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Title:

Protein adsorption and bioactivity of functionalized electrospun membranes for bone regeneration.

Short Title:

Adsorption of proteins for bone regeneration.

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Abstract

Objectives: To evaluate the adsorption of bone related proteins and bioactivity of experimental functionalized (carboxylated or aminated) polymeric membranes for bone regeneration.
Methods: Polymethylmethacrylate-based membranes functionalized with carboxyl or amino radicals were tested. Membranes were zinc loaded and the adsorption isotherms of zinc were studied. A commercially available polytetrafluoroethylene membrane was used as control. Human plasma proteins, bovine serum albumin, fibrinogen and fibronectin adsorption were measured with a spectrophotometer applying an acid determination protocol. Biomimetic calcium phosphate precipitation on polymeric membranes was also assessed trough simulated body fluid immersion. Scanning electron microscopy and elemental analysis by means of an energy dispersive system were used for mineral deposits identification.
Results: Both experimental membranes produced higher protein adsorption than the commercial control that does not adsorb proteins. Carboxylated membranes adsorbed significantly more albumin than the aminated ones, the opposite occurred with fibrinogen. With plasma and fibronectin proteins both type of membranes performed similarly. Only carboxylated membranes were bioactive and precipitated calcium and phosphate on their surfaces. *Conclusions:* The polymethylmethacrylate zinc-loaded membranes functionalized with carboxyl groups performed as high adsorbable membranes for bone regeneration related proteins. They also served as templates for mineralization of hydroxyapatite.
Clinical significance: Protein adsorption is the initial reaction after the implantation of a biomaterial into the body and will influence subsequent cell function and mineral deposition. Adsorption of those adherent bone related proteins on the implanted material surface could be tightly associated with its ability to induce new bone formation to promote bone regeneration.

Keywords: Membranes; Bone regeneration; Carboxyl; Amino; Adsorption; Proteins

1. Introduction

Periodontal disease results in creation of bone defects that may require regenerative surgical treatments which can include the utilization of barrier membranes [1]. Occlusive membranes for guided bone regeneration procedure are frequently utilized to regenerate bone. They attempt to obtain the protection of blood clots and the isolation from surrounding connective tissue, thus offering the access to a secluded space to the bone-forming cells for bone regeneration and vascularization [2,3]. They should also promote biocompatibility to allow integration with the host tissues without eliciting inflammatory responses, favoring proteins adsorption, cells adhesion and osseointegration through bioactivity [2]. The main disadvantage of resorbable membranes is their unpredictable resorption times and the liberation of undesirable degradation products [4,5]. In order to warrant the bone remodeling and maturation, non-resorbable membranes are preferred to protect the newly-formed bone from physiological stress and to exert spatio-temporal control over the wound-healing process [5].

Polytetrafluoroethylene (PTFE), a non-reabsorbable synthetic polymer, represents the gold standard for clinicians, due to its higher predictability if compared to reabsorbable membranes. Among others, one of the main disadvantages of PTFE are the low adhesiveness for plasma proteins and cells [7]. The critical shortcomings of PTFE-based nonresorbable and resorbable membranes, have led to studies of alternate membrane materials. Polymethylmethacrylates (PMMA) are non-reabsorbable but biocompatible and biostable polymers, which have been widely used in distinct medical applications [8]. PMMA enhance the mechanical behaviors of the hydrophilic structures, shows weak interactivity against tissue cells and promotes a biological response based on the formation of fibrotic tissue [8,9]. To enhance hydrophilicity, cell-membrane interactions, and osteogenic properties, a novel composite membrane is proposed. It is based on the electrospun of a mixture of $(MMA)_1$-co-$(HEMA)_1$ and $(MA)_3$-co-$(HEA)_2$. Electrospun nanofibrous scaffolds/membranes mimic closely the scale and morphology of the extracellular matrix (ECM) proteins (fibers with diameters ranging from 50 to 500 nm) [2]. Nanofibrous membranes prepared by electrospinning are also highly acknowledged in the field of protein filtration and enzyme immobilization [10].

Incorporation of zinc ions within the nanostructure of these membranes was performed as it allows for the modification of the biological and physical properties of the proposed tissue. It has been previously shown that membranes functionalized with zinc may enhance osteoblasts viability and proliferation, increase the rate of new bone formation and inhibit the bacterial biofilm formation [Toledano et al 20119 cloi; Osorio et al, 2020 J Dent; Bueno et al 2020 Dent Mater]. It has also been pointed out that polymer-based scaffolds/membranes loaded with zinc may display faster wound healing in bone regeneration, as shown in similar membranes tested in an animal model [Toledano et al 2019 cloi]. ojo Alvaro con las citas

Adsorption of proteins plays an important role in bioprocess recovery [10], as they are bioactive molecules [11]. This first event may determine the success or failure of the implanted device [12]. Protein adsorption occurs immediately on the biomaterial membrane surface as soon as it is implanted and comes in contact with blood [1]. Blood contains more than 1,000 types of plasma proteins. Simultaneous study of several

proteins adsorption over different materials would result in a myriad of data complicated to evaluate. Plasma proteins (PP), bovine serum albumin (BSA), fibrinogen (*Fg*) and fibronectin (*Fn*) have been selected as model proteins in this work for their important roles in the biological response to materials.

Factors affecting protein adsorption on material surfaces include, protein concentration, molecular weight (MW) of the protein, isoelectrical point (PI) of the protein and electrostatic charges, hydrophylicity or hydrophobicity of the material, presence of functional groups [12], chemical structure and the properties of the protein itself [10]. Protein adsorption occurs before cells arrive at the surface of the implanted membrane. The cells come in contact with the protein layer rather than with the membrane; hence, proteins get adsorbed onto the membrane determining the response towards tissues regeneration [1]. The bioactivity of biomaterials used for hard tissue repair are closely related to their adsorption capacities for bone-related proteins [14].

Carboxyl (COOH) and amino (NH_2) are among the main functional groups of amino acids that construct proteins. It has been previously shown that modifying materials surfaces with these specific chemical groups may affect proteins adsorption, cells adhesion and differentiation [24], or even initial apatite formation on biomaterials surfaces [15].

The aim of the study was to determine the adsorption of individual proteins related to bone regeneration and the ability of Ca/P deposition of experimental polymeric zinc-loaded membranes functionalized with carboxyl or amino groups. The tested null hypothesis is that proteins adsorption to the polymeric membranes or their ability to form biomimetic mineral deposits are not related to their chemical composition.

2. Material and Methods

2.1. Membranes preparation

Nanostructurated membranes (Tiss-OH) were acquired from NanoMyP (Granada, Spain). They were fabricated through electrospinning, with a commercial polymeric blend (PolymBlend®). PolymBlend® is composed of a mixture of two high molecular weight copolymers: methyl methacrylate-co-hydroxyethyl methacrylate (average Mw 200 kDa, PDI<2.5), and methyl acrylate-co-hydroxyethyl acrylate (average Mw 2,000 kDa, PDI<1.5). The percentage of the hydroxylated monomers in the polymer blend depended on the ratio in which the two copolymers were mixed. The recommended ratio for processing by electrospinning was 1:1, which corresponded to 41% of hydroxylated monomers. The employed monomers were approved for human use by the European Medicines Agency (EMA) and by the U.S. Food and Drug Administration (FDA). After fabrication, the surfaces of the membranes were hydrophobic. In order to make them hydrophilic, a reorientation of the hydroxyl groups (OH) towards the interface water-Tissue, took place. Then, the membranes were submerged in distilled water for 4 h at 60 ºC and dried.

To functionalize membranes whit carboxyl groups (Tiss-COOH), membranes were incubated for 30 minutes in an anhydrous sodium carbonate buffer (333 mM pH 12.5) under constant shaking, producing partial hydrolysis of the ester bonds. Then, the

membranes were subsequently washed 3 times with distilled water (15 minutes each wash, under constant shaking) and dried. To functionalize with amino groups (Tiss-NH_2) membranes were incubated for 40 minutes in a solution containing 0.35 M divinyl sulfone (DVS) in anhydrous sodium carbonate buffer (333 mM pH 12.5) under constant shaking. Membranes were subsequently washed 3 times in distilled water (15 minutes each wash, under constant shaking) and dried. In a second step, the preactivated membranes with vinyl groups were incubated for 3 h in a solution containing 0.9 M ethylenediamine (EDA) in 100 mM phosphate buffer (100 mM pH 7.4) under constant shaking in order to obtain Tiss-NH_2. After incubation, membranes were washed 3 times with distilled water (15 minutes each wash, under constant shaking) and dried.

All membranes were doped with zinc. To achieve zinc doping Tiss-NH_2 and Tiss-COOH membranes were incubated at room temperature and under continuous shaking in different aqueous solutions (pH=7) of $ZnCl_2$ (30, 80, 130, 180, 230, 280, 330, 380mgL^{-1}) during 90 min. To control for the functionalization degree the adsorption isotherms of Zn^{2+} on the membranes were studied. Experiments were performed in triplicate. Zinc isotherms are shown in Fig. 1. Zinc adsorption of 3 μg Zn/mg membrane was attained for the carboxylated membrane when immersed in 300 $[ZnCl_2]_0$ mgL^{-1}. Zinc adsorption values for the aminated membranes was 21 μg Zn/mg membrane in 300 $[ZnCl_2]_0$ mgL^{-1}. These conditions of maximum zinc quelation were selected for membranes preparation.

2.2. Bicinchoninic acid (BCA) assay protein quantification assay

Total serum protein adsorption on unmodified and modified samples was determined by using BCA assay [25]. It is an acid determination protocol in which a purple-colored reaction product is formed, by the chelation of two molecules of BCA with one cuprous ion.

Proteins adsorption was determined for zinc-doped Tiss-NH_2 and Tiss-COOH membranes. A commercially available non-resorbable PTFE-d membrane -Cytoplast TXT 200- (Osteogenics Biomedical, Lubbock, Texas, USA) was also included in the study. Protein solutions were prepared at 5% in order to be quantified at the standard curve. After being weighted, membranes were incubated with 3 mL of 5% protein solution for 3 hours at 37ºC under constant agitation. Once incubated, membranes were washed twice with 1.5 mL PBS 50 mM under constant agitation. Liquid excess was removed and the membranes were submitted to the elution process (they were added to a vial with 750 μL of the respective eluent) for 10 minutes at room temperature under constant agitation [26]. An equivalent volume of the elution samples to the standard curve samples was added to the working reagent and incubated 2 h. Once samples were measured at the spectrophotometer, their concentrations were quantified by extrapolation at the standard curve, while adjusting the results to the volume added to the elution process (750 μL). All sets of membranes incubated with 5% protein solution were accompanied by a blank membrane incubated with the same volume of PBS 50 mM. For all proteins the same procedure of incubation was used.

For bovine serum albumin (BSA) standard in the BCA kit, BSA determination was conducted with solid BSA (Acros Organics). This BSA was reconstituted with PBS 50 mM to a 5% concentration considering the blood protein concentration (80 mg/mL). Plasma proteins (PP) (Sigma-Aldrich) were reconstituted in phosphate buffered saline (PBS) 50 mM 5 mL and mixed by gentle shaking until homogeneity. The fibrinogen determination was assessed considering the *Fg* normal concentration in blood (3

mg/mL). The fibrinogen (Sigma-Aldrich) was reconstituted with PBS 50mM. The fibronectin determination was conducted after the complete reconstitution of all fibronectin (5 mg, Sigma-Aldrich) with 19 mL PBS 50 mM to a final concentration of 0.263 mg/mL. The fibronectin was diluted twice to a final concentration of 0.1315 mg/mL.

The standard curve was prepared using the BSA standard included in the BCA protein assay kit (containing BSA at 2mg/mL in 0.9% saline and 0.05% sodium azide). In this experiment, every standard curve sample was diluted using the eluent solutions, as seen in Table 1S. 0.1 mL of each standard were pipetted and added to 2.0 mL of the WR in each tube. At the given absorbance, the spectrophotometer did not find significant differences between samples. Thereby, sensitivity was increased by diluting every sample 11 times, and every sample contained the following composition: 1 mL WR + 50 µL sample + 500 µL diluents. Samples were incubated at room temperature (RT) for 2 hours and their absorbance was measured at 650–400 nm (with 562 nm absorbance peak). The BSA standard curve was applied in all cases.

BCA working reagent (WR) was prepared by mixing 50 parts of BCA reagent A (containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide) with 1 part of BCA reagent B (containing 4% cupric sulfate) (both included in Pierce BCA Protein Assay Kit (Thermo Scientific), *i.e.* for every milliliter of BCA Reagent A, 20 µL of BCA reagent B were required. Two elution solutions were used. Tris Triton Buffer solution eluent was prepared with the following reagents (with their respective concentrations adjusted for a final volume of 50 mL) [26], TRIS 10 mM pH 7.4 (The TRIS reagent must be first diluted adjusting the pH in 40 mL, approximately, before adding the rest of reagents), NaCl 100 mM, EDTA 1 mM, Glycerol 10% (from glycerol 86-88%), SDS 0.1% (0.1 g/100 mL), Triton X-100 1% (from Triton X-100 100%). Once all reagents were added, the solution was diluted to the mark with distilled water. The second solution was Tween 20 solution, which was prepared as follows: 1L distilled water, 8.5 g NaCl and 0.5 mL Tween20.

Mean adsorption values for each tested protein were analyzed by One-way ANOVA, being the independent variable the type of membrane. Multiple comparisons were performed by Student-Newman-Keuls. Statistical significance was set at $P<0.05$.

2.3. Acellular static in vitro bioactivity test

Three specimens of each experimental group (zinc-doped Tiss-NH_2 and Tiss-COOH, plus the PTFE-d membrane) were analysed. Membranes were soaked in 20 ml of simulated body fluid solution (SBFS) [pH 7.45] in sterile flasks for 7 days at 37ºC, all experimental conditions were as specified at the ISO standard 23317 [27]. Reagents per 1000 ml of SBF were: 8.035 g of NaCl, 0.355 g of $NaHCO_3$, 0.225 g of KCl, 0.231 g of $K_2HPO_4 \cdot 3H_2O$, 0.311 g of $MgCl_2 \cdot 6H_2O$, 39 g of 1M HCl,0.292 g of $CaCl_2$, 0.072 g of Na_2SO_4, 118 g of Tris, 0 to 5 ml of 1M HCl for final pH adjustment. After drying and carbon covering, surfaces were analyzed by Field Emission Electron Microscopy (FESEM) (GEMINI, Carl Zeiss SMT, Germany) at 3 Kv, 4.7 to 4.9 mm working distance. Elemental analysis was done by means of an energy dispersive analysis system (EDX) (Inca 300 and 350, Oxford Instruments, Oxford, UK).

3. Results

3.1. Proteins adsorption assessments

Proteins adsorption values attained on the tested membranes are displayed on Fig. 2. Both Tiss-NH_2 and Tiss-COOH membranes attained similar fibrotectin (*Fn*) [Tiss-NH_2: 7.27 μg prot/mg Tiss; Tiss-COOH: 8.71 μg prot/mg Tiss] and plasma proteins (PP) [Tiss-NH_2: 8.60 μg prot/mg Tiss; Tiss-COOH: 6.301 μg prot/mg Tiss] adsorption. Experimental membranes obtained higher values than those encountered for PTFE membrane [Fn: 0.50 μg prot/mg Tiss; PP 0.37 μg prot/mg Tiss]. Tiss-COOH membranes adsorbed significantly more albumin (BSA) [12 μg prot/mg Tiss] than Tiss-NH_2 [4.30 μg prot/mg Tiss]. PTFE membrane achieved the lowest amount of adsorbed BSA among the three membranes [0.48μg prot/mg Tiss]. Tiss-NH_2 membranes adsorbed significantly more *Fg* [7.87 μg prot/mg Tiss] than Tiss-COOH [3.44 μg prot/mg Tiss]. PTFE membranes did not adsorb *Fg* [0.00 μg prot/mg Tiss] (Fig. 2).

3.2. Acellular static in vitro bioactivity test

Representative FESEM images of the membranes after immersion in SBF for 7 days are shown in Fig. 3. Irregular crusts, amorphous clumps and platforms of deposits rich in calcium and phosphate were multilayered and precipitated onto the Tiss-COOH membranes. However, the Tiss-NH_2 and PTFE membranes lacked these deposits. Calcium and phosphate were identified after EDX analysis of Tiss-COOH membranes (Fig. 3f). The presence of COOH radicals, thereby, contributed to the bioactivity whilst the presence of NH_2 radicals resulted in lower or null calcium/phosphate deposits (Figs. 3c, 3i).

4. Discussion

Over 50 plasma proteins form the protein corona on the surface of biomaterials after their initial interaction with blood, and this interaction determines biomaterial integration at the surrounding tissue [17]. Protein adsorption is the initial reaction after the implantation of a biomaterial into the body and will influence subsequent cell function. Adsorption of those adherent bone related proteins on the implanted material surface could be tightly associated with its ability to induce new bone formation [14]. Hence, a surface protein layer can potentially have an impact on a variety of subsequent interactions of the colloids in the biological surrounding, due to the different protein functions such as, for example, signaling (growth factors) [28]. Protein corona formation is a dynamic process that is determined by forces between biomaterials and plasma proteins. Plasma proteins (PP), bovine serum albumin (BSA), fibrinogen (*Fg*) and fibronectin (*Fn*) have been selected as model proteins in this work for their important roles in the biological response to materials [17].

The BCA protein assay was selected, as it is a highly sensitive colorimetric assay that is used for quantification of total protein in solutions with detergents/surfactants. This method combines the well-known reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium (the biuret reaction) using a reagent containing bicinchoninic acid [25].

The osseointegration starts with a positive interaction between the biomaterial surface and surrounding tissues, which is facilitated by the adsorption of PP onto the biomaterial. Both membranes Tiss-NH_2 and Tiss-COOH adsorbed similar amount of PP (Fig. 2a). PP is a multi-protein system on protein adsorption [14], and during this process, abundant proteins bind to the surface quickly and are then replaced by less

abundant proteins with higher affinities. The lowest protein adsorption (Fig. 2) attained by the PTFE-d membrane was probably due to its hydrophobic nature, described by low surface energy and high contact angle (>110 degrees) to water-based compounds. The null protein adsorption that occurred on these PTFE membranes is in accordance with their clinical performance. Cells do not adhere to PTFE membranes, and they are never integrated into surrounding tissue, requiring a second surgery to be retrieved from the oral cavity. In order to offset this drawback, FN-silk protein has been aided to favor cell adherence and proliferation, but this attempt has been developed without conclusive results, at the moment, being in an experimental phase [6].

BSA adsorption is suitable for proteins attachment studies because of its high stability, its availability at high purity and its water solubility [16]. Its secondary structure consists of approximately 54% α-helix and 40% β-structure (β-sheet plus β-turns) and contains three binding domains, which are specific for metal ions, lipids, and nucleotides, respectively [17]. Tiss-COOH and Tiss-NH_2 were able to adsorb much higher concentration of BSA that the PTFE membranes; and a higher albumin adsorption was promoted on Tiss-COOH if compared with the attained on Tiss-NH_2 (Fig. 2b). It might be due to the charge that exhibited the carboxylated membrane and the charge heterogeneity of BSA [10,29]. Electrostatic force between the adsorbent and adsorbate play an important role in protein adsorption [10]. Materials bearing COOH chemical groups display a negative charged functionality to the surface [24]. BSA has an isoelectric point ranging from 4.7 to 5.5; thus the protein has an overall negative charge (−18e), due to the presence of a large number of carboxyl groups [11], at pH 7 [10,30]. Nevertheless, the occurrence of both negatively charged amino acids (glutamic acid, aspartic acid) and positively charged residues (lysine, histidine) on BSA can result in attachment to both positively and negatively charged surfaces [16]. Albumin has been considered as a three-dimensional cuboid with six faces, with the face with dominant positive or negative potential considered to be the adsorption site on oppositely charged functionalized surfaces [28]. An increase in pKa of the carboxylic functional group when adsorbed in the carboxylated surface can lead to the protonation of the carboxylic-terminated groups at neutral pH, resulting in the strong hydrogen bonding interactions with the BSA proteins [16]. Protein molecules near their isoelectric point generally adsorb more readily due to the reduced electrostatic repulsion between uncharged adsorbing molecules [14]. Under a clinical point of view, it should be stressed that the adsorbed BSA molecules undergo structural transformation and often denaturation to maximize their favorable interactions with the surface and the biomaterial. Distinct proteins such as albumin and IgG preferentially bind surfaces bearing strong basic or weak acid groups on their surface [31]. This finding is in line with the higher initial cell adhesion and proliferation promoted by the Tiss-COOH compared to the NH_2 surfaces after a rise in the expression of focal adhesion kinase and vinculin [24]. Abstiens *et al.* (2019) [32] also stated that albumin was likewise enriched in the corona of negatively charged carboxylated-nanomaterials; they also hold that although albumin is negatively charged at physiological pH, it contains 60 positively charged lysine groups, providing cationic patches on the surface that can interact with the carboxyl groups on the colloids. Moreover, albumin can in addition interact with positively charged proteins also present in the human plasma proteins, which initially adhere to the biomaterial, forming a second protein layer [32].

Fn is an abundant adhesion protein found in serum and in many extracellular matrices. *Fn* intermediates in cell adhesion and signalling [23]. Similar *Fn* adsorption

was found on both experimental membranes, and it was high above the adsorption values encountered for PTFE. Even when *Fn* binds better to hydrophobic surfaces than to hydrophilic ones [18]. *Fn* has an isoelectric point of 5.39 and is negatively charged at pH = 7.4 [34]. *Fn* is a multidomain protein whose monomer ranges in size from 230 to 270 kDa, or even ~440 kDa [35], and binds into dimers via two disulphide bonds at the C-terminus of the protein. Attending to clinical performance, not only PP but also *Fn* play a vital role in cell adhesion to an artificial material. *Fn* contains domains to interact with other extracellular matrix proteins [18]. ECM is very important for tissue formation during the wound healing. ECM associates cells together in tissues and guides their growth during wound healing, providing environmental signals to cells. *Fn* is one of the essential ECM components, which assembles into fibrils and guides cells in attaching to the ECM. It has also been stated that cell adhesion to ECM is crucial in the expression of the osteoblastic phenotype and bone tissue formation. So, *Fn* is essential for the bony tissue formation during the hard tissue healing (Biao et al, 2017). In this case, as well as protein quantity, the availability of specific protein binding sites is crucial in determining cell adhesion. Hence, enhanced protein adsorption and adsorption of *Fn* in optimal conformations on modified tissues may account for the increased cell adhesion [24]. *Fn* acquires a extended conformation for low availability in the media and is active when the N-terminal is accessible, which is favored by the extended conformation [12]. The presence of adsorbed BSA has also been related with the adsorption and activation of *Fn* [18], and surface passivation [19]. BSA not only can increase the binding of *Fn* to tissue culture plates but its biological activity [18].

Fg was also preferentially adsorbed to experimental membranes, if compared to PTFE ones. *Fg* is a rod-like molecule whose dimmers are composed of three different regions. Two of them bind to heparin and collagen and the third one contains an arginylglycylaspartic [ARG Gly-ASP (RGD)] aminoacids sequence which is a recognized cell binding domain [21]. *Fg* plays a critical role in blood clotting and promotes bone regeneration [22]. Both *Fg* and *Fn* are fibroid serum proteins that suffer conformational changes when they are adsorbed on the surface of a material it may determine their role in the biological response [12]. A relatively higher (double-fold) *Fg* adsorption on the positively charged Tiss-NH_2 membranes than in the negatively charged Tiss-COOH (Fig. 2c) indicated that, the protein–protein forces may play a more important role than in *Fn* [12]. *Fg* has been shown to form hydrogen bonds with amino groups (NH_2) and adhere to the surface of the membrane. *Fg* is negatively charged (5.2-7.6 net charge) under physiological conditions (pH = 7.4, I = 0.15 M) [20]. The adsorbent surface with opposite charge to that of the protein can be a better adsorbent than if they are of the same charges [10]. The structure of *Fg* contains a central hydrophobic domain connected to two other hydrophobic external domains through a coiled-coin chain. These domains are negatively charged on neutral pH conditions whereas there are also Arg and Lys residues positively charged and substantially more hydrophilic character [12]. NH_2 has also been shown to preferentially promote the exposure of high-density receptors as well as focal adhesion components allowing for adhesion. The amino group (NH_2) provides a positive charge to the biomaterial surface, bearing basic functional groups [24]. Adsorbed *Fg* does also exert a pro-osteogenic effect on human monocytes through its interaction with TLR-4 and subsequent production of BMP-2, elucidating two key aspects of the immunomodulatory action of adsorbed *Fg* in bone regeneration (Oliveira et al, 2017).

Calcium phosphate deposits were created on Tiss-COOH (Figs 3d, 3e, 3f) and it was stated a lack of bioactivity on the aminated and PTFE membranes (Figs 3a, 3b, 3c)

may be due to calcium ions in SBF, which are apt to be trapped by the double scissors of COO−function. Then, the still bounded calcium ions attracted negatively charged phosphate ions from the solution [36] to form a columnar Ca framework in calcium phosphate deposits [15]. Furthermore, Liu *et al.* (2006) [38] reported that HAP formation was successfully achieved with weaker acidic -PO_4H_2 and –COOH , but not with -OH and -$CH=CH_2$. Notably, the -COOH end group appeared to provide the optimal surface for nucleation and growth of biomimetic HAP. From EDX analysis, it may be inferred that formed minerals have a low Ca/P ratio (Fig. 3f), consistent with the possibility of calcium-deficient apatite formation, which is in accordance with previous findings on the deposition of HAP on polymer surfaces after SBF immersion [36,39]. However, this asseveration may only be confirmed by diffraction X-Ray analysis, that it is not feasible at the present experimental surfaces.

The affinity for interaction between polar side groups on the protein and positive (calcium) or negative (phosphate) sites is also of significant interest. Therefore, proteins display acidic functional groups such as carboxylic acid groups on substrate surfaces that effectively induce the heterogeneous nucleation of HAP in SBF. HAP obtained from SBF is a bone-like apatite with high biological affinity due to its small crystallites and a defective structure, which means that it is appropriate for uses as bioactive biomaterials [36]. Another important factor that influences calcium phosphate formation is the pH condition. HAP forms in alkaline conditions and Zn serves as co-factor for the enzyme alkaline phosphatase, which is involved in bone mineralization [40]. In strongly acidic conditions, the new mineral is mainly dicalcium phosphate dehydrate; and in weakly acidic conditions near pH 7.0, it could be tricalcium phosphate and octacalcium phosphate [15]. Hence this approach could be used to selectively enhance bone after the biomaterial implantation *in vivo* to repair skeletal defects. Since HAP is an ionic crystal, the attraction -and adsorption- of positively charged calcium ions would promote apatite formation.

In this study, the proteins were used separately to bring the interrelation between specific functionalized surfaces and the properties of individual proteins into focus. Protein–protein interactions or other unknown interfering reactions, as occur in complex systems, are hereby avoided [28]. This work has been driven by the pragmatic approach of understanding the influence of material surface features in the adsorption of the first protein layer toward prospective biomaterial design. Adsorption and competition among different proteins in solution or in a biological milleu (blood) is much more challenging and should be tackled in a future research. Thus, new strategies for selectively stimulating bone formation without promoting bone resorption should go through the specific design of materials to selectively adsorb the appropriate protein layer for the target cell/tissue/application.

It is known that proteins adsorption on biomaterials is involved in the bone formation and in the wound healing processes [] and also that mineral precipitation ability is a desirable property for bone regeneration materials []. However, it is hard to find research about the interaction between the membranes for guided bone regeneration and these proteins and about the capacity of these membranes to induce mineral precipitates. It is the main novelty of the present research, together with the introduction of an experimental non-resorbable membrane for bone regeneration able to produce the

two above mentioned effects. Moreover, these COOH-functionalized membranes have been shown to facilitate osteoblasts cell proliferation [JDent] and bone regeneration in an animal model research [CLOI].

For further *in vivo* research, it is important to highlight that surface chemistry has also been shown to influence focal adhesion assembly and downstream cell signaling. Focal adhesion kinase localizes to focal adhesions to activate several signaling pathways including cell migration, proliferation and differentiation. COOH surfaces were found to increase cell growth through up-regulation of focal adhesion components [24]. Even more, cell viability and DNA content over 14 days was also greater on the COOH modified biomaterials than on NH_2 modified, though NH_2 functionalization also increased cell growth [24]. The mineralization of HAP on the proposed polymer substrate surfaces has been controllable by the protein adsorption abilities of the polymers. It also has been shown that among the anionic moieties, carboxyl groups may represent effective binding sites for calcium ions [36], triggering bone formation and mineralization.

5. Conclusions

For all tested proteins, experimental membranes adsorbed higher concentrations than the commercial PTFE membrane which may lead to major cells adhesion, proliferation and membrane integration into the surrounding tissue. However, the clinical relevance of the encountered differences between the two experimental membranes (Tiss-COOH and Tiss-NH_2) remains to be ascertained. However, membranes functionalized with carboxyl groups will also facilitate calcium phosphate precipitation on the membrane surface, which may additionally lead to prompt membrane mineralization.

6. Disclosure

The authors report no conflicts of interest in this work.

7. Acknowledgements

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Legends for Figures

Figure 1. Attained zinc complexation values (µg Zn/mg membrane) for the different experimental membranes (n=3).

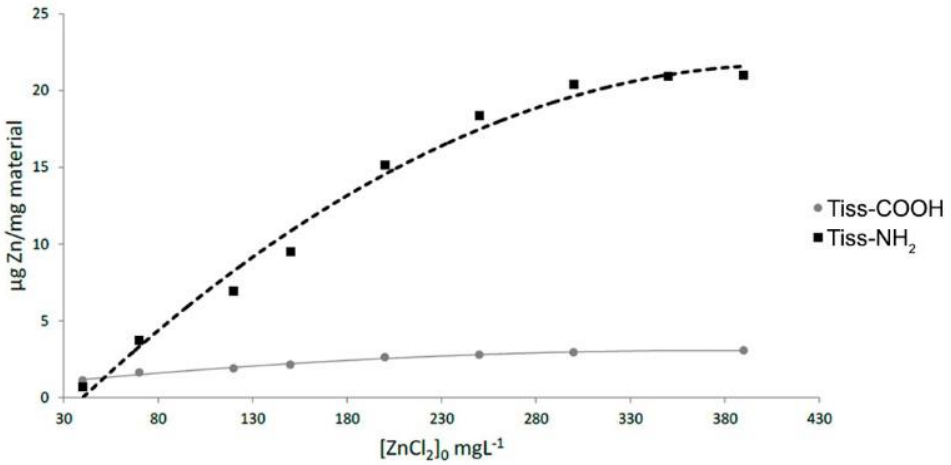

Figure 2. Attained human plasma proteins (PP), bovine serum albumin (BSA), fibrinogen (Fg) and fibronectin (Fn) on aminated (NH_2), carboxilated (COOH) and polytetrafluoroethylene (PTFE) membranes.

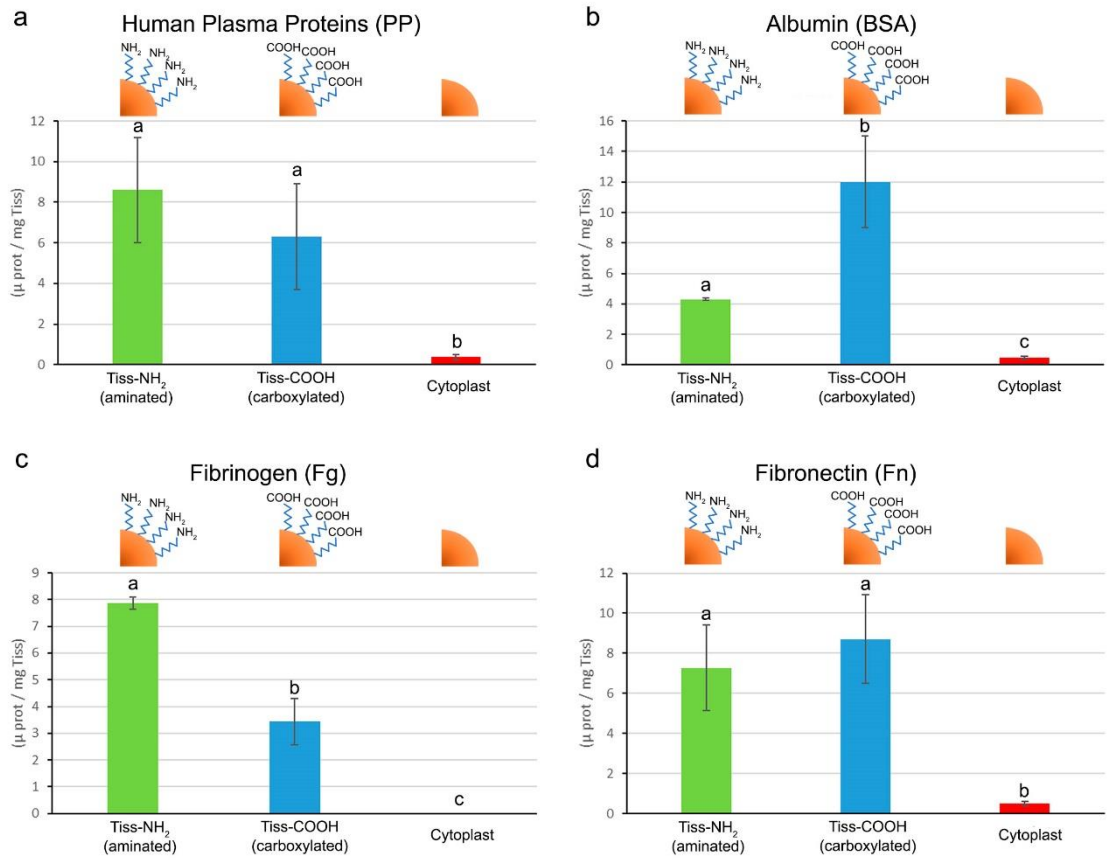

Figure 3. FESEM micrographs of membranes after 7 days of SBF immersion are presented in: a and b, Tiss-NH_2 membrane where very few or no mineral deposits were observed. The membrane showed its typical smooth appearance; d and e, Tiss-COOH membrane where a rougher surface characterized its appearance. Mineral deposits were uniformly distributed throughout the nanofiber surfaces; g and h, PTFE membrane without any mineral precipitation exhibited. Both are images of the surface designed to interact with the epithelial tissue; note the hexagonal surface texture, that according to the manufacturer helps to stabilize the membrane and the soft tissue flap**.** Calcium and phosphate were only identified after EDX analysis in Tiss-COOH membrane (f). In NH_2 (c) and PTFE (i) membranes, calcium and phosphorous were not encountered at the EDX spectra on nanofiber surfaces. At the presented EDX spectra, chloride and sodium are detected after SBFS immersion. Zinc was not detected in the EDX analysis as attained zinc concentration on membranes is out of the detection range of the performed EDX analysis.