several cutting-edge technologies: (a) unique spray mesh that can generate ultra-fine droplets; (b) laminar gas flow for a gentle drying process; (c) electrostatic particle collector capable of efficiently separating the dried particles from the gas stream [11–13]. Nano spray drying has several advantages over conventional spray drying in terms of ability to produce nanoparticles by generating far smaller droplets with narrower size distribution, smaller sample quantities and higher powder yields [13–15]. These outstanding features paved the way for numerous innovative applications, especially in the field of nanoencapsulation and targeted drug delivery [16].

The aim of this work is to prepare antibacterial and biocompatible nanocoatings for dental implants by means of nano spray drying. Poly (lactic-*co*-glycolic acid) (PLGA) was chosen due to its biodegradable, biocompatible and controlled release properties. Furthermore, PLGA is an FDA-approved polymer with a widespread variety of applications ranging from drug delivery to coating and surface modification of medical implants [17–20]. Norfloxacin (NFX) was used as broad-spectrum antibiotic that has a well-documented activity against staphylococci and several coliforms which can be involved in the development of peri-implantitis [2,3,21]. The prepared nanocoatings were subjected to several physicochemical characterizations to study drug content, drug release profile, morphology, particle size, morphological changes under physiological conditions and surface roughness. Moreover, their antibacterial activity and *in vitro* biocompatibility were investigated using *E. coli* and mouse fibroblasts (L929) respectively.

2. Materials and methods

2.1. Materials

Poly(lactic-*co*-glycolic acid) (Resomer® RG 503H, molecular weight 29,000 g/mol, lactide:glycolide 50:50) was supplied by Evonik Nutrition & Care GmbH (Essen, Germany). Sodium acetate (≥98.5%) was purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). Norfloxacin (≥98%) and 4′,6-diamidino-2-phenylindole (DAPI) were obtained from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany).

All solvents were of analytical or HPLC grade and were used as received. Ultrapure water from PURELAB® flex 4 equipped with a Point-of-Use biofilter (ELGA LabWater, High Wycombe, UK) was used for all experiments.

2.2. Bacterial strains and media

Escherichia coli DH5α (DSM 6897, DSMZ, Braunschweig, Germany) were cultured in Mueller Hinton broth and stored at −80 °C. One day prior to the bacterial experiments, the bacteria were thawed and then incubated overnight at 37 °C under gentle shaking (100 rpm) using an orbital shaker (Compact Shaker KS 15 A, Edmund Bühler GmbH, Bodelshausen, Germany).

2.3. Cell culture

Mouse fibroblasts (L929) were purchased from American Type Culture Collection (ATCC®, Manassas, USA). The cells were maintained at 37 °C and 8.5% CO_2 under humid conditions and were cultured in DMEM medium (Capricorn Scientific GmbH, Ebsdorfergrund, Germany) supplemented with 10% fetal bovine serum (Sigma-Aldrich Chemie GmbH) and 10 µl/ml antibiotic/antimycotic solution (Capricorn Scientific GmbH). The cells were cultivated in monolayers and passaged upon reaching 80% confluency.

2.4. Nanocoating preparation

Small titanium discs (Ti-discs) with defined surface area (1 cm^2) were cut from a titanium foil (0.25 mm thick, annealed, 99.5% (metals basis); Alfa Aesar, Karlsruhe, Germany). Prior to use, the discs were thoroughly cleaned using several solvents with the aid of ultrasound bath (Elmasonic P, Elma Schmidbauer GmbH, Singen, Germany). The Nano Spray Dryer B-90 (BÜCHI Labortechnik AG, Flawil, Switzerland) was used to produce nanoparticles which will coat the Ti-discs as previously mentioned [10]. The spray drying parameters were kept constant for all formulations: spray cap of 4.0 µm mesh diameter (45° angular position), gas flow 100 l/min, spray rate 50% and inlet temperature 70 °C. The Nano Spray Dryer B-90 was operated in the closed loop mode using nitrogen as drying gas. The feed solution was prepared by dissolving NFX and PLGA in a solvent mixture (acetonitrile and water (95:5) containing 0.005% w/v sodium acetate). Three nanocoatings (PLGA 2.5% NFX, PLGA 5% NFX and PLGA 10% NFX) with different theoretical norfloxacin loadings (2.5%, 5% and 10% wt. of PLGA) were prepared. Unloaded nanocoatings (PLGA 0% NFX) were also prepared following the aforementioned procedure without the addition of norfloxacin. The effect of the Ti-disc position on the produced nanocoatings was also investigated. Therefore, three different levels inside the particle collector were chosen (i.e. Top, Middle and Bottom) with approx. 6 cm distance between each of them. The coating process of the Ti-discs is illustrated in Supplementary materials, Fig. S1.

2.5. Amount of drug per cm^2 nanocoating

The nanocoatings were completely dissolved by placing coated Ti-discs in 24-well plates (Standard, F, Sarstedt AG & Co. KG, Nümbrecht, Germany) filled with 1 ml acetonitrile:0.1 M NaOH (1:1) per well. The 24-well plates were then gently shaken (150 rpm for 60 min) using an orbital shaker (KS4000 IC, IKA Werke GmbH & Co. KG, Staufen, Germany) at room temperature and under light protection. Subsequently, the concentration of norfloxacin was quantified by measuring the absorbance at λ = 274 nm using a microplate spectrophotometer (Multiskan™ GO, Thermo Scientific, Waltham, Massachusetts, USA).

2.6. Agar diffusion test

The overnight culture of *E. coli* was used to prepare a bacterial culture with an optical density (OD_{600}) of 0.025, which was incubated at 37 °C under gentle shaking (200 rpm) using an orbital shaker (Compact Shaker KS 15 A, Edmund Bühler GmbH). To reduce further growth, the bacteria were cooled on an ice bath after reaching an OD_{600} over 0.4. Subsequently, 100 µl of the bacterial culture was plated on BD™ Mueller Hinton II agar plates (BD GmbH, Heidelberg, Germany). The Ti-discs were then placed in the center of the agar plates (one disc per agar plate (Supplementary materials, Fig. S2)). Afterwards, the agar plates were incubated at 37 °C and ~90% RH (In-VitroCell ES NU-5841E, NuAire, Inc., Plymouth, Minnesota, USA). After 24 h, the antibacterial activity of the nanocoatings was evaluated by measuring the diameter of the zones of inhibition formed around the Ti-discs [22]. Each sample was analyzed in triplicates.

2.7. In vitro drug release studies

Norfloxacin release studies were performed over a period of 15 days under static conditions. The coated Ti-discs were placed in 5 ml glass vials (Schott AG, Müllheim, Germany) filled with 1 ml phosphate-buffered saline (PBS, filter-sterilized, pH 7.4) at 37 °C under light protection. After specific time intervals, 200 µl samples were withdrawn and replaced with equivalent volumes of fresh PBS. To avoid temperature variations, PBS was also stored at 37 °C under the same conditions. The concentration of norfloxacin was quantified by measuring the absorbance at $\lambda = 270$ nm using a microplate spectrophotometer (Multiskan™ GO, Thermo Scientific).

2.8. Morphology and surface properties

The morphology and surface structure of the nanocoatings were studied using SEM (Hitachi S-510, Hitachi-High Technologies Europe GmbH, Krefeld, Germany). The Ti-discs were fixed on aluminum pin stubs and sputter-coated with a gold layer (30 mA for 1 min) using Edwards S150 Sputter Coater (Edwards Vacuum, Crawley, UK). The samples were examined at an accelerating voltage of 5 kV. The micrographs were recorded digitally using DISS 5 digital image acquisition system (Point Electronic GmbH, Halle, Germany). Afterwards, mean particle size was determined by analyzing SEM micrographs using ImageJ software (version 1.47v, National Institutes of Health, USA).

The morphological changes of the nanocoatings under physiological conditions (in terms of pH and temperature) was investigated by placing coated Ti-discs in 5 ml tubes (Sarstedt AG & Co. KG) filled with 1 ml PBS (filter-sterilized, pH 7.4). After 15 days incubation at 37 °C, the Ti-discs were carefully taken out and dipped thrice in water to remove residual PBS. The Ti-discs were then left to dry at room temperature under a fume hood. Subsequently, the nanocoating morphology was studied using SEM following the same procedure mentioned above.

The surface roughness of the nanocoatings was analyzed using white light interferometry (NewView™ 7300 3D Optical Surface Profiler, Zygo Corporation, Middlefield, Ohio, USA). 10 different spots were randomly measured using a 20x objective, 2x zoom and a scan length of 20 µm. MetroPro 8.3.5 software (Zygo Corporation) was utilized to calculate the surface roughness.

2.9. Bacterial viability assay

Antibacterial activity of the nanocoatings was evaluated according to a procedure mentioned elsewhere with some modifications [23]. The overnight culture of *E. coli* was used to prepare a bacterial suspension with an OD_{600} of 0.2. 1 ml of this culture was added to 24-well plates (Standard, F, Sarstedt AG & Co. KG) containing the Ti-discs. The 24-well plates were incubated under static conditions at 37 °C and ~ 90% RH (In-VitroCell ES NU-5841E, NuAire, Inc.). After 24 h, the Ti-discs were taken out and immersed thrice in PBS (filter-sterilized, pH 7.4) to remove non-adherent bacterial cells. The Ti-discs were then transferred into new 24-well plates. To detach the bacteria from the surface of the Ti-discs, 1 ml trypsin-EDTA (0.5%, Capricorn Scientific GmbH) was added and the samples were gently shaken (100 rpm) at room temperature for 20 min using an orbital shaker (Compact Shaker KS 15 A, Edmund Bühler GmbH). In order to evaluate the bacterial viability (CFU/cm^2), different dilutions (1:10–1:1,000,000) of the samples were plated on BD™ Mueller Hinton II agar plates (BD GmbH) and the colonies were counted after 24 h incubation at 37 °C and ~90% RH (In-VitroCell ES NU-5841E, NuAire, Inc.). The samples were analyzed in three independent experiments.

2.10. In vitro biocompatibility

L929 cells were seeded onto 12-well plates (Standard, F, Sarstedt AG & Co. KG) containing Ti-discs at a seeding density of $1x10^5$ cells/3.65 cm^2 (per well). After predetermined time intervals (i.e. 24 h and 96 h), the Ti-discs were transferred into new 12-well plates and washed thrice with PBS containing Ca^{2+} and Mg^{2+} (filter-sterilized, pH 7.4), to remove non-adherent cells. For visualizing the cells adhered to the surface of the Ti-discs, the cells were fixed with 4% formaldehyde solution for 20 min after which the cell nucleus was counterstained with DAPI (0.1 µg/ml) for 20 min [24]. Finally, the Ti-discs were mounted onto microscope slides and examined under an inverted microscope (CKX53, Olympus Deutschland GmbH, Hamburg, Germany) equipped with light guide-coupled illumination system (U-HGLGPS, Olympus Deutschland GmbH) as fluorescence light source.

Cell counting experiments were performed according to a procedure mentioned elsewhere with some modifications [25]. L929 cells were cultured as mentioned above. After predetermined time intervals (i.e. 24 h and 96 h), the Ti-discs were transferred into 24-well plates (Standard, F, Sarstedt AG & Co. KG) and washed twice with PBS without Ca^{2+} or Mg^{2+} (filter-sterilized, pH 7.4), to remove non-adherent cells. Afterwards, the Ti-discs were incubated with EBSS without Ca^{2+} or Mg^{2+} for 10 min at 37 °C. The cells were detached from the surface of the Ti-discs using trypsin-EDTA (0.05%, Capricorn Scientific GmbH). After 10 min incubation at 37 °C, DMEM medium (supplemented with 10% fetal bovine serum) was used to gently wash down the cells from the Ti-discs. Finally, 100 µl of the cell suspension was mixed with 0.4% trypan blue solution (1:1) and the viable cells were directly quantified by pipetting 10 µl of this mixture into cell counting slide (R1-SLI, Olympus Deutschland GmbH) specially designed for the automated cell counter (Cell Counter model R1, Olympus Deutschland GmbH). Each sample was analyzed in three independent measurements.

2.11. Statistical analysis

All measurements were performed in triplicates and the values are presented as mean ± standard deviation, unless otherwise stated. Two-tailed Student's *t*-test was performed to identify statistically significant differences. Probability values of $p < 0.05$ were considered significant.

3. Results and discussion

3.1. Antibacterial activity

In a previous study, nano spray drying exhibited promising potential as a novel approach to prepare nanoscale coatings for dental implants. The wide range applicability of this technique was confirmed using three different model substances with different physicochemical properties, i.e. chitosan, PLGA and curcumin [10]. In this study, the capability of this technique to produce biocompatible nanocoatings with antibacterial activity was explored. Therefore, PLGA-based nanocoatings were prepared using norfloxacin as a model drug for broad-spectrum antibacterial agents [26–29]. The optimized spray drying process parameters were applied on Ti-discs, taking previous studies into consideration, wherein the spray mesh size and the solid concentration of the feed solution had the most pronounced influence on the size of the produced particles [14,30,31]. Therefore, all formulations were prepared with low solid concentration (1 mg/ml) and the smallest spray mesh available (4.0 µm) was used, thereby achieving the smallest possible particle size. Due to its crucial role in controlling the properties of the produced nanocoatings, Ti-disc position inside the particle collector was investigated. Therefore, the Ti-discs where fixed at three different positions (i.e. Top, Middle and Bottom).

Antibacterial activity of the nanocoatings was evaluated against *E. coli* in two stages: first, qualitatively, using agar diffusion test which is one of the standard methods commonly used for antimicrobial susceptibility testing [22]. The influence of the theoretical norfloxacin loading and Ti-disc position inside the particle collector were

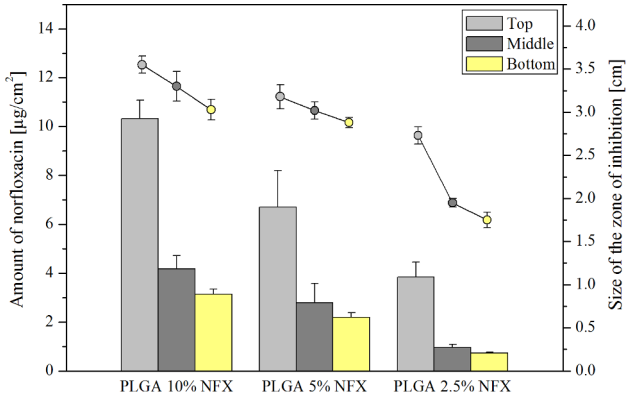

Fig. 1. Graphical representation of the relationship between the amount of norfloxacin per cm^2 of the nanocoating (bars) and the diameter of the zone of inhibition (dots). Nanocoatings with three different theoretical norfloxacin loadings (PLGA 2.5% NFX, PLGA 5% NFX and PLGA 10% NFX) were tested. The titanium discs were fixed at three positions inside the electrostatic particle collector (i.e. Top, Middle and Bottom).

investigated (Supplementary materials, Fig. S3). This approach served as a first step to explore the most suitable variant among different nanocoatings and reduce the number of samples. As expected, the largest zones of inhibition were observed in PLGA 10% NFX; however, they were comparable to those of PLGA 5% NFX. Moreover, at these loadings (i.e. 10% and 5%),

Ti-disc position inside the particle collector did not have a pronounced influence on the zones of inhibition. A significant decrease in the zones of inhibition was noticed in PLGA 2.5% NFX, especially Middle and Bottom. PLGA 0% NFX were used as negative control and did not show any antibacterial activity. Since the bacterial growth inhibition is related to the amount of antibiotic diffused through the agar [22], a direct relationship between norfloxacin content in the nanocoatings and the size of the zones of inhibition could be seen.

Spectrophotometric quantification showed that norfloxacin loading and Ti-disc position inside the particle collector significantly controlled the amount of norfloxacin per cm^2 of the nanocoating (Fig. 1). This can be attributed to the particle fractionation effect along the particle collector which was also observed in previous studies [10], wherein Ti-discs at the top part were covered with more and larger particles in comparison to those at the bottom part. As a result, the produced nanocoatings had a norfloxacin content ranged between 10.33 µg/cm^2 (PLGA 10% NFX Top) and 0.73 µg/cm^2 (PLGA 2.5% NFX Bottom).

3.2. *In vitro* drug release studies

Nano spray drying has been successfully utilized in several studies

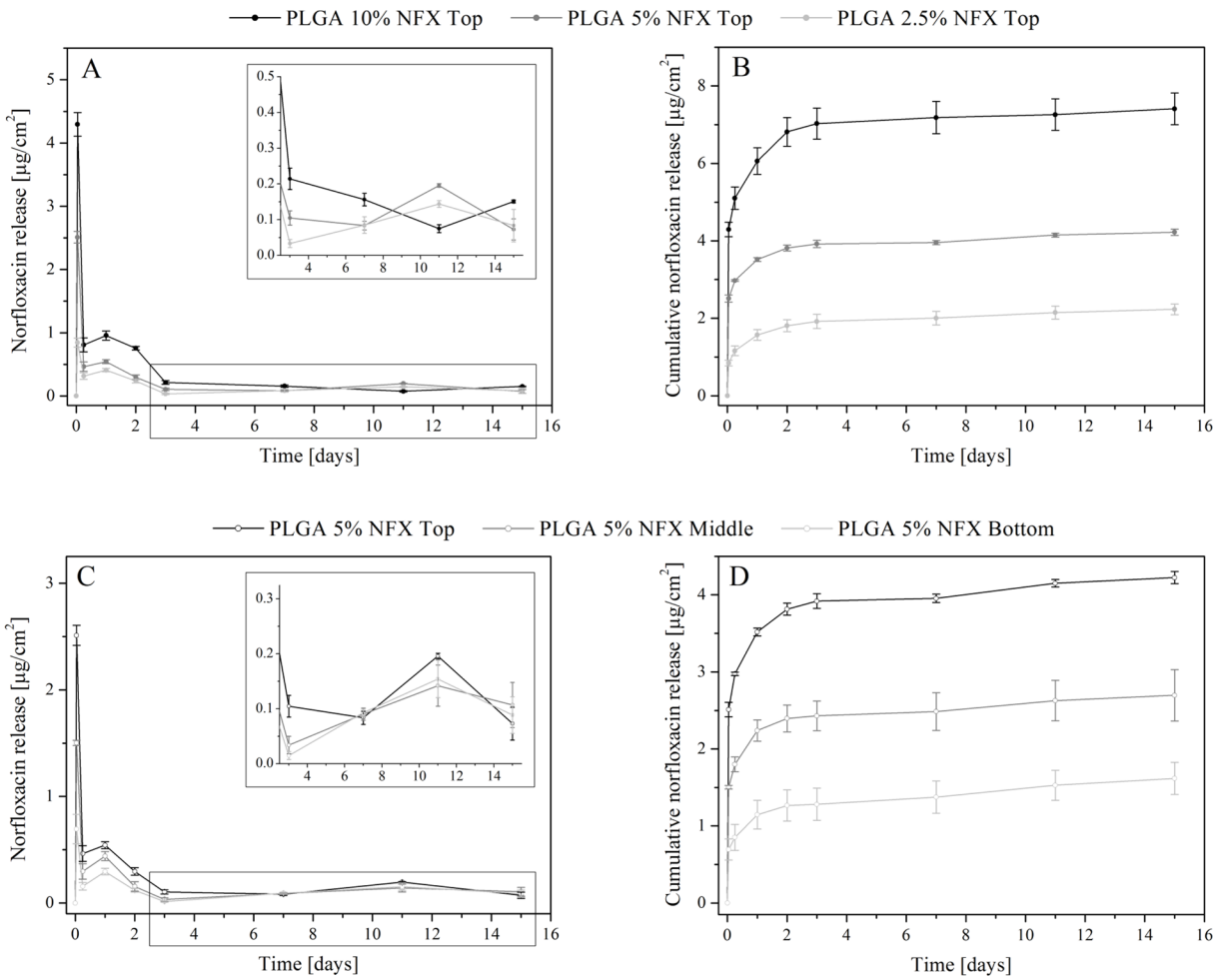

Fig. 2. *In vitro* release profile of norfloxacin over a period of 15 days in PBS (pH 7.4) at 37 °C. In the first experiment (A and B), the coated titanium discs were fixed at the same level inside the particle collector (i.e. Top); however, with three different theoretical norfloxacin loadings (i.e. 2.5%, 5% and 10%). In the second experiment (C and D), the coated titanium discs had the same theoretical norfloxacin loading (i.e. 5%); however, they were fixed at three different levels inside the particle collector (i.e. Top, Middle and Bottom). A and C: The released amount of norfloxacin after different time intervals. B and D: Cumulative release profile of norfloxacin.

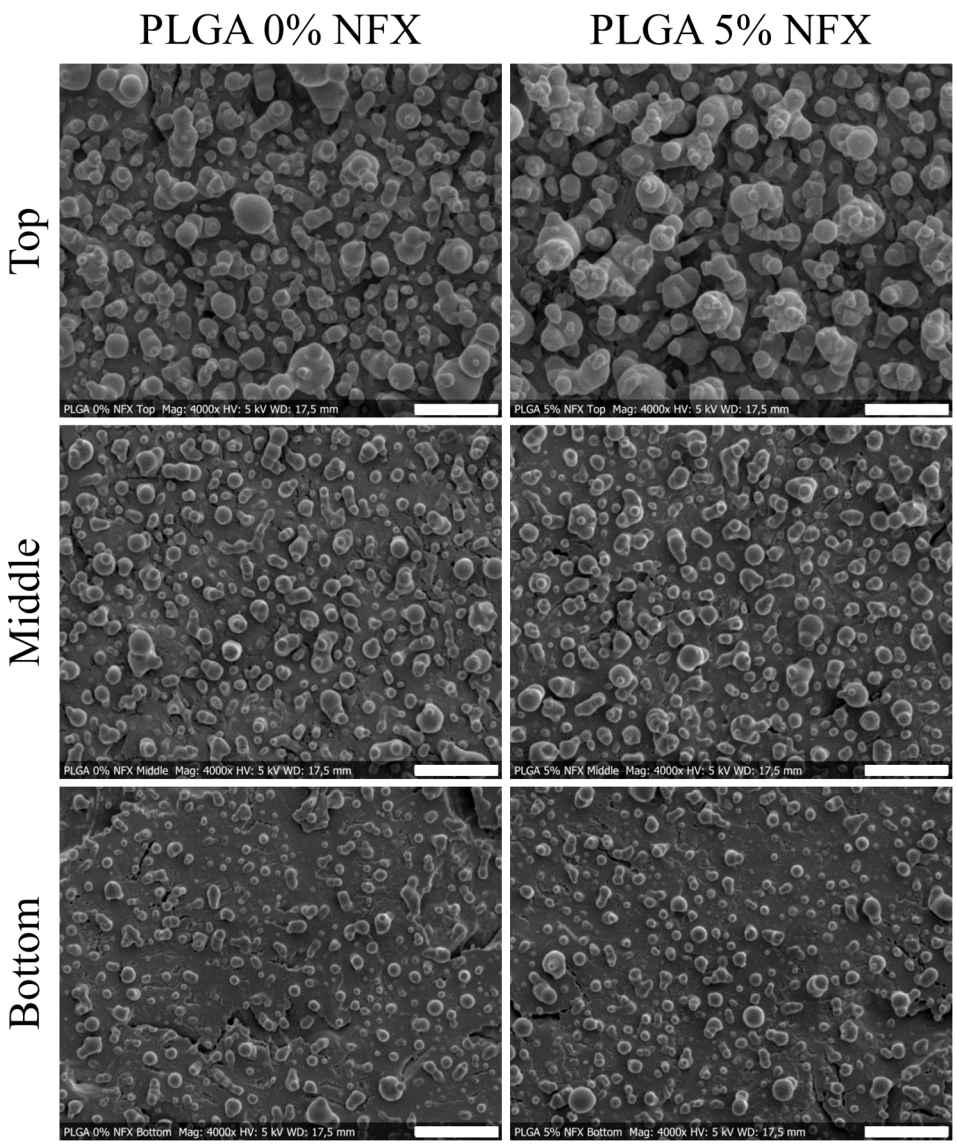

Fig. 3. SEM micrographs of coated titanium discs showing the morphology and surface structure of unloaded nanocoatings (PLGA 0% NFX) and norfloxacin loaded nanocoatings (PLGA 5% NFX). The titanium discs were fixed at three different positions inside the electrostatic particle collector (i.e. Top, Middle and Bottom). Scale bars represent 5 μm.

for the preparation of PLGA submicroparticles that exhibited controlled drug release properties [12,13,16,31]. Drug release rate was found to be influenced by several factors e.g. particle size [32], PLGA lactide:glycolide ratio and molecular weight [12].

In this work, the release profile of several nanocoatings was studied over a period of 15 days in PBS (pH 7.4) at 37 °C. To investigate the effect of theoretical drug loading on the release behavior, Ti-discs placed at the same level inside the particle collector (i.e. Top); however, with three different theoretical norfloxacin loadings (i.e. 2.5%, 5% and 10%) were examined (Fig. 2). Furthermore, the influence of Ti-disc position inside the particle collector was also explored; therefore, Ti-discs with the same norfloxacin loading (i.e. 5%), however, placed at three different levels inside the particle collector (i.e. Top, Middle and Bottom) were tested (Fig. 2). All nanocoatings exhibited similar release profiles that are typically observed with PLGA formulations encapsulating drugs with poor water solubility [18].

By observing the release graphs (especially Fig. 2A and C), the release profiles could be divided into two phases. In the first 48 h, an initial burst release was observed, which can be mainly due to drug desorption from the nanocoatings surface [33,34]. Moreover, the sharp peak noticed in all graphs after 1 h incubation can be also attributed to the fast dissolution of free nonencapsulated drug [35]. This “burst phase” was found to be directly influenced by the theoretical norfloxacin loading and it was most obvious at the highest norfloxacin loading (i.e. 10%) which is in agreement with previous studies [34]. Starting from the third day until the end of experiment period, a second phase was noticed, wherein norfloxacin was released at low but constant rate. According to previous studies, drug diffusion is the main release mechanism at this phase, possibly through the matrix of PLGA particles or water-filled pores [34–36]. However, drug release from PLGA formulations is a very complex process; therefore, other mechanisms might be also controlling the release at this phase [35]. This sustained release phase did not seem to be influenced by theoretical norfloxacin loading or Ti-disc position inside the particle collector. It is worth mentioning that no secondary burst release was observed during the test period.

Based on the results of agar diffusion test and *in vitro* release studies, PLGA 5% NFX were chosen for further characterizations. They were found to be more efficient in comparison to other nanocoatings (i.e. 2.5% and 10%), exhibiting similar release profiles and sufficient

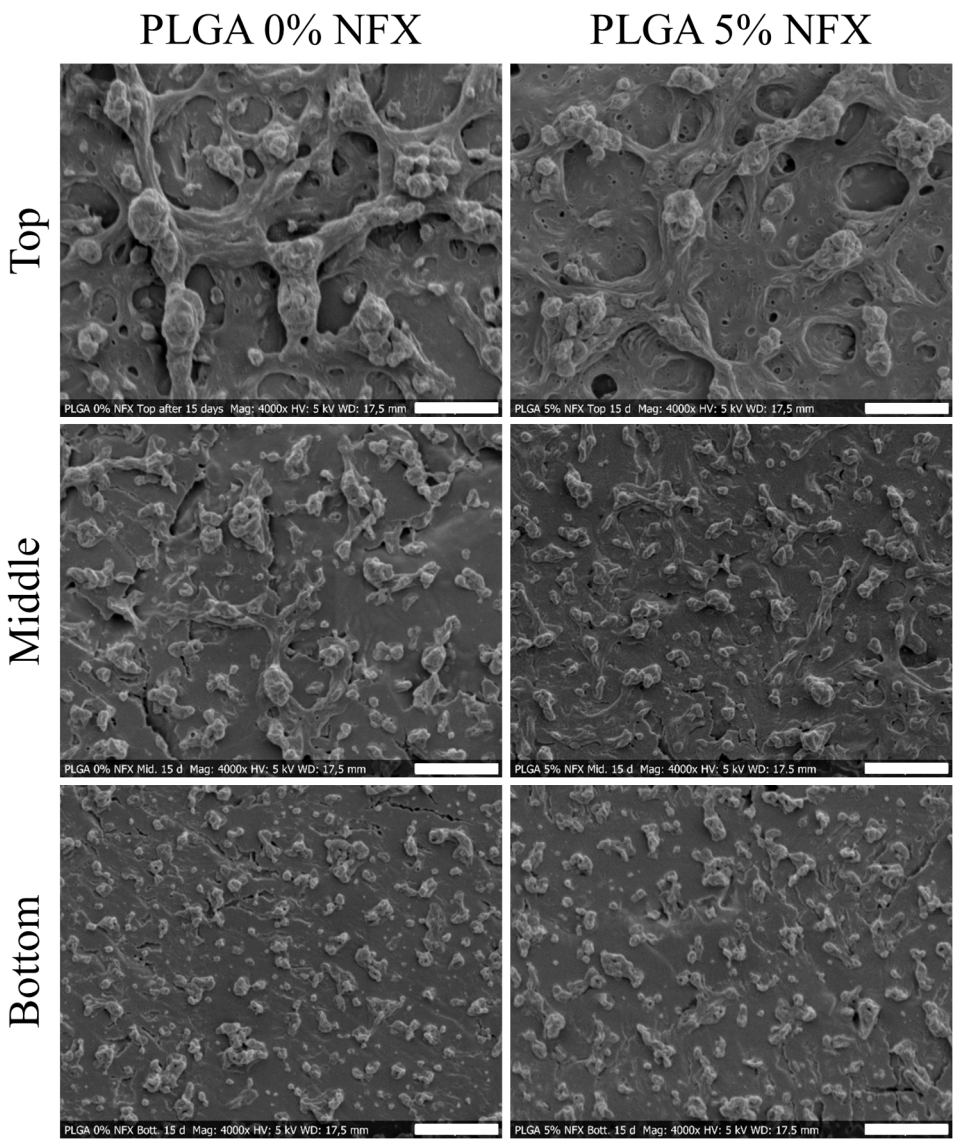

Fig. 4. SEM micrographs of the unloaded nanocoatings (PLGA 0% NFX) and norfloxacin loaded nanocoatings (PLGA 5% NFX) after 15 days incubation at 37 °C in PBS (pH 7.4). The titanium discs were fixed at three positions inside the electrostatic particle collector (i.e. Top, Middle and Bottom). Scale bars represent 5 μm.

antibacterial activity, yet without the need of higher norfloxacin loading.

3.3. Morphology and surface properties

SEM micrographs of the Ti-discs (Fig. 3) were similar to those of the preliminary experiments in terms of particle size distribution, morphology and surface structure which confirmed the reproducibility of this coating technique [10]. All produced particles were spherical and had a smooth surface. At the top part of the collector, the Ti-discs were covered with multilayered nanocoatings consisting of single particles and nanostructured microaggregates (Fig. 3 Top). On the contrary, the Ti-discs at the bottom part were coated with a monolayer of single particles with no obvious presence of any aggregates (Fig. 3 Bottom). Particle size analysis of the SEM micrographs revealed an average particle size around 600 nm for the nanocoatings at the top part of the collector with relatively broad particle size distribution and significant number of microparticles (Supplementary materials, Fig. S4 Top). Interestingly, at the bottom part a substantial decrease in the particle size was observed (around 400 nm) with a more homogenous size distribution wherein most of the particles were in the submicron range (Supplementary materials, Fig. S4 Bottom). These results can be explained based on the fact that bigger particles carry higher surface charge; therefore, they get captured easily by the electrostatic particle collector [11,13,37]. Thus, as the dried particles move downward, they will be fractionated along the electrostatic particle collector thereby having the smallest particles with the narrowest size distribution at the bottom part of the collector. It is noteworthy that SEM results revealed no significant difference between PLGA 0% NFX and PLGA 5% NFX in terms of morphology and particle size distribution.

SEM was also used to study the possible morphological changes the nanocoatings might experience after 15 days incubation in PBS (pH 7.4) at 37 °C. SEM micrographs showed that all examined nanocoatings (PLGA 0% NFX and PLGA 5% NFX) remained adhered to the surface of Ti-discs which was in agreement with previous studies [18]; however, substantial changes in the morphology and surface structure were observed (Fig. 4). Most particles were deformed and had corrugated surface. Significant number of aggregates was noticeable even in the Bottom level which was not the case before incubation in PBS. Interestingly, some particles lost their structure completely and were merged

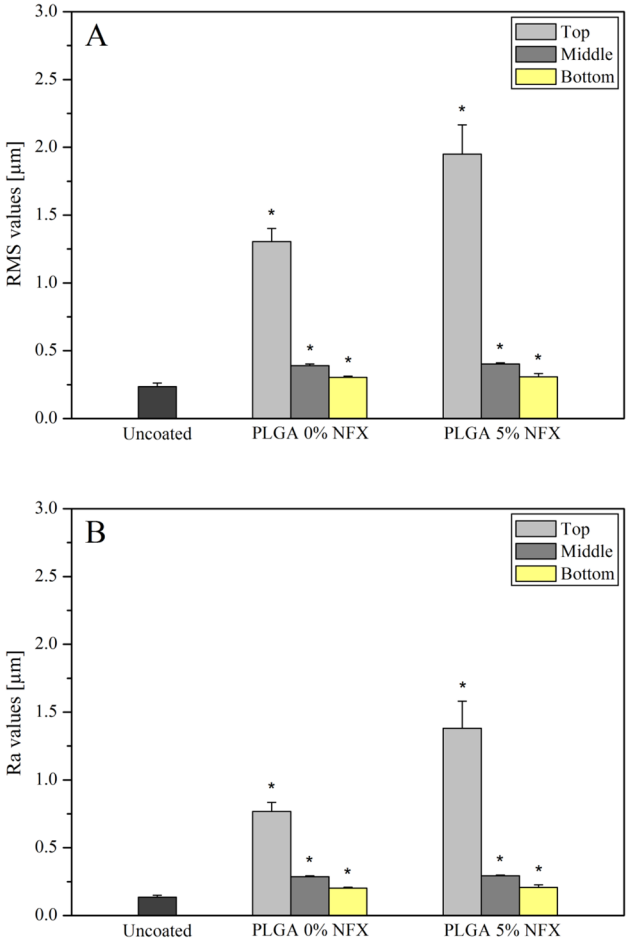

Fig. 5. Surface roughness RMS (A) and Ra (B) of unloaded nanocoatings (PLGA 0% NFX) and norfloxacin loaded nanocoatings (PLGA 5% NFX). The titanium discs were fixed at three different positions inside the electrostatic particle collector (i.e. Top, Middle and Bottom). Uncoated titanium discs were used as reference. The asterisk denotes probability values of $p < 0.05$ which were considered statistically significant.

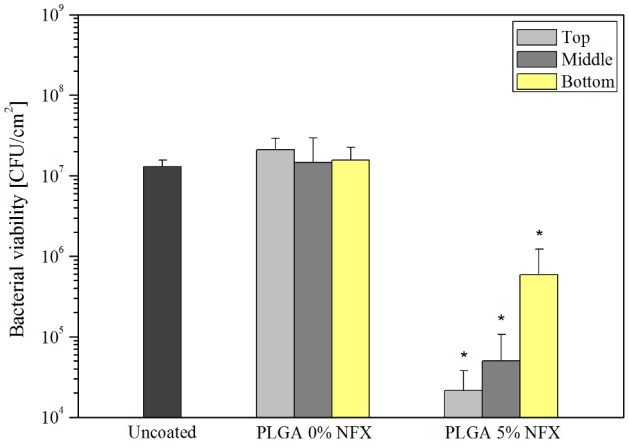

Fig. 6. Evaluation of *in vitro* antibacterial activity of unloaded nanocoatings (PLGA 0% NFX) and norfloxacin loaded nanocoatings (PLGA 5% NFX). The titanium discs were fixed at three positions inside the electrostatic particle collector (i.e. Top, Middle and Bottom). Uncoated titanium discs were used as negative control. *E. coli* viability is presented as colony-forming unit per cm^2 of the titanium discs. The asterisk denotes probability values of $p < 0.05$ which were considered statistically significant.

together forming sort of porous film. This phenomenon was obvious in all levels, especially Top and Middle where it appeared as a net-like structure.

Previous studies have related the causes of PLGA particle aggregation and deformation to the fact that PLGA polymer starts to degrade by means of hydrolysis upon contact with the incubation medium (i.e. PBS). As the degradation process continues, the molecular weight decreases causing a drop in the glass transition temperature (T_g). When the T_g reaches a critical value below the temperature of incubation medium (i.e. 37 °C), PLGA polymer will be in the rubbery state and become more flexible. Consequently, PLGA particles start to aggregate and lose their spherical shape [38–40].

Additionally, the surface roughness of the nanocoatings was studied due to its important role in the cellular interaction with the implant surface and significant influence on the early stages of biofilm formation [6,8]. The results were expressed as root mean square (RMS) and roughness average (Ra) (Fig. 5). According to RMS and Ra values, both PLGA 0% NFX and PLGA 5% NFX showed a substantial increase in the surface roughness when compared to the uncoated Ti-discs ($p < 0.05$). These values greatly varied depending on the Ti-disc position inside the particle collector. The high roughness values at the top position could be related to the significant presence of microparticles and the broad particle size distribution. On the other hand, a considerable decrease in the RMS and Ra values was noticed at the middle and bottom part of the collector where the nanocoatings were more homogenous and had a smaller particle size. These findings suggest a correlation between the surface roughness and the particle size distribution. Interestingly, a significant difference between the values of PLGA 5% NFX Top and PLGA 0% NFX Top was observed ($p < 0.05$), indicating a possible relationship between the norfloxacin loading and the surface roughness of the nanocoatings.

3.4. Bacterial viability assay

Since agar diffusion test delivers mainly qualitative results [41], further *in vitro* bacterial investigations with the chosen nanocoatings (i.e. PLGA 5% NFX) were performed to get a precise evidence about their antibacterial activity. Therefore, the coated Ti-discs were incubated with *E. coli* suspension for 24 h and the adhered bacteria were thereafter quantified. PLGA 5% NFX caused a significant reduction in the bacterial viability in comparison to uncoated Ti-discs ($p < 0.05$). This reduction ranged between 99.83% (PLGA 5% NFX Top) and 95.42% (PLGA 5% NFX Bottom) (Fig. 6). PLGA 0% NFX did not exhibit any antibacterial activity nor supported bacterial adhesion since no considerable difference in the bacterial viability was noticed ($p > 0.05$) regardless of the Ti-disc position inside the particle collector. Hence, the antibacterial activity of PLGA 5% NFX can be attributed only to norfloxacin without any pronounced influence from the surface properties of the nanocoatings.

3.5. In vitro biocompatibility

The nanocoating biocompatibility was evaluated using L929 cells as a standard sensitive cell line for assessing *in vitro* cytotoxicity [42,43]. The cell growth on the surface of the Ti-discs was qualitatively and quantitatively studied over a period of 4 days.

First, the cells were visualized using fluorescence microscopy wherein the cell nucleus was counterstained with DAPI (Fig. 7). All examined samples (i.e. uncoated Ti-discs, PLGA 0% NFX and PLGA 5% NFX) exhibited comparable results. A substantial increase in the cell number could be observed in the fluorescence micrographs of the Ti-discs after 96 h incubation where the cells covered most of the surface. These results gave only a visual confirmation of the nanocoating biocompatibility. Therefore, a quantitative assessment was still needed to understand the influence of norfloxacin loading and Ti-disc position inside the particle collector on the nanocoating cytotoxicity.

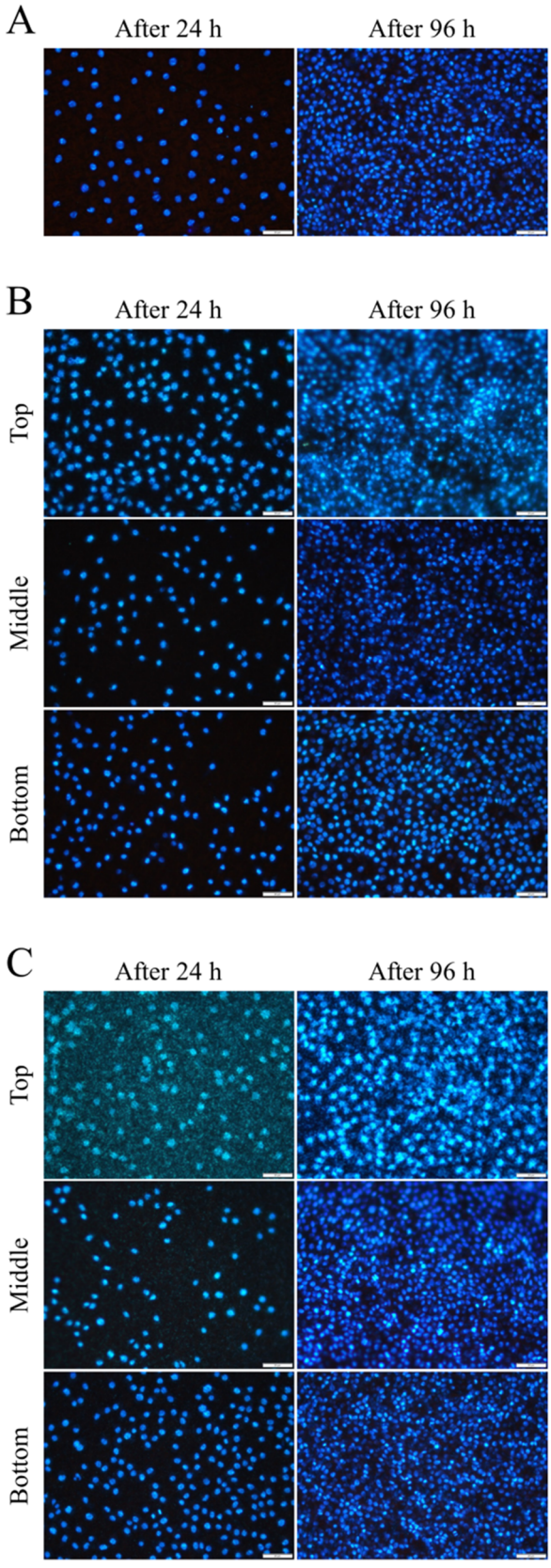

Fig. 7. Fluorescence microscope images showing the growth of L929 cells on titanium discs after 24 h and 96 h incubation. A: uncoated discs, B: unloaded nanocoatings (PLGA 0% NFX), C: norfloxacin loaded nanocoatings (PLGA 5% NFX). The titanium discs were fixed at three positions inside the electrostatic particle collector (i.e. Top, Middle and Bottom). The cell nucleus (blue) was counterstained with DAPI. Scale bars represent 80 μm.

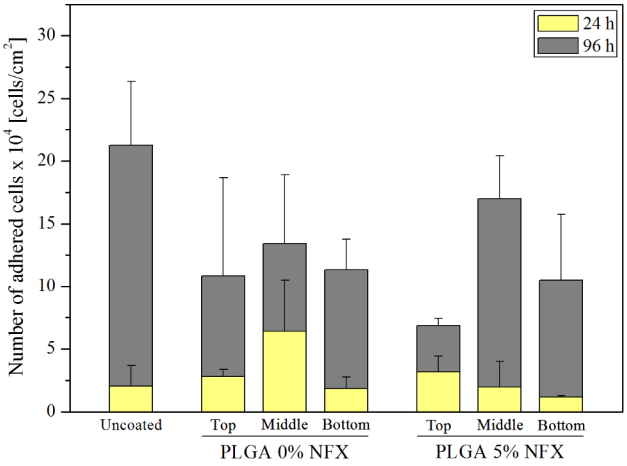

Fig. 8. Number of cells adhered to the surface of the titanium discs after 24 h and 96 h incubation. Cell counting assay were performed using mouse fibroblasts (L929) where unloaded nanocoatings (i.e. PLGA 0% NFX) and norfloxacin loaded nanocoatings (PLGA 5% NFX) were tested. The titanium discs were fixed at three positions inside the electrostatic particle collector (i.e. Top, Middle and Bottom). Uncoated titanium discs were used as a negative control.

This was achieved by counting the number of cells adhered to the surface of Ti-discs after 24 h and 96 h incubation (Fig. 8). Cell counting results of all samples after 24 h incubation were remarkably close with no significant difference between the coated (PLGA 0% NFX and PLGA 5% NFX) and the uncoated Ti-discs ($p > 0.05$). As seen previously in the visualization experiments, the cell number increased considerably in all samples after 96 h incubation. The number of the cells adhered to the coated (all nanocoatings except PLGA 5% NFX Top) and the uncoated Ti-discs was comparable ($p > 0.05$) indicating a good biocompatibility of the nanocoatings which did not exert any serious toxic effect that might influence the cell proliferation. These results are in agreement with previous studies which reported the use of PLGA nanoparticles as biocompatible coatings for medical implants [25,43].

The only noticed cytotoxicity was with PLGA 5% NFX Top which resulted in suboptimal cell growth when compared to the uncoated Ti-discs ($p = 0.04$). This could be related to the high norfloxacin content in the nanocoating which might have adversely affected the cell proliferation. These findings indicated the importance of adjusting the drug content in the nanocoatings to achieve a balance between the desired therapeutic effect and the cytotoxicity.

4. Conclusions

The outcomes of this study have proven the feasibility of preparing biocompatible nanocoatings with potent antibacterial activity using nano spray drying. This newly developed technique has the advantage of achieving nanoparticle production and implant coating in a single-step. Moreover, versatile nanocoatings can be produced by varying several process parameters, especially the implant position inside the electrostatic particle collector. This parameter has shown considerable influence on the nanocoating properties like morphology, surface roughness and drug content. Interestingly, all nanocoatings exhibited similar drug release profiles regardless of their position inside the particle collector. Due to the controlled release properties of PLGA, an advantageous biphasic release profile could be achieved. This will offer an initial burst release serving as an effective loading dose followed by constant sustained release providing a valuable maintenance dose. Such innovative nanocoatings will help developing nanostructured dental implants, thereby omitting the need of systemic administration of antibiotics and achieving an optimal implant-tissue interaction.

Conflict of interest

The authors declared that there is no conflict of interest.

Acknowledgments

We would like to express our gratitude towards Prof. Dr. Dr. Alexander W. Eckert (Department of Oral and Maxillofacial Plastic Surgery, Martin-Luther-University Halle-Wittenberg, Germany) for the fruitful scientific discussions. We would also like to thank Lili Duse, Eduard Preis, Benjamin Seitz and Eva Mohr (Department of Pharmaceutics and Biopharmaceutics, University of Marburg, Germany) for their help and technical assistance. Elias Baghdan is thankful to Brot für die Welt organization for the scholarship grant.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejpb.2019.03.003.

References

[1] F. Rodríguez Sánchez, C. Rodríguez Andrés, I. Arteagoitia, Which antibiotic regimen prevents implant failure or infection after dental implant surgery? A systematic review and meta-analysis, J. Cranio-Maxillofac. Surg. 46 (2018) 722–736.
[2] P. Kamolratanakul, P. Jansisyanont, A review of antibiotic prophylaxis protocols in oral and maxillofacial surgery, J. Oral Maxillofac. Surg. Med. Pathol. 30 (2018) 395–404.
[3] A.D. Pye, D.E.A. Lockhart, M.P. Dawson, C.A. Murray, A.J. Smith, A review of dental implants and infection, J. Hosp. Infect. 72 (2009) 104–110.
[4] B. Klinge, T. Flemming, J. Cosyn, H. De Bruyn, B.M. Eisner, M. Hultin, F. Isidor, N.P. Lang, B. Lund, J. Meyle, A. Mombelli, J.M. Navarro, B. Pjetursson, S. Renvert, H. Schliephake, The patient undergoing implant therapy. Summary and consensus statements. The 4th EAO consensus conference 2015, Clin. Oral Implants Res. 26 (2015) 64–67.
[5] Y.J.E. Spaey, R.M.A. Bettens, M.Y. Mommaerts, J. Adriaens, H.W. Van Landuyt, J.V.S. Abeloos, C.A.S. De Clercq, P.R.B. Lamoral, L.F. Neyt, A prospective study on infectious complications in orthognathic surgery, J. Cranio-Maxillofac. Surg. 33 (2005) 24–29.
[6] S. Ferraris, S. Spriano, Antibacterial titanium surfaces for medical implants, Mater. Sci. Eng., C 61 (2016) 965–978.
[7] J. Raphel, M. Holodniy, S.B. Goodman, S.C. Heilshorn, Multifunctional coatings to simultaneously promote osseointegration and prevent infection of orthopaedic implants, Biomaterials 84 (2016) 301–314.
[8] G. Mendonça, D.B.S. Mendonça, F.J.L. Aragão, L.F. Cooper, Advancing dental implant surface technology – From micron- to nanotopography, Biomaterials 29 (2008) 3822–3835.
[9] F. Rupp, L. Liang, J. Geis-Gerstorfer, L. Scheideler, F. Hüttig, Surface characteristics of dental implants: a review, Dent. Mater. 34 (2018) 40–57.
[10] E. Baghdan, S.R. Pinnapireddy, H. Vögeling, J. Schäfer, A.W. Eckert, U. Bakowsky, Nano spray drying: A novel technique to prepare well-defined surface coatings for medical implants, J. Drug Delivery Sci. Technol. 48 (2018) 145–151.
[11] X. Li, N. Anton, C. Arpagaus, F. Belleteix, T.F. Vandamme, Nanoparticles by spray drying using innovative new technology: the Büchi nano spray dryer B-90, J. Control. Release 147 (2010) 304–310.
[12] N. Schafroth, C. Arpagaus, U.Y. Jadhav, S. Makne, D. Douroumis, Nano and microparticle engineering of water insoluble drugs using a novel spray-drying process, Colloids Surf. B: Biointerfaces 90 (2012) 8–15.
[13] C. Arpagaus, P. John, A. Collenberg, D. Rütti, Nanocapsules formation by nano spray drying, in: Seid Mahdi Jafari (Ed.), Nanoencapsulation Technologies for the Food and Nutraceutical Industries, 10 Academic Press, 2017, pp. 346–401.
[14] S.H. Lee, D. Heng, W.K. Ng, H.-K. Chan, R.B.H. Tan, Nano spray drying: a novel method for preparing protein nanoparticles for protein therapy, Int. J. Pharm. 403 (2011) 192–200.
[15] D. Heng, S.H. Lee, W.K. Ng, R.B.H. Tan, The nano spray dryer B-90, Expert Opin. Drug Delivery 8 (2011) 965–972.
[16] C. Arpagaus, A. Collenberg, D. Rütti, E. Assadpour, S.M. Jafari, Nano spray drying for encapsulation of pharmaceuticals, Int. J. Pharm. 546 (2018) 194–214.
[17] F. Danhier, E. Ansorena, J.M. Silva, R. Coco, A. Le Breton, V. Préat, PLGA-based nanoparticles: an overview of biomedical applications, J. Control. Release 161 (2012) 505–522.
[18] E. Dayyoub, C. Hobler, P. Nonnweiler, M. Keusgen, U. Bakowsky, Nanostructured medical device coatings based on self-assembled poly(lactic-co-glycolic acid) nanoparticles, Mater. Sci. Eng., C 33 (2013) 3018–3024.
[19] H. Vögeling, S.R. Pinnapireddy, B. Seitz, U. Bakowsky, Indocyanine green loaded PLGA film coated coronary stents for photo-triggered in situ biofilm eradication, Colloid Interface Sci. Commun. 27 (2018) 35–39.
[20] M. Raschpichler, M.R. Agel, S.R. Pinnapireddy, L. Duse, E. Baghdan, J. Schäfer, U. Bakowsky, In situ intravenous photodynamic therapy for the systemic eradication of blood stream infections, Photochem. Photobiol. Sci. (2019).
[21] E.J.C. Goldstein, Norfloxacin, a fluoroquinolone antibacterial agent: classification, mechanism of action, and in vitro activity, Am. J. Med. 82 (1987) 3–17.
[22] M. Balouiri, M. Sadiki, S.K. Ibnsouda, Methods for in vitro evaluating antimicrobial activity: a review, J. Pharm. Anal. 6 (2016) 71–79.
[23] B.M. Al Meslmani, G.F. Mahmoud, T. Leichtweiß, B. Strehlow, F.O. Sommer, M.D. Lohoff, U. Bakowsky, Covalent immobilization of lysozyme onto woven and knitted crimped polyethylene terephthalate grafts to minimize the adhesion of broad spectrum pathogens, Mater. Sci. Eng., C 58 (2016) 78–87.
[24] S.R. Pinnapireddy, L. Duse, B. Strehlow, J. Schäfer, U. Bakowsky, Composite liposome-PEI/nucleic acid lipopolyplexes for safe and efficient gene delivery and gene knockdown, Colloids Surf. B: Biointerfaces 158 (2017) 93–101.
[25] B.M. Al Meslmani, G.F. Mahmoud, U. Bakowsky, Development of expanded polytetrafluoroethylene cardiovascular graft platform based on immobilization of poly lactic-co-glycolic acid nanoparticles using a wet chemical modification technique, Int. J. Pharm. 529 (2017) 238–244.
[26] P.C. Appelbaum, P.A. Hunter, The fluoroquinolone antibacterials: past, present and future perspectives, Int. J. Antimicrob. Agents 16 (2000) 5–15.
[27] E. Dayyoub, M. Frant, S.R. Pinnapireddy, K. Liefeith, U. Bakowsky, Antibacterial and anti-encrustation biodegradable polymer coating for urinary catheter, Int. J. Pharm. 531 (2017) 205–214.
[28] B.S. Seitz, N. Plenagl, M. Raschpichler, H. Vögeling, M. Wojcik, S.R. Pinnapireddy, J. Brüßler, U. Bakowsky, Nanoparticles and liposomes for the surface modification of implants: a comparative study of spraying and dipping techniques, Phys. Status Solidi (a) 215 (2018) 1700847.
[29] H. Vögeling, L. Duse, B.S. Seitz, N. Plenagl, M. Wojcik, S.R. Pinnapireddy, U. Bakowsky, Multilayer bacteriostatic coating for surface modified titanium implants, Phys. Status Solidi (a) 215 (2018) 1700844.
[30] K. Schmid, C. Arpagaus, W. Friess, Evaluation of the nano spray dryer B-90 for pharmaceutical applications, Pharm. Dev. Technol. 16 (2011) 287–294.
[31] M. Beck-Broichsitter, C. Schweiger, T. Schmehl, T. Gessler, W. Seeger, T. Kissel, Characterization of novel spray-dried polymeric particles for controlled pulmonary drug delivery, J. Control. Release 158 (2012) 329–335.
[32] M. Beck-Broichsitter, I.E. Paulus, A. Greiner, T. Kissel, Modified vibrating-mesh nozzles for advanced spray-drying applications, Eur. J. Pharm. Biopharm. 92 (2015) 96–101.
[33] A. Kumari, S.K. Yadav, S.C. Yadav, Biodegradable polymeric nanoparticles based drug delivery systems, Colloids Surf. B: Biointerfaces 75 (2010) 1–18.
[34] H. Gasmi, F. Siepmann, M.C. Hamoudi, F. Danede, J. Verin, J.F. Willart, J. Siepmann, Towards a better understanding of the different release phases from PLGA microparticles: dexamethasone-loaded systems, Int. J. Pharm. 514 (2016) 189–199.
[35] S. Fredenberg, M. Wahlgren, M. Reslow, A. Axelsson, The mechanisms of drug release in poly(lactic-co-glycolic acid)-based drug delivery systems—a review, Int. J. Pharm. 415 (2011) 34–52.
[36] C. Wischke, S.P. Schwendeman, Principles of encapsulating hydrophobic drugs in PLA/PLGA microparticles, Int. J. Pharm. 364 (2008) 298–327.
[37] R.C. Suryaprakash, F.P. Lohmann, M. Wagner, B. Abel, A. Varga, Spray drying as a novel and scalable fabrication method for nanostructured CsH_2PO_4 Pt-thin-film composite electrodes for solid acid fuel cells, RSC Adv. 4 (2014) 60429–60436.
[38] N. Badri Viswanathan, S.S. Patil, J.K. Pandil, A.K. Lele, M.G. Kulkarni, R.A. Mashelkar, Morphological changes in degrading PLGA and P(DL)LA microspheres: implications for the design of controlled release systems, J. Microencapsul. 18 (2001) 783–800.
[39] W. Friess, M. Schlapp, Release mechanisms from gentamicin loaded poly(lactic-co-glycolic acid) (PLGA) microparticles, J. Pharm. Sci. 91 (2002) 845–855.
[40] J. Panyam, M.M. Dali, S.K. Sahoo, W. Ma, S.S. Chakravarthi, G.L. Amidon, R.J. Levy, V. Labhasetwar, Polymer degradation and in vitro release of a model protein from poly(d, l-lactide-co-glycolide) nano- and microparticles, J. Control. Release 92 (2003) 173–187.
[41] L.B. Reller, M. Weinstein, J.H. Jorgensen, M.J. Ferraro, Antimicrobial susceptibility testing: a review of general principles and contemporary practices, Clin. Infect. Dis. 49 (2009) 1749–1755.
[42] ISO 10993-5:2009(en) Biological Evaluation of Medical Devices—Part 5: Tests for In Vitro Cytotoxicity, 2009.
[43] B.M. Al Meslmani, G.F. Mahmoud, U. Bakowsky, Immobilization and characterization of PLGA nanoparticles on polyethylene terephthalate cardiovascular grafts for local drug therapy of associated graft complications, J. Drug Delivery Sci. Technol. 47 (2018) 144–150.