Functionalised nanoscale coatings using Layer-by-Layer assembly for imparting antibacterial properties to polylactide-co-glycolide surfaces

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Abstract

In order to achieve high local biological activity and reduce the risk of side effects of antibiotics in the treatment of periodontal and bone infections, a localised and temporally controlled delivery system is desirable. The aim of this research was to develop a functionalised and resorbable surface to contact soft tissues to improve the antibacterial behaviour during the first week after its implantation in the treatment of periodontal and bone infections. Solvent-cast poly(D,L-lactide-co-glycolide acid) (PLGA) films were aminolysed and then modified by Layer-by-Layer technique to obtain a nano-layered coating using poly(sodium4-styrenesulfonate) (PSS) and poly(allylamine hydrochloride) (PAH) as polyelectrolytes. The water-soluble antibiotic, metronidazole (MET), was incorporated from the ninth layer. Infrared spectroscopy showed that the PSS and PAH absorption bands increased with the layer number. The contact angle values had a regular alternate behaviour from the ninth layer. X-Ray Photoelectron Spectroscopy evidenced two distinct peaks, N_{1s} and S_{2p}, indicating PAH and PSS had been introduced. Atomic Force Microscopy showed the presence of polyelectrolytes on the surface with a measured roughness about 10 nm after 20 layers deposition. The drug release was monitored by Ultraviolet-visible spectroscopy showing 80% loaded-drug delivery in 14 days. Finally, the biocompatibility was evaluated in vitro with L929 mouse fibroblasts and the antibacterial properties were demonstrated successfully against the keystone periodontal bacteria Porphyromonas gingivalis, which has an influence on implant failure, without compromising in vitro biocompatibility. In this study, PLGA was successfully modified to obtain a localised and temporally controlled drug delivery system, demonstrating the potential value of LbL as a coating technology for the manufacture of medical devices with advanced functional properties.

Keywords: antibacterial**;** Layer-by-Layer; metronidazole; periodontitis; PLGA.

1. Introduction

The use of antimicrobial biomaterials is becoming increasingly important in medicine and dentistry, in which the elimination of bacteria and device-associated biofilms is essential for effective treatment [1, 2]. For instance, the conventional treatment of periodontitis by scaling and root planing is advantageously accompanied by the adjuvant administration of antibiotics, which can be applied by systemic or local administration [3]. Compared to systemic drug delivery, local administration in periodontology is considered to be more effective, since the pathogen-specific drug can be placed directly in the periodontal pocket achieving effective concentrations for a sufficiently long period of time. In addition, the risk of undesired side effects caused by high systemic doses or resistance development can be reduced [3, 4]. It is therefore beneficial to use local delivery systems that control the release of their agents and guarantee lasting drug concentrations in the pocket in spite of high sulcular fluid rates [3]. Among antibiotics, metronidazole (MET) is highly effective for the management of anaerobic infections, such as intra-abdominal and gynecologic infections, septicemia, endocarditis, bone and joint infections, central nervous system infections, respiratory tract infections, skin and skin-structure infections. Moreover, MET is largely used for treating infections by anaerobic bacteria associated with periodontal diseases due to the low minimum inhibitory concentration it requires [5]. For treatment of mixed aerobic and anaerobic infections, metronidazole should be used in combination with other antibacterial agents that are appropriate for the treatment of the aerobic infection, because MET is ineffective against aerobic bacteria [6, 7].

For treating periodontitis, a number of resorbable drug delivery systems were developed during recent decades, such as drug loaded hydroxypropyl cellulose films [8], or drug carrying gels such as Elyzol® (Dumex GmbH, Bad Vilbel, Germany) dental gel, based on melted glycerol mono-oleate [9, 10]. However, for these systems, the periodontal milieu often poses the major problem that the required period of drug exposure (7–10 days) cannot be achieved [11]. Also in the field of periodontal surgery, as in the transplantation of a mucous membrane [12], resorbability of the

scaffold material is important to avoid inflammatory effects and surgical removal. Therefore, the use of films or membranes represents a promising approach in periodontal treatment to promote tissue regeneration by avoiding migration of epithelial cells into the periodontal pocket and incorporating antibiotics to inhibit the related bacteria growth [13]. Different barrier membranes with a graded functionalised structure are proposed in literature in order to obtain tuned mechanical and degradation characteristics [14, 15]. Bottino et al. [16] have proposed multilayered membranes consisting of three layers: an inner layer based on poly(DL-lactide-co-ε-caprolactone) (PLCL) and two outer functionalised layers, based on ternary polymeric blends (gelatin, PLCL and PLA) in contact with the surrounding tissues. The outer layer, in contact with bone, was loaded with nano-hydroxyapatite to enhance bone regeneration; while the other layer, in contact with epithelial tissue, was functionalised with metronidazole, incorporated in the layer bulk. Interestingly, the direct incorporation of antibiotics to these membranes has given promising results in terms of inhibition of bacteria growth [7, 17, 18]. However, although the antibacterial effects of these drugs are well known, recent findings have warned of the potentially toxic effects of these highly concentrated antibiotic drugs on dental pulp stem cells and dental pulp fibroblasts [19, 20]. Furthermore, the direct drug release from polymeric substrates undergoes bulk degradation, modifying dramatically the physico-chemical properties of the device, mainly in terms of mechanical properties [21].

Therefore, the purpose of this research was to propose an innovative localised and controlled delivery system in the treatment of dental and periodontal infections in order to: (1) reduce the released antimicrobials amount to the gum surface, preventing both bacteria resistance and drug-related systemic side effects, and (2) preserve the physico-chemical properties of the barrier membrane without incorporating the antibiotic into the bulk of the material. Specifically, in this study, a periodontal biodegradable membrane was coated by Layer-by-Layer (LbL) technique to obtain discrete nanoscale layers to incorporate and to control the release of the antibiotic drug with minimal interaction with the biomaterial substrate.

LbL assembly, firstly introduced by Decher [22], is an alternative surface modification technique to Langmuir Blodgett deposition and Self-Assembled Monolayers (SAMs). LbL electrostatic assembly of charged polymers has been widely used as a versatile technique for the formation of multilayered thin films with tailored structure and composition in a wide range of electrical, magnetic, biological and optical applications [23]. LbL assembly is based on the alternating exposure of a charged substrate to solutions of positively and negatively charged polyelectrolytes. A rinsing step is included between the two previously described adsorption processes, to remove excess as well as to prevent cross-contamination of the polyelectrolyte solutions [24]. The LbL technique allows fine control of the coating properties and the obtainment of homogeneous multi-layered structures. It is also applicable to substrates of any shape, it is environmentally-friendly and it allows room temperature processing and low-cost manufacturing [25]. Polyelectrolyte multilayered films have therefore been considered for biomedical application such as capsules for drug delivery [26, 27], immunosensing [28], regenerative neurobiology [29], antibacterial and anti fungal protection [30, 31]. Despite its potential, LbL has not yet been translated for routine application in the manufacture of medical devices, and further research is required to demonstrate its practical value. In this study, nano-structured multilayered coatings were deposited on resorbable dense membranes based on a biocompatible and biodegradable synthetic polymer (poly(D,L-lactide-co-glycolide acid), PLGA) using the LbL method. The antibiotic drug was incorporated into the polyelectrolyte layers, based on poly(sodium4-styrenesulfonate) (PSS) and poly(allylamine hydrochloride) (PAH), after optimisation of the process to obtain appropriate drug release kinetics. The nano-coating was characterised by morphological, physico-chemical and biological analyses in order to evaluate the multilayered correct deposition, and the biological and antibacterial behaviour.

2. Material and Methods

2.1 Materials

Poly(D,L-lactide-co-glycolide) (PLGA; lactide:glicolide (75:25), Mw = 66,000-107,000), Poly(sodium4-styrenesulfonate) (PSS average Mw ~ 70,000; powder), Metronidazole (MET; Mw: 171.15), and Ethylenediamine (ED) were purchased from Sigma-Aldrich, UK. Poly(allylamine hydrochloride) (PAH) was purchased from Alfa Aesar, UK. As solvent, acetone (99.8%) was purchased from Fisher Scientific, UK. All materials and chemicals were used as received without any additional purification.

2.2 PLGA film preparation and functionalisation

The compact film was prepared by a solvent casting process. Briefly, PLGA was dissolved in 20 mL of acetone (3 % w/v) at room temperature by vigorous stirring, then cast on Petri dishes (φ= 8 cm, Duroplan) and dried under fume hood for 48 hours.

Aminolysis. PLGA compact films were aminolysed by immersion in 0.05 M ED solution and allowed to react at 20 °C for 15 minutes to obtain a positive charge on the surface by -NH_2-grafting. Treated films were washed three times with ice cold water, followed by soaking for a further hour in fresh water on ice, dried in a oven at 37 °C for 12 h, and, finally, stored in a desiccator over silica gel until use.

Layer-by-Layer. The deposition of PSS/PAH multilayered films (shown in Figure 1) was carried out at room temperature. PSS and PAH solutions were prepared as 5 mg/mL solutions in 0.1 M NaCl and the pH was adjusted to 5.5. Prior to LbL deposition all the polymer solutions were filtered through 0.45 µm Polyvinylidene fluoride (PVDF) membrane (Fisher). The ζ-potentials of the polyelectrolytes solutions was measured by laser Doppler electrophoresis (Zetasizer Nano, Malvern instrument, USA). Aminolysed PLGA films (size 5 × 5 cm with a thickness ~ 180 µm) were first immersed in PSS solution (5 mL) and left in incubation for 15 min. Then, they were rinsed with 0.1 M NaCl water solution at pH 5.5 for 5 min. The, PLGA films were finally immersed in PAH solution (5 mL) and left in incubation for 15 min, following the same rinsing procedure. The

procedure was repeated to obtain 20 layers (10 bilayers of PSS/PAH). The antibiotic drug, metronidazole, was incorporated into the nano-layers from the ninth layer following the same process described above, dissolving 0.1 % w/v MET in PSS and PAH solutions. At the end of the LbL procedure, the samples were rinsed with deionised water at pH 5.5 for 10 min. The films were dried in an oven at 37 °C and then stored at 3 °C.

2.3 Physico-chemical characterisation

Surface composition was determined by X-Ray Photoelectron Spectroscopy (XPS) on Theta Probe (Thermo Scientific, East Grinstead, UK), which uses a micro- focused AlKa X-ray source (1486.6 eV), operated with a 400 µm spot size (100 W power). Survey spectra were collected at a pass energy of 200 eV, a step size of 1 eV and a dwell time of 50 ms, with the spectrometer operated in standard (not angle-resolved) lens mode. Three points were analysed on each sample surface as received. Charge neutralisation was used throughout the analysis. High resolution regional spectra were collected using a pass energy of 40 eV, a step size of 0.1 eV and a dwell time of 200 ms. High resolution spectra envelopes were obtained by curve fitting synthetic peak components using the software CasaXPS.

Contact angle analysis in static conditions was carried out using a CAM 200 KSV instrument, using Drop Shape Analysis System DSA 10 software. For all analyses, bidistillate water drops (6 µL) were used. At least 9 measurements for samples were averaged.

Chemical analysis of functionalised coated films was performed by ATR-FTIR over a wavenumber range of 4000–550 cm^{-1} using a Nicolet iS10 spectrometer (resolution 4 cm^{-1}; 32 scans).

Atomic Force Microscopy measurements were acquired on a Multimode Nanoscope (V) (Veeco Instruments, Santa Barbara, CA) scanning force microscopy operating with the conventional Amplitude Modulation (AM) mode. Samples were attached to AFM magnetic discs using double sided tape, and the scanning was performed in air. The images were scanned using cantilevers with a normal spring constant of 26 N/m. Scanning was performed at a speed of 1 Hz, with a scan size of 1µm and a resolution of 512 points per line was selected for a higher resolution.

2.4 In vitro drug release

Functionalised drug-loaded films (size 1.5 × 1.5 cm with a thickness ~ 180 µm) were immersed in glass vials containing 5 mL of Phosphate Buffered Solution (PBS; pH 7.4, Sigma-Aldrich). The vials were sealed to minimise the changes in the initial pH and incubated at 37 °C without stirring. The blank control (coated films without MET incorporation) were analysed under the same experimental conditions. The medium was withdrawn at different time point for the measurement and replaced with fresh buffer. UV-Vis spectrophotometer (Lambda 2S Perkin Elmer) was used to determine the drug content using maximal absorption peaks for MET at 320 nm. Six replicates were measured, and the results were averaged with standard deviation. The initial content of the antibiotic drug was measured by UV-Vis after dissolving the coated PLGA film in acetone.

2.5 Cell tests

2.5.1 Cell seeding

In vitro cell tests were performed on pure PLGA films and after LbL process with and without the incorporation of the antibiotic drug. Before cell seeding, material samples (φ= 1.2 cm) were sterilised in 1-isopropanol (Sigma-Aldrich) under UV light for 30 min in 24-well plates, washed several times in PBS, and incubated with 0.5 ml fetal calf serum (FCS) for 30 minutes. The FCS was then discarded. L929 fibroblast cells (ATCC, Rockville, MD) were grown in a controlled atmosphere (5 % CO_2; T= 37 °C) in Iscove's modified Dulbecco's medium (DMEM) supplemented with 10 % fetal calf serum (Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich), penicillin (100 U/mL), and streptomycin (100 µg/mL; Sigma-Aldrich) and 0.1 mM nonessential amino acids (NEAA, Lonza, UK) for 3 weeks. Cells from up to three passages were used for all experiments. A total of 100,000 cells/cm^2 were seeded onto the film specimens in 1 ml DMEM and allowed to adhere and proliferate up to 7 days.

2.5.2 PrestoBlue Colorimetric Assay

After culturing cells for 1, 4 and 7 days, the medium was removed and the sample were transferred to new multiwell plates; 10 % PrestoBlue solution (5 mg/mL in DMEM; Fisher Scientific) was

added to the cell monolayers; the multiwell plates were incubated at 37 °C for an additional 1 h. After discarding the supernatants, the dark blue solution was removed (0.2 mL) and quantified spectrophotometrically at 560 nm (Leica DM2500). The results are reported as fluorescent unit. The mean and the standard deviations were obtained from five different experiments.

2.6 In vitro bacterial assays on pure and coated PLGA films

The periodontopathogen Porphyromonas gingivalis W50 was maintained in Fastidious Anaerobe Agar (FA; Lab M) containing 5 % (v/v) oxalated Horse blood (Oxoid) at 37 °C under anaerobic conditions (10 % CO_2, 10 % H_2 and 80 % N_2) at 37 °C in an anaerobic chamber (Don-Whitley, minimacs station). For agar diffusion assays bacteria were grown in liquid culture in brain–heart infusion broth (BHI; Difco Laboratories) supplemented with 0.5 % (w/v) yeast extract, haemin (5 μg/mL), vitamin K (0.5 μg/ml) and cysteine (0.1 % w/v) anaerobically. Firstly, sterile 1.6 cm discs (n= 3) of all experimental materials (pure PLGA films, LbL coated films without and with MET incorporation) were placed onto FA Top-Agar supplemented with 5 % Horse Serum (Oxoid) including the same additions (Yeast Extract, Cysteine, haemin and vitamin K). The liquid grown bacteria (approx. 10^8 bacteria) were then inoculated into molten FA Agar supplemented with 5% Horse Serum (same as above) and incubated for five days before zones of inhibition were measured.

2.7 Statistical analysis

Experiments were run at least in triplicate for each sample. All data were expressed as mean ± SD. Statistical analysis was determined by using Analyse-it v2.22 software. The statistical differences between groups were calculated using Kruskal-Wallis One Way Analysis of Variance on Ranks (ANOVA). Statistical significance was declared at $p< 0.05$.

3. Results

3.1 Physico-chemical characterisation of the functionalised nanoscale coating

The aminolysis treatment on the PLGA film surface was monitored by XPS, evaluating the nitrogen (N) content related to the formation of primary/secondary amine groups (Figure 2a). A distinct N_{1s} peak at 399.5 eV in the spectrum of the treated sample indicates that PLGA surface has been successfully treated by aminolysis, as evidenced also by the increase of the nitrogen/carbon atomic ratio from 0 for the non-treated films to 0.36 for the treated films. Moreover, the deconvolution peak of C_{1s} can be further curve-fitted into its peak components that revealed C_{1s} binding energies for amines (~285.5 eV) and amides (~288.1 eV), which were not observed for pure PLGA, confirming the chemical treatment (data not shown) [32]. Bioactive multilayered coatings incorporating the antibiotic drug were deposited successively by LbL on the positive charged film surfaces and monitored by XPS, contact angle measurement, ATR-FTIR spectroscopy and AFM analysis.

XPS spectra showed a N_{1s} peak at 399.5 eV and S_{2p} peak at 168 eV, indicating PAH and PSS have been successfully introduced (Figure 2b-f). Furthermore, Figure 3 shows the surface sulphur/nitrogen atomic ratio (calculated by XPS survey spectra) as function of the number of layers. S/N ratio evidenced an alternating regular behaviour showing the PSS and PAH layer deposition. The wettability of the pure polyelectrolytes, aminolysed PLGA substrate and coated samples was evaluated by static contact angle analysis (Figure 4). The substrate was found to be hydrophilic, with a contact angle of 62 ± 3°, while PSS and PAH had a contact angle of 66° ± 2° and 78° ± 4° respectively. Moreover, the contact angle increased after the polyelectrolytes incorporation from 62 ± 3° to 79 ± 3°.

ATR-FTIR analysis was performed on aminolysed film and on each layer of the coated samples (Figure 5). The spectra showed the appearance of the typical absorption bands of PAH and PSS increasing the layer number. In detail: for PSS: stretching vibration of H_2O at 3700 - 3000 cm^{-1}; aromatic =C-H stretching vibrations at 3100 cm^{-1}; alkyl C-H stretching vibrations at 2920 cm^{-1};

aromatic C-H out of plane bending vibrations at 1810 and 1925 cm^{-1}; O-H bending vibrations of H_2O at 1640 cm^{-1}, aromatic -C=C- stretching vibrations at 1600, 1500, 1450 and 1410 cm^{-1}. $-SO_3^-$ asymmetric stretching vibrations at 1190 and 1130 cm^{-1} and $-SO_3^-$ symmetric stretching vibrations at 1040, and 1005 cm^{-1}. For PAH, it was observed: stretching vibration of N-H at 3360 cm^{-1}; alkyl C-H stretching vibrations at 2920 cm^{-1}; N-H asymmetric scissoring vibrations at 1580 cm^{-1}: N-H symmetric scissoring vibrations at 1490 cm^{-1}, and N-H asymmetric stretching vibrations at 1330 cm^{-1}. Moreover, the observation of the characteristic bands of MET at 3100 cm^{-1} (asymmetric stretching vibrations of $C_{14}H_2$ unit), and 2982 and 2956 cm^{-1} (symmetric stretching vibrations of $C_{14}H_2$ unit and symmetric stretching vibrations of CH_3, respectively) confirmed the incorporation of the drugs into nano-layered structure (layer 19 and 20 in Figure 5). However, no other MET characteristic bands are evidenced due to the overlap with the PSS and PAH bands.

The surface morphology of the multilayered coatings as a function of the layer number was investigated by AFM analysis in tapping mode (Figure 6). The surface of pure PLGA dense film was smooth with some irregularities (holes ca. 2-3 nm deep and 400-600 nm in diameter) due to the process of solvent casting, as shown in Figure 6a. After aminolysis treatment (Figure 6b), some inhomogeneities were observed. These took the form of raised features, with a height of ca. 1 nm. that exhibited dark contrast in phase images. Pure PLGA surface (6a) showed a homogeneous contrast in phase, suggesting a surface of uniform composition, but after the aminolysis treatment (6b), the phase image revealed different contrasts resulting from different components at the surface that were formed by the aminolysis process. The substrate coated with only two layers of PSS and PAH (Figure 6c) exhibited a somewhat different morphology than the aminolysed PLGA film (6b), suggestive of a less uniform coverage following the introduction of these first two layers. Samples with 20 layers showed the presence of polyelectrolytes on the sample surface (Figure 6d). The measured roughness was about tens of nanometers after 20 layers deposition. The MET incorporation did not show significant difference in the final coating morphology (Figure 6e).

3.2 In vitro drug release test

In vitro MET release, after immersion of the functionalised films in PBS for different times, was displayed in Figure 7. The initial content of the antibiotic drug was around 85 ± 25 (μg/film 1 cm^2), calculated by UV-Vis spectrophotometry, with an efficiency of drug incorporation approximately of 40-50%. The MET-loaded films showed a three phase (triphasic) release profile: an initial linear pattern with about 10 % of the drug released during the initial 12-24 h of incubation, followed by a sustained linear and controlled MET release (75-80 % release of the loaded-drug), before a linear zero-order release was observed after 14 days.

3.3 Cell tests

The biocompatibility of PLGA films before and after LbL coating (without/with MET incorporation) was studied, evaluating the adhesion and proliferation of L929 fibroblast cells, with particular attention to the effect of the antibiotic drug on the cell response. The characterisation of L929 fibroblast cells behaviour was based on PrestoBlue assay to evaluate their viability (Figure 8). The test showed an increase of cell viability along the cell incubation period. In particular, cell viability showed no significant differences among samples at 24 h incubation. The presence of LbL coating on the aminolysed surface enhanced significantly the cell proliferation compared to their un-functionalised counterparts, as observed after 4 and 7 days of incubation. Moreover, the presence of MET resulted in no significant difference in terms of cell proliferation compared to functionalised films with only LbL coating (fluorescence units for coated PLGA films with and without MET incorporation were 34,578 ± 1,853 and 36,787 ± 2,455 after 7 days of cells seeding). Furthermore, after 7 days it was observed cell confluence in all samples.

3.4 In vitro bacterial assays

The antibacterial activity of the released MET was investigated using bacterial disc-diffusion overlay growth inhibition experiments using the keystone periodontal pathogen Porphyromonas gingivalis (Pg) as an indicator strain. These assays were designed with the nature of the in vivo environment in which it might be required to act, i.e. a hard surface (the implant material) in contact

with an aqueous/ gel like matrix (i.e. oral secretions rich in mucinous material). Therefore we considered this assay where a small disk of our MET material, and controls, were placed on an agar surface that was then overlaid with agar. The data clearly show that the growth of Pg after 5 days is inhibited only by the PLGA disc that has had MET incorporated (Figure 9, sample 3) with a zone of inhibition of 24 ± 2 mm compared to none for the other materials.

4. Discussion

In this study, an innovative delivery system was prepared and shown to be capable of localised and controlled antibiotic drug release without degrading the physico-chemical properties of the barrier membrane. Specifically, PLGA dense films, prepared by solvent casting, were functionalised in two steps: (1) surface modification by controlled aminolysis to produce primary and secondary amine groups on the surface for charging positively the surface, and (2) layered nano-structure coating, based on well known biocompatible polyelectrolytes used in biomedical application (PSS and PAH) [33], by LbL technique to incorporate and control the antibiotic drug release. In this work, the final number of 20 layers was selected after optimisation of different parameters in order to achieve an appropriate metronidazole release kinetics: (1) number of layers (10, 15, 20 or 25); (2) the dipping time into the polyelectrolytes solutions (5, 10 or 15 minutes); (3) the polyelectrolytes molar concentration (0.05, 0.1 or 0.5 M); and (4) the MET incorporation (from the first, fifth or from ninth layer). The strong interaction between PSS and PAH favoured complete complexation. Indeed, electrophoresis measurements showed that PAH and PAH-MET complex solutions were positively charged with ζ-potentials of +14.5 and +13.7 mV, while PSS and PSS-MET solutions were negatively charged with ζ-potentials of -18.8 and -16.2 mV, respectively. The antibiotic drug, metronidazole, existing mainly (about 90%) as a zwitterion [34], was added to both polyelectrolytes.

XPS, contact angle measurement, ATR-FTIR spectroscopy and AFM analysis were performed to confirm the successful obtainment of the nano-coating on the PLGA film surface. XPS spectra in

Figure 2 evidenced the N_{1s} and S_{2p} peaks, indicating PAH and PSS have been successfully introduced. Moreover, S/N ratio, as contact angle, evidenced an alternating regular behaviour, characterised by values as a function of the layer number. These results suggest changes in the surface chemistry due to the LbL assembly, clearly indicated that the PSS and PAH layers were stratified. In particular, the S/N ratio (Figure 3) was lower when the PAH was the top layer, in fact nitrogen was the characteristic element of PAH, while sulphur was the characteristic element of PSS. Furthermore, PAH top layer had a contact angle value higher than PSS top layer, in fact the contact angle fluctuated between 70° and 80° (Figure 4). The same trend was found in different paper in literature, in which contact angles of LbL films generally vary between the values of the pure components of each layer [24, 35].

To observe the changes in the chemical and morphological characteristics of the surfaces of coated PLGA films, ATR-FTIR spectroscopy and AFM analysis were performed. Infrared spectra (Figure 5) showed (1) the polyelectrolytes coating with their typical absorption bands that increased with layer number (i.e. $-SO_3^-$ stretching vibrations at 1130 cm^{-1} for PSS, and N-H scissoring vibrations at 1580 cm^{-1} for PAH), and (2) the incorporation of the drug into nano-layered structure with the characteristic bands of MET (i.e. asymmetric and symmetric stretching vibrations of $C_{14}H_2$ unit at 3100 and 2982 cm^{-1} respectively).

Atomic force microscopy provided further evidence for the step-by-step formation of nanoscale LbL coatings on PLGA surfaces from the aminolysis chemical process. After deposition of the first bi-layer (Figure 6b), it appeared that the adsorption of these two layers is mainly onto the amino groups, since dark islands in the phase image were almost uncovered. Dark zones probably correspond to residual alcohol groups from the aminolysis treatment (Figure 6a). With increasing the layer number, the roughness of coated surface is increased, as also reported in literature [36-38] In particular, Guo et al. [36] obtained a similar rather smooth coated surfaces after deposition of 10 layers, when PAH-PAA (poly(acrylic acid)) complexes were the outmost layer. In contrast, they observed a rough surface morphology when PSS was the outmost layer.

Metronidazole was incorporated onto the multilayered structure with the aim of preventing new or re-infection. Among antibiotics, MET is used in the oral cavity for treating infections by anaerobic bacteria associated with periodontal diseases due to its low minimum inhibitory concentration [5]. In this study, we established controlled release, biocompatibility and antibacterial behaviour of MET. In preliminary testing (data not shown), the incorporation of MET after either the first or the fifth layers appeared to reduce the initial release rate for MET and produced a longer-term release profile that was considered undesirable, perhaps as a result of strong interaction of the drug with the substrate biomaterial. However, with the MET incorporation after the ninth layer, in vitro drug release tests showed three different phases (Figure 7), with an initial linear pattern, followed by a sustained linear and controlled drug release, that evidenced that MET was diffusing rapidly through the coated nano-layers, facilitated by the water uptake of these samples [21] during the mid-phase. The release of MET (~ 80%), before 14 days, was therefore through diffusion. Furthermore, at two weeks of immersion in PBS the surface roughness, measured by AFM (data not shown), decreased slightly, indicating a suitable stability of the multi-layered coating. At the latter phase (after 14 days), drugs were released as the nano-coating underwent degradation giving a zero-order release. The results are superior to commercially available products (e.g., ELYZOL®, a MET containing gel) that need to be applied with greater drug concentrations over a 7-day period [39, 40]. Furthermore, in this work we reported a strong decrease of MET-loaded content, comparing with the data reported in literature. Recently, Pichayakorn et al. [41] prepared metronidazole-loaded chitosan microparticles (MET-MPs) via an emulsion cross-linking process, and evaluated the in vitro release of MET from hydrogels and films, containing the drug in forms of MET-MPs. They reported a consistent release of MET (~ 2-3 mg/film 1 cm^2 and ~ 1.5 mg/hydrogel 5 g) after 6 h of immersion in PBS. Furthermore, the antibiotic drugs did not interfere with the process of cell adhesion or proliferation within the functionalised films as shown in Figure 8, indicating good biocompatibility for the prepared nanoscale system.

Finally, for the antibacterial experiments we chose the periodontal pathogen Porphyromonas gingivalis [42], one of the major virulent pathogens associated with periodontal disease and some device-associated infections such as peri-implantitis [43, 44]. It is also a well-established intracellular pathogen and considered a model indicator organism for the treatment and reduction of periodontal inflammation and damage. In this work, we observed that only the nano-layered PLGA discs, incorporating the antibiotic, inhibited the growth of Pg after 5 days of bacteria culture (Figure 9). These data indicate that the MET material not only releases MET in an aqueous/ gel-like environment but also in 3-dimensions and prevents any growth on or near the MET incorporated disks, indicating that it may be effective in the oral cavity where it would be employed. Moreover, we estimated that our materials contained 85 µg MET per cm^2 and released this at a rate of 8 µg per 24 hour period in an aqueous environment. The MIC for MET of this strain has been estimated to be around 60-125 ng/mL while a level of only of 1 µg/ml MET in the sulcus fluid has been reported to be necessary for effective elimination of the most relevant obligate anaerobic periodontal pathogenic bacterial strains [45, 46]. In addition, we observed no resistant colonies in this assay, indicating that the level of release of MET in our in vitro antibacterial assay from the nano-layered structure coupled with its lack of toxicity to human cells might represent a potentially effective therapeutic efficient dose. Future work may be directed at biofilm tests to simulate the more conditions in which the films may need to be effective work in clinical use.

5. Conclusion

Functionalised bioresorbable films were placed on a biocompatible biodegradable polymer, and these may be modified successfully to obtain a localised and temporally controlled delivery system for MET. The LbL technique was therefore effective for creating a multilayered coating on PLGA surface film to incorporate antibiotic. The advantages of the low temperature LbL fabrication technology described here are evident, providing an opportunity to prepare structures with predictable physico-chemical and biological properties but without degrading the physico-chemical

properties of the barrier membrane. Results of in vitro release and antibacterial experiments demonstrated that the drug-loaded films were capable of effectively delivering MET in a controlled way, with 80 % drug released within two weeks of incubation, i.e. the period in which most implant-associated infections are initiated. Looking forward to the intended clinical use, the main advantage of the system reported here was the relatively small amount of loaded drug needed to achieve effective concentrations. Antibiotic concentrations that are tailored for relatively rapid release to achieve a therapeutic effect also minimise the risk of systemic side effects. This study has therefore demonstrated that the LbL technique may be applied to the manufacture of medical devices with advanced functionality based on the improved release of sensitive biologically active molecules.

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Figure captions

Figure 1. Diagram showing the LbL method for the modification of PLGA substrate.

Figure 2. Survey XPS spectra after aminolysis and after LbL treatment.

Figure 3. Sulphur/nitrogen atomic ratio of the multilayer assembly surface as a function of the layer number: layers of odd numbers have PSS as the outermost layer; layers of even number have PAH as the outermost layer (layer zero is the aminolysed treated PLGA). Reported data are the average values whereas the bars are the standard deviation (n = 6).

Figure 4. Static contact angle as a function of the layer number: layers of odd numbers have PSS as the outermost layer; layers of even number have PAH as the outermost layer (layer zero is the aminolysed treated PLGA). Reported data are the average values whereas the bars are the standard deviation (n = 9).

Figure 5. ATR infrared spectra of the coated PLGA surfaces (from 1 to 20 nano-layers).

Figure 6. AFM topography (left), amplitude error, and phase 1x1 µm images, and z-range cross section of (a) PLGA pure film; (b) PLGA with the attachment of an aminolysed film; (c) 2-layered surface of PSS^- and PAH^+ molecules; (d) 20-layered surface of PSS^- and PAH^+ molecules and (e) 20-layered film incorporating metronidazole.

Figure 7. In vitro release kinetics of MET from nano-functionalised PLGA films. Each data point represents a mean ± standard deviation (n = 6).

Figure 8. L929 fibroblast cells viability on fabricated dense PLGA films before and after LbL coating without/with incorporation of metronidazole after 24 hours, 4 and 7 days of culture. Data show statistical difference respect to the control * ($p < 0.05$).

Figure 9. Representative macro-photograph showing growth inhibition of Porphyromonas gingivalis at day 5 by pure PLGA film (Sample 1), nano-functionalised PLGA film without (Sample 2) and with MET incorporation (Sample 3).

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