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**Abstract**

Novel researches are focused on the prevention and management of post-operative infections. To avoid this common complication of implant surgery, it is preferable to use new biomaterials with antibacterial properties. Therefore, the aim of this work is to develop a method of combining the antibacterial properties of antibiotic-loaded poly(3-hydroxybutyrate) (PHB) nano- and micro-spheres and poly(ethylene glycol) (PEG) as an antifouling agent, with titanium (Ti), as the base material for implants, in order to obtain surfaces with antibacterial activity. The Ti surfaces were linked to both PHB particles and PEG by a covalent bond. This attachment was carried out by firstly activating the surfaces with either Oxygen plasma or Sodium hydroxide. Further functionalization of the activated surfaces with different alkoxy silanes allows the reaction with PHB particles and PEG. The study confirms that the Ti surfaces achieved the antibacterial properties by combining the antibiotic-loaded PHB spheres, and PEG as an antifouling agent.
Modification of titanium surfaces by adding antibiotic-loaded
PHB spheres and PEG for biomedical applications

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Abstract Novel researches are focused on the prevention and management of post-operative infections. To avoid this common complication of implant surgery, it is preferable to use new biomaterials with antibacterial properties. Therefore, the aim of this work is to develop a method of combining the antibacterial properties of antibiotic-loaded poly(3-hydroxybutyrate) (PHB) nano- and micro-spheres and poly(ethylene glycol) (PEG) as an antifouling agent, with titanium (Ti), as the base material for implants, in order to obtain surfaces with antibacterial activity. The Ti surfaces were linked to both PHB particles and PEG by a covalent bond. This attachment was carried out by firstly activating the surfaces with either Oxygen plasma or Sodium hydroxide. Further functionalization of the activated surfaces with different alkoxyamines allows the reaction with PHB particles and PEG. The study confirms that the Ti surfaces achieved the antibacterial properties by combining the antibiotic-loaded PHB spheres, and PEG as an antifouling agent.

1 Introduction

One of the most common problems after implant surgery is postoperative bacterial infection. Bacteria attach and proliferate on surfaces of biomedical devices and implants. Early phase infection after implantation is the riskiest time for infection for only some implants. Dental implants, for instance, pose a much higher risk of complication after long periods of implantation. The growing colony encapsulates itself with a protective exocellular bacterial polysaccharide layer, thus creating a biofilm, which is much harder to eliminate than circulating bacteria [1, 2]. These infections can pose serious problems causing the failure of the implant, often requiring re-operation and replacement of the infected device. This produces, besides considerable pain and discomfort for the patient, the associated costs. Despite considerable research and development efforts, the problem of infections related to biomedical devices and implants persists. Therefore, there is a strong need to mitigate bacterial colonization by providing the surfaces of biomedical devices and implants with features that are unfavorable for bacterial attachment and proliferation [3]. In this work, antibacterial coatings have been designed to prevent the initial adhesion of bacteria and their proliferation onto the implant surface. In order to accomplish this, surfaces with antibacterial properties were developed with the combination of different materials.

On one hand, Ti and its alloys are well known to be biocompatible and used as a base material for many types of implants. Ti, due to its high strength, biocompatibility
and corrosion resistance, can be used as a hard tissue implant such as artificial bone, hip joint, dental implant or bone plate. The biocompatibility of Ti implants can be attributed to their excellent resistance corrosion and to a surface protein layer formed under physio-logical conditions. This protein layer actually makes the surface suitable for bacterial colonization and biofilm formation. In the early phase after implantation, the local defense system is severely disturbed by the surgical trauma, and so it is the most dangerous time for infection. The reduced defense mechanism facilitates colonization of bacteria and infection may result [4, 5]. On the other hand, PHB is a biopolymer from the family of polyhydroxyalkanoates (PHAs), which is produced by microorganisms from removable resources [6]. Among natural and synthetic biodegradable polymers such as polyglycolic acid or polylactic acid, PHB is found to be remarkable for its applications in drug delivery due to its excellent biocompatibility and biodegradability [7, 8]. In addition, the biopolymer production via microbial fermentation prevents the presence of toxic products in the synthetic polymerization process [9]. Also, the hydrolytic degradation of PHB leads to obtaining the monomer α-3-hydroxybutyric acid which is a common blood constituent (a ketone body produced by the liver from fatty acids, ketogenic) [10].

Therefore, this biopolymer was used in a previous work for the formation of Doxycycline (Dox)-loaded micro- and nano-spheres [13]. Doxy is a well-known broad-spectrum antibiotic, which is effective against both gram-positive and gram-negative bacteria, protozoa, and various anaerobes. As a tetracycline analogue, it can work as a bacteriostatic which is capable of inhibiting the bacterial protein synthesis at the ribosomal sites. It has been frequently used in treating destructive periodontal diseases such as juvenile periodontitis or acute periodontal abscesses. In order to avoid possible side effects such as gastro-intestinal disturbance and photosensitivity, and reach the infection with an effective drug concentration, Doxy was entrapped in a PHB matrix [14].

The antifouling properties of PEG-based coatings have been widely reported in the literature [15–17]. PEG is an inert, water-soluble polymer that has extensive application as a biocompatible coating. PEG-polymers resist protein and polysaccharide adsorption on the surfaces, and consequently hinder cellular adhesion. Thus, PEG inhibits biofilm formation and prevents biofouling. The protein resistance conferred to a surface by attaching PEG is related to unfavorable elastic and osmotic stresses which generate a repulsive force, the magnitude of which depends on the surface density and chain length of the PEG. Some studies reveal that protein repulsion is stronger with a long PEG chain length and a high surface density [18, 19]. Some of the techniques that have been used to attach PEG to surfaces are physical and chemical adsorption, covalent attachment, and block or graft copolymerization [20].

Altogether, the aim of the present work is to develop new strategies of combining the antibacterial properties of Doxy-loaded PHB nano- and micro-spheres with Ti, as a base material for implants, in order to obtain surfaces with antibacterial activity. Moreover, a novel approach to observing the synergy effects of PEG chains together with Doxy-loaded PHB spheres has been studied.

### 2 Materials and methods

#### 2.1 Materials

Ti bar was obtained from c.p. grade 2 Ti provided by TECHNALLOY. The bar was cut into disks that were 3 mm thick and 10 mm in diameter. Doxy-loaded PHB nano- and micro-spheres were obtained from a previous work [13]. From that previous work, the spheres used in this study were the corresponding ones labeled US/TU13 with a diameter size of (17.43 ± 6.01) μm and US/TU14 with a diameter size of (0.32 ± 0.16) μm, for micro- and nano-spheres respectively. Poly(ethylene glycol) bis(3-aminopropyl) (PEG) (Mn ~1500) was supplied by Sigma-Aldrich. The solid bacterial media used was Chromocult® for Escherichia coli. Tryptone Soya Broth (TSB) was used as a liquid medium bacterial culture.

#### 2.2 Preparation of Ti disks

Mirror-like, smooth surfaces (Ra ≤ 40 nm) were achieved by grinding with SiC papers of a decreasing grit size (from P800 to P2400—European P-grade standard), followed by polishing with suspensions of alumina particles (6 and 1 μm particle size) on cotton cloths. Prior to functionalization, samples were ultrasonically rinsed with cyclohexane, isopropanol, distilled water, ethanol and acetone. Samples were stored dry under a vacuum.

#### 2.3 Ti surface treatments

##### 2.3.1 Surface activation

2.3.1.1 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 purging with 99.5 % pure oxygen and high vacuuming (3 times) the samples were exposed to low electromagnetic radiofrequency radiation (between 8 and 12 MHz) for 10 min.
2.3.1.2 Sodium hydroxide treatment (NaOH) The Ti disks were immersed in 5 M of previously prepared sodium hydroxide in closed polypropylene flasks. They were placed into a furnace at 60 °C for 24 h. Afterwards the samples were cleaned and immersed in Milli-Q water for 30 min, then rinsed with the same water and acetone. They were dried with nitrogen and, finally, stocked in a vacuum.

2.3.2 Silanization with 3-aminopropyltriethoxysilane (APTES)

In order to covalent link the Ti surfaces with Doxy-loaded PHB spheres, a silanization reaction was first carried out. Ti surfaces activated with either oxygen plasma or NaOH solution were placed, with a magnetic stirrer, at the bottom of an Erlenmeyer which was previously properly cleaned with H2O and acetone, and dried with nitrogen. 10 ml of anhydrous toluene and 0.2 ml of APTES 0.04 M were added into the Erlenmeyers closed with a rubber stopper, with vacuum and purged nitrogen gas. The reaction mixture was left under agitation for 1 h at 70 °C. Afterwards, samples were transferred to a beaker containing toluene, and were sonicated for 5 min in order to eliminate the loosened silane. Subsequently, the samples were washed three times with toluene, once with acetone, isopropanol, and ethanol, and three times with acetone, and then dried with nitrogen. Finally, the samples were placed in a petri dish and the silanes were cured at 120 °C for five minutes. Thus, the samples were ready for reaction with Doxy-loaded PHB micro- and nano-spheres.

2.3.3 Silanization with 3-chloropropyltriethoxysilane (CPTES)

In order to covalent link the Ti surfaces with PEG and the further addition of the Doxy-loaded PHB spheres a silanization reaction was carried out CPTES. Ti surfaces activated with either plasma or NaOH were placed, with a magnetic stirrer, at the bottom of an Erlenmeyer which was previously properly cleaned with H2O and acetone, and dried with nitrogen. 10 ml of anhydrous toluene, 0.1 ml of diisopropylethylamine (DIEA) 0.06 M and 0.2 ml of CPTES 0.08 M were added into the Erlenmeyers closed with a rubber stopper, with vacuum and purged nitrogen gas. The reaction mixture was left under agitation for 1 h at 70 °C. Afterwards, samples were transferred to a beaker containing toluene, and were sonicated for 5 min in order to eliminate the loosened silane. Samples were then washed with toluene, isopropanol, water, ethanol, and acetone (three times with each solvent), and dried with nitrogen. Thus, the samples were prepared for reaction with PEG.

2.3.4 Ti surfaces reaction with PEG

Following the silanization of the Ti surfaces with APTES, the samples were placed, with a magnetic stirrer, into a hermetically sealed beaker. Then, 0.26 g of PEG, 0.01 g of sodium carbonate, and 40 ml of mille-Q water were added. The reaction was left for 24 h at 200 rpm and at room temperature. Thus, the samples were ready for reaction with Doxy-loaded PHB micro- and nano-spheres.

2.3.5 Ti surfaces reaction with Doxy-PHB spheres

All Ti surfaces with plasma or NaOH activation treatments as well as with or without PEG were linked together with the Doxy-loaded PHB micro- and nano-spheres. The silanized samples were immersed in dimethylformamide (DMF) 15 mM, and N,N-diisopropylethylamine (DIEA) 60 mM. Micro- and nano-spheres were added separately in the amount necessary to obtain a concentration of 15 mM. O-Benzotriazole-N,N,N,N'-tetramethyl-uronium-hexafluorophosphate (HBTU) (45 mM) was tested as a coupling agent [21]. The reaction was carried out at room temperature for 2 h with orbital agitation. The samples were then washed in DMF twice, rinsed with water and acetone, and dried with nitrogen. Finally, they were stored in a vacuum for further experiments. Figure 1 shows the scheme reaction between the benzotriazole based ester (HBTU) of PHB with a primary amine.

Figure 2 shows a summary scheme of the reactions proposed in this study for the attachment of Doxy loaded-PHB spheres via APTES (Fig. 2a) and via CPTES and difuntionalised-PEG (Fig. 2b).

2.3.6 Sphere stability

Ti surfaces with PHB particles were exposed to high vortex-agitation for 5 min. The presence of spheres was observed before and after the agitation via FESEM and the loss of spheres was quantified by analyzing images.

2.4 Field Emission Scanning Electron Microscope (FESEM)

The Ti surfaces were platinum/palladium coated (coating thickness between 10 and 15 nm) under a vacuum by means of a CRESSINGTON sputter coater (208 HR, Germany) and scanning electron micrographs were obtained using a Jeol (JSM-7001F, Japan) scanning electron microscope, operating at 15 kV. Energy Dispersive X-ray Spectroscopy (EDS) was also used for the elemental analysis of the surfaces.
2.5 Contact angle (CA)

A Wettability study on the Ti surfaces was performed by the sessile drop method, using a 1 L with a deposition rate of 1 L/s, and working at room temperature (22 °C) and in a controlled atmosphere. The measurements were taken using a Contact Angle System OCA15 Plus (Dataphysics, Filderstadt, Germany) with the sessile drop method.

Ultrapure distilled water (Millipore Milli-Q, Merck Millipore Corporation, USA) and diiodomethane (DII) (Sigma-Aldrich, Spain) were used as working fluids.

2.6 X-ray photoelectron spectroscopy (XPS)

X-ray photoelectron spectroscopy (XPS) was used to analyze the chemical composition of the samples surface. XPS spectra were acquired with an XR50 Mg anode source operating at 150 W and a Phoibos 150 MCD-9 detector (D8 advance, SPECS Surface Nano Analysis GmbH, Germany). High-resolution spectra were recorded with a pass energy of 25 eV at 0.1 eV steps and pressure below 7.5-10-9 mbar. Binding energies were referred to the C 1 s signal. Two samples were studied for each working condition.

2.7 In vitro antibacterial assay

Ti treated surfaces were tested in vitro with Escherichia coli as a gram-negative bacterium. The bacterial strain was cultivated for 24 h. From it, precultures were incubated overnight in 10 mL tubes containing 5 mL of TSB at 37 °C. Then, a bacterial solution was prepared with TSB, adjusting the OD to 0.2 at 600 nm (bacterial concentration about 10⁸ CFU/ml). The samples were introduced in a multi-well plate with 1 mL of the bacterial suspension and incubated for 2 h at 37 °C. Upon continued vortexing (5 min) of the Ti samples, viable cell counts in the supernatant were determined by cultivating them in the respective solid media for 24 h at 37 °C, and counting the colonies formed [22].

3 Results

3.1 Surface activation

In this study the nano-texture of the surfaces treated with NaOH and the smoothness of the ones treated with plasma are confirmed through FESEM observations. Figure 3 shows the FESEM micrographics’ of both Ti samples treated with plasma and with NaOH. The contact angle of these surfaces shows that the Ti surfaces treated with plasma are more hydrophilic than the ones treated with NaOH (Fig. 4). The relative intensities (RI) from XPS results are shown in Table 1. The titanium surfaces treated with plasma show an increase in the OH group percentage from the total oxygen. The OH ratio for Plasma treatment is higher than the ratio for NaOH treatment (OH⁻/O²⁻ = 0.59 for NaOH and OH⁻/O²⁻ = 0.92 for plasma).

3.2 Silanization with 3-aminopropyltriethoxysilane (APTES)

The results of contact angle and FESEM-EDS give evidence of the silanization reaction with APTES on the activated Ti surfaces. On one hand, there is an increase in the hydrophobicity of the surfaces due to the amino group at the end of the molecule chain of APTES (compare Fig. 4 Ti + PLASMA with Ti + PLASMA + APTES and Ti + NaOH with Ti + NaOH + APTES). On the other hand, the elemental analysis spectra from FESEM-EDS of the Ti surfaces treated with APTES show the presence of Nitrogen, which also corresponds to the primary amine of the alkoxysilane. Therefore, the change in the hydrophobicity and the presence of Nitrogen on the surfaces are evidence that the silanization reaction took place.

Once the silanization reaction was confirmed, the attachment of the Doxy-loaded PHB spheres was performed. The presence of PHB spheres on the Ti surface was confirmed by FESEM. In the case of Ti samples activated with plasma and then silanized with APTES for the covalent attachment of the PHB spheres, the FESEM results show that only nano-size particles were attached.
Figure 5a, b, c, d, i, j, k, l show titanium surfaces treated with APTES on which micro- and nano-size spheres were added. The presence of small spheres on the Ti surfaces where micro-PHB particles were added to the reaction can be explained due to the high particle-size dispersion of the Doxy-loaded PHB micro-particles \([(17.43 \pm 6.01) \mu m]^{[13]}\). On the Ti surfaces treated with plasma, only PHB-loaded spheres in the nano-scale were attached (Fig. 5a and c). However, it was possible for micro-spheres to be attached to the titanium surfaces activated with NaOH.
This can be compared in Fig. 5a with i and c with k, which correspond to FESEM micrographs’ of micro-spheres added to Ti surfaces treated with plasma and NaOH (taking into account the different magnification of the micrographs). The coupling agent HBTU was studied in order to optimize this reaction, however only a minor difference between the samples treated with HBTU and without it can be observed. Samples using HBTU as a coupling agent show a slightly larger amount of spheres on the surface (for instance, compare Fig. 5a with c).

### 3.3 Silanization with 3-chloropropyltriethoxysilane (CPTES)

As mentioned above, a variation in the contact angle is evidence of surface modification on substrates. The contact angle of the Ti surface treated with CPTES decreased compared to the non-treated surface (Fig. 4). Comparing both alkoxylanes, APTES and CPTES, a higher contact angle was obtained on the Ti surface silanized by APTES. Theoretically, the chlorine group from CPTES is less hydrophilic than the primary amine group in APTES [23]. In our study, both silanes provided hydrophobicity to the surfaces (comparing the contact angle of the Ti samples activated with plasma or NaOH, with the same surfaces treated with APTES and CPTES). This result is in accordance with the literature [31]. The greater hydrophobicity of the surfaces with APTES is probably due to the tendency that it has to form multilayers and, consequently, to have a higher proportion of alkyl chains. In both cases, the change on the contact angle is an indication of the silanization reaction. More evidence of these reactions is the presence of Nitrogen (0.39 keV) and Chlorine (2.62 keV) in the FESEM-EDS spectra of the treated Ti surfaces. Regarding the XPS results, a significant increase in silicon percentage content relative to the control sample (untreated) was observed in all silanized samples with both APTES and CPTES.
CPTES. This increase is indicative of the presence of the organosilane on the surface metal and suggests that the silanization process has occurred. It is important to underline that a new component appeared for silanized surfaces corresponding to siloxane bond O–Si–O at 103.3 eV, thus demonstrating that silane molecules were chemically bound to alloy surfaces via residual ethoxy groups on the metal substrate and not physisorbed. For example, Fig. 6a and b show the deconvolution of the Cl 2p and Si 2p spectra, respectively; while Fig. 6c shows the chemical composition present on the surface silanized with CPTES.

Afterwards, functionalized PEG was added to the system in order to react with the surface silanized with CPTES. The hydrophobicity of the samples corroborates the attachment of PEG on the Ti surfaces, since the samples with PEG are more hydrophilic than the ones with only CPTES. Therefore, the contact angle slightly decreases for samples containing PEG compared to the Ti surface with only CPTES (Fig. 4). Also, the FESEM-EDS spectra and XPS results show the presence of Nitrogen, not showing any peak corresponding to the Chlorine group in the EDS spectra and a very small one in the XPS results corresponding to residual Chlorine. Figure 7 shows the high resolution N 1 s spectra as well as the chemical composition on the surface. The deconvolution (Fig. 7a) consisted of two components: a small peak at 401.84 eV corresponding to protonised amines (NH$_3^+$) and a larger one at 400.08 eV corresponding to a primary free amine not protonised (NH$_2$).

The last step was the addition of Doxy-loaded PHB spheres on the Ti surface treated with PEG. Particles were observed on all Ti surfaces under electron microscopy, FESEM. Figures 5e, e’, f, g, h, m, m’, o, p show micro- and nano-size spheres added on the titanium surfaces treated with difunctionalised PEG. As in the previous case with APTES, only PHB-loaded spheres in the nano-scale were attached to the surfaces activated with Oxygen plasma (compare Fig. 5e, g) (figure e’ shows a micrograph at higher magnifications ~30000×—from Fig. 5e). Also, it was possible for micro-spheres to be attached to the titanium surfaces, but only when the surface was activated with NaOH (compare Fig. 5e with m and g with o). Regarding the coupling agent, the presence of HBTU does not make much difference in the sphere attachment (for instance, compare Fig. 5e with g, f with h or m with o).

### 3.4 Sphere stability

In order to study the stability of the spheres, Ti surfaces were exposed to high agitation. Results confirmed that PHB particles were still on the Ti surfaces after the agitation process and FESEM image analyses show a loss of PHB-sphere of 2.7 % for samples silanized with APTES and 4.2 % for samples treated with functionalized PEG by vortex-agitation for 5 min. It was observed that when HBTU was added to the bonding reaction between APTES with PHB and difunctionalised-PEG with PHB, the sphere loss decreased, 1.3 and 2.2 %, respectively. Figure 5m shows the sample Ti + NaOH + CPTES + PEG + HBTU + MICRO/PHB after the stability test. The amount of spheres slightly decreases (2.2 %) compared to the same sample before the stability test (Fig. 5m). The shape of the spheres varies showing a coarse texture probably due to the polymer. The fact that the particles were observed on the surface after being exposed to high agitation is an indication of the covalent attachment between the metal and the biopolymer via silanization. This also confirms the stability of the particles on the surface.

### 3.5 In vitro bacterial assay

For the case of Ti surfaces modified by APTES and Doxy-loaded PHB spheres, Fig. 8 shows the number of bacteria (CFU/mm$^2$) attached to these surfaces for the two activation treatments, Oxygen plasma and NaOH. A significant improvement in the antibacterial skills of the surfaces can be observed in all samples. This improvement is more evident for the pre-treated surfaces with NaOH solution. Only the sample Ti + Plasma + APTES + PHB with micro-spheres and HBTU shows a slightly higher CFU compared to the other samples, explained by the fact that micro-spheres were not able to properly attach on the Ti surface as observed in the FESEM macrographs in Fig. 5a.

In the case of Ti surfaces modified by CPTES with PEG and Doxy-loaded PHB spheres added, Fig. 9 shows the bacterial count (CFU/mm$^2$) for both Ti surface pretreatments. Ti surfaces treated with CPTES and PEG already show a significant decrease on the bacterial counting. This corroborates the antifouling effect of the PEG. Once the PHB spheres were added to the system the treated surfaces show even better antibacterial activity. This behavior is clearer in the samples treated previously with NaOH.

### 4 Discussion

In order to obtain surfaces with antibacterial activity, spheres of PHB loaded with Doxy as an antibiotic were used to cover Ti surfaces. PEG was also studied in this work for its antifouling skills. In this case, a difunctionalised-PEG was used as a linker to connect the Ti surfaces with Doxy-loaded PHB spheres. Two different PHB-sphere sizes, on the micro- and nano-scale, were used in order to study their effect. The novelty of this work lies in the combination of three different biomaterials with different...
properties by means of a covalent linkage via chemical reaction with alkoxylines (Fig. 2).

4.1 Activation treatments

Plasma cleaning and sodium hydroxide treatments were used to activate Ti surfaces. On one hand, the oxygen plasma technique allows modification of surfaces by attachment or adsorption of functional groups to tailor surface properties for specific applications. In particular, it removes organic contaminants by chemical reaction with highly reactive oxygen radicals and it promotes surface oxidation and hydroxylation (OH groups) increasing its surface wettability [24]. On the other hand, chemical treatment in alkali solutions is a strong contender to impart submicron- or nano-roughness to Ti surfaces [25–27]. Recently reported experiments also show that sodium hydroxide treatment of the Ti increases the surface energy, and consequently the bioactivity is higher, as is the number of OH groups on the surface [28]. In this work, contact angle and XPS results corroborate that the plasma treatment incorporates more OH groups on the Ti surfaces than the NaOH treatment as it is more hydrophilic, which coincides with what is reported [29].

4.2 Silanization with 3-aminopropyltriethoxysilane (APTES)

Silanization is the most widely used method of immobilizing biological molecules on model surfaces [23, 30]. In this study, APTES was first bonded to the Ti activated surfaces. Then, Doxy-loaded PHB spheres were attached to these surfaces by condensation reaction between the carboxylic acid at the end of PHB chains and the primary amine, to obtain an amide bond (Fig. 2a). HBTU was studied as a coupling agent to improve this linkage (Fig. 1).

Once the silanization reaction was confirmed by contact angle, FESEM-EDS and XPS results and the amine groups available to bond with the carboxylic groups of the PHB, the antibiotic-loaded PHB spheres were added to the surface for the linking reaction to take place. The results of PHB sphere attachment can be attributed to the different activation treatments and their impact on the surface topography: specifically surface and roughness. Spheres in the nano-scale were attached to the surface of Ti activated with plasma, and spheres in the micro-scale were also attached to the Ti surfaces activated with NaOH treatment. The increase in surface roughness due to the application of NaOH activation treatment provides the Ti with more specific surface and anchoring points, both electrostatic and covalent, yielding the necessary stability for the micro-spheres to be able to attach to the Ti surface. In general, nano-sized spheres were better linked on the titanium surfaces than the micro-sized ones, as they have a more specific surface and are therefore more stable.

Consequently, two strategies for attaching either micro- or nano-size Doxy-loaded PHB spheres were found in this first part of the study. This makes it possible to choose the particle size depending on the application. Ti implants with a smooth surface can be covered with nano-sized spheres by applying a plasma treatment as for example the neck of dental implants, while implants, which may need a rougher surface can be covered with both micro and nano sized particles like blasted implants or porous implants such as intervertebral discs. In both cases, the antibacterial activity of the material makes a contribution.

4.3 Silanization with 3-chloropropyltriethoxysilane (CPTES)

CPTES has the capability to bind nucleophiles such as amines directly in basic conditions due to the presence of the chlorine group at the end of its chain which acts as an electrophilic center. The free primary amine has a free pair of electrons to share, thus making it a center with an excess of electronic density. This allows CPTES to react with peptides and proteins [23, 28]. However, neither peptides nor proteins were used in this study. The antifouling properties that PEG possesses were expected to work in this study to avoid the bacterial attachment to the surface together with the antibacterial activity that the PHB-spheres have due to the Doxy contained. Thus, a PEG containing two primary amine groups was used to react with CPTES on one side, and to bind with the PHB spheres on the other side. Figure 2b shows a scheme of the chemical reaction of CPTES with the Ti surfaces activated with either Plasma or NaOH treatment.

The silanization reaction with CPTES was verified by means of contact angle, FESEM-EDS spectra and XPS
results. XPS results especially confirmed that the reaction took place. Compared to other published studies such as the work of Sevilla et al. [31], the values of the Chlorine and Silicon percentages were as expected for the reaction between the activated Ti surfaces and the CPTES. Therefore, these results confirm the silanization reaction.

Once it was confirmed that CPTES was properly and covalently attached to the Ti surfaces, difunctionalised PEG was added to the system. The different methodologies used in this study confirm and prove the attachment of PEG on the Ti surfaces: the hydrophobicity of the samples, the presence of Nitrogen peak and absence of Chlorine peak on FESEM-EDS spectra and the presence of Nitrogen XPS results. Regarding the hydrophilicity and the contact angle results, PEG reduced the contact angle of the surfaces because it is highly hydrophilic. The PEG added to the Ti surface tends to decrease the contact angle, as can be observed in the Fig. 4 with the samples activated with NaOH. This effect is not so evident for the samples treated with plasma. This might be because they have a less
specific surface, and probably less PEG chain density. This hypothesis can be corroborated by the antibacterial efficiency of the surfaces. If Fig. 9a (Ti + Plasma + CPTE-S + PEG + PHB) is compared with Fig. 9b (Ti + NaOH + CPTES + PEG + PHB), the best results are obtained with the Ti surfaces treated with NaOH. With this activation treatment, a higher specific surface is obtained and therefore better linkage with PEG, as well as with the PHB micro-spheres. Considering XPS results, the presence of two peaks in the deconvolution of the Nitrogen (a small peak corresponding to protonised amines and a larger one corresponding to a primary free amine not protonised) points out that the PEG chains are in the required and expected position for further PHB attachment [32–34]. Finally, Doxy-loaded PHB spheres were added to the modified Ti surfaces and, as showed in the FESEM micrographs, micro- and nano-spheres were attached to the surfaces, showing good stability after being exposed to a high shaking process. Thus, the attachment of the Doxy-loaded PHB spheres via CPTES with PEG was successful.
It was found that high concentrated polyethylene glycol solutions have significant antibacterial activity against various pathogenic bacteria, including *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Staphylococcus aureus*. This antibiotic effect of PEG can be attributed to two effects: lowering of water activity and the specific action of high concentrated PEG molecules on bacterial cells [35]. However, in this study the antimicrobial activity of PEG might be related to its hydrophilic property since the PEG layer does not provide a high PEG concentration.

The novelty of this study is focused on this last step. After the Ti surface functionalization with the two different alkoxy silanes, the addition of Doxy-loaded PHB spheres and PEG took place. Only a few works have been found with the combination of PHB and PEG and all of them are related to polymer blending [30] or copolymerization [32, 36, 37]. Only Wang [38] combined PHBV with PEG in a similar reaction, but with the aim of attaching polypeptides to the surface to improve the biocompatibility of the substrate. Therefore, this is the first time that this combination has been arranged in order to give antibacterial properties to a Ti surface by combining the antibiotic-loaded PHB particles with PEG as an antifouling agent.

### 4.4 Coupling agent (HBTU)

HBTU is a popular in situ activating reagent commonly used in solid phase peptide synthesis. Activation with HBTU is cheap and it is much faster and more complete than carbodiimide-mediated reactions and results in shorter cycle times and increased coupling efficiency. It is reported to convert carboxylic acids into amides efficiently and practically. This process may be used with a wide range of carboxylic acids including N-protected amino acids [21, 23, 29]. When HBTU reacts with a carboxylic acid it forms an active benzotriazole based ester which can react with a primary amine. Figure 1 shows the scheme reaction between the benzotriazole based ester of PHB with the primary amine from APTES or from the difunctionalised PEG. This chemical reaction is detailed somewhere else [21]. In this study, HBTU does not considerably increase the amount of spheres added to the Ti surfaces. However, it was observed in the stability test that it helps to covalently add the Doxy-loaded PHB spheres.

### 4.5 In vitro bacterial assay

In this work, a gram-negative bacterial strain was used to study the effect of the titanium surface treated with Doxy-loaded PHB spheres and PEG. *E. coli* is one of the common gram-negative isolates from post-operative wounds [39]. The in vitro antibacterial assay shows very appealing results, since all the surfaces tested with the attachment of antibiotic-loaded PHB spheres presented antibacterial activity.

In this study there have been two main strategies for the production of antibacterial Ti surfaces. On one hand, the
attachment of PHB micro- and nano-spheres loaded with an antibiotic such as Doxycine by the silanization with APTES. These surfaces show antibacterial properties, especially the ones previously activated with NaOH. This improvement can be explained by the fact exposed before that NaOH activation treatment provides highly rough surfaces with more specific surface and anchoring points for the antibiotic-loaded PHB spheres to attach to on the surface. On the other hand, the use of PEG as an antifouling agent together with Doxy-loaded PHB-spheres was the second strategy for the production of antibacterial Ti surfaces. The antifouling effect of PEG was observable since there was a decrease in the bacterial attachment in both surface pretreatments [16]. Furthermore, there was an improvement in the antibacterial properties of the surfaces when PHB spheres were added to the surfaces. Therefore, the synergy of using PEG chains with Doxy-loaded PHB-spheres has been successful.

Regarding the cito-toxicity, in principle, the coating can be considered harmless. On one hand, the main chemical compounds like CPTES, APTES, and the coupling agent have already been used successfully in many studies [40, 41] and are not considered toxic. On the other hand, there exist advances in the applications of polyhydroxyalkanoate microspheres for drug delivery systems [42, 43], and it is well reported that PHB polymer is biodegradable and non-cito-toxic [44, 45]. However, it would be desirable to carry out further tests to confirm this.
out studies such as the specific drug release as well as studies of cytotoxicity for the proposed strategies in this study in the future, in order to ensure that the new coating system will not result in harmful effects.

5 Conclusion

The general objective of the study was successfully achieved since novel methods for the preparation of titanium surfaces with antibacterial properties have been developed. Covalent attachment of Doxy-loaded PHB spheres was successfully achieved using APTES as an alkoxysilane as well as using the synergistic effect of the PEG together with Doxy-loaded PHB spheres. In vitro bacterial assays confirm that both strategies lead a titanium surface with antimicrobial properties. The synergistic effect of the PEG together with Doxy-loaded PHB spheres reduces bacterial adhesion (Escherichia coli and Staphylococcus aureus). However, the best results were obtained by adding antibiotic-loaded PHB spheres via covalent attachment with the alkoxysilane APTES. In this case, plasma treatment for the activation of titanium surfaces can be considered a good alternative for attaching nano-PHB particles, while NaOH treatment can be used for the attachment of both micro- and nano-spheres.

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Reference

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