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Surface & Coatings Technology 441 (2022) 128571

Contents lists available at ScienceDirect

Surface & Coatings Technology

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

Chemical, physical, and mechanical characterization of chitosan coatings on a chemically pre-treated Ti6Al4V alloy

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ARTICLE INFO

Keywords:
Titanium
Chitosan
Coating

ABSTRACT

The coating of titanium with chitosan is a promising way to reduce the infection risk and to modulate the inflammatory response of bone implants, but no effective coating procedure exists at the moment. Two titanium surfaces are tested in this research: a mechanically polished (as reference) and a chemically pre-treated one. Three coating strategies are followed: the direct linking at different pH (acidic and neutral), covalent bonding through a chemical activation of the titanium substrate (tresyl chloride) or through a linker molecule (polydopamine). The obtained coatings have been characterized by means of SEM, AFM, FT-IR spectroscopy, zeta potential titration curves, tape adhesion test, and soaking at physiological pH for 2 weeks. The best results, in terms of the degree of the surface coverage and stability of the interface, are obtained on the chemically pre-treated surface. Comparing the different coating procedures, in descending order of efficacy, we find the direct linking at acidic pH, covalent bonding through chemical surface activation, use of a linker molecule, and direct linking at neutral pH. The combination of a proper coating procedure and pre-treatment of the surface is effective in enhancing the electrostatic bonding of the coating to titanium.

1. Introduction

Chitosan is considered as being a non-toxic and biologically compatible polymer. It is a natural biopolymer derived from the deacetylation of chitin, which is the structural element in the exoskeleton of crustaceans (such as crabs and shrimps) and cell walls of fungi. However, certain modifications implemented on chitosan could limit the biocompatibility and any residual reactants should be carefully removed. The biocompatibility of chitosan is largely attributed to its similarity to the extracellular matrix glycosaminoglycans [1]. Generally speaking, the chitosan with higher degree of deacetylation (91–95%) and lower average molecular weight (100,000–200,000) is preferred for scaffolds, because it has higher hydrophilicity, cell adhesion and proliferation. On the other side, also cytotoxicity is higher at high degree of deacetylation and more strongly related to the molecular weight than at lower degree of deacetylation [1]. In this work, a chitosan with a low degree of deacetylation is selected according to the good outcome in terms of the enhancement of the natural bone remodeling mechanism [2]. The tests performed about the use of this biopolymer in the human body do not report any allergic reaction after implantation, injection or ingestion. The degradation of chitosan occurs by enzyme action or hydrolysis [3]. The cost of chitosan itself as a component in high-value biomedical applications is considered marginal.

Chitosan exhibits low immunogenicity, excellent mechanical properties, and proven antimicrobial activity (for instance against *Staphylococcus aureus* and *Escherichia coli* [4]) generally explained on the basis of its cationic nature [5] [6]. The low molecular weight chitosan penetrates the bacterial cell walls, binding DNA, and inhibiting its transcription [7], while the high molecular weight chitosan binds the negatively charged components of the cell walls forming a barrier against the nutrients transport into the cell. The amount of the chitosan bonded with the bacterial cell wall depends on the environmental pH value and the degree of acetylation. A low environmental pH increases the positive charge of chitosan that favours binding to the bacterial cell wall [8]. The eventual development of the bacterial resistance against chitosan upon its increasing use is still to be investigated in details.

Because the peri-implant infections are still a serious issue for the dental and orthopedic implants, a lot of methods have been explored to coat implants with chitosan [9]. The different employed techniques are based on physical/electrostatic adsorption or covalent bonding [10].

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https://doi.org/10.1016/j.surfcoat.2022.128571
Received 18 April 2022; Received in revised form 18 May 2022; Accepted 24 May 2022
Available online 27 May 2022

Solution casting is a simple dipping method based on electrostatic interactions between positively charged chitosan and the negative charged oxide layer on titanium surfaces. It can be improved by surface pretreatments of the titanium surface by sandblasting or acid etching to increase roughness and surface charge. Solution casting is particularly interesting as it is cheap, fast, and does not require special equipment [11], but the bonding strength of the coating to the titanium surface is currently inadequate for clinical applications: a surface chemical treatment able to largely increase the negative surface charge on titanium is explored in this research to overcome the current limits. The chitosan can be also deposited on a surface using an electrophoretic process because the chitosan dispersed into an acidic solution (pH below 6) is positively charged and attracted to a negatively charged surface through an electric field [12,13]. This method often includes the blending of the chitosan with other materials such as CaP (calcium phosphate) for a better bone integration. The electrophoretic deposition can also be achieved via a layer-by-layer approach [14] such as by using alginate or heparin. The electrostatic interactions have been used [15] to attach the quaternized chitosan on polymers and metals, through its permanent positively charged ammonium groups, but this process involves different toxic reagents. Considering that process parameters of the electrophoretic process strongly affect the final coating, a time-consuming optimization is necessary, which is presently inefficiently accomplished by trial-and-error methods [16]: this techniques has not been considered in this research. Since the quaternized chitosan is water-soluble, a polymerized matrix such as an acrylic acid or multivalent anionic acids (sodium tripolyphosphate, sodium pyrophosphate, and sodium hexametaphosphate) is needed or a fast release must be expected [15,16].

A coating on a bone implant needs to be firmly bounded in order to withstand the mechanical forces derived from implant placement and to secure the implant position during osseointegration. Silanes or covalent bonding with quaternized chitosan have been used to guarantee a better attachment of the chitosan to a titanium surface [17,18]. Silanization is based on the reaction of an amino silane with the titanium oxide layer, resulting in covalent bonds. In a second step, glutaraldehyde reacts with the surface-bound silane and simultaneously presents aldehyde groups for interaction with the chitosan polymer. This process results in significantly superior bonding strength as compared to solution casting methods, and allows for direct control of the coating thickness. However, residual traces of glutaraldehyde could be released during biodegradation of the coating and damage the surrounding tissue [11]. Following the same principle, dopamine and tresylchloride linkers are here used instead of an amino silane to covalently bond chitosan coatings to titanium.

The aim of this work is characterize the effects of three different approaches for attachment of chitosan to polished or chemically pretreated titanium alloy and to characterize the resulting coatings. The selected chemical pre-treatment of the titanium substrate is expected to enhance the adhesion of the chitosan coating because of both the surface topography (multiscale micro and nano roughness) and chemistry (high density of hydroxyl functional groups). Three approaches are used for forming the chitosan coating: direct grafting (at acidic or neutral pH), the use of 2,2,2-trifluoroethanesulfonyl chloride (tresyl chloride) to introduce a good leaving group (surface chemical activation) [19] or polydopamine as a linker for covalent bonding [20] because polydopamine exhibits high reactivity towards the biomolecules containing the amide, amino, and thiol functional groups.

2. Materials and methods

2.1. Polishing and chemical treatment of the Ti6Al4V substrate

The Ti6Al4V titanium alloy was purchased as cylindrical bar (ASTM B348, gr5, Titanium Consulting and Trading) and cut into disks (1 cm diameter and 2 mm thick). The disks were polished using SiC papers, from 320 to 4000 grit, and subsequently washed in acetone (Supelco) and ultrapure water (MilliQ), once for 5 and twice for 10 min, respectively. After drying, they were stored in air. The polished samples are labelled PT.

The chemically treated substrates were prepared according to an already published and patented method [21–23]. Briefly, the specimens were polished as previously described, immersed for 1 min into a diluted hydrofluoric acid (Sigma Aldrich), washed, and treated for 2 h in a solution of hydrogen peroxide (PanReac) at 60 °C. The oxidation resulted in a nanoporous oxide layer with violet or green colour. The samples treated in this way are labelled CT.

2.2. Chitosan coating

The chitosan polymer used in this work was supplied by Genis hf. (Siglufjörður, Iceland). Acetic acid, hydrochloric acid, PBS, NaOH, tresyl chloride, dopamine hydrochloride, and tris(hydroxymethyl)aminomethane (Tris) were obtained from Sigma Aldrich.

2.2.1. Method 1 – direct coating

Two procedures were tested for the direct attachment of chitosan to the titanium surface: the dissolution of chitosan in acetic acid and the suspension of chitosan in a neutral phosphate buffered saline.

In the first procedure, 1% *w/v* chitosan was dissolved in 1% acetic acid. The chemically treated Ti6Al4V specimens (CT) or the polished ones (PT) were immersed into the solution for 5 min and then dried overnight at room temperature in air. The specimens were neutralized in a solution of 0.25 M NaOH for 0.5 h and then washed with deionized water (the name of these samples is CT_D_AA and PT_D_AA). In the second procedure, a 0.5% *w/v* PBS solution with pH 7.32 was prepared and stirred overnight with a magnetic stirrer; the concentration of the solution is limited by the solubility of chitosan at this pH. The CT specimens were immersed into the suspension for 3 min and then dried (the name of these samples is CT_D_PBS). The coated samples were stored in a desiccator.

2.2.2. Method 2 –surface chemical activation through tresyl chloride

Tresyl chloride was used to convert the surface hydroxyl groups of the chemically treated substrates (CT) into a sulfonyl ester groups that is as a good leaving group. The CT samples were soaked into tresyl chloride and stored at 37 °C for 2 days. Subsequently, the samples were washed with deionized water, a solution of deionized water and acetone (50:50), and acetone. A suspension of chitosan in PBS was prepared, as described above, and the CT samples were immersed in the solution for 24 h at 37 °C (the name of these samples is CT_TC_37). After the immersion, the samples were rinsed three times with deionized water, dried at room temperature, and stored in a desiccator.

Chitosan dissolved in acetic acid cannot be used to coat tresylated surfaces. The amino groups on chitosan are protonated in acetic acid and this prohibits the nucleophilic attack of the amino functional group necessary to displace the tresyl leaving group and to form a bond with the surface.

2.2.3. Method 3 – polydopamine treatment

Polydopamine is spontaneously formed by the pH-induced oxidative polymerization of the dopamine hydrochloride in an alkaline solution (pH > 7.5). 2 mg/ml of dopamine hydrochloride were dissolved into a 10 mM Tris solution and pH adjusted to 8.52 with HCl. The CT samples were soaked in the dopamine hydrochloride solution for 4 h at room temperature with continuous stirring. They were rinsed with deionized water and dried at room temperature. 0.5% *w/v* chitosan was dispersed in deionized water and the polydopamine coated samples were incubated in this solution for 24 h at room temperature with stirring. The name of these samples is CT_PD_W. In this case, chitosan was dispersed in water in order to preserve the pre-coating with polydopamine.

Chitosan dissolved in acetic acid cannot be used to coat polydopamine modified surfaces. Acidic conditions inactivate the chitosan

amino functional groups with respect to bond-forming with the polydopamine functional groups.

2.3. Characterization methods

A Supra 25 FE-SEM, Zeiss was used for Scanning electron microscopy (SEM) observation of the as prepared samples. A JCM-6000PLUS, JEOL was used for SEM observation of the coated samples after the tape test. Prior to the observation, the samples were gold coated in a sputter coater S150B (Edwards). The surface morphology was visualized with the SEM and image analysis (Image J - Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA) was used to quantify the degree of the surface coverage by the chitosan coating.

An atomic force microscope (AFM) XE-100 from Park Systems was used in the non-contact mode. The scan sizes were 1 × 1 μm^2 and 5 × 5 μm^2. The software Gwyddion [24] was used to extract topographical information from the AFM images. The roughness was measured on 1 μm × 1 μm AFM images.

The IR spectra were measured by ATR/IR, Nicolet iZ10 MX/microscopic IR, Nicolet iN10 MX (Thermo Scientific). Measurement was conducted in ATR mode between 400 and 4000 cm^{-1} with 32 measurement cycles.

Zeta potential titration curves as a function of pH were obtained by means of electrokinetic measurements (SurPASS, Anton Paar). Two samples were positioned face to face (into an adjustable gap cell) at the distance of 100 μm and a 0.001 M KCl electrolyte solution (starting pH around 5.6) was fluxed between the surfaces. The pH was varied by addition of 0.05 M HCl or 0.05 M NaOH. 4 measurements were acquired for each pH value. The acidic and basic ranges were measured through two separate measurements both starting from pH 5.6. The measurement in the acidic range was not possible on the coated samples because of swelling of chitosan, as expected.

The tape test was performed according to ASTM D3359-17 [25] in order to measure the adhesion of the coatings to the substrates. A grid of parallel cuts was obtained on each sample by a cutter, the surface was gently cleaned with a brush, the tape was positioned on all the surfaces and removed to test the coating adhesion. The surface was then visually inspected by SEM and optical microscopy and compared with the reference schemes reported in the standard, to estimate the coating removal within the areas delimited by the grid.

2.4. Stability in PBS

Selected samples were incubated in PBS for 2 weeks at 37 °C in order to test the stability of the coatings in an environment close to the physiological pH. The samples were subsequently characterized by SEM. The incubated samples are labelled as CT_D_AA_2w, CT-TC_37_2w, and CT_PD_W-2w.

3. Results

3.1. The surface of the substrates

The chemically treated Ti6Al4V (CT) is characterized by SEM and AFM. Fig. 1 shows the SEM images of the chemically treated surface, exposing a titanium oxide layer with a multiscale roughness, as observed earlier [22]: the micro scale roughness is due to the acid etching, while the nanoporosity is due to the following oxidation process in hydrogen peroxide. The 2D and 3D representations of the AFM acquisitions are reported in Fig. 1. The mean roughness (S_a) of the CT surface is 17.9 nm.

The polished substrate (PT) has a conventional grooved surface due to the mechanical polishing (images not reported); the roughness parameters measured through AFM are reported in Table 1.

All these data concerning the substrates are useful for a comparison with the coated surfaces (data reported in Table 1 and Fig. 2-3).

3.2. Coating results

3.2.1. Morphology and topography of the coatings (SEM and AFM)

Fig. 2a-h shows a comparison of the surfaces obtained through the different coating methods of the CT substrate at two magnifications. The appearance of the coated polished substrate (PT_D_AA) is analogous to

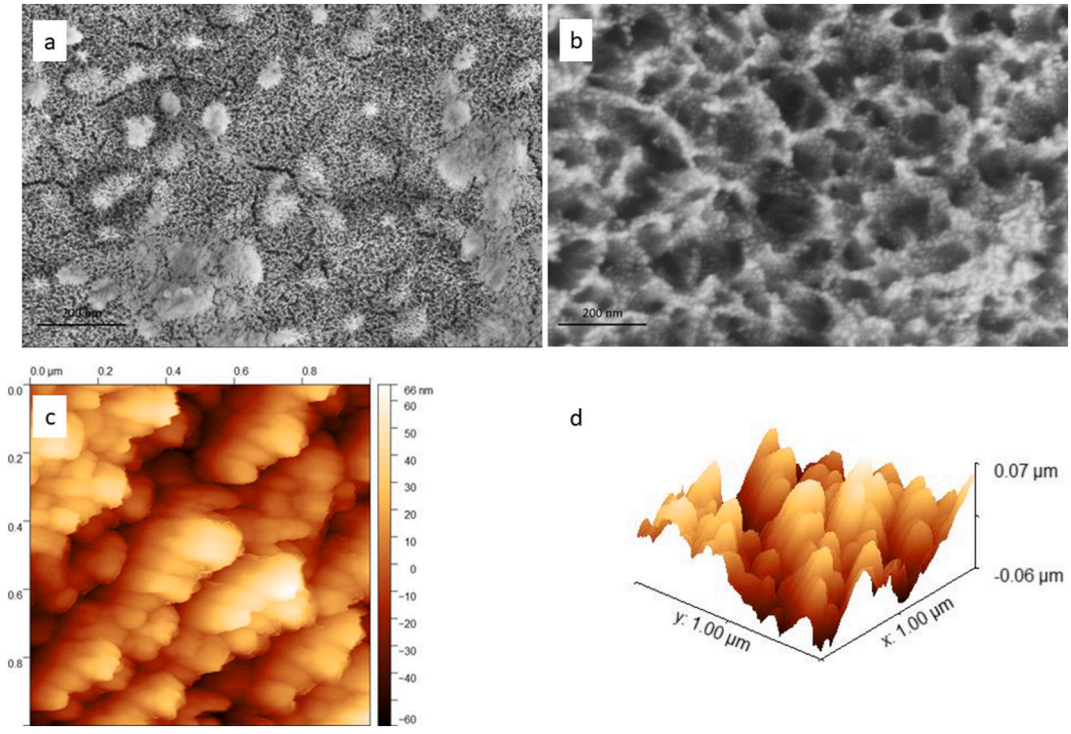


Fig. 1. The chemically treated CT surface (substrate). Top: SEM images at different enlargements. Bottom left: AFM 2D image. Bottom right: 3D-view of the AFM data, note that the *Z*-axis is expanded 4-fold.

Table 1
Roughness parameters of the substrates and coated samples. The data were collected on a 1 × 1 micron area.

	Sa [nm]	Sq [nm]
PT	5.5	6.9
CT	17.9	21.7
CT_D_AA	1.6	2.0
CT_D_PBS	8.1	10.2
CT_TC_37	21.4	26.2
CT_PD_W	14.2	16.8

CT_D_AA and it is not reported. The comparison of Figs. 1 and 2 evidences a clear change of the surface morphology of the CT substrate after the chitosan coating. The CT peculiar micro and nanosurface texture is no more visible where there is the chitosan coating. Because of this, it is easy to calculate the degree of surface coverage from the SEM images; the percentage of covered surface is also reported in the figure near the name of the samples.

The data reveal a high variation in the percentage of the coated surface depending on the used coating method. The best result, concerning the degree of coverage, is obtained with the direct coating (CT_D_AA, PT_D_AA, coverage 100%). The coating on CT_D_AA completely covers the surface and the topography of the titanium oxide layer is not observable even at higher magnifications (Fig. 2e); PT_D_AA looks similar. The coverage is lower in the case of the surface chemical activation (CT_TC_37, coverage 72%) and the use of a linker molecule (CT_PD_W, coverage 65%). The lowest degree of coverage is that of the samples obtained by direct coating at neutral pH (CT_D_PBS, coverage 41%).

Looking at larger magnification at the samples, a fibrous appearance of the coating is evident on CT_PD_W, CT_TC_37, and CT_D_PBS (Fig. 2f-h). Some uncoated areas are also clearly visible on these samples. The chitosan coating on CT_D_AA has a different appearance and it is quite smooth (Fig. 2e). This difference with respect to the other coatings (which are fibrous), is attributable to the higher solubility of the chitosan powder in acetic acid with respect to PBS (as it is used in the preparation of CT_TC_37 and CT_D_PBS) or water (as in the case of CT_PD_W). If the pH is not as low as it is in acetic acid, a suspension of chitosan is obtained rather than a solution, as expected, and it results in

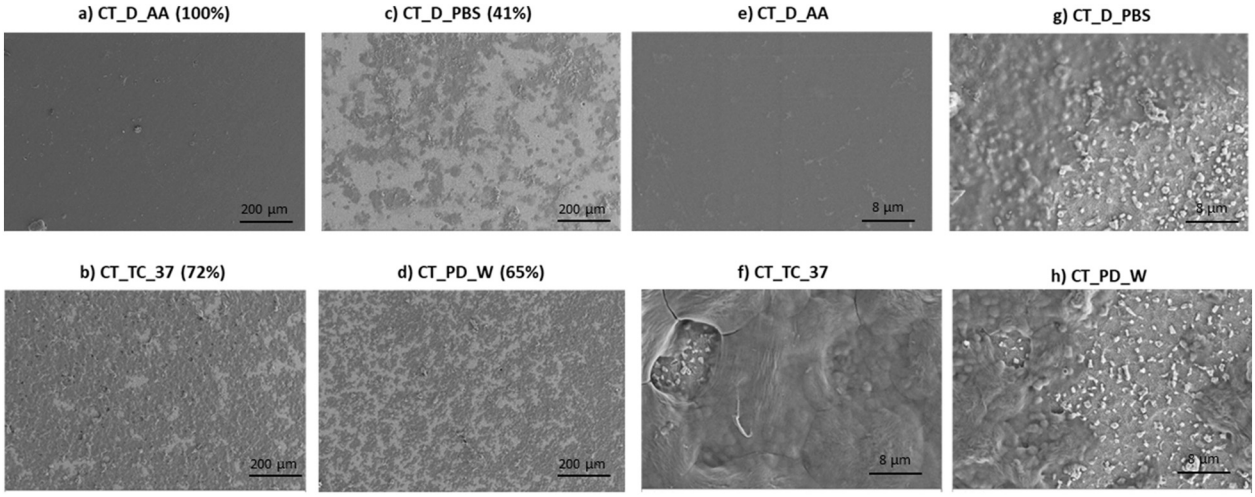


Fig. 2. SEM images showing the chitosan coated Ti6Al4V surfaces. The percentage of surface covered with chitosan is given near the name of the sample. Images e-h are larger magnification pictures.

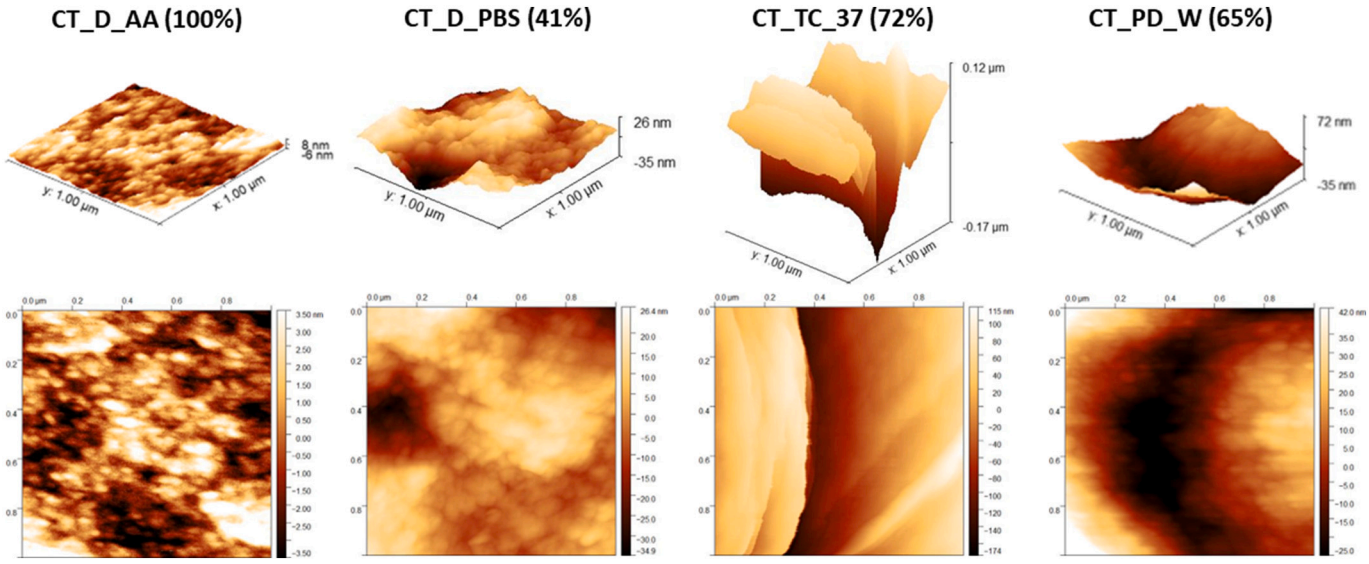


Fig. 3. 3D (with an enlarged vertical scale for a better visualization) and 2D representations of the AFM data of the samples CT_D_AA, CT_D_PBS, CT_TC_37, CT_PD_W. The percentage of surface covering is reported for each sample.

a fibrous appearance of the coating.

Table 1 reports semi-quantitative roughness data (Sa and Sq) obtained on the different samples by AFM. Fig. 3 shows 3D (with an enlarged vertical scale for a better visualization) and 2D representations of the AFM data. Both the substrates (PT and CT) have low roughness; it is expected to be larger on CT because of the chemical treatment. It is evident that the chitosan coating induces a very smooth surface with roughness around 2 nm (on the measured area). CT_D_AA has the smoothest surface among the tested samples in agreement with a complete covering of the topography of the substrate and evident lower roughness parameters respect to the substrate (CT). The roughness parameters obtained on the other samples are higher and affected by the partially uncoated surface areas. The inhomogeneity of the coatings in CT_D_PBS, CT_TC_37 and CT_PD_W does not allow AFM images representative of a large area to be collected as the height differences are out of the range of the instrument. Considering that a Sq/Sa ratio of 1.25 is expected for a Gaussian profile, it can be noted that this is the case of the PT and CT_D_AA samples, while the real Sq values are slightly lower than what is expected for a Gaussian profile in the case of the CT substrate and the partially coated samples. This is in agreement with the presence of nanosized pores on CT and on the CT samples with a partial covering of the surface by the coating.

3.2.2. FT-IR analysis

FT-IR analysis is performed in order to check if the applied process affects the surface chemistry of the coatings; one coating for each process has been analysed. It must be remembered that this analysis does not average the signal from a wide area and it can be different from point to point: it cannot be used to quantify the degree of surface coverage by the coating.

No signal is detected on PT samples, as expected. The reference spectrum of CT is reported in [23]: a broad signal centred at 3400 cm^{-1} due to the presence of -OH groups in the surface oxide layer formed during the chemical treatment is registered. The FT-IR spectrum of chitosan is largely reported in literature and generally shows the vibrational patterns typical of polysaccharides [26,27]. Starting from this literature, the following characterizing peaks can be assigned: the peak due to -C=O vibration at 1653 cm^{-1}, the peak due to bending of the $-NH_2$ group at 1560 cm^{-1}, and the broad band centred at 3400 cm^{-1} due to axial stretching of -OH groups. A contribution to the peak related to the -OH groups comes also from the chemically treated substrate. A sharp peak at 2900 cm^{-1} is due to axial deformation of $-CH_2-$ from the glucosamine unit structure. The three peaks at 1420–1320 cm^{-1} are due to $-CH_3$ bending vibrations and the one at 1100 cm^{-1} to -C-O-. At 945 cm^{-1} a small peak is due to -C-O-C- stretching and it is typical of polysaccharides.

All the expected peaks (-OH and -C=O stretching, $-NH_2$ bending) are detected on the CT_D_AA and CT_D_PBS samples (Fig. 4); when chitosan is dissolved in acetic acid during the coating process, the characteristic peaks are more intense in the coating, in agreement with the higher surface coverage of this sample. The peak around 2250 cm^{-1} in the CT_D_AA spectrum is an interference effect, not related to functional groups in the sample.

Analysing the FT-IR spectra of samples treated with tresyl chloride (CT_TC_37) or polydopamine (CT_PD_W), all the characterizing peaks of chitosan can be evidenced, but not as intense as they are on CT_D_AA. This observation agrees with the lower coverage degree measured by image analysis of the SEM pictures.

3.2.3. Zeta potential titration curves

Zeta potential titration curves allow to measure the IsoElectric Point (IEP) of the different surfaces, as reported in Fig. 5. These measurements can be used to estimate the presence of the coating assuming that the more the IEP is similar to the one of chitosan (IEP 6–8) [28], the more uniformly coated is the surface. According to the results obtained by SEM and AFM, the CT_D_PBS sample was not tested through this technique because of the low degree of surface covering by the coating.

Zeta potential of polished (PT) and chemically treated (CT) Ti6Al4V are reported as reference. Polished Ti6Al4V (PT) shows an isoelectric point at pH 4.7, that is what is expected for a surface almost free of surface functional groups. The chemically treated sample has a shift of the isoelectric point to more acidic values (by interpolation it is set around pH 2 – Fig. 5) and a plateau in the basic region (onset at pH 4.5) [29]. Both things evidence the presence of acidic -OH groups on the surface of the chemically treated substrate which act as a strong acid in contact with a solution, cause a shift of the IEP, and are completely deprotonated at pH above 4.5. The standard deviation values registered on PT and CT are around 10 mV, as expected for a metal surface measured through an electrokinetic measurement as a result of surface conductivity [30].

As it can be seen in the table reported in Fig. 5, the different coating procedures result in different zeta potential titration curves. The zeta potential of chitosan at acidic pH is expected to be significantly positive due to the protonation of the amino groups in the chain. In the case of chitosan, the IEP is the pH value at which there is an equilibrium of opposite charges and a balance between deprotonated and protonated

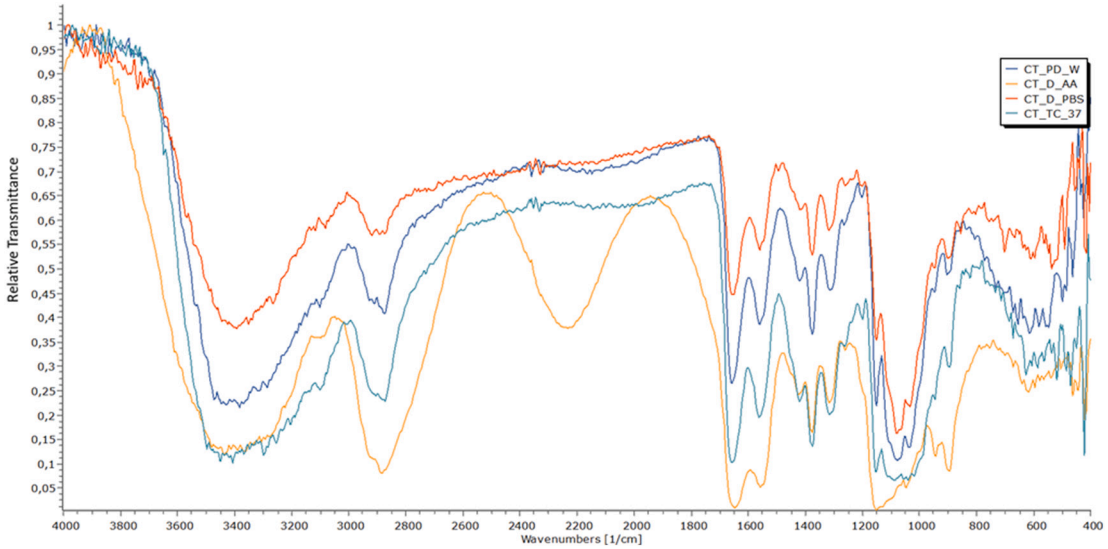
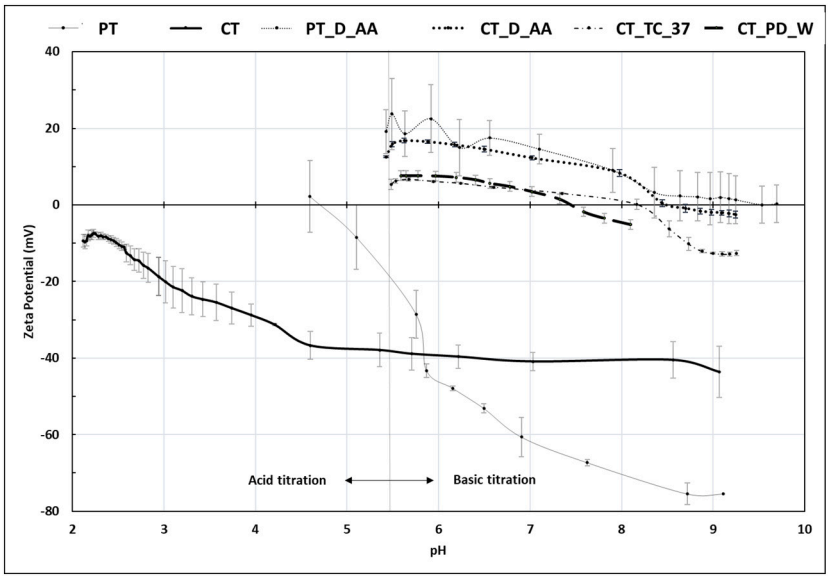


Fig. 4. FTIR spectra of the coated samples: CT_D_AA, CT_D_PBS; CT_TC_37, CT_PD_W.



SAMPLE	IEP
PT	4.7
CT	≈ 2.0
PT_D_AA	9.5
CT_D_AA	8.5
CT_TC_37	8.2
CT_PD_W	7.3

Fig. 5. Zeta potential titration curves of the samples polished (PT), chemically treated (CT), and coated with chitosan (PT_D_AA, CT_D_AA, CT_D_TC_37, CT_PD_W).

functional groups: IEP at pH as high as 8 indicates a clear prevalence of strong basic functional groups on the surface, attributable to a large surface covering by chitosan and to a great number of -NH_3^+ functionalities at the surface of the chitosan coating.

Considering that the PT and CT substrates have a low isoelectric point, a shift of the IEP of the coated samples towards higher pH values is related to a higher degree of covering of the surface by the coating. Comparing the obtained values of IEPs, it can be deduced that the coating of chitosan covers the substrate with an increasing degree moving from CT_PD_W (the lowest covering) to CT_TC_37, and then to PT_D_AA and CT_D_AA which have the highest covering. These results agree with the SEM observations. This suggests that amino groups are protonated when the coating procedure is performed in acetic acid and contribute to a strong attraction of the coating to the negative charged CT substrate.

As comparison, Amaral et al. [31] measured the zeta potential for chitosan with different degree of deacetylation (DDA), finding that the 50% DDA form has a zeta potential value around 15 mV at pH 5.5. It is evident in Fig. 5 that the samples with a zeta potential value much closer to the value measured by Amaral are the samples coated with chitosan dissolved in acetic acid (16 mV), while for the other samples the zeta potential is lower (6 mV for CT_TC_37; 7 mV for CT_PD_W).

A plateau is always observable on the coated samples between pH 5.5 and 6 related, as expected, to the strong basic amino groups which are completely protonated at pH lower than 6. A plateau is also observable in the curves of the coated samples around pH 9 and it can be ascribed to -OH groups of chitosan which are completely deprotonated only at pH higher than 9.

As last, it can be observed that all the curves of the coatings formed on a CT substrate have a very low standard deviation (1–2 mV): this reveals that these coatings are chemically very stable in the tested pH range (pH 5–9.5). On the other side, it can be noted that the PT_D_AA sample has very high standard deviation (up to 25 mV): this sample shows a high surface coverage by the coating, but it can be assumed to be chemically/mechanically instable and the coating is detached during the zeta potential titration because of the flux of the electrolyte.

3.2.4. Tape test

This test is performed to evaluate the mechanical adhesion of the coating to the substrate on the PT_D_AA, CT_D_AA, and CT_TC_37 samples according to the previous results; CT_D_PBS and CT_PD_W are not considered because of the low fraction of covered surface by the coating. The classification of the coating adhesion on the basis of the tape test is based on the observation of the surface after the test. Due to the transparency of chitosan, it is rather difficult to assign a classification value to the coated samples by visual observation and the samples were observed by SEM (Fig. 6). In the case of CT_D_AA, classification is 5B (ASTM Standard) with no observed removal of the coating in the areas delimited by the grid. In the case of the chitosan coating on the samples activated by tresyl chloride (CT_TC_37), the classification is 0B because the uncoated area after the test is 83%. In the case of PT_D_AA, the coating was completely removed by the tape during the test apart from a very small area on the edge of the sample (Fig. 6). The larger adhesion of the chitosan coating on the CT substrate (registered on CT_D_AA) can be related both to the multiscale roughness of the etched surface and to the electrostatic attraction between the negative charged titanium oxide layer and the -NH_3^+ groups of chitosan during the coating formation in an acidic environment. Such a strong adhesion does not occur on PT_D_AA and CT_TC_37 because in the first case the substrate has neither a porous morphology nor functional groups negatively charged and, in the second case, the coating formation occurred in a neutral environment.

3.2.5. Stability in PBS at 37 °C for 2 weeks

A first evaluation of stability of the coatings in a relevant environment has been performed through soaking in PBS for 2 weeks. The samples with higher degree of surface coverage and mechanical stability have been selected for this test, according to the previous results.

The samples are characterized after soaking through SEM observation and FTIR spectra: the results are reported in Figs. 7-8. No relevant change was detected by comparing the as-prepared coatings with the soaked ones in terms of percentage of the surface area covered by the coatings and functional groups exposed. The tested coating procedures reveal to produce stable coatings at physiological pH. A further characterization will include increasingly complex environments containing proteins, cells of different types, inflammatory conditions, and simulated infection risk.

4. Discussion

A chitosan coating on a titanium alloy can induce benefits in terms of the biological response, coupled to the good mechanical resistance and Young's modulus of the substrate, in view of an application in a biomedical implant. Even if this type of coating has been already investigated in literature [32], stability of the coating to the titanium

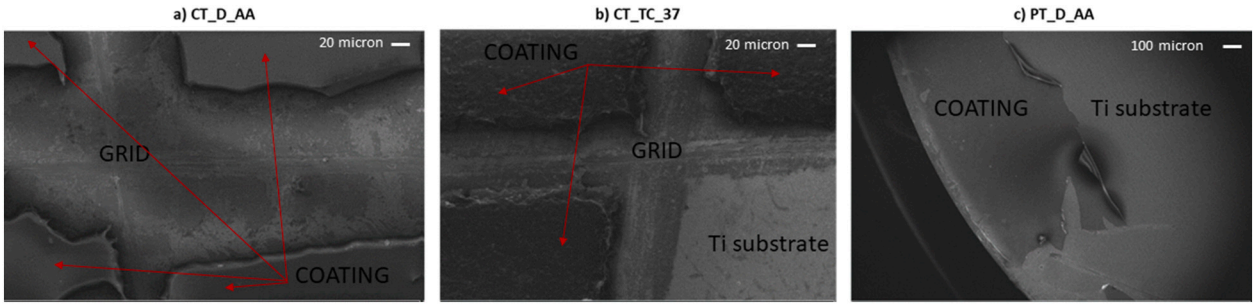


Fig. 6. SEM observation of the CT_D_AA (a), CT_TC_37 (b) and (c) PT_D_AA samples after the tape test.

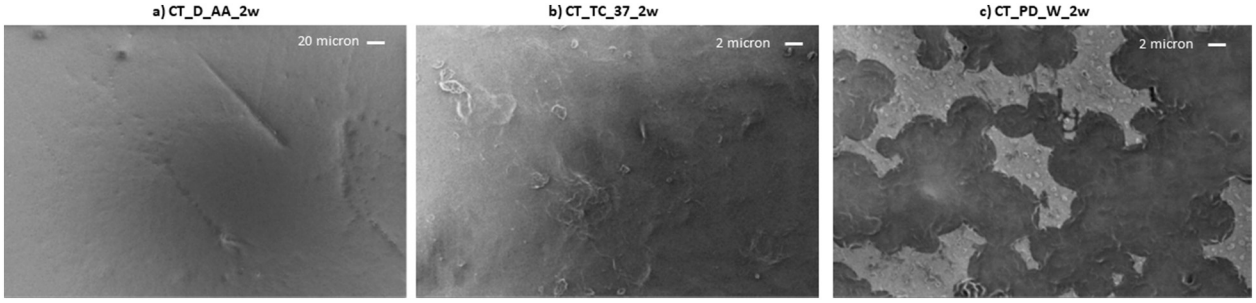


Fig. 7. SEM observations of the samples soaked for 2 weeks in PBS solution: CT_D_AA_2w, CT_TC_37_2w, CT_PD_W_2w.

substrate is not satisfactory without the use of potentially toxic linkers, yet. Different methods of coating (dissolution of chitosan in acetic acid or prepared with PBS, use of a good leaving group or a linker) and different surface finishing of the substrate (PT and CT) are here tested in order to screen the best ones. Three main coating procedures are applied.

In the first procedure, chitosan is prepared into two solutions, acetic acid and PBS. The chitosan used in this research resulted to be differently soluble in the different solvents used: it can be completely dissolved in acetic acid solution, while a suspension was obtained in PBS at pH 7.2–7.4. This pH is not far from the expected IEP of chitosan, while chitosan is supposed to be positively charged in an acidic solution (acetic acid). The effect of the different charge and dissolution degree of chitosan on the coating procedure can be evaluated by comparing CT_D_AA and CT_D_PBS. On the other side, the chemically treated Ti used as substrate (CT) has a very low isoelectric point (pH 2) and it has a negative charge in neutral and acidic environments. A direct graft of positively charged chitosan to a negatively charged surface can be expected in a solution of acetic acid [33]. As reference, a polished Ti sample (PT) was also coated with chitosan dissolved in acetic acid to investigate the effect of the substrate surface by comparing CT_D_AA and PT_D_AA. This comparison reveals a good degree of surface coverage in both cases and formation of a smooth surface because of the high dissolution degree of chitosan in the acetic acid. On the other side, there is a strong effect of the substrate surface topography and chemistry on the mechanical and chemical stability of the coating. In fact, it is easily detached in the tape test and it is instable during a zeta potential titration curve in the case of PT_D_AA. On the contrary, both nano sized porosity and presence of deprotonated hydroxyl functional groups have a role in the achievement of a stable coating on CT_D_AA. The comparison between CT_D_AA and CT_D_PBS confirms the positive role of a high solubility of chitosan in the solution used for the coating and of an electrostatic attraction between the coating and substrate for a good adhesion. The coating has a fibrous appearance, smaller surface coverage, and lower mechanical adhesion in the case of CT_D_PBS.

The second and third procedures were selected in order to get a covalent bonding of the coating with the substrate avoiding the use of any toxic compound such as glutharaldehyde. The second procedure involves an activation of the CT titanium surface with tresyl chloride, as performed by Hayakawa et al. [34], and then functionalization with chitosan suspended in PBS at 37 °C (for a higher reactivity of tresyl chloride). Tresyl chloride is used as a leaving group to enhance the adhesion of the coating at pH 7.4 and it is expected to be removed during the coating procedure with chitosan. This procedure (CT_TC_37) results in a higher degree of surface coverage with respect to direct coating at the same pH (pH 7.4 - CT_D_PBS) revealing a positive outcome of activation through the leaving group, but it is not as effective as the electrostatic attraction obtained through the direct link at acidic pH is (CT_D_AA) in terms of coverage of the surface and mechanical stability of the interface.

In the third procedure, the substrate is coated with polydopamine as described by Messersmith et al. in his study on mussel-inspired functionalization technique and other research works [20,35–38]. In this case, the presence of polydopamine as a linker is expected. This procedure results in a larger surface coverage with respect to direct coating at the same pH (pH 7.4 - CT_D_PBS), but in a lower enhancement of coating efficacy with respect to the use of a good leaving groups (CT_TC_37).

The realised coatings reveal a good stability at physiological pH for 2 weeks. Further work will be needed to explore the biological response of the coatings in a much more complex environments including proteins, different type of cells, bacteria, and inflammatory conditions. Several variable factors must be considered in terms of degree of deacetylation and molecular weight of the chitosan used for the coating starting from the already acquired results obtained on the selected type of chitosan in form of scaffold or membrane [2,39].

5. Conclusions

Different strategies have been followed to get a continuos and stable coating of chitosan on a titanium substrate: direct coating at different pH, covalent bonding through a good leaving group or a linker molecule. Two types of surface finishing of the titanium substrate are compared: a polished and a chemically treated one. The best result is obtained by

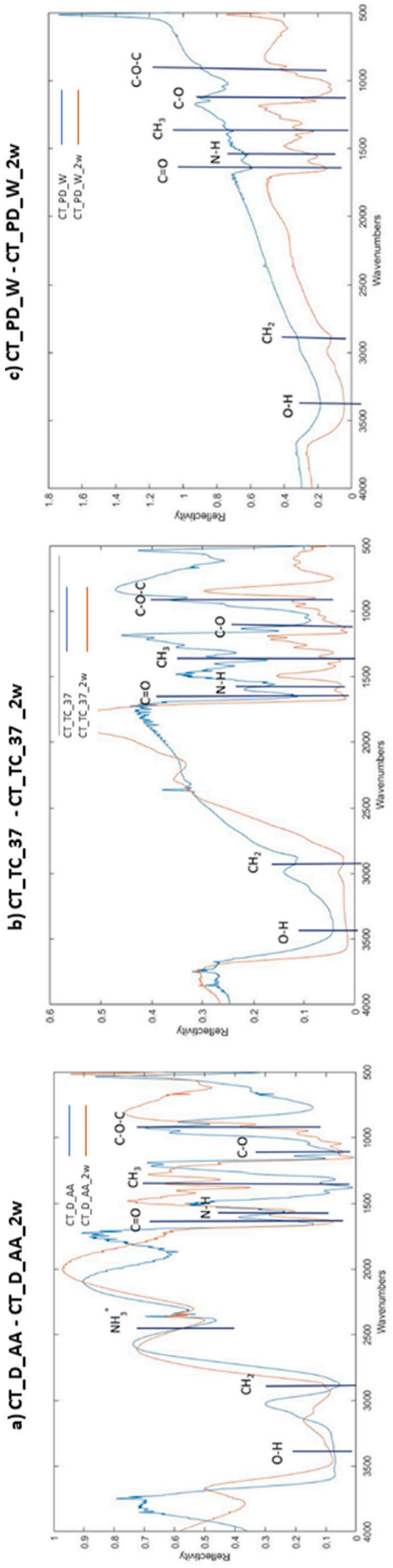


Fig. 8. FTIR spectra of the samples soaked for 2 weeks in PBS solution: CT_D_AA_2w, CT_TC_37_2w, CT_PD_W_2w.

direct coating at acidic pH on a pre-treated titanium substrate because of nano sized porosity of the substrate and strong electrostatic attraction between the positively charged chitosan and deprotonated hydroxyl groups of the substrate. The obtained coating fully covers the substrate and it is mechanically and chemically stable. A direct grafting of chitosan obtained from food production byproducts (shrimp shells) allows the development of a natural coating, without the employment of synthetic linkers, with a sustainable and green approach.

CRediT authorship contribution statement

S. Ferraris: Investigation, Methodology, Writing - original draft.

G. Örlygsson: Conceptualization, Funding acquisition, Writing - original draft.

C.H. Ng: Conceptualization, Funding acquisition, Writing - original draft.

G. Riccucci: Investigation, Writing - original draft.

S. Spriano: Conceptualization, Funding acquisition, Project administration, Supervision, Writing - original draft.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Silvia Spriano reports financial support and administrative support were provided by European Commission.

Chuen How Ng is an employee of Genis and this company is commercializing chitosan products.

Acknowledgements

The European Commission and the National Agencies (MIUR, The Icelandic Technology Development Fund) are acknowledged for funding the project NAT4MORE within the call M.ERA-NET Joint Call 2016 (Reference Number: project 4169).

References

[1] J.D. Bumgardner, R. Wiser, P.D. Gerard, P. Bergin, B. Chestnutt, M. Marini, V. Ramsey, S.H. Elder, J.A. Gilbert, Chitosan: potential use as a bioactive coating for orthopaedic and craniofacial/dental implants, J. Biomater. Sci. Polym. Ed. 14 (2003) 423–438, https://doi.org/10.1163/156856203766652048.

[2] L. Kjalarsdóttir, A. Dýrfjörd, A. Dagbjartsson, E.H. Laxdal, G. Örlygsson, J. Gíslason, J.M. Einarsson, C.-H. Ng, H. Jónsson, Bone remodeling effect of a chitosan and calcium phosphate-based composite, Reg. Biomater. 6 (2019) 241–247, https://doi.org/10.1093/rb/rbz009.

[3] M. Dash, F. Chiellini, R.M. Ottenbrite, E. Chiellini, Chitosan - a versatile semi-synthetic polymer in biomedical applications, Prog. Polym. Sci. 36 (2011) 981–1014, https://doi.org/10.1016/j.progpolymsci.2011.02.001.

[4] W.A. Sarhan, H.M.E. Azzazy, High concentration honey chitosan electrospun nanofibers: biocompatibility and antibacterial effects, Carbohydr. Polym. 122 (2015) 135–143, https://doi.org/10.1016/j.carbpol.2014.12.051.

[5] R. Muzzarelli, R. Tarsi, O. Filippini, E. Giovanetti, G. Biagini, P.E. Varaldo, Antimicrobial properties of N-carboxybutyl chitosan, Antimicrob. Agents Chemother. 34 (1990) 2019–2023, https://doi.org/10.1128/AAC.34.10.2019.

[6] L.-Y. Zheng, J.-F. Zhu, Study on antimicrobial activity of chitosan with different molecular weights, Carbohydr. Polym. 54 (2003) 527–530, https://doi.org/10.1016/j.carbpol.2003.07.009.

[7] C.-L. Ke, F.-S. Deng, C.-Y. Chuang, C.-H. Lin, Antimicrobial actions and applications of chitosan, Polymers 13 (2021) 904, https://doi.org/10.3390/polym13060904.

[8] J. Rhoades, S. Roller, Antimicrobial actions of degraded and native chitosan against spoilage organisms in laboratory media and foods, Appl. Environ. Microbiol. 66 (2000) 80–86, https://doi.org/10.1128/AEM.66.1.80-86.2000.

[9] J.J.T.M. Swartjes, P.K. Sharma, T.G. van Kooten, H.C. van der Mei, M. Mahmoudi, H.J. Busscher, E.T.J. Rochford, Current developments in antimicrobial surface coatings for biomedical applications, Curr. Med. Chem. 22 (2015) 2116–2129.

[10] S. Kumari, H.R. Tiyyagura, Y.B. Pottathara, K.K. Sadasivuni, D. Ponnamma, T.E. L. Douglas, A.G. Skirtach, M.K. Mohan, Surface functionalization of chitosan as a coating material for orthopaedic applications: a comprehensive review, Carbohydr. Polym. 255 (2021), 117487, https://doi.org/10.1016/j.carbpol.2020.117487.

[11] R. Lieder O. E. Sigurjonsson, "Chitosan as Coating Material for Titanium Implants" in book "Chitin and Chitosan Derivatives: Advances in Drug Discovery and Developments", Publisher: CRC Press, Editors: SK Kim.

[12] J. Redepenning, G. Venkataraman, J. Chen, N. Stafford, "Electrochemical preparation of chitosan/hydroxyapatite composite coatings on titanium substrates, J. Biomed. Mater. Res. A 66 (2003) 411–416, https://doi.org/10.1002/jbm.a.10571.
[13] X. Pang, I. Zhitomirsky, Electrodeposition of composite hydroxyapatite-chitosan films, Mater. Chem. Phys. 94 (2005) 245–251, https://doi.org/10.1016/j.matchemphys.2005.04.040.
[14] J. Zhou, G. Romero, E. Rojas, L. Ma, S. Moya, C. Gao, Layer by layer chitosan/alginate coatings on poly(lactide-co-glycolide) nanoparticles for antifouling protection and Folic acid binding to achieve selective cell targeting, J. Colloid Interface Sci. 345 (2010) 241–247, https://doi.org/10.1016/j.jcis.2010.02.004.
[15] D. Mitra, M. Li, R. Wang, Z. Tang, E.-T. Kang, K.G. Neoh, Scalable aqueous-based process for coating polymer and metal substrates with stable quaternized chitosan antibacterial coatings, Ind. Eng. Chem. Res. 55 (2016) 9603–9613, https://doi.org/10.1021/acs.iecr.6b02201.
[16] J. Kodama, H. Chen, T. Zhou, J. Kushioka, R. Okada, H. Tsukazaki, D. Tateiwa, S. Nakagawa, Y. Ukon, Z. Bal, H. Tian, J. Zhao, T. Kaito, Antibacterial efficacy of quaternized chitosan coating on3D printed titanium cage in rat intervertebral disc space, Spine J. 21 (2021) 1217–1228, https://doi.org/10.1016/j.spinee.2021.02.016.
[17] R. Gupta, N.K. Chaudhury, Entrapment of biomolecules in sol–gel matrix for applications in biosensors: problems and future prospects, Biosens. Bioelectron. 22 (2007) 2387–2399, https://doi.org/10.1016/j.bios.2006.12.025.
[18] S. Kumari, H.R. Tiyyagura, Y.B. Pottathara, K.K. Sadasivuni, D. Ponnamma, Timothy E.L. Douglas, Andre G. Skirtach, M.K. Mohan, Surface functionalization of chitosan as a coating material for orthopaedic applications: a comprehensive review, Carbohydr. Polym. 255 (2021), 117487, https://doi.org/10.1016/j.carbpol.2020.117487.
[19] T. Hayakawa, Biochemical surface modifications to titanium implants using the tresyl chloride activated method, Dent. Mater. J. 34 (2015) 725–739, https://doi.org/10.4012/dmj.2015-067.
[20] H. Lee, S.M. Dellatore, W.M. Miller, P.B. Messersmith, Mussel-inspired surface chemistry for multifunctional coatings, Science 318 (2007) 426–430, https://doi.org/10.1126/science.1147241.
[21] S. Spriano E. Verné S. Ferraris, "Multifunctional Titanium Surfaces for Bone Integration". European Patent 2214732 (2007).
[22] S. Ferraris, A. Cochis, M. Cazzola, M. Tortello, A. Scalia, S. Spriano, L. Rimondini, Cytocompatible and anti-bacterial adhesion nanotextured titanium oxide layer on titanium surfaces for dental and orthopedic implants, Front. Bioeng. Biotechnol. 7 (2019) 103, https://doi.org/10.3389/fbioe.2019.103.
[23] S. Ferraris, A. Vitale, E. Bertone, S. Guastella, C. Cassinelli, J. Pan, S. Spriano, Multifunctional commercially pure titanium for the improvement of bone integration: multiscale topography, wettability, corrosion resistance and biological functionalization, Mater. Sci. Eng. C 60 (2016) 384–393, https://doi.org/10.1016/j.msec.2015.11.049.
[24] D. Nečas, P. Klapetek, Gwyddion: an open-source software for SPM data analysis, Cent. Eur. J. Phys. 10 (2012) 181–188, https://doi.org/10.2478/s11534-011-0096-2.
[25] ASTM D3359-17, Standard Methods for Rating Adhesion by Tape Test, ASTM International, West Conshohocken, PA, 2017.
[26] E.B. Ibitoye, I.H. Lokman, M.N.M. Hezmee, Y.M. Goh, A.B.Z. Zuki, A.A. Jimoh, Extraction and physicochemical characterization of chitin and chitosan isolated from house cricket, Biomed. Mater. 13 (2018), 025009, https://doi.org/10.1088/1748-605X/aa9dde.
[27] C.A. Breda, D.L. Morgado, O.B.G. Assis, M.C.T. Duarte, Processing and characterization of chitosan films with incorporation of ethanolic extract from 'pequi' peels, Macromol. Res. 25 (2017) 1049–1056, https://doi.org/10.1007/s13233-017-5143-4.
[28] T. López-León, E.L.S. Carvalho, B. Seijo, J.L. Ortega-Vinuesa, D. Bastos-González, Physicochemical characterization of chitosan nanoparticles: electrokinetic and stability behavior, J. Colloid Interface Sci. 283 (2005) 344–351, https://doi.org/10.1016/j.jcis.2004.08.186.
[29] S. Ferraris, M. Cazzola, V. Peretti, B. Stella, S. Spriano, Zeta potential measurements on solid surfaces for in vitro biomaterials testing: surface charge, reactivity upon contact with fluids and protein absorption, Front. Bioeng. Biotechnol. 6 (2018), https://doi.org/10.3389/fbioe.2018.00060.
[30] T. Luxbacher, The ZETA Guide Principles of the Streaming Potential Technique, Anton Paar, 2014.
[31] I.F. Amaral, A.L. Cordeiro, P. Sampaio, M.A. Barbosa, Attachment, spreading and short-term proliferation of human osteoblastic cells cultured on chitosan films with different degrees of acetylation, J. Biomater. Sci. Polym. Ed. 18 (2007) 469–485, https://doi.org/10.1163/156856207780425068.
[32] Y. Yuan, B.M. Chesnutt, L. Wright, W.O. Haggard, J.D. Bumgardner, Mechanical property, degradation rate, and bone cell growth of chitosan coated titanium influenced by degree of deacetylation of chitosan, J. Biomed. Mater. Res. B Appl. Biomater. 86B (2008) 245–252, https://doi.org/10.1002/jbm.b.31012.
[33] F.M. Fowkes, Acid-base interactions in polymer adhesion, Tribol. Ser. 7 (1981) 119–137, https://doi.org/10.1016/S0167-8922(08)70878-2.
[34] T. Hayakawa, M. Yoshinari, Fibronectin immobilization onto titanium using tresyl chlorideactivation technique, Nano Biomed. 1 (2009) 34–40, https://doi.org/10.11344/nano.1.34.
[35] H. Li, C. Luo, B. Luo, W. Wen, X. Wang, S. Ding, C. Zhou, Enhancement of growth and osteogenic differentiation of MC3T3-E1 cells via facile surface functionalization of polylactide membrane with chitooligosaccharide based on polydopamine adhesive coating, Appl. Surf. Sci. 360 (Part B) (2016) 858–865, https://doi.org/10.1016/j.apsusc.2015.11.077.
[36] J. Liu, H. Xu, X. Tang, J. Xu, Z. Jin, H. Li, S. Wang, J. Gou, X. Jin, Simple and tunable surface coatings via polydopamine for modulating pharmacokinetics, cell uptake and biodistribution of polymeric nanoparticles, RSC Adv. 7 (26) (2017) 15864–15876, https://doi.org/10.1039/C7RA01354J.
[37] X. Yu, J. Walsh, M. Wei, Covalent Immobilization of collagen on titanium through polydopamine coating to improve cellular performances of MC3T3-E1 cells, RSC Adv. 4 (2013) 7185–7192, https://doi.org/10.1039/C3RA44137G.
[38] D. Zheng, K.G. Neoh, Z. Shi, E.T. Kang, Assessment of stability of surface anchors for antibacterial coatings and immobilized growth factors on titanium, J. Colloid Interface Sci 406 (2013) 238–246, https://doi.org/10.1016/j.jcis.2013.05.060.
[39] R. Lieder, M. Darai, M.B. Thor, C.-H. Ng, J.M. Einarsson, S. Gudmundsson, B. Helgason, V.S. Gaware, M. Máasson, J. Gíslason, G. Örlygsson, Ó. E. Sigurjóonsson, In vitro bioactivity of different degree of deacetylation chitosan, a potential coating material for titanium implants, J. Biomed. Mater. Res. A 100A (2012) 3392–3399, https://doi.org/10.1016/j.jcis.2013.05.060.