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Osteoblast, fibroblast and in vivo biological response to poly(vinylidene fluoride) based composite materials

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Abstract Electroactive materials can be taken to advantage for the development of sensors and actuators as well as for novel tissue engineering strategies. Composites based on poly(vinylidene fluoride), PVDF, have been evaluated with respect to their biological response. Cell viability and proliferation were performed in vitro both with Mesenchymal Stem Cells differentiated to osteoblasts and Human Fibroblast Foreskin 1. In vivo tests were also performed using 6-week-old C57Bl/6 mice. It was concluded that zeolite and clay composites are biocompatible materials promoting cell response and not showing in vivo pro-inflammatory effects which renders both of them attractive for biological applications and tissue engineering, opening interesting perspectives to development of scaffolds from these composites. Ferrite and silver nanoparticle composites decrease osteoblast cell viability and carbon nanotubes decrease fibroblast viability. Further, carbon nanotube composites result in a significant increase in local vascularization accompanied an increase of inflammatory markers after implantation.

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1 Introduction

Novel materials are continuously developed with the objective of being used in biomedical applications. Among these, polymer based materials have confirmed to be a good choice as biomaterials in tissue engineering applications, smart prostheses, and sensors, among others [1, 2]. In the last years, the potential of electro active polymers has been recognized for biomedical applications due to its ability to convert mechanical, thermal, or magnetic signals into electrical ones. It this sense, these materials can be used as smart scaffolds to stimulate cell growth and compatibility, biosensors, mechanical sensors, and actuators, among others [3]. From the short choice of electro active polymers, including poly(L-lactic acid) (PLLA) and poly-(hydroxybutyrate) (PHB), poly(vinylidenefluoride) (PVDF) and its co-polymers are still the ones with the best electro active performance, showing the largest piezo, pyro, and ferroelectricity responses [4]. Poly(vinylidenefluoride) can be obtained in different crystalline phases, known as α, β, γ, and δ, depending on the processing conditions. The β-phase is the one with the best electro active properties [4, 5]. The possibility of tailoring PVDF properties and microstructure, allows new and challenging applications in the biomedical area, not only in device applications but also induce targeted cell responses [6]. Previous studies investigated the biocompatibility of PVDF films and showed the PVDF as a very promising material for biomedical applications [6, 7]. In this sense, the effect of the phase of PVDF and its polarization state has been previously studied [6]. Low et al. [8] studied the in vitro attachment and metabolic activity of L929 cells on PVDF films and showed that α-phase PVDF supports higher cell metabolic activity and cell spreading compared to β-phase PVDF. The preparation of the most recent PVDF-based composites aims to improve the polymer piezoelectric properties and/or to add new and

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interesting properties for distinct applications. In particular, the addition of magnetic nanoparticles allows to obtain magnetoelectric and multiferroic composites for sensors and cell stimulation [9]; the addition of Ag particles allows larger dielectric response and antimicrobial properties [10]; the zeolites addition allows increasing dielectric properties, functional properties, and controlled drug release [11]; the ceramic fillers to improve electroactivity [12] and the addition of carbon nanotubes increases dielectric and mechanical properties [13]. On the other hand, once fillers have been introduced into the polymer matrix, biomaterial–cell interaction is modified and novel bioactivity or even suppression of biocompatibility can occur [14, 15].

In the tissue engineering field, the knowledge of cell/biomaterial interactions is the key issue [16], being the knowledge of the material biocompatibility a fundamental parameter for successful membrane and scaffold development [17]. Cell culture techniques have been extensively used for the study of cell/biomaterial interactions allowing the investigation of the biomaterial cytotoxicity and biocompatibility. The cell attachment on the biomaterials is considered to be one of the most important stages in cell/biomaterial interaction [18]. The in vivo interactions are more complex than those in vitro, which do not reflect the whole body response of the organism such as immune and inflammatory response [19]. The in vivo biomaterial implantation is assumed to require regular wound healing mechanisms, a complex process involving the dynamic interaction of different cell types like inflammatory cells, fibroblasts, vascular smooth muscle cells, and endothelial cells. Fibroblasts are ubiquitous in organs and play a crucial role in tissue regeneration provided its ability to produce extracellular matrix components like collagen. In addition, these cells are also able to release growth factors that promote tissue homeostasis. Angiogenesis is a crucial step in this cascade of events as it provides the formation of new blood vessels from preexisting ones to ensure the transport of oxygen, nutrients, and growth factors to promote tissue vascularization and remodeling at the implanted site [20, 21]. However, exacerbated angiogenesis comprises regular wound healing by a persistent inflammatory reaction being critical for the success of many tissue engineering applications [20, 22].

As the viability of PVDF and its phase and polarization state has been previously addressed [6], the objective of this investigation is to evaluate the potential of several PVDF-based composites for tissue engineering applications by investigating the biological response of two cell types, Mesenchymal Stem Cells differentiated to osteoblasts and Human Foreskin Fibroblast 1, which are present in the implanted site and are responsible to cover the developed materials. Cell viability and proliferation were investigated in an in vitro environment, whereas biocompatibility of PVDF nanocomposites was also studied in an in vivo environment. The composites were investigated in the phase in which they appear after processing, through the α to β phase transformation can be achieved in all of them by a conventional stretching procedure in order to obtain piezoelectric composites [23, 24].

2 Materials and methods

2.1 Preparation of samples

Poly(vinylidenefluoride) (Solef 1010 from Solvay) was dissolved in *N,N*-dimethyl formamide (DMF) (20 wt% PVDF) and α-PVDF films were obtained by spreading a PVDF solution on a glass slide as described previously in [23, 24]. In order to obtain β-phase, the conventional stretching procedure was achieved on α-PVDF films [24, 25]. The PVDF composites were obtained by the addition of filler into the PVDF solution. The selected fillers were purchased from different providers: $CoFe_2O_4$ from Nanoamor; Zeolite NaY from Zeolist; Carbon nanotubes from *Applied Sciences Inc*.; and Montmorillonite from Sigma–Aldrich. In some cases, such as the cases of ferrite nanoparticles and clays, the electro active β-phase of the polymer is directly nucleated. It is to notice, that in all cases, the β-phase can be obtained by stretching [23, 24]. The filler content was chosen in order to maximize the respective effect on the PVDF matrix (increase of the dielectric constant, piezoelectric phase, and mechanical properties, among others). It is also to notice that the phase of the polymer has no effect on its biocompatibility [6].

In particular, the pure polymer samples and polymer composites indicated in Table 1 were prepared. In Table 1, references are also given where the main characteristics of the prepared samples can be found.

2.2 Cell culture

Mesenchymal stem cells (MSC) were obtained from ATCC. Mesenchymal stem cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with Glutamax (Gibco BRL®, NY, USA), supplemented with 10 % Fetal Bovine Serum (FBS) (Gibco BRL®, NY, USA), and 100 U/mL penicillin/streptomycin (P/S) (Gibco BRL®, NY, USA). After that, the MSC cells were differentiated in osteoblast before the experiments.

Human Foreskin Fibroblast cell line (HFF1) was purchased on ATCC. Human Foreskin Fibroblast cells were grown in high-glucose DMEM medium (Sigma, USA) supplemented with 15 % FBS (Invitrogen Life Technologies), and 100 U/mL P/S (Gibco BRL®, NY, USA). Both cell lines were maintained at 37 °C in a humidified 5 %

Table 1 Specification of the PVDF samples

Matrix	Filler	Filler concentration (%)	Reference	Identification
β-PVDF	No filler	–	[25]	β-PVDF
	$CoFe_2O_4$	40	[9]	β-PVDF/Co-40
α-PVDF	No filler	–	[25]	α-PVDF
	$(PbZr_{0,53}Ti_{0,47})O_3$	10	[26]	α-PVDF/PZT-10
	Zeolite NaY	32	[11]	α-PVDF/NaY-32
	Carbon nanotubes	5	[27]	α-PVDF/NTC-5
	Ag nanoparticles	0.005	[28]	α-PVDF/Ag-0.005
	Ag nanoparticles	0.02	[28]	α-PVDF/Ag-0.02
γ-PVDF	Montmorillonite	4	[29]	γ-PVDF/MT-4

CO_2 atmosphere. The medium was replaced every 2 days and when the cells reached the confluence, they were trypsinized and subcultured at 1:3 ratios. Cells between passages seven and ten were used for all experiments.

2.3 Cell viability

Human Foreskin Fibroblast and osteoblast cells (1 x 10^5 cells/mL) were seeded in 96-well cell culture plates on top of each sample and allowed to grow for 24 h. As a control, cells were grown directly in the bottom of the culture plates. Cell viability was assessed using Cell Titer 96® Aqueous ONE Solution Reagent [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS) colorimetric assay (Promega, Madison, USA), according to the manufacturer instructions. After 24 h in culture, MTS was added to each well plate and incubated at 37 °C protected from light. The optical density was then measured at 492 nm using a microplate reader (Thermo Electron Corporation, Multiskan Ascent). All samples were assayed in triplicate and results are presented as mean ± SEM and expressed as the percentage of control.

2.4 Cell proliferation

To determine the cell proliferation potential of each composite an incubation of Methyl-^{3}H thymidine (88 μCi/μmol) (Amersham Biosciences®; Buckinghamshire; UK) was conducted. Cells were seeded in 24-well cell culture plates (3 x 10^4 cells/well) in a final volume of 500 μL incomplete culture medium (without FBS). After 24 h in contact with the different composites, cells were then incubated with Methyl-^{3}H thymidine in the final concentration of 0.5 μCi/well in 200 μL incomplete culture medium (without FBS) for 4 h at 37 °C. The medium was removed and cells were fixed with 10 % trichloroacetic acid (TCA) (Merck®, Darmstadt, Germany) and washed twice with PBS to remove the unbound radioactivity. Cells were lysed with 0.1 % Triton/PBS and then the lysate was added to scintillation fluid (PerkinElmer®, Waltham, USA). The DNA synthesis was measured by incorporation of Methyl-^{3}H thymidine in a liquid scintillation beta counter (RackBeta, Turku, Finland), as previously described in Miranda et al. [30] and the results are presented. The counts (disintegrations per min) of each treatment were averaged and expressed as the percentage of control.

2.5 Animal studies

To evaluate the compounds biocompatibility an in vivo assay using 6-week-old C57Bl/6 mice (Charles River Laboratories Inc., USA) was conducted to directly determine the effect in skin angiogenesis and systemic inflammation triggered by the contact of composites. Animals were maintained under controlled conditions of temperature 23 ± 5 °C and humidity of 35 ± 5 % under a 12 h dark/light cycle and allowed free access to regular chow diet and water ad libitum. To perform this study, animals were randomly divided into nine groups (one for each compound), n = 3. After anaesthesia, equal dimensions of compounds were subcutaneously implanted (±0.5 cm) into the dorsal skinfold. Control group consists only in the surgical procedure without implantation. After 2 weeks, the animals were euthanized with a lethal dose of pentobarbital (30 mg/kg). Blood samples were collected and centrifuged (2,000×*g* for 15 min) and evaluation of systemic inflammatory markers was performed, namely *N*–acetyl-ß-D-glucosaminidase (NAG) and nitric oxide (NO) in plasma.

A biopsy of skin tissue surrounding the implant was fixed in 10 % neutral-buffered formalin, processed by dehydration incubating through a graded series of ethanol, xylol and finally embedded in paraffin blocks. Three-micrometer-thick tissue sections were used for histological and immunohistochemistry analysis for CD31 marker to access angiogenesis. All animal care and procedures were

in accordance to the Portuguese Act 1005/92 (number 3, iii) and European Community guidelines (86/609/EEC) for the use of experimental animals.

2.6 Systemic inflammatory markers analyses

2.6.1 N-acetyl-ß-D-glucosaminidase (NAG) assay

N-acetyl-ß-D-glucosaminidase is a lysosomal enzyme highly expressed in activated macrophages, being its plasmatic presence an important inflammatory marker. To perform this assay, in a 96-well plate, 100 µL of rat serum was incubated with equal volume of the substrate *p*-nitrophenyl-*N*-acetyl-ß-D-glucosaminide solution at 37 °C. The reaction was stopped with a freshly 0.2 M glycine buffer (pH 10.6) and the substrate hydrolysis was measured at 405 nm in a spectrophotometer plate reader (Thermo Electron Corporation, Multiskan Ascent). All samples were assayed in triplicate. Results were expressed as NAG concentration (nmol/mL).

2.6.2 Nitric oxide (NO) assay

The detection of total NO levels, considering total amount of their metabolites, nitrate and nitrite, was performed in the serum by colorimetric assay using Griess reagent method. In a 96-well plate, 100 µL of rat serum was incubated with equal volume of Griess Reagent, for 15 min at room temperature on the shaker. Then, the optical density was measured at 550 nm in a spectrophotometer microplate reader (Thermo Electron Corporation, Multiskan Ascent). All samples were assayed in triplicate. Results were expressed as NO concentration (µM).

2.7 Immunohistochemistry

Sections of skin tissue surrounding material implantation were used for capillary endothelial cells immunostaining. Endogenous peroxidase activity was blocked with 4 % hydrogen peroxide in PBS for 30 min at room temperature. To retrieve antigen, sections were placed in 10 mM citrate buffer (pH 6) and heated at 98 °C. After blocking with 10 % BSA in PBS for 1 h, sections were incubated with primary antibody against CD31 (1:100) (Abcam, Cambridge, UK) overnight at 4 °C. Then, anti-rabbit secondary antibody (1:200) (Santa Cruz Biotechnology, USA) was applied for 30 min. Avidin–Biotin Complex (ABC) complex method (Vectastain ABC kit, Vector, Burlingame, CA, USA) was used according to the manufacturer's instructions. The antigen–antibody reaction was developed using diaminobenzidine (DAB) (DAB substract kit, Abcam, Cambridge, UK) as peroxidase substrate, rendering CD31 positive cells with a brown staining. Sections were counterstained with hematoxylin (Sigma-Aldrich, Portugal), dehydrated and coverslipped. CD31-expressing microvessels were counted in the three most vascularized areas with magnification of 200×, and the data were averaged and normalized to the total area of the tissue section. Any positive-staining endothelial cell or endothelial cell cluster that was separated from adjacent microvessels was considered an individual vessel [31].

2.8 Statistical analysis

Results are expressed as mean ± SEM. All the assays were performed in $n \geq 3$. Statistical significance of the difference between different materials was evaluated by one-way analysis variance (ANOVA) followed by the Bonferroni test. Statistical analyses were performed using GraphPad Prism software (GraphPad Software version 5.0, San Diego, CA, USA). Significance was set at $P \leq 0.05$.

3 Results and discussion

3.1 In vitro assays

To evaluate the biological in vitro compatibility and possible cytotoxic effects of different polymer composites, two human cells types, fibroblasts and osteoblast, were included. The choice of these cells was done since fibroblasts and osteoblasts can be found in physiological locations for which implants based on smart materials can be directed, as one the potential of electro active materials is in particular for osteoregeneration and smart prosthesis applications as sensors and actuators [32, 33].

3.1.1 Cell viability

The cell viability of osteoblast and HFF1 cells on different composites was analyzed after 24 h with MTS test and it is shown in Fig. 1.

The osteoblast viability decreases significantly in contact with β-PVDF/Co-40 composite comparing to the control (without composite) and α e β-PVDF. The composite α-PVDF/Ag-0.02 shows also a reduced viability compared to the same samples (Fig. 1a). No significant differences in cell viability were observed for the others composites, when compared to control.

Human Foreskin Fibroblast cells were also seeded into composites for 24 h. In Fig. 1b, it is possible to notice that there is no cytotoxic effect upon incubation of different composites, with the exception of α-PVDF/NTC-5 as compared to the β-PVDF.

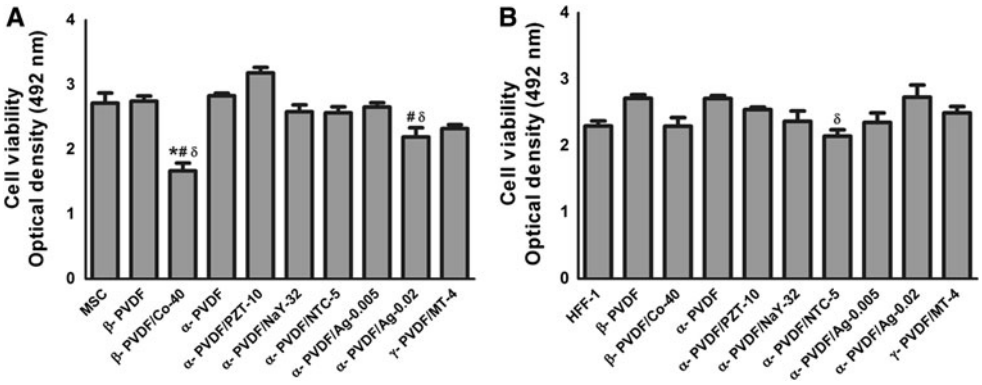

Fig. 1 **a** Osteoblast and **b** fibroblast viability after incubation with different composites. Each value represent the mean ± SEM, n = 6. * $P \leq 0.05$ vs MSC or HFF-1 in the absence of compounds; # $P \leq 0.05$ vs α- PVDF; δ $P \leq 0.05$ vs β- PVDF

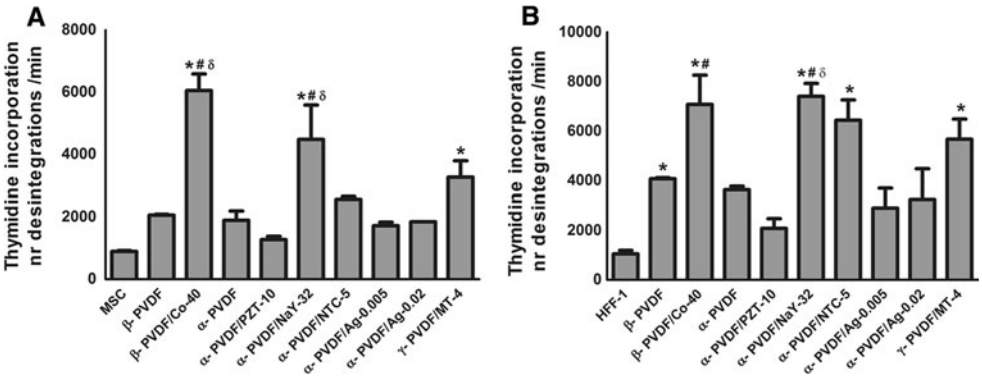

Fig. 2 **a** Osteoblast and **b** fibroblast proliferation assessed by radioactive thymidine incorporation upon incubation with different composites. Each value represent the mean ± SEM, n = 6. * $P \leq 0.05$ vs MSC or HFF-1 in the absence of compounds; # $P \leq 0.05$ vs α- PVDF; δ $P \leq 0.05$ vs β- PVDF

3.1.2 Cell proliferation

Cell proliferation of osteoblast and fibroblast was analyzed after 24 h of culture on different composites and the results are shown in Fig. 2a, b, respectively. It is to notice that although cell viability (Fig. 1) is related to the number of cells (proliferation), the correlation between cell viability and proliferation can only be performed by studying the evolution of the cell culture over time and not for a single culture time.

Depending on the used type of cells, the effect of the biomaterial on the proliferation rate is quite different. According to Fig. 2a, the β-PVDF/Co-40 and α-PVDF/NaY-32 composite increases the osteoblast proliferation after 24 h, comparing to α- and β- PVDF. Furthermore, the osteoblast proliferation on γ-PVDF/MT-4 composite increases significantly when compared with the control.

In Fig. 2b, we can denote that the β-PVDF/Co-40, γ-PVDF/MT-4, α-PVDF/NaY-32, α-PVDF/NTC-5, and β- PVDF composites show significantly higher proliferation as compared to the control. The fibroblast proliferation increase induced by β-PVDF/Co-40 was statistically significant compared with α- PVDF, which also occurs with the composite α-PVDF/NaY-32 as compared to β- PVDF.

Observing the results, the γ-PVDF/MT-4, β-PVDF/Co-40, and α-PVDF/NaY-32 composites increase the cell proliferation in both cell cultures, which is quite beneficial for the successful of biological implants. However, contrarily to the expected, the composite β-PVDF/Co-40 shows an increase of osteoblast and fibroblast proliferation but a decrease on osteoblast viability, compromising their biomedical applications.

3.2 In vivo assays

The promising results with PVDF in cell cultures prompted us to examine whether this composites interferes with inflammatory and vascular processes, two features associated during tissue regeneration. The in vivo biocompatibility evaluation of the PVDF composites was therefore analyzed in angiogenic and systemic inflammatory processes.

3.2.1 Immunohistochemistry

A controlled and effective angiogenic process is crucial for the success of biomaterials implantation. We evaluated the microvascular sprouting by the quantification of the number of vessels present in skin sections that were in contact with different composites for a period of 14 days (Fig. 3a). According to the results, it was found that α-PVDF/NTC-5 composite reveal a pro-angiogenic potential upon CD31 labeling (Fig. 3a, b). No significant differences of microvessel density comparing with the control were detected on the other composites.

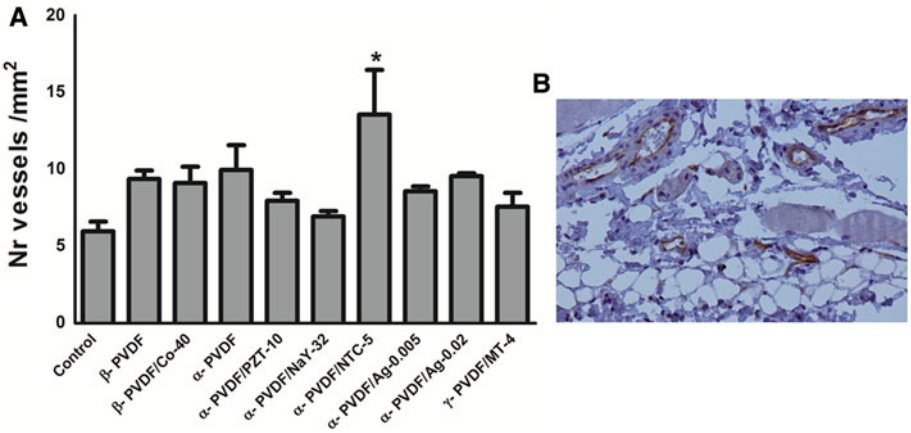

Fig. 3 **a** Angiogenesis evaluation of skin vessels number, in contact with implant, by CD31 labeling. Each value represent the mean ± SEM, n = 6. * $P \leq 0.05$ vs control; # $P \leq 0.05$ vs α- PVDF; δ $P \leq 0.05$ vs β- PVDF. **b** Representative image of immunodetection with CD31 endothelial marker. Magnification of ×200

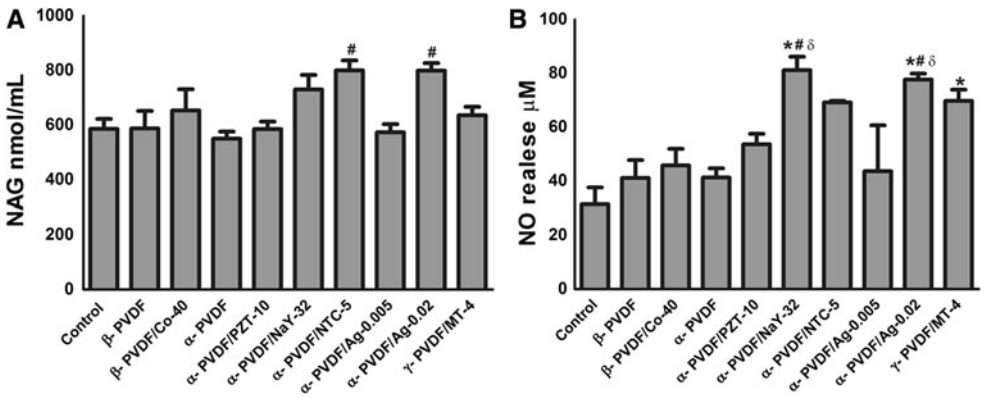

Fig. 4 Plasma quantification of NAG enzyme **(a)** and NO release, inflammatory process mediators, after 2 weeks of subcutaneous implants procedure **(b)**. Each value represent the mean ± SEM, n = 6. * $P \leq 0.05$ vs control; # $P \leq 0.05$ vs α- PVDF; δ $P \leq 0.05$ vs β- PVDF

3.2.2 Systematic inflammatory markers analyses

To understand the composites biocompatibility, inflammatory markers such as *N*-acetyl-ß-D-glucosaminidase enzyme and nitric oxide were determined.

3.2.3 N-acetyl-ß-D-glucosaminidase (NAG) assay

The mononuclear cells infiltration into the implants was quantified measuring the lysosomal enzyme *N*-acetyl-ß-D-glucosaminidase (NAG) levels that characterized the presence of active macrophages. Figure 4a shows that α-PVDF/NTC-5 and α-PVDF/Ag-0.02 composites induced an increase of NAG enzyme compared to the α-PVDF. α-PVDF/Ag-0.005 show the lower NAG quantity of all composites.

3.2.4 Nitric oxide (NO) assay

Nitric oxide is produced by many cell types and is an important effector molecule for several processes [34, 35]. The NO plasma quantification after 2 weeks of PVDF composite contact with subcutaneous implants is shown in Fig. 4b. Differences in NO production between cells proliferating on control, α- and β- PVDF and PVDF composites were observed. After contact with γ-PVDF/MT-4, α-PVDF/NaY-32, and α-PVDF/Ag-0.02 composites, a significant increase of NO inflammatory marker was verified compared with the control. α-Poly(vinylidenefluoride)/NaY-32 and α-PVDF/Ag-0.02 composites also show a significant increase compared with α- and β- PVDF.

It is to notice that quantitative profiles of the two inflammatory markers showed no correlation to each other as these two inflammatory markers operate in different physiological processes: whereas NAG is a lysosomal enzyme highly expressed in activated macrophages and involved in the breakdown metabolism of glycoproteins, NO is produced by many cells types and is an important effector molecule for several processes, being NO production the first step of a cascade of events that prevents blood coagulation and thrombus formation.

4 Discussion

Body movements impose dynamic loads that are convenient to be measured in implanted mechanical devices providing valuable information on the healing process and

on the device varying environment [36, 37]. Further, dynamic loads can be also taken to advantage in active tissue engineering strategies [6]. Implant strain and stress monitoring, as well as cell stimulation can take advantage of the use of electroactive materials such as PVDF. Further, research in tissue engineering has been working to develop new materials which could enhance or inhibit NO production due to its relevance to physiological functions. A cascade of events that prevents blood coagulation and thrombus formation was started with the NO production [38]. Nitric oxide production in bone cells has been linked to their proliferation and differentiation, than can be improved by the use of electro active materials due to the piezoelectricity of bone itself. Moderate NO synthesis in osteoblasts is necessary for the maintenance of their growth [39].

The purpose of this study was to examine the biocompatibility of several composites based on PVDF, the biocompatible polymer with the best piezoelectric response. Provided that osteoblasts and fibroblasts are the most abundant cells in the implantation sites, the effect of different compounds in these two cell cultures has been investigated.

It was verified that β-PVDF/Co-40 and α-PVDF/Ag-0.02 composites decreased osteoblast viability and α-PVDF/NTC-5 composite decreased fibroblast viability. Although these are in vitro findings, and hence restricted to how composites affect one cell type rather than the whole tissue, these two composites may compromise the behavior of osteoblasts and fibroblasts. Moreover, implantation of α-PVDF/NTC-5 resulted in a significant increase in local vascularization, accompanied by significant augmented in systemic inflammatory markers, confirming therefore the inadequate use of these materials in living organisms. In this way, whenever used for in vivo applications, the materials should be properly encapsulated. In contrast, γ-PVDF/MT-4 and α-PVDF/NaY-32 composites increased the proliferation of both cell cultures and did not show a significant in vivo pro-inflammatory effect, which renders both of them attractive for biological applications and tissue engineering, opening good perspectives to development of scaffolds with these compounds. These biomaterials are supposed to improve patient's mobility without body rejection. Implantation of foreign materials is often accompanied by exacerbated inflammatory systemic markers with increased macrophages release that secrete pro-inflammatory cytokines. Given the cross-talk between inflammation and angiogenesis, there is also a strong increase in neovascularization at the implantation site leading to tissue damage. In this sense, the composites γ-PVDF/MT-4 and α-PVDF/NaY-32 besides the good performance in vitro, did not promote inflammatory reaction, accompanied by a controlled vascular bed at the implanted site.

5 Conclusions

Smart polymer and in particular electro active materials can be taken to advantage for the development of sensors and actuators or to novel tissue engineering strategies. A large set of composites based on PVDF, the polymer with the largest piezoelectric response have been evaluated with respect to their biological response. Cell viability and proliferation were performed in vitro both with Mesenchymal Stem Cells differentiated to osteoblasts and Human Foreskin Fibroblast 1. In vivo tests were also performed using 6-week-old C57Bl/6 mice. Taken together, both in vitro and in vivo findings it can be concluded that for the biological applications in direct contact with the cells, zeolite and clay composites are identified as biocompatible materials promoting cell response and not showing in vivo pro-inflammatory effects, which renders both of them attractive for biological applications and tissue engineering, opening good perspectives to development of scaffolds from these composites. From the remaining composites, ferrite and silver composites decrease osteoblast cell viability and carbon nanotubes decreased fibroblast viability. Further, carbon nanotube composites resulted in a significant increase in local vascularization, accompanied by significant augmented in systemic inflammatory markers after implantation.

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