Biofunctionalization of titanium surfaces for osseointegration process improvement

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Abstract: This study aims to improve the osseointegration of titanium implants through surface immobilization of peptides that induce a beneficial biological response. This was carried out biofunctionalizing titanium surfaces by silanization and subsequent covalent binding of a peptide with a sequence that promotes cell adhesion. Objective: The development of a new technique of immobilization of oligopeptides on the surface of titanium by using 3-chloropropyltriethoxysilane (CPTES) as bonding agent between the surface of titanium and the peptide. Materials and methods: A physicochemical characterization of the surfaces through the techniques of XPS, ToF-SIMS and contact angle was performed. Also cell adhesion studies have been conducted to evaluate in vitro biological response. Results: Through the process of silanization the titanium surface is completely covered with CPTES, which allows the subsequent accession of oligopeptides. The cell adhesion results show a higher cell adhesion and cell extension on biofunctionalized samples. Conclusions: We developed a system of covalent binding of oligopeptides on titanium surfaces that can modify the biological response of the attached cells.

Keywords: biofunctionalization, Titanium, Osseointegration.

1. Introduction

Every year millions of metallic implants are implanted in patients, at a cost of billions of dollars in the whole world, to restore bodily functions that have degenerated due to old age, disease or trauma [1, 2]. When these elements are brought into contact with the physiological environment, it starts a chain of events that are dominated by protein adsorption and subsequent adhesion of platelets and macrophages, inducing inflammation of the affected area. This nonspecific response to the implant is considered not optimal for a good performance of the implant in the organism [3]. The cascade of reactions developed by the human immune system depends on the physicochemical properties of the surface. The acceptance of an implant by the human body is given by the durability, integrity and surface characteristics of the implant, as
well as chemical and biological reactions which occur within the tissue-implant interface [4, 5]. Several approaches to develop a new generation of metallic implants and medical devices are currently being studied. These new devices are designed to interact with the host body in a controlled and predictable way to replace diseased or damaged tissues. Some of these proposals lie in the incorporation of biomacromolecules, such as peptides, proteins, enzymes, lipids or DNA plasmids, on the surface of the implant material [6-11]. Of these proposals, the use of peptides to get a direct connection between the implant and cell surface receptors has gained wide acceptance and seems to have great potential due to the ease of modification of a broad range of materials [12]. Often, the peptides are designed to mimic small domains found in proteins present in the extracellular matrix of living tissue to promote cell activity. Modifying the surface prior to implantation, the material ensures an interaction with specific cell types through biomolecular recognition processes, thus avoiding nonspecific interactions between the implant and the surrounding tissue. This level of specificity in cell interaction and growth of native extracellular matrix significantly reduces recovery time after implantation. Accelerating the healing process, the patient may use faster the implant in service. This strategy has received in recent years, the name of biomimetics [14]: "manufacture materials that mimic the composition, structure or process of synthesis of natural materials." More specifically the surfaces modified with these processes are called biomimetic surfaces. In this work we have developed a new method for binding short peptides on the surface of titanium to obtain biomimetic surfaces based on the use of peptides capable of controlling cell activity both in vitro and in vivo.

2. Materials and Methods

Starting from commercially pure grade two titanium bars, 10 mm diameter and 2 mm thick disks were machined. The titanium discs were trimmed with silicon carbide paper and polished with colloidal silica to obtain a surface roughness Ra between 10 and 15 nm. Subsequently, the discs were washed three times with the following solvents: cyclohexane, isopropanol, distilled water and acetone, sonicated for 15 minutes each and were finally dried with nitrogen. In order to activate titanium surface for further silane binding the titanium discs were treated with plasma cleaning (Plasma Cleaner, Sterilizer PDC-002, Harrick Scientific Corporation, USA) for 5 minutes. Immediately after the disks were silanized by soaking in a solution of 0.85 ml of pentane, 0.075 ml of Diisopropylethylamine (DEA) and 0.15 ml of 3-chloropropyl triethoxysilane (CPTES) per disk. To avoid the incorporation of water into the solution, the silanization was carried out in an inert atmosphere of nitrogen for 1 hour with gentle shaking. After silanization, titanium disks were washed in a series of ethanol, isopropanol, acetone and distilled water and dried with nitrogen. Then titanium disks were immersed in an aqueous solution at pH 11.0 with 100 µmol of dissolved peptide per sample for 18 h, and finally washed in ethanol, isopropanol, distilled water and acetone and dried with nitrogen. The peptides used for the study were provided by GenScript USA Inc. The peptide amino acid sequences selected were: NH2-KGRGDS-COOH, NH2-KGRDGS-COOH and NH2-KGGGRGDS-COOH. The RGD sequence has the potential of cell adhesion [13]. To contrast the information obtained, clean titanium control samples and non silanized samples exposed to the different peptide solutions were also studied.

The physicochemical characterization of the obtained surfaces was carried out with X-ray Photoelectron Spectroscopy (XPS), Time of Flight Secondary Ion Mass Spectroscopy (ToF-SIMS), fluorescence microscopy and contact angle. The XPS analysis was performed using a spectrometer NanoESCA (Omicron Nanotechnology, Germany) imposing a step energy of 150 eV. To offset the effects of residual carbon peak the spectra was referenced to 284.8 eV. TheToF-SIMS analysis was performed with a ToF SIMS IV (Ion Tof, Germany) using cesium ion as a surface ionizer. The contact angle measurements were performed using a Goniometer OCA 15+ (Dataphysics, Germany). For each measure the sessile drop method with 300 µl of distilled water for each measure was used.
To observe the cellular response, samples were biofunctionalized with the three different peptides previously mentioned. Titanium samples with peptides adsorbed on the surface were prepared for comparison purposes. The cells selected for adhesion testing were human osteoblasts MG-63 line. Prior to exposure of cells, titanium samples were washed in ethanol for 1 h and then were blocked with bovine serum albumin 6% in PBS for 1 h. Osteoblasts were exposed to titanium surfaces for 3 h in serum free medium and then were fixed and stained with phalloidin and Hoechst (Sigma Aldrich, USA) for 45 minutes at 37 °C to reveal its actin and nuclei respectively.

3. Results

XPS: Table 1 shows the chemical composition present on the surface of the samples. The presence of chlorine on silanized surfaces is an indicator of the presence of CPTES on the surface. There is also an increase of nitrogen on the samples that have been exposed to a peptide. Since the only molecule that contains nitrogen in the whole system of study is the peptide, this data indicates the presence of the peptide on the surface.

| % at | Ti | Ti+CPTES | Ti+CPTES+RGD | Ti+RGD |
|------|----|----------|--------------|--------|
| O1s  | 49.6 | 41 | 35.5 | 43.9 |
| C1s  | 26.1 | 39.1 | 42.7 | 34.9 |
| Ti2p | 20.8 | 13.7 | 12.7 | 18.8 |
| Si2p | 2.2 | 3.3 | 4.3 | nd |
| N1s  | 1.1 | 0.5 | 2.5 | 2.3 |
| Cl2p | 0.1 | 2.4 | 2.2 | nd |

ToF-SIMS: Positive ion spectra obtained by ToF-SIMS is shown in figure 1. For the silanized and peptide KGRGDS biofunctionalized samples sharp spikes in values of mass/charge 28, 30, 42, 56, 70, 85, 97 and 111, corresponding to ions CH2N+, CH4N+, C2H4N+, C3H6N+, C4H8N+, C5H4N+, C7H13N+ and C6H11N+ respectively. All these species contain nitrogen, indicating the presence of the peptide KGRGDS on the surface. In the case of the biofunctionalized sample without silane these peaks are also present although with less intensity.

![Figure 1: ToF-SIMS spectra obtained on the treated and non treated samples.](image-url)
Contact angle: The results of contact angle (Fig. 2) show significant differences between silanized and non silanized samples, increasing the contact angle in the first case. The alkyl chain of the CPTES has hydrophobic properties increasing the contact angle of silanized samples as compared to the contact angle of clean titanium. The reduction of the contact angle of the silanized and biofunctionalized samples with the peptide KGRGDS when compared to contact angle of silanized titanium indicates the presence of the peptide on the surface because it is hydrophilic. There were no statistically significant differences between the contact angle of clean titanium and biofunctionalized titanium without CPTES, suggesting that the amount of peptide adsorbed, in the last case, is lower than in the case of silanized titanium with CPTES.

![Figure 2. Contact angle results obtained on treated and non treated titanium samples.](image1)

Cell adhesion: Cell adhesion results (Fig. 3) showed a higher cell affinity for the biofunctionalized samples with the RGD sequence. Table 2 shows the number of cells per area unit and surface area covered by them. These two values are indicative of cell adhesion on the studied surfaces. Although the greatest number of cells adhered to the surface on the samples biofunctionalized with the peptide KGRGDS there is a greater cell extension on the biofunctionalized sample with the peptide KGGGRGDS.

![Figure 3. Fluorescence micrographs of the MG-63 attached to different studied surfaces. A: Clean titanium. B: Ti+CPTES. C: Ti+CPTES+KGRGDS. D: Ti+CPTES+KGGGRGDS.](image2)
Table 2. Cell adhesion on the studied samples.

| Sample Description          | Cell number/cm² ± SD | Cell extension (%) ± SD |
|-----------------------------|----------------------|-------------------------|
| Clean Ti                    | 657.2 ± 118.8        | 41.4 ± 10.7             |
| Ti+CPTES                    | 45.0 ± 7.2           | 16.7 ± 13.1             |
| Ti+KGRGDS                   | 589.7 ± 78.4         | 34.5 ± 10.1             |
| Ti+KGGGRGDS                 | 625.7 ± 58.7         | 37.5 ± 11.3             |
| Ti+KGRDGS                   | 270.1 ± 11.7         | 28.8 ± 12.7             |
| Ti+CPTES+KGRGDS             | 1960.60 ± 159.0      | 48.3 ± 15.5             |
| Ti+CPTES+KGGGRGDS           | 847.7 ± 114.4        | 58.2 ± 20.9             |

4. Discussion

The XPS results have shown the presence of a CPTES layer on the surface of titanium in silanized samples. The shallow penetration depth of this technique is about 5 nm. As it can be seen comparing the clean titanium sample and the completely biofunctionalized titanium, the presence of titanium element is significantly reduced with the Biofunctionalization treatment but the XPS still detects a significant amount of it. This indicates that the layer formed has a thickness of less than 5 nm, possibly a monolayer with a thickness of about 1 nm is formed [15]. This thickness seems to increase by the addition of the peptide because the amount of titanium detected is even lower. On the other hand, the increase in the percentage of nitrogen in the samples indicates the presence of peptide itself. Moreover, although the ToF-SIMS technique is not quantitative, the results allow inferring that there is a greater amount of peptide on silanized surfaces than in those non-silanized.

It has also been observed that the silanized samples have a more hydrophobic behavior than clean titanium. Although it is not the only factor involved, this can significantly influence the cell adhesion as has been observed in cell adhesion tests. Silanized samples without peptide presented a minimum cell adhesion; it means that cell adhesion is mediated, mainly, by the peptides present on the titanium surfaces. Comparing KGRDGS and KGGGRGDS peptides on the sample surfaces, there were a higher number of cells adhering to the surface in the case of silanized samples and biofunctionalized with KGRGDS peptide. Nevertheless cell extent in the silanized biofunctionalized samples with the peptide KGGGRGDS is somewhat higher. The RGD sequence is recognized by cells through their integrins, mainly \( \alpha_5\beta_1 \) integrin binding to this sequence and hence to the titanium surface. RDG sequence is a sequence of control which has no cellular adhesion potential. The inclusion of one or three glycines in the peptide can give differences in cell adhesion since it is possible that the peptide KGGGRGDS, having the RGD sequence further away from the surface may allow easier access for cellular recognition. These data could indicate a greater accessibility of the peptides KGGGRGDS for the cells.

5. Conclusion

A new method of immobilization of oligopeptides on titanium surfaces has been developed. The silanization with CPTES before biofunctionalization allows a more selective cell adhesion than in the case of a biofunctionalized titanium surface without prior silanization.

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