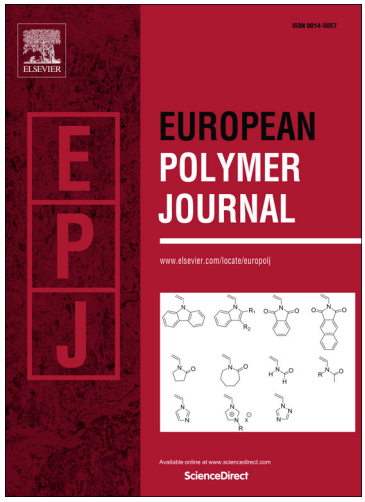
Accepted Manuscript



Antibacterial PHAs coating for titanium implants

Alejandra Rodríguez-Contreras, Yolanda García, José María Manero, Elisa Rupérez

PII: S0014-3057(16)31417-3
DOI: http://dx.doi.org/10.1016/j.eurpolymj.2017.03.004
Reference: EPJ 7753

To appear in: *European Polymer Journal*

Received Date: 31 October 2016
Revised Date: 25 February 2017
Accepted Date: 1 March 2017

Please cite this article as: Rodríguez-Contreras, A., García, Y., María Manero, J., Rupérez, E., Antibacterial PHAs coating for titanium implants, *European Polymer Journal* (2017), doi: http://dx.doi.org/10.1016/j.eurpolymj.2017.03.004

Antibacterial PHAs coating for titanium implants

Alejandra Rodríguez-Contreras[1,*], Yolanda García[1], José María Manero[1,2] , Elisa Rupérez[2]

[1] Departament de Ciència dels Materials i Enginyeria Metal·lúrgica, Laboratori de Microscòpia Electrònica, Universitat Politècnica de Catalunya-Barcelona TECH. Avda. Diagonal, 647-08028, Barcelona (Spain)

[2] Departament de Ciència dels Materials i Enginyeria Metal·lúrgica,, Biomaterials, Biomechanics and Tissue Engineering Group, Universitat Politècnica de Catalunya-Barcelona TECH, Avda. Diagonal, 647- 08028, Barcelona (Spain)

*Corresponding author:

Dr. Alejandra María Rodríguez Contreras
Departament de Ciència dels Materials i Enginyeria Metal·lúrgica- Laboratori de Microscòpia Electrònica Avda. Diagonal, 647
Pavelló E (Etseib)-Planta 0 08028 Barcelona
Tels.: 0015145687012/0034651569562

E-mail address: Sandra8855@hotmail.com

Abstract

Biomaterial-associated infection is a serious complication of modern implantation surgery. Thus, the improvement of implant surfaces is required to avoid the first stage for biofilm formation, bacterial adhesion. The current research addresses this issue by developing drug delivery systems (DDS) consisting of antibiotic-loaded polyhydroxyalkanoates (PHAs) coatings on titanium implants. Dip-coating technique was used to achieve optimal coatings with biodegradable biopolyesters, polyhydroxybutyrate (PHB) and its copolymer, polyhydroxybutyrate-*co*-hydroxyvalerate (PHBV). The coatings were completely characterized (wettability, topography, thickness and roughness), and studies of drug delivery, toxicity, antibacterial effect, and cell adhesion were performed. For both of biopolymers, surfaces were partially covered with 1 and 3 immersions, while with 6, they were completely covered. Although both antibiotic-loaded biopolymer coatings assure the protection against bacteria populations, PHBV coatings are closer to the desired release profile; its faster degradation provides for a greater and more stable drug release for a given period of time compared to PHB coatings. The use of coatings with different drug concentration per layer results in more controlled and homogeneous releases. The DDS designed not only assure toavoid the first stage of bacterial adhesion, but also their proliferation and biofilm formation, since the coatings degrade with time under physiological conditions, guaranteeing a prolonged drug release.

Keywords: Drug delivery systems, biodegradable biopolymers, gentamicin, dip-coating technique, polyhydroxyalkanoates

1. INTRODUCTION

Biomaterial-associated infection is a serious complication of modern implantation surgery which often leads to prolonged patient pain and functional losses. The removal of an infected implant is the final outcome of most of these infections, generating high costs for the health-care system and discomfort for the patient. Biomaterial infections are developed on the implant surface due to the ability of microorganisms to get attached and to further form biofilms [1]. Therefore, the study and the improvement of the implant surface to avoid the first stage for the biofilm formation of the pathogenic microorganism is required. To overcome implant-related infection and bacterial load on the implant surface, antibiotic drug incorporation and its controlled release can be a promising strategy. Implantable drug-eluting devices (or implantable drug delivery systems-DDS) offer several advantages over conventional drug delivery methods. For instance, they can provide localized, site-specific drug delivery, which is especially important in applications such as cardiology and oncology, where targeted delivery can improve the effectiveness of treatment and minimize side effects or damage to healthy tissue [2]. The design of DDS highly relies on polymers, which is driven by the suitability of physicochemical, mechanical and processability of the polymer-based formulations [3]. Synthetic polyesters, poly(lactic acid) (PLA), poly(glycolic acid) (PGA) and their copolymers, polycaprolactone (PCL), poly(lactic–co-glycolic acid) (PLGA) are examples of biodegradable polymers used for DDS [4,5]. Lately, natural polyesters polyhydroxyalkanoates (PHAs) emerged as a vast family of potential candidates as biodegradable polymers for DDS. Polyhydroxybutyrate (PHB) and its copolymer Poly(3-hydroxyvalerate) (PHBV) which is less crystalline, and more flexible than PHB itself [6], are members of this family of biopolymers. PHAs are primarily characterized by the

fact that they are produced by microorganisms from removable resources, and, therefore, they exhibit superior traits with respect to synthetic polymers such as the prevention of toxic products from chemical polymerization [7]. Furthermore, the by-products of their hydrolytic degradation make it possible to obtain common blood constituents (a ketone body produced by the liver from fatty acids, ketogenesis) [8]. Pas have performed well in a number drug delivery applications due to their excellent biocompatibility, non-toxicity and biodegradability [3,9,10,11,12,13]. Metal-based drug eluting systems have received little attention in the literature in comparison to polymer-based systems. So far, only intramedullary nails with a PDLLA coating containing gentamicin were placed on the market [14]. For example, metal implants such as plates and wires have been coated with a polymer matrix layer loaded with a microbial antibiotic, while others have studied different therapeutic agents including triclosan and chlorhexidine (CHX) [15,16**,**17]. In such studies, however, *in vitro* antibiotic release experiments showed that most of the drug was released within several hours after exposure to an aqueous environment. Even in the most favorable case, the system was only able to provide the desired drug release for less than 12 days [18,19]. Therefore, long-term release drug delivery systems are required. In this research, we develop and study novel gentamicin-loaded PHAs coatings on titanium implants designed to serve as devices to prevent implant-associated infections by a gentamicin-controlled release phase. We pay special attention to maintaining suitable concentrations of antibiotics for long periods of time, since it is essential for treating highly resistant infections.

2. MATERIAL AND METHODS

2.1 . Materials

Titanium bar was obtained from c.p. grade 2 Ti provided by Technaloy S.A. The bar was cut into disks that were 3 mm thick and 10 mm in diameter. PHB and PHBV were kindly provided by Dr. Martin Koller (Institute of Biotechnology and Biochemical Engineering-Graz University of Technology). These PHAs were biosynthesized by *Cupriavidus necator* and glucose was the main carbon source used in their fermentations. The used antibiotic was gentamicin sulfate (Genta; $C_{21}H_{43}N_5O_7$, MW 477.596 g/mol, freely soluble in water), which was purchased by Sigma-Aldrich. Chemical products such as hydrogen peroxide, Trichloromethane (TCM), poly(vinyl alcohol) (PVA) were purchased from Sigma Chemical Co. and used without further purification.

2.2. Biopolymer Characterization

The molecular masses of the biopolymers were analyzed via gel performance chromatography (GPC). Chloroform was utilized as an eluent at a flow rate of 0.80 ml min^{-1} with a stabilization pressure of 35 bars and a sample concentration of 1.5 mg ml^{-1}. A Waters Styragel HT column for midrange molecular-mass distributions was used, and samples of polystyrene with different molecular masses were used as standard. The chemical structure of both polymers and monomer composition of PHBV were determined by Proton Nuclear Magnetic Resonance (^{1}H NMR)[20]. Spectra were recorded at 25°C on a Bruker AM300 spectrometer. The polymer samples were dissolved in chloroform and a drop of TMS (tetra methyl silane used as internal standard for calibrating chemical shift for ^{1}H) was added as reference. 10 mg of the sample dissolved in 1 ml of deutered solvent was used. Proton spectra were recorded at 300.1 MHz with a spectrum of 32 K data points. A total of 64 scans were utilized with a relaxation delay of 1 s.

2.3. Surface treatment procedures

Mirror-like, smooth surfaces were achieved by grinding with SiC papers of decreasing grit size (from P800 to P2400–European P-grade standard), followed by polishing with suspensions of alumina particles on cotton cloths containing hydrogen peroxide (6 μm and 1 μm particle size). Prior to chemical activation, samples were ultrasonically rinsed with cyclohexane, isopropanol, distilled water, ethanol and acetone. Samples were stored dry under a vacuum. ***Oxygen plasma technique (Plasma):*** Surface cleaning and activation were carried out by means of plasma cleaning (Plasma Cleaner, Sterilizer PDC-002, Harrick Scientific Corporation, USA). After oxygen purging and high vacuum (3 times) the samples were exposed at low electromagnetic radiofrequency radiation (between 8 and 12 MHz) for 10 min. ***Sodium hydroxide treatment (NaOH):*** The Ti disks were immersed in 5 M of previously prepared NaOH in closed polypropylene flasks. They were placed into a furnace at 60 ºC for 24 h. Afterwards the samples were cleaned and immersed in Milli-Q water for 30 min, then rinsed with water of the same quality and acetone. They were dried with nitrogen and, finally, stocked in a vacuum.

2.4. Drug/ biopolymer matrix preparation

Gentamicin dispersion on biopolymer solution: Gentamicin sulfate particles were incorporated in 10% and 30% (w/w) of the biopolymer mass to a saturated solution of biopolymer in chloroform under conditions of constant stirring. ***Emulsion Preparation (Water in oil (W/O) emulsion technique):*** The used method was based up on that reported by **Chen and Davis** [21] and further modification by **Rodríguez-Contreras *et. al*** [12,13]. Simple emulsion was carried out by PHB solving in chloroform (10 mL) (organic phase). Then, 2 mL of aqueous solution of Genta with 2% PVA was emulsified into the previous organic solution under ultrasonic homogenizer at 5W (3 min at 60%) (Bandelin Sonopuls with microtip MS 73). Different concentrations of Genta (10% and 30% (w/w) of the polymer mass) and polymer (5, 10, 20 and 40 mg/mL) were studied.

2.5. Dip-Coating process

The previously described solutions (gentamicin dispersion and daily fresh prepared W/O emulsion) were used to coat Ti surfaces by dip-coating technique. KSV NIMA Dip Coater (Nima instruments, Stockholm, Sweden) was used with a single vessel and KSV NIMA Dip Coaters software. The substrate was immersed with a constant rate of 10 mm/min and it was extracted with a controlled output rate of 70 mm/min. The process was performed at room temperature (22 ºC). Different numbers of immersions were studied (1, 3 and 6 times dip-coating), being assured that each coating was totally dried between every immersion. Different combinations of immersions were also tested, with a range of concentrations of gentamicin sulfate in each immersion. **Figure 1** shows a summary scheme of the PHAs coating on titanium surface.

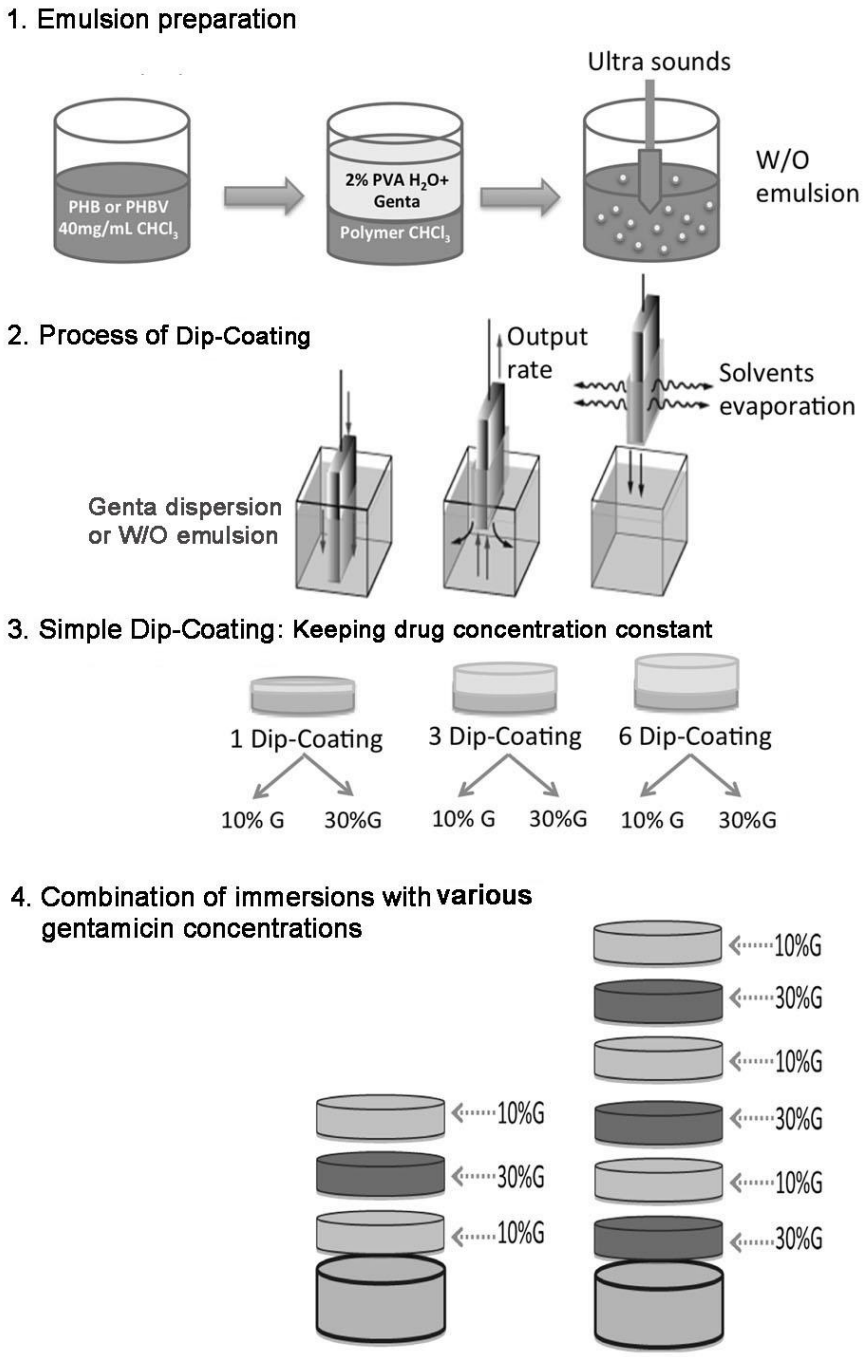


Figure 1.

2.6. Physicochemical characterization

Wettability and contact angle (CA): Static contact angles were measured with water and biopolymer solution respectively using the sessile drop method (Contact Angle System OCA15 Plus; Dataphysics, Germany). Measurements were acquired in triplicate for three samples at room temperature, with a volume of 3 µL and a dose of 1 µL /s.

Biopolymers solutions (40 mg/mL in Chloroform for the highest polymer concentration) were prepared to study their wettability and affinity to the Ti-activated surfaces. ***Field Emission Scanning Electron Microscope (FESEM):*** Scanning electron micrographs were obtained using a Jeol (JSM-7001F, Japan) scanning electron microscope, operating at 20 KV. Energy Dispersive X-ray Spectroscopy (EDS) was also used for the elemental analysis of the surfaces. ***Roughness analysis*:** White light interferometry (Wyko NT1100 Optical Profiler, Veeco Instruments, USA) was used to evaluate roughness of the samples. The average height (Ra) of each sample was measured in three randomly chosen points of the disk. Finally, sample thickness was measured by perfilometry (Veeco, Dektak 150).

2.7. Drug delivery characterization

Release of gentamicin sulphate from the coated Ti surfaces was evaluated. *In vitro* release profiles were obtained by immersing coated samples in 10 mL phosphate buffer solution (PBS) (pH 7.40) under conditions of mild stirring. The vials were incubated at 37 ºC. At predetermined intervals, 1 mL of release media was taken to assay drug concentration, while a fresh PBS replaced the same volume [22]. In order to determine the amount of released drug, mass spectroscopy was used (Varian 500-MS) using a Zorbax Eclipse XVB-C18 column (4,6x150 mm, 5µm, 80Å). This evaluation was performed three times per sample.

2.8. Biological characterization

Human osteoblast-like Saos-2 cells were cultured in McCoy's 5A medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin (50 U/mL and 50 µg/mL, respectively), 2 mM L-glutamine, 1 mM sodium pyruvate and 20 mM HEPES buffer at 37 ºC in a humidified atmosphere at 5% CO_2, all from Invitrogen. Cells were harvested at confluence using TrypLE (Invitrogen) and seeded at a cell

density of 1×10^4 cells/well. ***Cell citotoxicity assay***: Indirect *in vitro* cytotoxicity tests of the treated surfaces of Saos-2 cells were carried out by analyzing the activity of the lactate dehydrogenase (LDH) enzyme by the Cytotoxicity Detection Kit LDH. Each specimen was immersed in DMEM for 72 h at 37 ± 2 °C with an extraction medium area/volume ratio of 0.5 cm^2/1 mL (according to ISO 10993-5). Afterward, the extraction medium was removed and diluted with DMEM (dilution 1/0; 1/1; 1/10; 1/100; 1/1000). As a reference for 100% maximum survival, cells were placed in TCPS (Tissue culture polystyrene). ***In vitro antibacterial assay:*** A commonly used procedure for studying the antibacterial action was followed [23,24]. The assay was performed with both Gram-positive and Gram-negative bacteria (*Staphylococcus aureus* and *Escherichia coli*, respectively). The overnight-incubated bacteria solutions were diluted with sterile PBS solution until it reached an absorbance value of 0.2 at 600 nm (bacterial concentration about 10^8 CFU/ml). The solutions were used to inoculate (100 µL) on Petri plates with Chromocult® (Merck) media for *E. coli* and Triptoy Soy Agar (TSA) media for *S. aureus*. The discs were placed in the middle of the inoculated surface. The plates were incubated at 37 ºC for 24 hours, when the diameter of inhibition was measured. The controls were carried out with drug-free disks. ***Cell adhesion assay:*** SAOS P31-Sarcoma Osteogenic cells were seeded onto titanium samples, which had been previously sterilized with ethanol (70%, v/v), at a density of 20,000 cells/disk and incubated at 37 °C and 5% (v/v) CO_2 containing atmosphere. 4 h post-seeding non-adherent cells were washed off by gently rinsing with PBS, and remaining cells were lysed with 200 µl/well of M-PER® (Pierce, Rockford, IL, USA). Enzymatic activity of lactate dehydrogenase(LDH) was quantified by colorimetric assay (Cytotoxicity Detection Kit (LDH), Roche Diagnostics, Mannheim, Germany), using amultimode microplate

reader (Infinite M200 PRO, Tecan GroupLtd., Männedorf, Switzerland). Cell number was obtained using a standard curve.

2.9. Statistical studies

Surface characterization and biological results were expressed as a mean value of standard deviation (SD) for each sample. T-test was used with a 95% confidence interval to evaluate statistical differences.

3. RESULTS AND DISCUSSION

In order to obtain surfaces with antibacterial activity, coatings of PHAs loaded with Genta as an antibiotic were used to cover Ti surfaces. There has been an increasing interest in the use of natural materials as drug delivery vehicles due to their appealing properties. The natural origin, biodegradability, and biocompatibility of PHAs make them suitable for a variety of applications in the health industry [10,25,26]. Particularly because of their property of biodegradation, PHAs were studied as a matrix for the antibiotic and as a controlled drug delivery system.

3.1. Biopolymer characterization

The biopolymer molecular masses were analyzed by GPC and the results show similar MW for both biopolyesters: 240 kDa for PHB and 270 kDa for PHBV with polydispersity index (PDIs) of 8.6 and 3.4, respectively (**figure 2 C and D**). H^1 NMR was carried out to verify the biopolymers structure (**figure 2 A and B**). The characteristic peaks at 0.99 ppm and 1.25 ppm for methyl group for valerate and hydroxybutyrate monomers, respectively, can be used to determine the valeric composition in the copolymer PHBV sample according to equation 1 [27,28]:

$$\text{3HV monomeric percentage}(\%) = \frac{\text{Area } CH_3 \text{ (3HV)}}{\text{Area } CH_3 \text{ (3HV)} + \text{Area } CH_3 \text{ (HB)}} \text{ x } 100 \text{ [1]}$$

It was confirmed that PHBV has 19 mol% of the monomer HV. The presence of this monomer decreases the polymer crystallinity and, thus, its mechanical properties change (high elastic modulus and flexural strength with low tensile strength and elongation at break) [29] and degradability [30]. Such different properties directly influence the features of the coatings with both polymers.

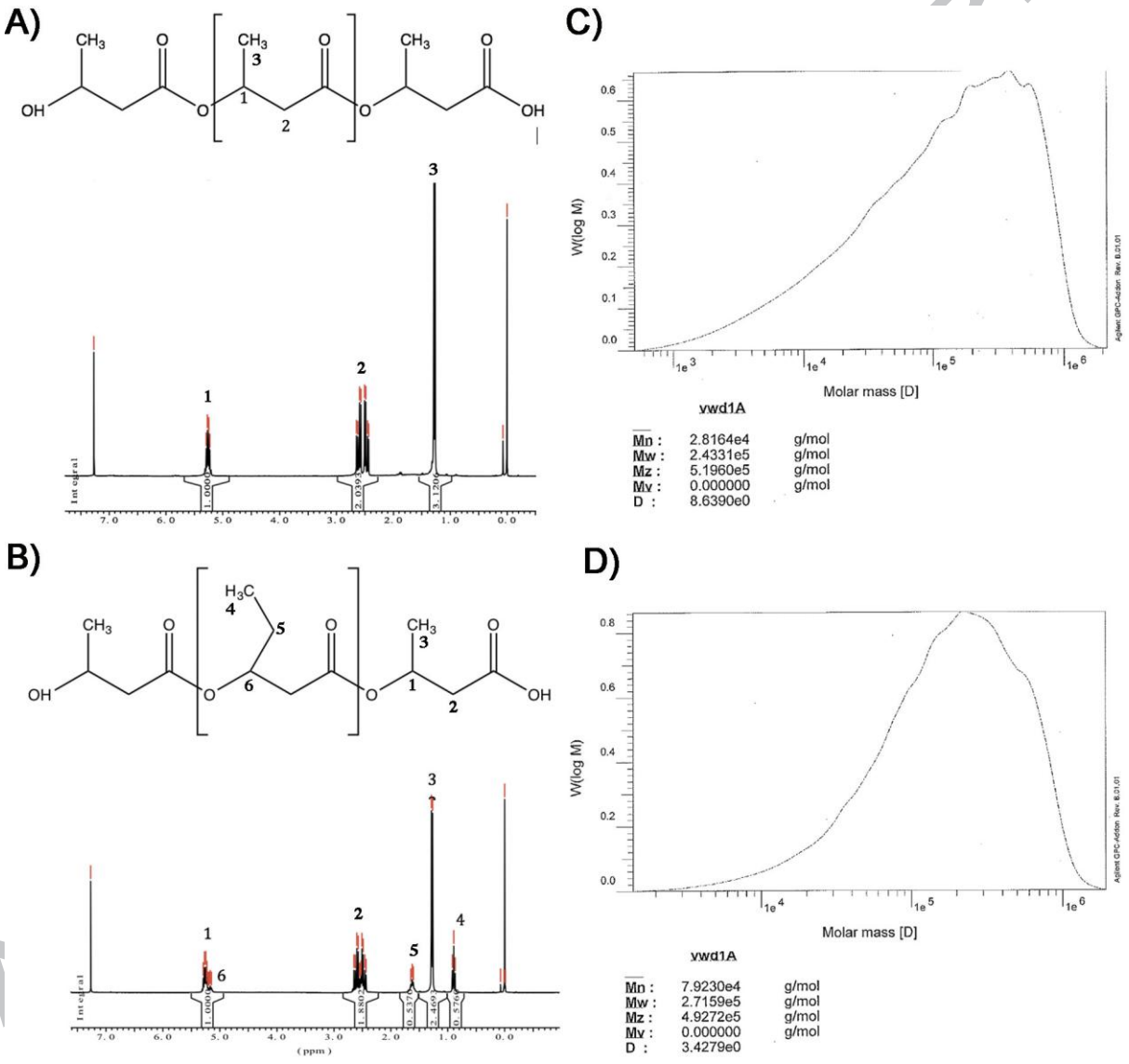


Figure 2.

3.2 Ti activation technique characterization

Oxygen plasma and NaOH treatments were used to modify Ti surfaces. Both methods allow modification of surfaces by attachment or adsorption of functional groups to tailor

surface properties for specific applications. In particular, both methods chemically remove organic contaminants and promote surface oxidation and hydroxylation (OH groups) by increasing surface wettability [31]. Especially, chemical treatment in alkali solutions is a strong contender to impart submicron or nano-roughness to Ti surfaces [32,33,34]. NaOH treatment generates a homogeneous roughness on Ti surface with a very intricate and nano-porous morphology, whereas plasma cleaning did not modify the surface roughness (compare **figure 3 B and C**). The corresponding profile roughness parameter Ra has been included on the micrographs. Biopolymers solutions (PHB and PHVB) were prepared to study their wettability and affinity to the activated Ti surfaces. The contact angles of both biopolymer solutions are significantly lower on the Ti surface treated with NaOH (**figure 3D**). This improvement of surface affinity to the biopolymer solutions is explained due to the incorporation of higher OH groups on the Ti surfaces, which allow the bounding with the carboxylic groups at the end of the biopolymers chain via Van der Waals bounds. This affinity is greater with PHB, probably due to its lower MW and higher PDI than PHBV. This lower MW and higher PDI leads to a greater number of shorter polymer chains. Therefore, PHB shows a greater number of ending groups, carboxylic group, which easily bond with OH groups, than PHBV. According to the literature, NaOH etching is the most efficient activation method for promoting surface hydroxylation (incorporation of OH groups) increasing its surface wettability and its specific area. Higher roughness and consequently more specific surface for samples chemically treated, probably, benefit the physical absorption of the biopolymer solutions. Therefore, chemical activation with NaOH solution was the activation method selected for further studies.

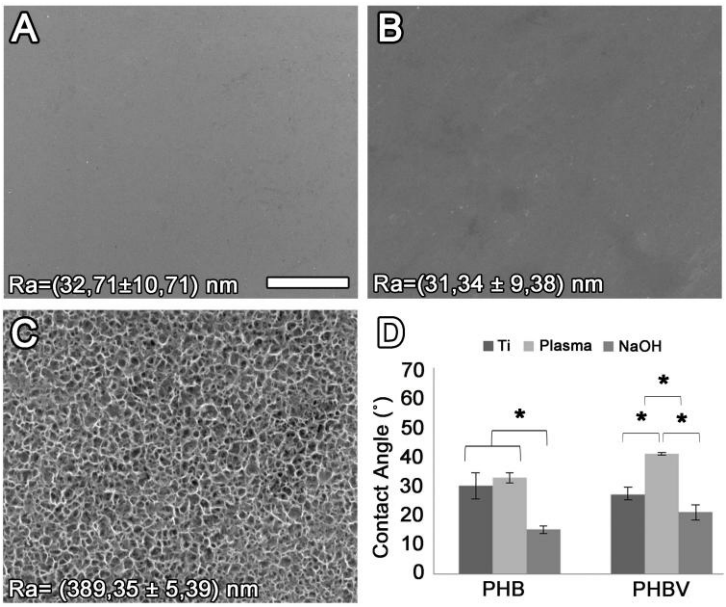


Figure 3.

3.3. Emulsion preparation

Since gentamicin is not soluble in the same solvents as PHB and PHBV, a *dispersion* of the particles in the biopolymers solution was prepared and used in a first attend to further cover the Ti surface by dip-coating. Unfortunately, a homogeneous distribution of gentamicin particles dispersed in the polymer coating was not achieved; Genta particles were in most cases too big and they tended to cluster. **Figure 4** shows Ti surface after dip-coating with Genta dispersed in PHB/chloroform solution. In order to obtain an optimal homogeneity of the antibiotic in the biopolymer matrix, emulsions of the drug in the biopolymer solution were proposed as fluids for the process of surface covering via dip-coating. Following previous reported studies for PHA emulsion preparation [13], an aqueous solution of Genta with 2% PVA was emulsified into the organic solution (biopolymer in chloroform) using an ultrasonic homogenizer. The strategy of using water in oil (W/O) emulsions as a fluid for dip-coating technique was appropriate to obtain uniform distributions of gentamicin sulfate in the coating.

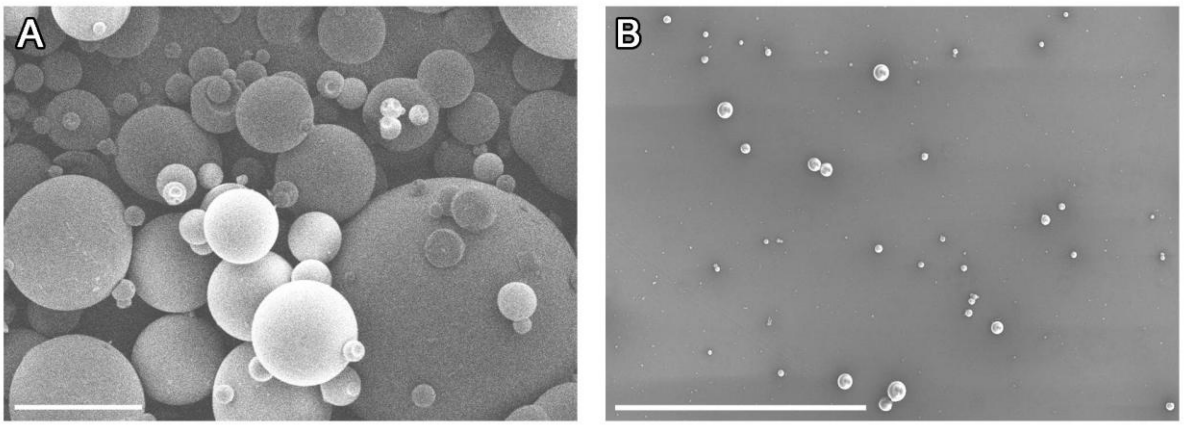
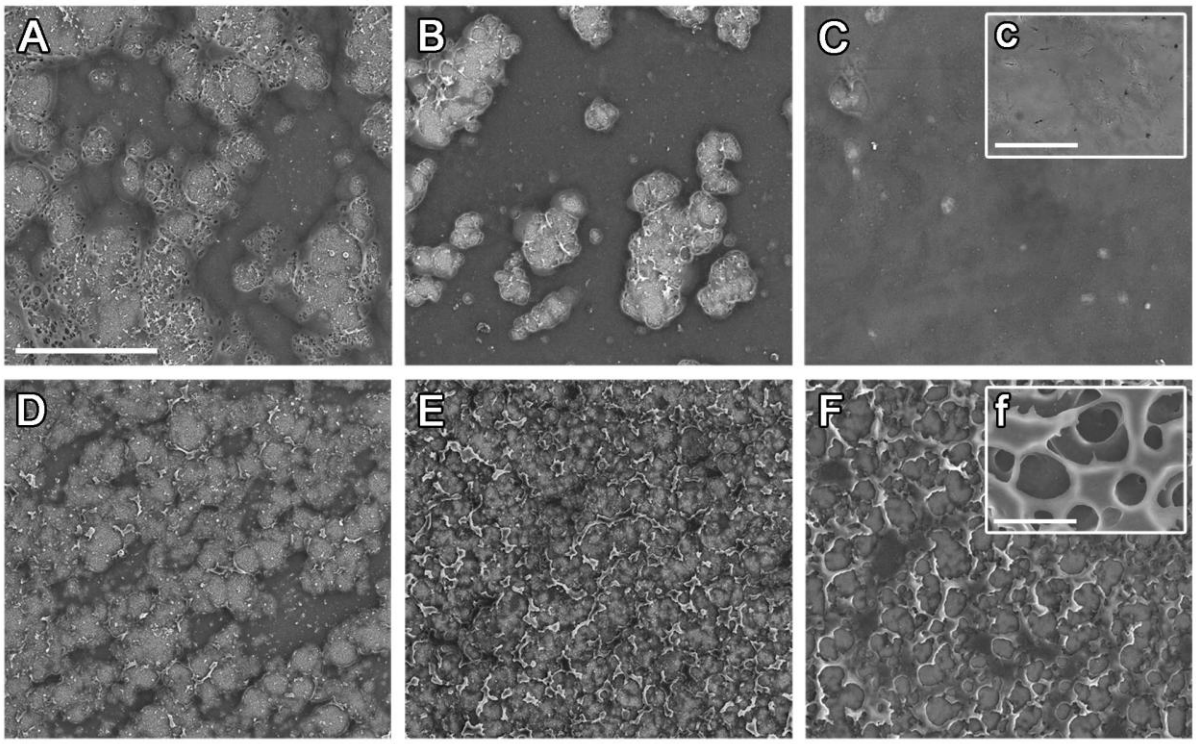


Figure 4.

3.4. Dip-Coating process

Different biopolymer concentrations and numbers of dip-coating (1, 3 and 6 immersions) were tested to study the covering of the Ti surfaces (**figures 5**). More homogeneous coatings were obtained with the higher concentration of biopolymer (40 mg/mL) in the emulsions. Therefore, all subsequent studies of Ti surface coating were carried out with this concentration. Regarding the number of dip-coatings, the surfaces were entirely covered by the biopolymers after 6 immersions; confirmed by FESEM images (**figure 5 c and f**). Both biopolymer coatings show very different topographies. PHBV forms highly porous surfaces (average of pores diameter around 50 µm), while PHB shows more compact, thicker and heavier ones (**figures 5 G and H**). **Figures 5f** and **5c** show FESEM images at high magnification, where it can be confirmed that the biopolymer coating totally covers the Ti surfaces. Regarding PHBV coatings, a porous network is being added in each immersion, thus increasing the roughness and the volume of the coating with the number of immersions (**figure 5G**). This porous structure will promote drug release, since it shows higher specific surface, exposing the biopolymer to a higher degradation. These porosities or cavities could also provide for space for the cells to grow among the coating, thus improving their biointegration. The amount of Genta in the emulsion did not modify this results. The water contact angle was measured on the PHB

and PHBV coating surfaces. They normally show high contact angles of 60-90 degrees [36]. However, a drop of water was immediately absorbed by the substrate, possibly due to the topography of the surfaces.



G

Property	Polymer	3D	6D
Roughness Ra (µm)	PHB	21.34 ± 0.02	27.94 ± 0.04
	PHBV	31.06 ± 0.06	36.32 ± 0.02
Thickness (µm)	PHB	39,12 ± 0.08	42.60 ± 0.03
	PHBV	12.45 ± 0.02	13.22 ± 0.01

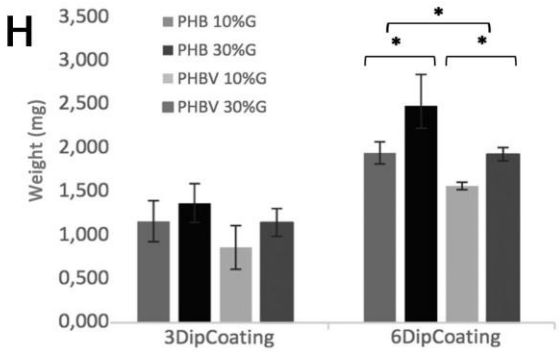


Figure 5.

3.5. Toxicity assay and cell adhesion

The conditions studied were the ones exhibiting great risk of toxicity; coatings with six times the process of dip-coatings and 30% gentamicin sulfate, since the antibiotic content is the highest. **Figure 6** shows the results of the toxicity test. According to UNE-EN ISO 10993-5, the percentage of cell survival should exceed 80% in the first dilutions to ensure that the system is not toxic. In the case of the study, all samples tested have

exceeded this percentage and, consequently it can be confirmed that the proposed systems are not toxic. Furthermore, the non-toxicity of the biopolymer coatings was confirmed by the results from cell adhesion test. Osteoblastic cells adhered to PHB and PHBV coatings in the same number as polished Ti surfaces (results show no significant differences between samples and Ti surfaces) (data not shown).

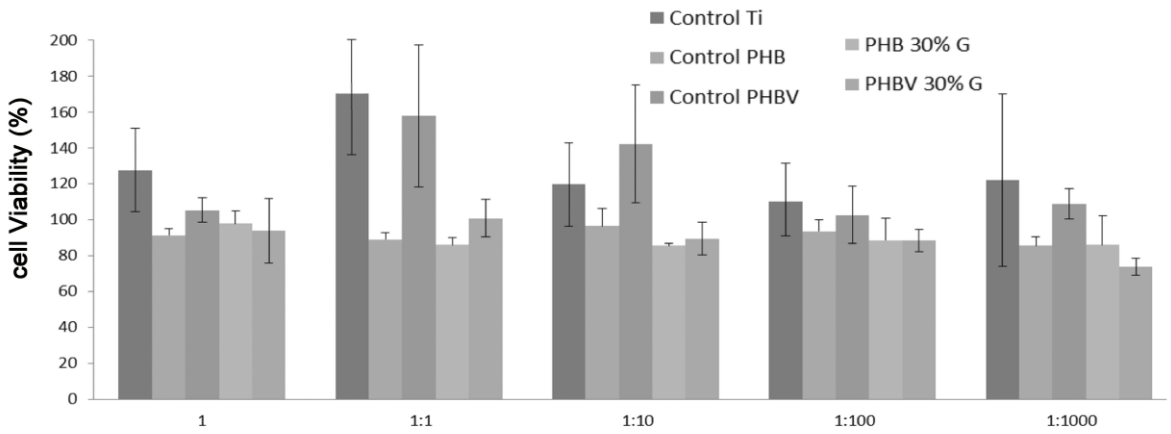


Figure 6.

3.6 Drug delivery studies

The technique of delivery must guarantee a rapid release of the antibiotic from the carrier, and local drug levels above the minimal inhibitory concentration (MIC). For gentamicin, these limits are: MIC50=0.5 µg/mL and MIC90=32.0 µg/mL [37]. Genta release profiles from PHB and PHBV coatings of 3 and 6 immersions are shown in **figure 7A**. In terms of initial drug release and release rate, there are no remarkable differences between coatings made with 3 or 6 immersions. As expected, lower amount of drug is released when the number of dip-coating is less, since there is fewer antibiotic incorporated to the bulk of the polymer matrix. The release profiles show a fast liberation of gentamicin the first 24 hours of incubation, afterward drug release rate decreases, and it is maintained until the end of the incubation. According to these experimental results, release of the active principle from the biopolymer matrix takes place in two stages. In the early hours of incubation, gentamicin is released from the surface of the coating.

Once the external gentamicin is totally released, then the polymer matrix must be degraded to liberate the remaining active principle inside the bulk. Comparing both PHAs, the initial drug released from PHB coatings is higher than from PHBV ones, regardless of the number of immersions. While having the same number of dip-coatings and the same drug concentration, PHB samples incorporate more active principle than PHBV. This can be corroborated by the coating weight, which is higher for the PHB coatings (**figure 5H**), and it can be related to the chemical or physical interaction between the drug and the biopolymer. PHB has lower MW and higher PDI than PHBV, consequently it shows a higher number of carboxyl groups placed at the end of the biopolymer chains. This chemical group can easily bond with the hydroxyl and amine groups from gentamicin, giving to PHB coatings more affinity to the active principle.

In the second stage of drug release, PHB samples do not reach the minimum inhibitory concentrations (MIC50 = 0.0005 mg/mL) required for antimicrobial properties in some cases, while samples with PHBV show drug release above MIC limit in all cases. This can be explained by two reasons. First, the better affinity (and linkage) that PHB exhibits for gentamicin sulfate compared to PHBV, prevents it from being readily released. PHBV can more easily loosen its chemical bonds with gentamicin, thus, releasing it easily. The second reason is related with the different degradation rates of both biopolymers, since the polymer matrix has to be degraded in order to release the drug located inside the coating. PHBV has shown faster degradation than PHB due to its lower crystallinity [38]. It is reported that when stored at 37°C for 6 months in human blood serum PHB degrades very slowly with a weight loss of 5% [39]. Moreover, the high porosity that PHBV shows affects its degradation process, and, consequently, the drug delivery. This has been confirmed by studying the stability of biopolymer coatings. After two weeks of incubation under physiological conditions (PBS and 37°C) the initial

morphology of biopolymer coating surfaces changed, revealing early signs of degradation (Figure 7B). Holes can be observed on the surfaces of PHB coating after their exposure to PBS for two weeks, while smooth surfaces can be detected on initial coatings (**figure 5C**). The more rapid rate of degradation of PHBV with respect to PHB can be inferred from both the range of holes sizes and the roughness of the surface of the highly porous network of PHBV coatings (**Figure** 7B, PHBV inset). Given the drug profile of PHBV, and, in consideration of the results described above it can be confirmed that confirmed that PHBV coatings allow the release of enough quantity of drug (above MIC value) to maintain antimicrobial properties for longer periods of time.

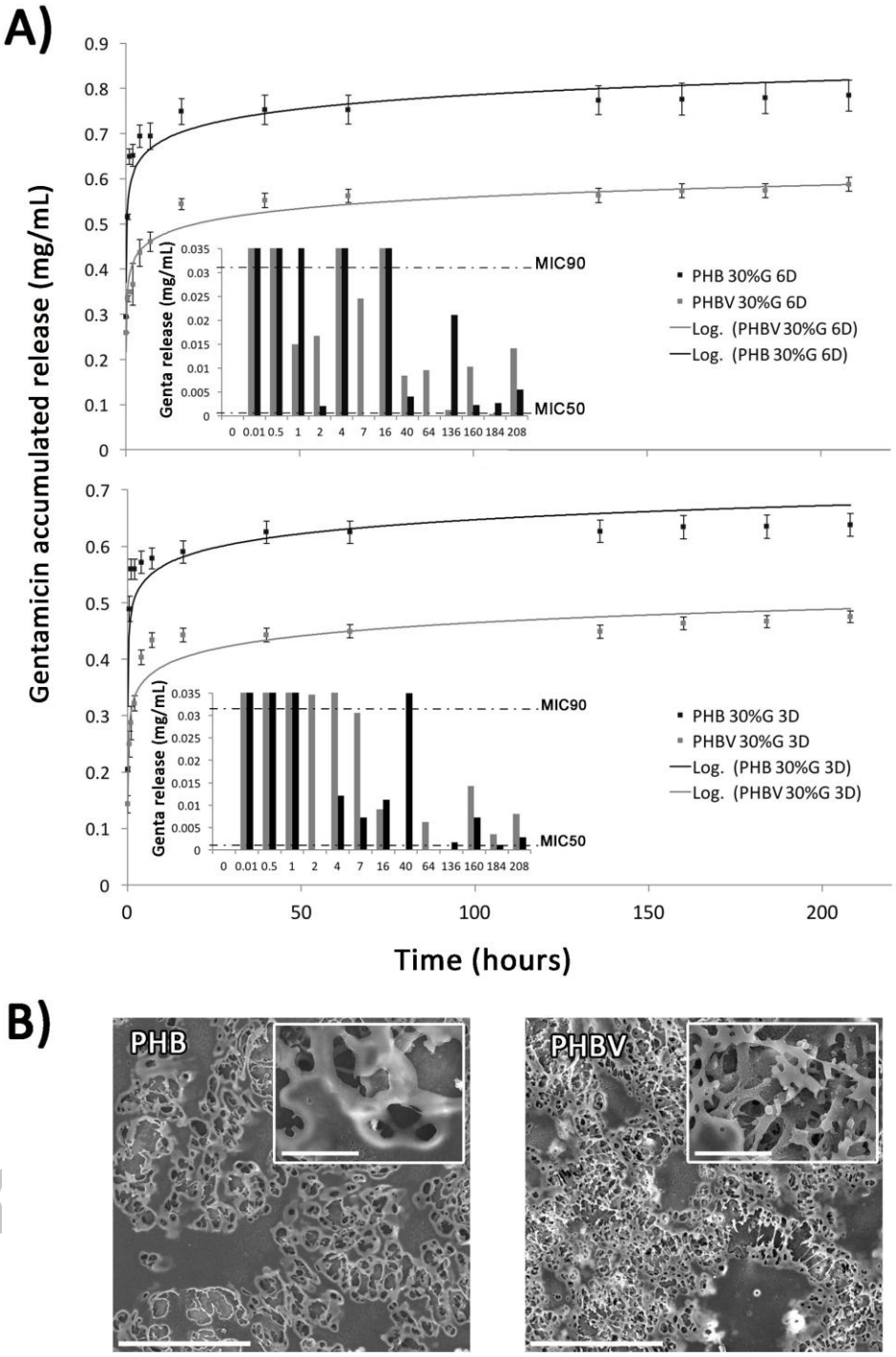


Figure 7.

3.7. Drug delivery studies of PHAs coatings with combined % of Genta per Dip-Coating

An advantage of dip-coating process is that it allows the combination of different layers of polymers and active principle amounts. Thus, a variety of dip-coatings with a range of gentamicin concentrations were studied in order to prevent the quick first release of active principle and to acquire drug release profiles more homogeneous and constant with PHBV. In all cases, the amount of gentamicin in the outer layer was 10% in order to control the burst release of drug at short times of incubation. When 6 dip-coatings were applied, the release rate of gentamicin sulfate considerably decreased (by 78%) in the first hours of incubation compared to the release rate of the samples which have uniform amount of gentamicin (**Figure 8**). However, this decrease of the drug release rate for coatings with 3 dip-coatings is less (6% slower for coatings with alternating concentrations of gentamicin). After the first hours of incubation, the amount of drug released continues to be above the MIC for 20 days, showing a more consistent release profile than the samples with the same concentration of gentamicin in all layers. Thus, it was possible to attain a more stable and homogeneous drug release for the period of time studied, and, in particular, after the first hours of incubation.

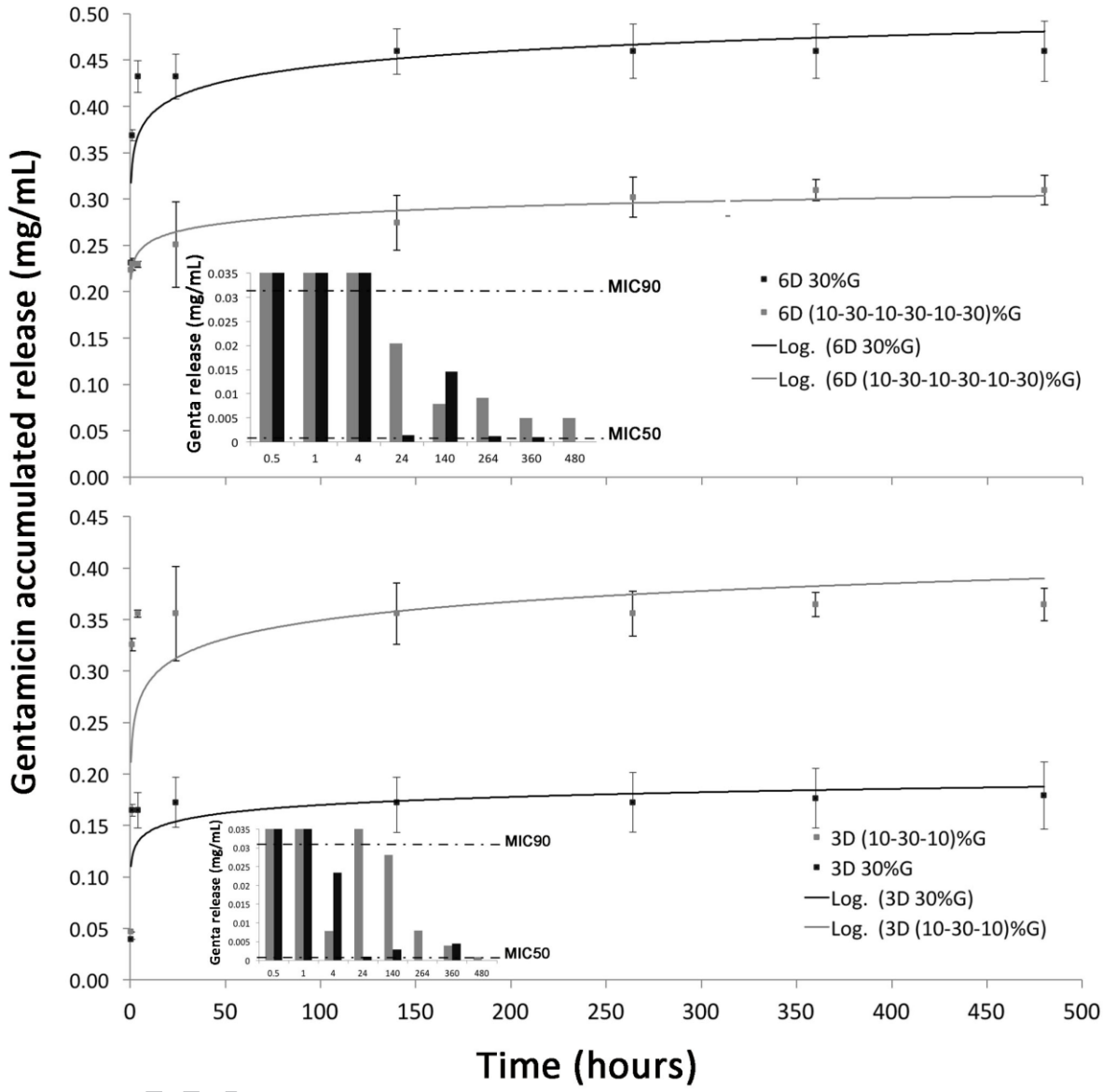


Figure 8.

3.8. *In vitro* antibacterial assay

Determination of bacterial growth inhibition was performed with both Gram-positive and Gram-negative bacteria (*Staphylococcus aureus* and *Escherichia coli*, respectively). All cases tested (PHBV coatings with 1, 3 and 6 immersions and with 10 and 30% Genta) show growth inhibition of both microorganisms, indicating that Genta released is above 0.0005 mg/mL, according to the literature [37]. There was a slight increase in the diameter of the inhibitory growth zone when the concentration of Genta was higher and also when the number of dip-coatings increased. The diameter of the halo is then proportional to the percentage of drug

concentration, and the number of immersions. The effect of the antibiotic on both bacteria is very similar (without significant differences), although gentamicin is reported to have a stronger effect on Gram-negatives such an *E. coli* [40]. Ti discs with PHBV coating with different dip-coatings (3 and 6) and with both, simple (30%) (**figure 9A**) and combined (10-30%) (**figure 9B**) Genta concentration per layer, were tested after drug release analyses to confirm their antibacterial properties. After 24, 264 and 480 hours of incubation in physiological conditions at 37 ^{O}C, samples show different sizes of the inhibition zone diameter (**figure 9C**). In particular after 20 days (480 h) of incubation, the coatings provide Ti surfaces with a significant antibacterial effect (compare the diameter of the inhibition zone of the samples with the controls: Ti surfaces without coating and with biopolymer coating without Genta) after drug release through the period of time studied.

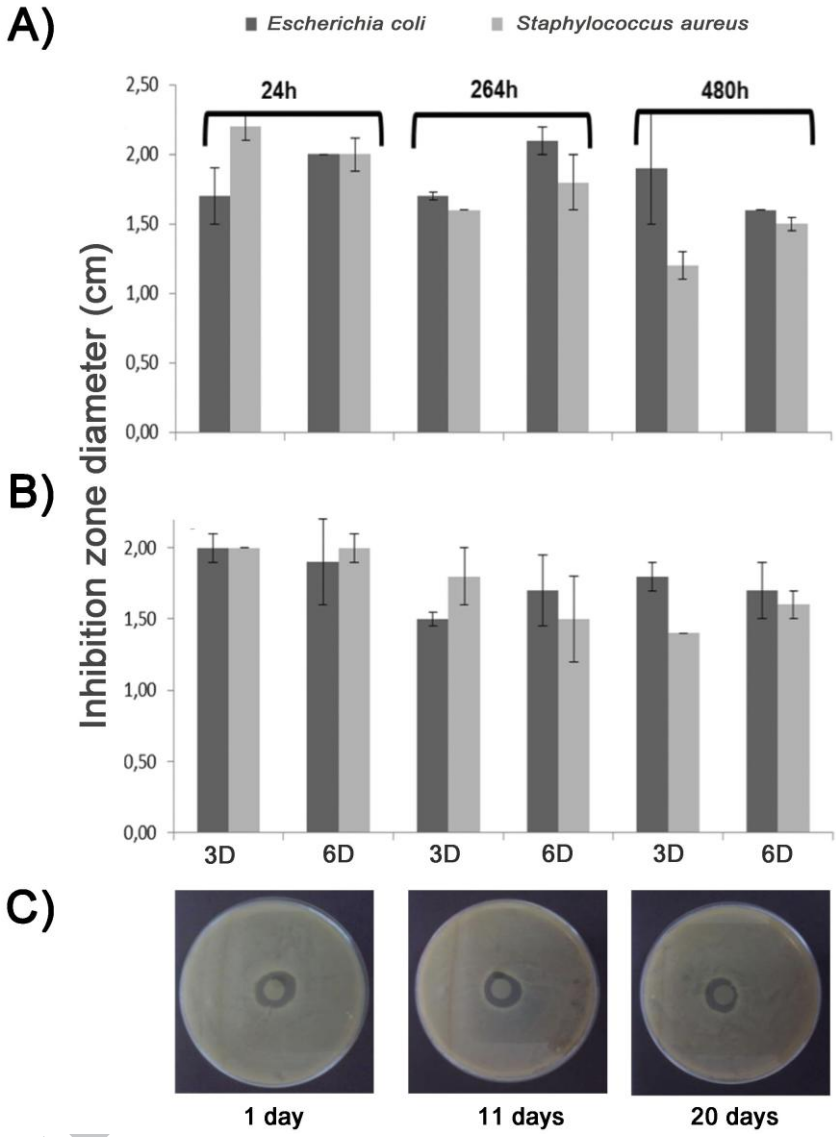


Figure 9.

4. Conclusions

A drug delivery system for an application involving gentamicin was developed in this study by using a dip-coating technique to achieve an optimum coating on Titanium surfaces using biodegradable biopolymers, PHB and PHBV. The dip-coating technique makes it possible to combine layers with varying drug concentrations in order to obtain distinct coatings with a range of drug release profiles. In this study, the optimal conditions for applying dip-coating technique for obtaining an optimal drug delivery were achieved. The optimal release profiles obtained exceeded, for all intervals of time tested, the minimum concentration for the

antibiotic to be effective, without surpassing the limits of toxicity. The system designed for drug delivery not only assures the elimination of the first stage of bacterial biofilm formation (bacterial adhesion), but also their proliferation, since the biopolymer coating with antibiotic is able to degrade with time under physiological conditions guaranteeing a controlled drug release. Specifically, the use of PHBV for the coating of Ti surfaces with different active principle concentration layers, results in a more constant and homogeneous drug release.

Acknowledgments

Authors acknowledge the Spanish Government for Financial Support through project MAT2015-67183-R (MINECO/FEDER, UE) and the Agency for Administration of University and Research Grants of the Government of Catalonia [2014 SGR 1333]. Authors would like to acknowledge Dr. Martin Koller from Institute of Biotechnology and Biochemical Engineering-Graz University of Technology for the providing of the biopolyesters.

Bibliography

[1] J. Gallo, M. Holinka, C.S. Moucha, Antibacterial Surface Treatment for Orthopaedic Implants, Int. J. Mol. Sci. 15 (2014) 13849-13880.
[2] A.J. Zaki, M, S.K. Patil, D.T. Baviskar, D.K. Jain, Implantable Drug Delivery System: A Review. Int. J. Pharm. Tech. Research 4(1) (2012) 280–292.
[3] R. Nigmatullin, P. Thomas, B. Lukasiewicz, H. Puthussery, I. Roy, Polyhydroxyalkanoates, a family of natural polymers, and their applications in drug delivery, J. Chem. Technol. Biotechnol. 90(2015)1209–1221.
[4] Z. Lan, Y.Lyu, J. Xiao, X. Zheng, S. He, G. Feng, Y. Zhang, S. Wang, E. Kislauskis, J. Chen, S. McCarthy, Laham R, X. Jiang, T. Wu, Novel Biodegradable Drug-Eluting Stent Composed of Poly-L-Lactic Acid and Amorphous Calcium Phosphate Nanoparticles Demonstrates Improved Structural and Functional Performance for Coronary Artery Disease. J. Biomed. Nanotechnol. 10 (2014) 1194-204.
[5] A. Srivastava, T. Yadav, S. Sharma, A. Nayak, A. Kumari, N. Mishra, Polymers in Drug Delivery. J. Biosci. Med. 4 (20160 69-84.
[6] G.T. Köse, H. Kenar, N. Hasirci, V. Hasirci, Macroporous poly(3-hydroxybutyrate-*co*-3 hydroxyvalerate) matrices for bone tissue engineering. Biomat. 24 (2003) (11) 1949-58.

[7] M. Koller, L. Maršálek, M. Miranda de Sousa Dias, G. Braunegg. Producing microbial polyhydroxyalkanoate (PHA) biopolyesters in a sustainable manner. New Biotechnology (2017). Online ahead of print. http://dx.doi.org/10.1016/j.nbt.2016.05.001

[8] D.H. Williamson, J. Mellanby, H.A. Krebs, Enzymic determination of D(-)-β-hydroxybutyric acid acetoacetic acid in blood. Biochem. J. 82 (1961) (1) 90-96.

[9] P-Y. Tian, L. Shang, H. Ren, Y. Mi, D-D. Fan, M. Jiang, Biosynthesis of polyhydroxyalkanoates: Current research and development. Afr. J. Biotechnol. (2009) 709-714.

[10] K.P. Luef, F. Stelzer, F. Wiesbrock. Poly(hydroxyalkanoate)s in Medical Applications. Chem Biochem Eng Q 29 (2015) (2), 287-297.

[11] Y-C. Xiong, Y-C. Yao, X-Y. Zhan, G-Q. Chen, Application of Polyhydroxyalkanoates Nanoparticles as intracellular sustained drug-release vectors. J. Biomat. Scienc. 21 (2010) 127-140.

[12] A.M. Rodríguez-Contreras, M.S. Marqués-Calvo, F.J. Gil, J.M. Manero, Modification of titanium surfaces by adding antibiotic-loaded PHB spheres and PEG for biomedical applications. J. Mater. Sci.: Mater. Med. (2016) 27:124.

[13] A.M. Rodríguez-Contreras, C. Canal, M. Calafell-Monfort, M.P. Ginebra, G. Julio-Moran, M.S. Marqués-Calvo, Methods for the preparation of doxycycline-loaded phb micro- and nano-spheres. Europ. Pol. J. 49 (2013) (11) 3501–3511.

[14] T. Fuchs, R. Stange, G. Schmidmaier, M.J. Raschke, The use of gentamicin-coated nails in the tibia: preliminary results of a prospective study. Arch. Orthop. Trauma Surg. 131 (2011) 1419-25.

[15] M. Lucke, G. Schmidmaier, S. Sadoni, B. Wildemann, R. Schiller, N.P. Haas, M. Raschke. Gentamicin coating of metallic implants reduces implant-related osteomyelitis in rats. Bone 32 (2003) 521–531.

[16] M. Lucke, B. Wildermann, S. Sadoni, C. Surke, R. Schiller, A. Stemberger, M. Raschke, N.P. Haas, G. Schmidmaier. Systematic versus local application of gentamicin in prophylaxis of implant-related osteomyelitis in a rat model. Bone (2005) 770–778.

[17] M. Aviv, I. Berdicevsky, M. Zilberman, Gentamicin-loaded bioresorbable films for prevention of bacterial infections associated with orthopedic implants. J. Biomed. Mater. Res. A 83 (2007) 10–19.

[18] J.S. Price, A.F. Tencer, D.M. Arm, G.A. Bohach. Controlled release of antibiotics from coated orthopedic implants. J. Biomed. Mater. Res. 30 (1996) 281–286.

[19] H. Gollwitzer, K. Ibrahim, H. Meyer, W. Mittelmeier , R. Busch, A. Stemberger, Antibacterial poly(D,L-lactic acid) coating of medical implants using a biodegradable drug delivery technology. J. Antimicrob. Chemother. 51 (2003) 585–591.

[20] J. Jagur-Grodzinski, Polymers for tissue engineering, medical devices, and regenerative medicine. Concise general review of recent studies. Polym. Adv. Technol. 17 (2006) 395–418.

[21] J. Chen, S.S Davis, The release of diazepam from poly(hydroxybutyrate-hydroxyvalerate) microspheres. J. Microencapsul. 19 (2002) 191-201.

[22] Donlan RM. Biofilms and device-associated infections. Emerg Infect Dis 2001;7:277–281

[23] M.T. Madigan, J.M. Martinko, J. Parcker. Brock Biology of microorganisms. Prentice Hall Companion, ninth edition, 2000.

[24] K. Nonsee, C. Supitchaya, W. Thawien. Antimicrobial activity and the properties of edible hydroxypropyl methylcellulose based films incorporated with encapsulated clove (*Eugenia caryophyllata* Thunb.) oil. Int. Food Res. J. 18 (2011) (4) 1531-1541.

[25] B.D. Ulery, L.S. Nair, C.T. Laurencin. Biomedical applications of biodegradable Polymers. J. Polym. Sci. B. Polym. Phys. 49 (2011) (12) 832–864

[26] Muhammadi, Shabina, M. Afzal, S. Hameed. Bacterial polyhydroxyalkanoates-eco-friendly next generation plastic: Production, biocompatibility, biodegradation, physical properties and applications. Green Chemistry Letters Reviews 8 (2015) 56–77

[27] H. Liu, M. Pancholi, J. Stubbs, D. Raghavan, Influence of Hydroxyvalerate Composition of PolyhydroxyButyrate Valerate (PHBV) Copolymer on Bone Cell Viability and *in vitro* Degradation. J. Appl. Pol. Sci. 116 (2010) 3225–3231.

[28] F. Masood, F. Hasan, S. Ahmed, P. Chen, A. Hameed, Biosynthesis and Characterization of Poly-(3-hydroxybutyrate-*co*-3-hydroxyvalerate) from *Bacillus cereus* S10. J. Polym. Environ. 20 (2012) 865–871.

[29] E. Bugnicourt, P. Cinelli, A. Lazzeri, V. Alvarez, Polyhydroxyalkanoate (PHA): Review of synthesis, characteristics, processing and potential applications in packaging. Pol. Letters 8 (2014) (11) 91–808

[30] M.J. Yaszemski, D.J. Trantolo, K-U. Lewandrowski, V. Hasirci, D.E. Altobelli, D.L. Wise, Tissue Engineering and Novel Delivery Systems. CRC Press 2003.

[31] V. Paredes, E. Salvagni, E. Rodriguez, J. Gil, J.M. Manero, Assessment and comparison of surface chemical composition and oxide layer modification upon two different activation methods on a cocrmo alloy. J. Mat. Sci.: Mater. in Medicine 25 (2013) (2) 311-20.

[32] H.M. Kim, F. Miyaji, T. Kokubo, T. Nakamura, Preparation of bioactive Ti and its alloys via simple chemical surface treatment. J. Biomed. Mater. Res. (1996) (32) 409-417.

[33] X.X. Wang, S. Hayakawa, K. Tsuru, A. Osaka, Improvement of the bioactivity of H_2O_2/$TaCl_5$-treated Ti after a subsequent heat treatment. J. Biomed. Mater. Res. (2000) 171-176.

[34] D.K. Pattanayak, S. Yamaguchi, T. Matsushita, T. Kokubo, Nanostructured positively charged bioactive TiO_2 layer formed on Ti metal by NaOH, acid and heat treatment. J. Mater. Sci.: Mater. in Medicine 22 (2011) 1803-1812.

[35] M. Godoy-Gallardo, Z. Wang, Y. Shen, J.M. Manero, F.J. Gil, D. Rodriguez, M. Haapasalo, Antibacterial Coatings on Titanium Surfaces: A Comparison Study Between *in vitro* Single-Species and Multispecies Biofilm. ACS Appl. Mater. Interfaces 7 (2015) 5992−6001.

[36] A.C. Mottin, E. Ayres, R.L. Oréfice, J.J.D. Câmara. What Changes in Poly(3-Hydroxybutyrate) (PHB) When Processed as Electrospun Nanofibers or Thermo-Compression Molded Film? Mat. Research (2016) 19:1.

[37] S.A. Salmon, J.L. Watts, Minimum inhibitory concentration determinations for various antimicrobial agents against 1570 bacterial isolates from turkey poults. Avian Dis. 44 (2000) (1) 85-98.

[38] I. Vroman, Lan Tighzert, Biodegradable Polymers. Materials 2 (2009) 307-344.

[39] A.P. Bonartsev, V.L. Myshkina, D.A. Nikolaeva, E.K. Furina, T.A. Makhina, V.A. Livshits, A.P. Boskhomdzhiev, E.A. Ivanov, A.L. Iordanskii, G.A. Bonartseva Biosynthesis, biodegradation, and application of poly(3- hydroxybutyrate) and its copolymers - natural polyesters produced by diazotrophic bacteria. Formatex. 2007.

[40] S. Chopra, V. Manyanga, J. Hoogmartens, E. Adams, LC with electrochemical and UV detection for analysis of a formulation containing gentamicin and parabens. Anal. Methods 5 (2013) 2491–2496

Figure Legends:

Figure 1. Scheme of the process of Dip-Coating.

Figure 2. H^1 NMR of homopolymer PHB spectra (A): a doublet at 1,274 ppm attributed to the methyl group ($-CH_3$) coupled with one proton (signal 3), a doublet of quadruplet at 2.520 ppm which is attributed to a methylene group ($-CH_2$) adjacent to an asymmetric carbon atom bearing a single proton (signal 2), and a multiplet at 5.260 ppm characteristic of the methane group (-CH) (signals 1). H^1 NMR of copolymer PHBV (B): the presence of valerate monomer incorporates the signals 4, 5 and 6, attributed to the methyl group ($-CH_3$) at 0.99 ppm, a methylene ($-CH_2$) resonance at 1.6 ppm and methyne (-CH) resonance at 5.260 ppm, respectively. GPC spectra of PHB (C) and PHBV (D).

Figure 3. Ti surface characterization: FESEM micrograph and surface roughness of Ti surface polished (A), after Plasma treatment (B) and after treatment with NaOH (C). Scale bars correspond to 600 nm. Contact angle of PHB and PHBV solutions on the different Ti surfaces. There are significant differences between the contact angle form with non-treated PHB solution on Ti surfaces and oxygen plasma treated with NaOH. *$p < 0.05$: statistically significant (D).

Figure 4. FESEM images of gentamicin sulfate spheres. Average of particle size correspond to (45,70 ± 23,24) μm with a normal distribution. Scale bars correspond to 40 μm (A). Non-homogeneous Genta spheres deposition on Ti surfaces with PHB as drug carrier. Scale bars correspond to 500 μm (A).

Figure 5. Characterization of biopolymer coatings. FESEM images of biopolymer coatings with different immersions: (A) PHB partial coating with 1 immersion, (B) PHB partial coating with 3 immersion and (C) PHB total coating with 6 immersion, (D) PHBV partial coating with 1 immersion, (E) PHBV partial coating with 3 immersion and (F) PHBV total coating with 6 immersion. Scale bars correspond to 300 μm. (c, f) FESEM images of totally covered coatings of PHB and PHBV at higher magnifications. Scale bars correspond to 40 μm. (G) Values of roughness and thickness of the coatings. (H) PHB and PHBV coating with 3 and 6 immersions. *$p < 0.05$: statistically significant.

Figure 6. Cytotoxicity results (UNE-EN ISO 10993-5)

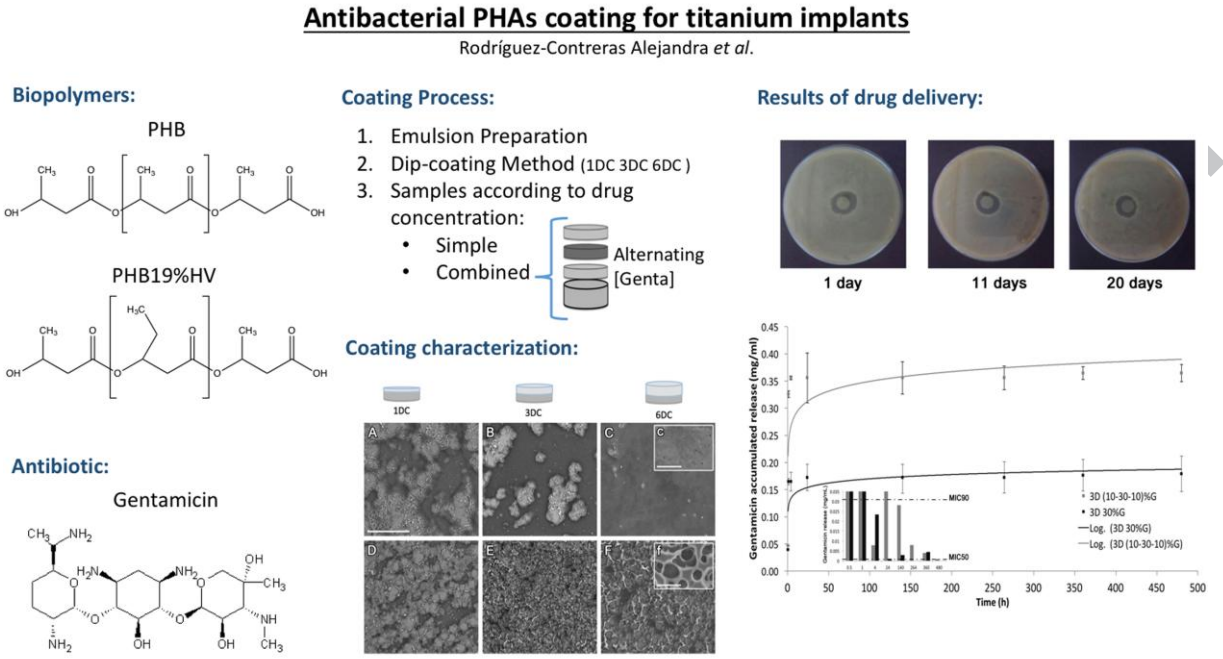
Figure 7. Drug release of PHB and PHBV coatings of different immersions. Main graphics show gentamicin accumulated release with time, while the graphic insets show the quantity of gentamicin released in every time (A). FESEM micrograph's of PHB

(left) and PHBV (right) coatings (6D with 30% Genta) after 2 weeks in PHS and 37 ^{o}C. Scale bars correspond to 300 μm and the one in the inset 40 μm.

Figure 8. Comparison of drug release profiles of PHBV coatings between 30% Genta amount in all layers, and coatings with alternating concentrations of gentamicin per immersion. The primary graphics show gentamicin accumulated release over time, while the graphic insets show the quantity of gentamicin released in every mesurement.

Figure 9. Diameter of halo of inhibition growth of *E. coli* and *S. aureus* of PHBV samples with 3 (3D) and 6 (6D) dip-coating, combined (10-30%) (A) and with simple (30% Genta) (B) gentamicin concentration. Controls: Ti surfaces without coating and Ti surfaces with PHBV coating without Genta did not show any halo of inhibition growth. (C) Image of the petri plates showing the halos of inhibition growth for *E. coli* after 1 day, 11 days and 20 days of incubation of Ti surfaces coated with PHBV (3 immersions and 30% Genta in all coatings).

Graphical abstract



Highlights:

1. Optimum coating on Ti surfaces with PHB and PHBV for drug delivery
2. Different dip-coatings layers offer different drug release profiles
3. The designed biopolymer systems prevent the bacterial attachment