Cell study of the biomimetic modifications on a CoCrMo alloy for biomedical applications

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Abstract

The number of cells adhered and their morphology on implants are indicative of its adaptation. Accordingly, the field of cellular behavior and biomimetic modifications in inorganic materials for biomedical applications show a notable increase in scientific studies. Whence, in this study, the immobilization of peptide sequences and the biological behavior on CoCrMo alloy will be evaluated. To validate the biomimetic modifications carried out on the surfaces we were proposing as hypothesis that a higher density of biomolecules bring about better cellular response. The methodology employed included 2 stages: I) Surfaces biomimetic modification: samples were initially bio activated with nitric acid, followed samples were biofunctionalizated with APTES+Maleimide and finally peptide sequences were immobilized of surface (RGD, FHRRIKA, PHSRN RGD/FHRRIKA or RGD/PHSRN). II) To evaluate the biomimetic modification, were performed studies of cell adhesion. The techniques used for characterization were X-ray photoelectron spectroscopy (XPS), contact angle (CA), immunofluorescence, scanning electron microscope (SEM). The results indicated that samples with RGD and RGD/FHRRIKA exhibited higher number of cells adhered and also cells more spread on the surface, which suggests that they were adapted to the surface, it is demonstrated that the biomimetic modifications carried out are able to successfully induce the bone tissue repair process.

Keywords: Cell response, biomimetic modification, peptide sequences.

Introduction

Often, bones suffer damage due to accidents, aging and other causes, which makes surgical procedures for replacement with artificial materials nowadays become a common practice [**1**]. Additionally, worldwide, the increase in fractures related to osteoporosis and bone loss represents one of the biggest problems in the field of orthopedics and it is expected that in the future more than 50% of people over 60 years of age suffer from degenerative joint diseases [**2**].

For a long time, metallic materials to replace parts of bones have been used, fracture fixation devices, stents, dental implants, etc. The advantage of metals over polymers or ceramics lies in greater strength and hardness, in addition, these can be easily processed [**3**]. The most used metal biomaterials today are stainless steels, Ti, Co, Ta, Mo, W, its alloys, and noble metals (Au, Pd, dental amalgams-Hg-Ag-Cu-Sn). All these materials have a high resistance to corrosion in the fluids of the human body, given their noble nature and/or spontaneous passivation; Therefore, they are considered bioinert materials [**4-9**].

About metallic orthopedic devices, they usually are made of steel or titanium. Therefore, after the healing process, metal device must be removed, which increases the risk of infection, a new fracture or bone morbidity. Add, other failures leading to a second operation have been reported, as such as: (i) screw plate systems, which have been used for the reduction and fixation of fractures, (ii) the systems of distal femoral locking plates. Where the implant may not working properly due to early mechanical failure, such as: loosening of the locking screws, loss of fixation, plate flexion, among other factors [**10-12**].

In summary, bone damage is multi-causal and often requires the use of implants or fixation elements. However, materials traditionally used throughout history have presented numerous disadvantages as aseptic loosening and/or structural factors related to the design of the material, or hypersensitivity reactions due to corrosion processes; which lead to the release of metal ions at levels higher than those admitted by the body, among others [**13-17**].

The foregoing demonstrates that it is essential to focus implant studies on their ability to promote tissue

regeneration; taking advantage of the knowledge of materials science and biology, and in this way contribute to tissue engineering. Thereby, it will be of vital importance to use biochemical agents to accelerate the process of bone remodeling, given that a high percentage of patients who require implants are adults older than 50 years. Therefore, they are prone to suffer from osteoporosis.

In addition, by obtaining faster bone remodeling, patient recovery is facilitated in less time. Similarly, as it has been mentioned, the growth in the number of small osteoinductive molecules may represent the next generation of clinical therapies for bone repair and regeneration [**18-20**].

For this reason, in this work we study the influence of different short sequences of peptides: RGD (Arg-Gly-Asp), PHSRN (Pro-His-Ser-Arg-Asn), FHRRIKA (Phe-His-Arg) -Arg - Ile-Lys-Ala) and two 50/50 mixtures: RGD / PHSRN and RGD / FHRRIKA, in the cellular behavior on the surface of a commercially accepted CoCrMo alloy for biomedical applications. Thus, in this way, we will evaluate how biomimetic modifications can affect the cellular response [**21-28**].

Experimental

Materials

A CoCrMo alloy (ASTM F1537) purchased from the Technalloy Company (**Table 1**) was used. The diameter of the material was 12 mm and the samples were cut with a thickness of 2 mm. With silicon carbide abrasive paper of 600 and 1200 grains, the samples were polished. Immediately, using polishing discs and colloidal alumina material (1.00 to 0.05 μm) were given a mirror polish. Subsequently, ultrasonic baths with acetone, water and ethanol were used to degrease them. Finally, the samples were dried with compressed air.

Table 1. Chemical composition for CoCrMo alloy as provided by manufacturer.

Element	Co	Cr	Mo	Mn	Fe	Si	Ni
% weight	61.63	31.84	5.42	0.38	0.32	0.26	0.15

To remove the impurities from the cutting and polishing process, when talking about the control sample, it refers to polished CoCr samples, which were to place in ultrasonic bath with cyclohexane, isopropanol, ethanol, deionized water and acetone.

In previous studies, the cleaning surface was evaluated using the resolved X-ray photoelectron spectroscopy (AR-XPS) technique that allows a more sensitive surface analysis obtaining detailed information on the chemical composition of the external surface at different angles (generally from 3 to 10 nm). As surfaces initially presented a high carbon content, coming from environmental pollution, it was necessary to perform a cleaning and activation process, this step showed a considerable reduction in carbon pollution compared to the control, with statistically significant differences [**29**].

Biomimetic modification

The process of biomimetic modification consists of three stages: activation, silanization and biomolecules immobilization.

Previously the authors optimized the surface activation process, their results indicated that when comparing the nitric acid etching (NA) and oxygen plasma (OP), both methods (OP, NA) removed surface contaminants and increased oxygen concentration at the surface, however, the ratio OH^-/O^2 proved that NA etching introduced a higher amount of active hydroxyl groups in the metal oxide film [**29**].

On the silanization process, this requires incubation for 1h in a basic solution of pentane containing organosilane (3-aminopropyltriethoxysilane (APTES)), then is modified with 3-(maleimido)-propionic acid N-hydroxysuccinimide ester for crosslinker. (CoCr+NA+APTES+Maleimide = CoAM).

Finally, for the biomolecules immobilization (peptides), the samples were incubated overnight at room temperature, in a phosphate buffer solution (pH = 7), which contained some of the following short sequences of peptides: CGGRGDS, CGGPHSRN, CGGFHRRIKA, or mixtures (50/50) of CGGRGDS/ CGGFHRRIKA and CGGRGDS/CGGPHSRN. The peptides used were provided by GenScript [**22-27**]. For the characterization of the surface, different techniques were used: contact angle (CA) and X-ray photoelectron spectroscopy (XPS).

In vitro biological response

Before to mesenchymal cell culture, all samples were immersed for 30 minutes in a solution of phosphate buffer (PBS) and bovine serum albumin (BSA (1%)) to prevent nonspecific protein uptake. Then the sterilization of the samples was carried out by immersing them in ethanol for 10 min. Finally, the surfaces were washed three times with sterile PBS to remove residual ethanol [**24, 30-32**].

The cell adhesion process was carried out in a time of 6 h and at a temperature of 37°C. In this assay, these types of samples were used: (i) Two control samples without biomimetic modification; one of them with PBS- BSA (1%) called CoCr+BSA, and another without PBS-BSA (1%) called CoCr, (ii) silanized samples with short peptide sequences, their nomenclature was: CoAM+RGD (CoAMR), CoAM+FHRRIKA (CoAMF), CoAM+PHSRN (CoAMP), CoAM+RGD+FHRRIKA (CoAMRF), CoAM+RGD+PHSRN (CoAMRP).

For the cell adhesion assay, $6x10^3$ cells per sample were used. Which in a serum-free medium were incubated. The characterization techniques used were: (i) Immunofluorescence, through the staining of its nuclei with DAPI (4,6-diamino-2-phenol-dihydrochloride), for its subsequent observation through a confocal microscope, which will determine the number of cells per unit of area, and ii) SEM, to observe the morphology and the area of the cells adhered on the surfaces [**33, 34**].

Results and discussion

Silanization process

The authors previously evaluated the behavior of 3-chloropropyltriethoxysilane (CPTES) or 3-glycidoxypropyltriethoxysilane (GPTES) and 3-Amino propyl triethoxy-silane modified with 3-maleimido acid propioinic n-hydroxysuccinimide ester (APTES+Ma). Resulting that both CoCr alloy and Ti alloy showed the best results with APTES + Ma, in terms of Si percentage and silane stability [**35, 36**].

In this process, samples increased the contact angle from 28.6° up 88.5°. Thus, silanization produced a homogenous surface modification, due that drops were placed in different position of samples, and all samples had similar results. By XPS was possible to confirm the presence of silanes on the surface, because a peak of Si 2s of 7% $\pm$ 0.5 appear on the silanazed surface. Also there were intensification in atomic concentration of: (i) carbons contents from 36% $\pm$ 0.4 up 59% $\pm$ 3.1, due to carbon chains of silanes, and ii) an increase of the atomic percentage of N from 4% $\pm$ 0.3 to 9% $\pm$ 0.7, it is due to the fact that the presence of imide group belongs of crosslinker (maleimido) [**37-41**]. Finally the oxygen peak had decreased due the silanization process to covered the oxide layer [**42-44**].

Similar results were obtained in previous studies using a TiHfNb nobel alloy. In this case, Ti+APTES+Ma alloy samples showed higher values for the Si peak (12% $\pm$ 1.0) and for C (55% $\pm$ 3.7). In addition, in the case of the Ti alloy, there was an increase in N (from 1% $\pm$ 0.1 to up 10% $\pm$ 0.9) and the peak O decreased from 60% + 2.9 to 23% $\pm$ 1.9 [**45**].

Peptide immobilization

To induce a specific cell adhesion, different short peptide sequences were immobilizes [**46**]. The short peptide sequences are conformed of one cysteine (Cys) and three glycine (Gly); as spacers, because the Cys contains a sulfur atom in its side chain, which is highly reactive. Also, the peptides were blocked by reacting the terminal amino group; Then, they have the ability to react with silanes through the side chain of cysteine (-SH).

Results confirmed the presence of peptide, due to all samples with peptides showed the presence of S 2p (**Table 2**). The silane peak (Si 2s) also disappeared, since it has been completely coated with biomolecules. Lastly, there was an increase in the percentage of O 1s, it is due either the carboxyl groups (COOH) of the terminal chain peptides or the peptide bonds (-OC-NH-). Finally, the presence of S 2p revealed that Cys was able to react with silanes through the side chain of cysteine (-SH).

Table 2. Results of Peptide immobilization by XPS (atomic percentage).

Element	AM	AMR	AMF	AMP	AMRF	AMRP
S 2p	0+0.0	5+0.2	5+0.3	5+0.5	4+0.3	5+0.4
Si 2s	7+0.5	0+0.0	0+0.0	0+0.0	0+0.0	0+0.0
O 1s	25+1.7	32+2.1	35+1.3	36+2.3	37+2.7	37+2.2

If these results are compared with previous studies on the Ti alloy, there is a notable difference, because in the Ti alloy all the samples with biomolecules showed only 1% of S [**45**]. In addition, samples with APTES + Ma always obtain a higher concentration of biomolecules with respect to samples with CPTES or GPTES. Therefore, it seems that the absorption of biomolecules could be influenced by the chemical composition of the substrate and the type of silane.

In vitro biological response

Cell adhesion assay was evaluated by immunofluorescence (DAPI) to determine the number of cells per area (**Fig. 1**).

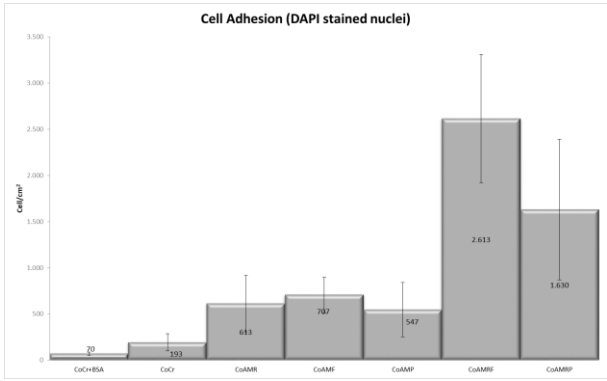

Fig. 1. Cell Adhesion by immunofluorescence.

The best results occurred in samples with mixtures, because these sequences have higher presence of amine groups. Also in these surfaces there should be more groups -NH_2, -COOH, which cause greater adherence cellular [**47**]. About to RGD + FHRRIKA, this mixture improved adhesion and even studies indicate that influence the process of proliferation [**26**]. It is also appreciated that the control sample (CoCr) has some cells adhered. Thus, it is necessary to evaluate the cell morphology, knowing that an optimal result will be the one that has the best relationship between the numbers of cells adhered, its area and its morphology.

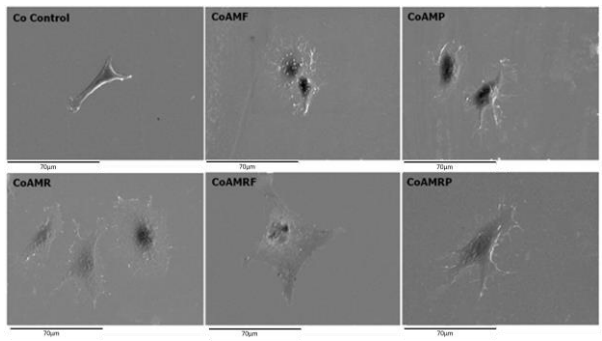

Fig. 2. SEM micrographs showing rat mesenchymal cells.

In SEM images (**Fig. 2**) is possible to appreciate the effect of biomolecules on cell morphology. Samples without peptide look contracted. However, samples with RGD and mixtures show cells more spread, and

filopodia. Then, results confirm that RGD sequence allowed that cells feel more comfortable, due that on surface with this peptide sequence (alone or in mixtures) cells are more widespread, largest area and exhibit filopodia. Confirming that this peptide sequence (RGD) promotes cell adhesion and to mix this with FHRRIKA or PHSRN peptide improve cell adhesion response [**26, 48**-**56**].

The quantification of cell area (**Fig. 3**) confirmed that samples with RGD, and RGD+FHRRIKA presented the best results with differences statistically representative to be compared with another sequences.

The results showed that the concentration of biomolecule adsorbed on the surface is a dependent variable of the cell adhesion process, because when both alloys were silanized with APTES+Ma always had the highest concentration of biomolecules and better cell response. Also in all cases, surfaces with APTES + Ma + RGD (alone or in mixtures) showed better results of cell adhesion in vitro [**35, 57**].

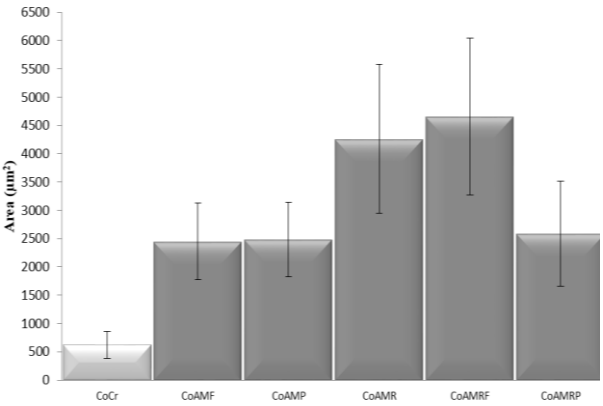

Fig. 3. Cell Area estimated through SEM micrographs.

Conclusion

Evaluating the biomimetic modification, results confirm that activation process proved to be effect in terms of cleaning and chemical surface modification, through to the incorporation of OH- group on the surface. By XPS could be confirmed the presence of silane, because appeared peaks of Si on silanized samples and the presence of biomolecules on the surface due that there is a new peak of sulphur on samples with peptide.

The results indicated that samples with RGD and RGD/FHRRIKA exhibited higher number of cells adhered and also cells more spread on the surface, which suggests that they were adapted to the surface, it is demonstrated that the biomimetic modifications carried out are able to successfully induce the bone tissue repair process.

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