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Biocompatibility of novel polymer-apatite nanocomposite fibers

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Abstract: On the basis of the bioactivity of hydroxyapatite (HA) and the excellent mechanical and biocompatible performance of polyethylene terephthalate (PET), composite microfibers made of nanograde HA with PET was designed and fabricated to mimic the structure of biological bone, which exhibits a composite of nanograde apatite crystals and natural polymer. The PET/HA nanocomposite was molded into fibers so that the bulk structures' mechanical properties can be custom tailored by changing the final 3D orientation of the fibbers. This study focused on the *in vitro* biocompatibility evaluation of the PET/HA composite fibers as potential bone fixation biomaterial for total hip replacement prosthesis surfaces. The MTT assay was performed with the extracts of the composite fibers in order to evaluate the short-term effects of the degradation products. The cell morphology of L929 mouse fibroblast cell line was analyzed after direct contact with the fiber scaffolds for different time periods, and the cell viability was also analyzed by the Alamar Blue assay. The release of the inflammatory cytokine, tumor necrosis factor-alpha (TNF-α), from RAW 264.7 macrophages in the presence of fiber extracts and fibers was used as a measure of the inflammatory response. The ability of the fiber matrices to support L929 attachment, spreading, and growth *in vitro*, combined with the compatible degradation extracts and low inflammation potential of the fibers and extracts, suggests potential use of these fibers as load-baring bone fixation biomaterial structures. © 2007 Wiley Periodicals, Inc. J Biomed Mater Res 84A: 44–53, 2008

Key words: biocompatibility; inflammatory response; polymer fibers; nanocomposites; hydroxyapatite

INTRODUCTION

The concept of bioactive particulate reinforced polymer composite as bone analogue was first introduced in the early 1980's by Bonfield and coworkers[1–3] with the development of hydroxyapatite (HA) reinforced high density polyethylene (HDPE) composite HAPEX™ used to produce bulk isotropic 3D products. This pioneering work inspired many other bioactive polymer/HA composites, designed in an attempt to match the properties and structure of those of the bone for ultimate orthopedic applications.[4–7] However, these particulate composites do not present mechanical properties, namely the strength and stiffness, of cortical bone, which have limited their applications as load-bearing bone substitutes. Considering the excellent mechanical properties of polyethylene terephthalate (PET)[8] as a matrix and the expected stiffening and strengthening effects of ceramic nanofillers,[9] microfibers of PET/HA nanocomposite are a promising biocomposite that can be molded into 3D anisotropic structures with tailored properties by controlling the microfiber 3D orientation. A very interesting application of these nonresorbable microfibers is a composite coating for total hip replacement prosthesis or other load-baring biomaterials in order to improve their osteointegration.

Because of the HA similarity to the main mineral component of hard tissues, as well as its osteoconduction and bone binding properties, successful applications as bone substitutes with excellent bioactivity and biocompatibility have resulted.[10] However, in order to preserve HA attractive properties in a HA/polymer nanocomposites, new variables come into play. Namely, the physico-chemical properties of the composite; which

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are highly affected by: (a) the chemical interactions between HA particles and matrix and (b) the structural organization of the matrix itself. For this purpose, a stress induced process for the alignment of nanoparticles in polymeric suspension under a high shear rate has been used to compound the HA nanocrystals using a twin-screw (TSE) and using this compound to produce oriented fibers from polymer/HA by melt spinning. The polymer, PET, has been successful for cardiovascular grafts since the 1950's in critical procedures where high strength and predictable long-term performance is needed,[11] mainly due to its chemical structure, which promotes resistance to hydrolysis due to the hydrophobic aromatic groups and its high crystallinity.[12] Paradoxically, the same characteristics that allow PET its long history in vascular related human implantation, renders it bio-inert in terms of bone related functions. It is anticipated that by loading oriented PET fibers with increasing amounts of bioactive inorganic HA particles, the nanocomposite fibers could theoretically function as bioactive material due to the known tendency of HA nanocrystals to aggregate. Our second hypothesis concerning the HA loading of the fibers is that they will be able to sustain the physiological loads required from surface bone fixation material for total hip replacement prosthesis. The PET fibers were charged with a maximum of 10 wt % HA proportion as higher HA fills induced fiber breakage during the manufacturing process. Lastly, the process by which these fibers are apposed to the surface of the total hip prostheses and their resulting mechanical properties are under investigation and will be published elsewhere.

One of the first stage in the development of the PET/HA nanocomposite microfibers is to evaluate their cytotoxicity. It is desirable that these nanocomposite microfibers elicit a minimal cytotoxicity response and facilitate cell attachment for their use as a tissue engineering scaffolds. *In vitro* cytotoxicity testing provides a convenient and reliable method to assess the biological response to a biomaterial and also serves as an initial screening process for future *in vivo* studies. The initial step in an *in vitro* biocompatibility study is the evaluation of the *in vitro* cytotoxicity of a biomaterial based on the morphological examination of cell damage and growth when in direct or indirect contact with the materials. Toxicity of the proposed biomaterials involves disturbance of the cellular homeostasis leading to a multiplicity of biochemical changes. High importance is given to cell death, cell proliferation, cell morphology, and cell adhesion all being parameters directly correlated with *in vitro* toxicity.[13] Additionally, the inflammatory response is a significant element of the host response to biomaterials as it also contributes to the phenomenon of aseptic loosening of orthopedic prosthesis and as such, is used as an assessment of biocompatibility.[14] Macrophages Raw 264.7 were cultured *in vitro* to determine the direct and indirect effect the PET/HA composites on the production and release of tumor necrosis factor (TNF-α). TNF-α was selected based on its well known control over local inflammation, cellular activation, and chemotaxis[15] as well as its established role as a potent stimulator of bone resorption via its inhibitory effect on osteoblasts,[16] and its ability to activate osteoclasts.[14,17] Furthermore, the addition of HA reinforcement presents contradictory results as some authors report improved performance of HA reinforced implants,[18] while others report that ceramic coatings may produce particulate wear debris, enhanced the production of cytokines, and induce osteolysis.[19,20]

In vitro experiments can be particularly beneficial for polymer/ceramic composites because they can individually test all three phases that occur in the materials life cycle during implantation, namely, unreacted macromers (if any), cross-linked network, and possible degradation products. In the present study, we evaluated the *in vitro* cytotoxicity of these three phases of the novel nanocomposites PET/HA fibers. We hypothesized that PET/HA nanocomposites with 10% HA would demonstrate acceptable biocompatibility *in vitro* and superior surface properties for cell proliferation.

MATERIALS AND METHODS

PET-HA nanocomposite preparation

The polyethylene terephthalate (PET) used in this study to prepare the fibers was obtained from M&G Polymers (Traytuff 8506) with an intrinsic viscosity of 0.85 (dL/g). The hydroxyapatite (HA) particles were obtained from Plasma Biotal (UK, Captal 30). A master batch of PET and HA containing 38 wt % HA was compounded in the form of pellets using a twin screw extruder at 280°C. Scanning electron microscopy (SEM) indicated a good dispersion of HA in the PET matrix.[9] The master batch was then diluted by dry blending prior to feeding it in a fiber spinning line to prepare PET fibers with 0, 2, 4, 6, 8, and 10 wt % HA, designated in Table I. The fiber spinning line consisted of a single screw extruder equipped with a 15 cm linear die with 150 holes of 380 μm each. The fibers were drawn from the die using a roller positioned at about 2 m from the die exit. The extrusion temperature was 285°C. The final diameter of the fibers was in the range of 25–80 μm. X-ray diffraction analysis of the fibers revealed that the PET was essentially amorphous.[9] Optical micrographs observation of the PET0 and PET10 nanocomposite fibers are shown in Figure 1.

Ultrasonic cleaning and sterilization of nanocomposite fibers

Equal amount of samples (2 g) of each fiber batches were cleaned by a two-step ultrasonification procedure

TABLE I
Morphological Features of Fiber Nanocomposites

Nanocomposite Fibers	HA Fraction (%)	Fiber Density (g/cm^3)	Fiber Diameter (μm)	Porosity (%)
PET0	0	1.30	52	88.7
PET2	2	1.34	58	89.0
PET4	4	1.37	54	89.3
PET6	6	1.41	53	89.5
PET8	8	1.44	64	89.8
PET10	10	1.48	57	90.0

involving 99.9% ethanol and 98.9% acetone for 10 min cycles. The samples were then wrapped in plastic sterilization pouches and sterilized using pure ethylene oxide (EtO). EtO sterilization was carried out in SteriVac® (3M), with a 4-h cycle followed by 24-h aeration to remove residual EtO.

Biocompatibility—Effect of material extracts

Cell culture

Murine L929 fibroblast and RAW 264.7 macrophage cell lines (ATCC, Rockville, MD) were used in this study. Cells were grown at 37°C in a 5% CO_2 humidified atmosphere in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, Mississauga, ON, Canada), supplemented with 3.7 g/L of sodium bicarbonate, 10% heat-inactivated (56°C for 30 min) fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin (Gibco Laboratories, Burlington, ON, Canada).

Preparation of the extracts

Extracts were prepared from the material samples in agreement with the ISO specification (10993–5) governing *in vitro* tests.[21] Each polymer nanocomposite was immersed in serum free DMEM at a ratio of 0.2 g/mL and incubated for 24 h at 37°C under constant agitation (250 rpm). After this period, the medium was harvested and kept at −90°C until used. The extracts were used undiluted and supplemented with 10% FBS.

Cytotoxicity of fiber extracts

The cytotoxicity of fiber extracts was evaluated against L929 fibroblasts using the methyl tetrazolium (MTT) assay in 96-well plates as described by the manufacturer (Sigma-Aldrich). The MTT assay is based on the ability of living cells to convert a water-soluble yellow dye, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) into purple formazan crystals. Briefly, L929 cultured cells were seeded in 96-well plate (2.5×10^5 cells/mL, 200 μL/well) and allowed to adhere for 24 h at 37°C in a 5% CO_2 humidified atmosphere. The culture medium was replaced by the previously prepared extracts, and the plates were further incubated for 24, 48, and 72 h. Control samples consisted of L929 cells grown on tissue culture plastic supplemented with complete DMEM, but not in contact with fiber extracts, as previously described for the study of glass–ceramics[22] and nano-sized HA.[23] After the incubation periods, the extracts were removed and each well was treated with the MTT solution for 4 h at 37°C. Liquid was then removed, solubilization solution added, and microplate was shaken for 15 min before reading at 550 nm on a microplate reader. Cytotoxicity was calculated as the percentage of negative control cell viability. Results are the mean ± standard deviation of three (3) experiments performed in triplicate.

TNF-α release

Murine RAW 264.7 macrophages were seeded in 24-well culture plates at a density of 2×10^4 cells/well in 1 mL of DMEM supplemented with 10% heat-inactivated FBS,

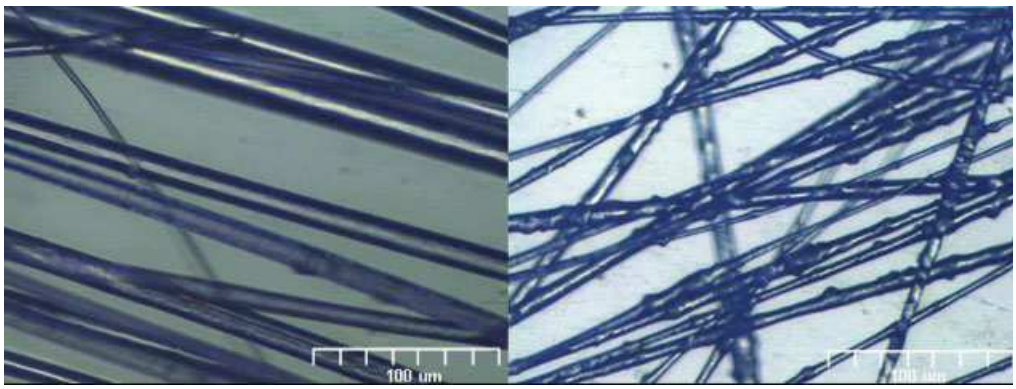

Figure 1. Optical microscope images of PET0 (left) and PET10 (right) fibers. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

100 units/mL penicillin, and 100 μg/mL streptomycin. After overnight equilibration, the medium was replaced by the polymer nanocomposite fiber extracts. Supplemented DMEM was used as a negative control while 10 μg/mL of lipopolysaccharide (LPS, *E. coli*; Sigma-Aldrich) was used as a positive control. Supernatants were harvested for assaying the levels of tumor necrosis factor-alpha (TNF-α) with sandwich ELISA as prescribed by the manufacturer (Biosource, Nivelles, Belgium). The optical density was then determined using a microplate reader set to 450 nm and corrected at 570 nm. The minimum detectable levels were 3 pg/mL. Results are the mean ± standard deviation of three (3) experiments performed in triplicate. Numerical data were analyzed statistically using Student *t* tests. Statistical significance was considered at $p < 0.05$.

Biocompatibility—Direct contact assay

Fiber scaffold preparation

Specimen of nonwoven fiber scaffolds for direct contact assay tests were prepared by compression molding using a laboratory Carver press. The scaffolds were heated between the press platens at a temperature of 85°C under a pressure of 1 metric ton applied for 1 min and then under 2 metric tons for 2 min. Heating was then stopped to allow specimens to cool down until room temperature is reached. The fiber scaffolds had a high porosity of ~90% to mimic the porosity of spongy (trabecular) bone. Details of the scaffolds morphology are given in Table I.

Cells and matrix seeding

To test the long-term biocompatibility of the polymer nanocomposite fibers, the fibers were placed in 24-well plates and sterilized by EtO. After the 5 days aeration time, the fiber scaffolds were promptly soaked in phosphate buffered saline (PBS) and then soaked overnight in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin prior to L929 fibroblast seeding (1×10^4 cells/cm^2). This procedure facilitates protein absorption and cell attachment onto the fibers. L929 fibroblasts cultured on the regular polystyrene surface (tcp, tissue culture plate) were used as control. Cells were maintained in culture for up to 14 days. Medium was changed every 3 days.

Cell morphology

Cell morphology, spreading, orientation, and growth on the surfaces of the fiber scaffolds were evaluated using the common qualitative technique, Field Emission Gun Scanning Electron Microscope (FEG-SEM) on a Hitachi S-4700 apparatus (Hitachi High-Technologies Canada Rexdale, Ontario). Harvested L929 fibroblasts were washed twice with PBS and fixed with 1% glutaraldehyde, first for 1 h at room temperature, then overnight at 4°C. The samples were rinsed with PBS for 30 min and then dehydrated through a series of graded alcohol solutions. The specimens were air-dried overnight and the dry cellular constructs were finally sputter coated with palladium and observed under the FEG-SEM at an accelerating voltage of 2.0 kV.

Cell proliferation

Cell proliferation was monitored using the Alamar Blue™ assay as specified by the manufacturer (Biosource, Nivelles, Belgium). The assay is based on a fluorometric/colorometric growth indicator that detects metabolic activity. Specifically, the system incorporates an oxidation–reduction (REDOX) indicator that both fluoresces and changes color in response to chemical reduction of growth medium resulting from cell growth.[24] The directly plated fibroblasts were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. At selected time points of 1, 3, 7, and 14 days, medium was removed and 1 mL aliquots of Alamar Blue (diluted 1:10 in phenol red-free medium) were added to each well and incubated for a further 4 h at 37°C, 5% CO_2. Wells without cells were used as the blank control and L929 cells grown on tissue culture plastic (tcp) supplemented with complete DMEM were used as a negative control as reported elsewhere.[22] Following the incubation 3 × 100 aliquots from each well were taken and transferred to a 96-well plate for reading. Absorbance was measured on an ELISA microplate reader at 570 and 600 nm. The intensity of red color (570 nm) is proportional to the percent reduced of Alamar Blue that can than be related to the metabolic activity of the cell population through the following:

$$\%\ \text{metabolic activity} = \frac{\varepsilon_{ox}(\lambda_2)A(\lambda_1) - \varepsilon_{ox}(\lambda_1)A(\lambda_2)}{\varepsilon_{red}(\lambda_1)A'(\lambda_2) - \varepsilon_{red}(\lambda_2)A'(\lambda_1)} \times 100 \quad (1)$$

where ε_{ox} is the molar extinction coefficient of Alamar Blue oxidized form (BLUE), ε_{red} is the molar extinction coefficient of Alamar Blue reduced form (RED), A is the absorbance of test wells, A' is the absorbance of negative control well, λ_1 is given by 570 nm and λ_2 by 600 nm. Results are the mean ± standard deviation of three (3) experiments performed in triplicate. Numerical data were analyzed statistically using Student *t* tests. Statistical significance was considered at $p < 0.05$.

TNF-α release

To test the polymer nanocomposite fibers potential in macrophages activation, murine RAW 264.7 macrophages were seeded on PET0 and PET10 fiber scaffolds at a density of 2×10^5 cells/well in 1 mL of DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin. Supplemented DMEM was used as a negative control while 10 μg/mL of lipopolysaccharide (LPS, *E. coli*; Sigma-Aldrich) was used as a positive control. Supernatants were harvested for assaying the levels of TNF-α with sandwich ELISA as prescribed by the manufacturer (Biosource,

Nivelles, Belgium). The optical density was then determined using a microplate reader set to 450 nm and corrected at 570 nm. The minimum detectable levels were 3 pg/mL. Results are the mean ± standard deviation of three (3) experiments performed in triplicate. Numerical data were analyzed statistically using Student *t* tests. Statistical significance was considered at $p < 0.05$.

RESULTS

Biocompatibility—Effect of material extracts

Cytotoxicity

Figure 2 shows the short-term effects of the extractable products on L929 fibroblasts. Cytotoxicity was calculated as the percentage of cell viability over control values. Results clearly show that, at 24 h, the extracts have no significant effect on the cell viability, as it remains roughly constant around 100% for all nanocomposite fibers. At 48 and 72 h, the cellular viability also remains around 100% for all fiber composition. No statistical differences can be observed with respect to incubation time.

TNF-α release from extracts

The effects of the material extracts on TNF-α release by RAW 264.7 macrophages are shown in Figure 3.

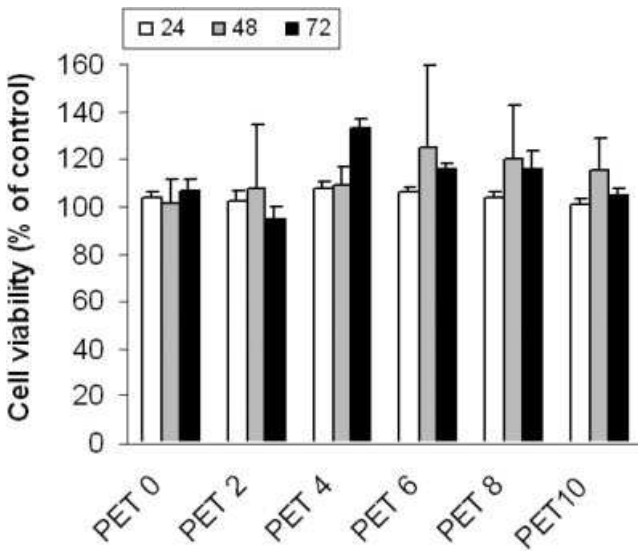

Figure 2. Effect of PET/HA fiber extracts on the viability of L929 fibroblast cells as determined by the MTT assay. L929 cells were incubated in the presence of undiluted fiber extracts (0.2 g/mL) and the fibroblast viability was determined by the MTT assay at 24, 48, and 72 h. L929 cells grown on tissue culture plastic (tcp) supplemented with complete DMEM was used as the negative control. Results are expressed as percentage of negative control and are the mean ± standard deviation of three different experiments.

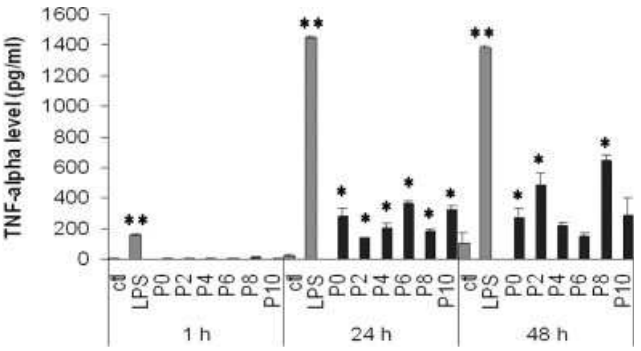

Figure 3. Effect of fiber extracts on TNF-α release. Raw 264.7 macrophages were incubated for 1–48 h with undiluted extracts (0.2 g/mL) of PET0 to PET10 fibers. Supplemented DMEM was used as a negative control while 10 μg/mL of lipopolysaccharide (LPS) was used as a positive control. Results are the mean ± standard deviation of three experiments.

Significant variations according to the percentage of HA and the time of culture were seen. However, an unequivocal dose-response as a function of HA in the materials could not be established. All nanocomposite fibers had no effect on TNF-α release (compared with control) after 1 h in presence of materials extracts. However, the extract from the polymer without HA (PET0) significantly stimulated 10 and 2 times (compared to negative control) respectively the release of TNF-α after 24 h (279 pg/mL vs. 27 pg/mL) and 48 h (270 pg/mL vs. 107 pg/mL). The presence of HA in the polymer (PET2 to PET10) had few additional effect on TNF-α release after 24 h. At 48 h, the release of TNF-α (compared with negative control) was significantly increased in presence of 8% HA (642 pg/mL) and decreased with 4% HA (221 pg/mL) and 6% HA (149 pg/mL). PET10, the fibers with the highest amount of HA, stimulated 12 and 2 times (with respect to negative control) the release of TNF-α after 24 h (326 pg/mL) and 48 h (288 pg/mL), representing 25% of the positive control LPS at both 24 and 48 h. As a control, LPS stimulated TNF-α release (with respect to control) by 23 times (160 pg/mL), 55 times (1450 mL), and 13 times (1380 mL) after 1, 24, and 48 h, respectively.

Biocompatibility—Direct contact assay

Cell proliferation

Figure 4(a) shows the proliferation of L929 fibroblasts on PET0 to PET10 fiber scaffolds after 3 days in culture, as determined by Alamar Blue. The pure polymer fiber (PET0) reduced the metabolic activity of L929 fibroblasts by 35%. The presence of HA increased this metabolic activity in a dose-dependent manner with maximal level reached with 10% HA

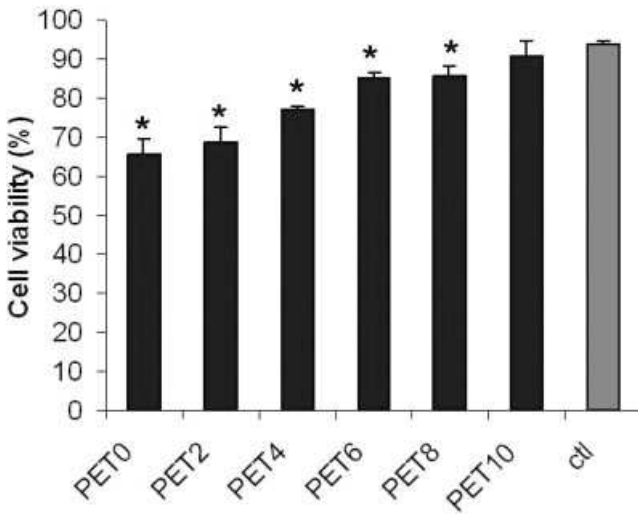

Figure 4. Effect of HA nanoparticle dosage in the PET fibers on the proliferation of L929 fibroblast cells. (a) L929 fibroblasts were seeded on PET0 to PET10 fiber scaffolds and their viability was assessed after 3 days by the Alamar Blue assay. Negative control samples consisted of L929 cells grown on tissue culture plastic (tcp) supplemented with complete DMEM. Results are expressed as % reduced of Alamar Blue and are the mean ± standard deviation of three different experiments. (b) L929 fibroblasts were seeded on PET0 and PET10 fiber scaffolds and their viability was assessed after 1, 3, 7, and 14 days by the Alamar Blue assay. Negative control samples consisted of L929 cells grown on tissue culture plastic (tcp) supplemented with complete DMEM. Results are expressed as percentage of the reduced of Alamar Blue and are the mean ± standard deviation of three different experiments.

(91% of control). Proliferation of L929 fibroblasts was then assayed for 1 day up to 14 days on all composite fibers, but only PET0 and PET10 are shown in Figure 4(b). Significant differences were observed between PET0 and PET10 after 1 and 3 days in culture. This difference disappeared after 7 days with metabolic activities reaching 93 and 99% of control for PET0 and PET10, respectively.

Cell adhesion

The interactions between L929 fibroblasts and nanocomposite fibers were studied *in vitro* by FEG-SEM up to 14 days in culture (Fig. 5). Although all fibers supported healthy attachment and spreading of L929 fibroblasts, the cell-fiber scaffold interactions varied for different fiber composition. At day 14, PET10 demonstrated a denser and greater cell sheet after 14 days than the PET0 fibers indicating the differences in cell proliferation.

TNF-α release after direct contact with fiber scaffolds

The effects of the nanocomposite fibers on TNF-α release by RAW 264.7 macrophages are shown in Figure 6. The trends are comparable to the previously discussed for the stimulation of TNF-α by the fiber extract. Indeed, macrophages plated on fiber scaffolds had no effect on TNF-α release after 1 h. However, PET0 stimulated nine times the release of TNF-α after 24 h (296 pg/mL) and 48 h (226 pg/mL), respectively. As opposed to PET0, PET10, the main focus of this study, significantly decreased the TNF-α release down to 0.8 and 1.5 times after 24 h (25 pg/mL) and 48 h (41 pg/mL), representing a fraction of the positive control LPS. As a control, LPS stimulated TNF-α release by 3 times (26 pg/mL), 18 times (566 pg/mL), and 23 times (588 pg/mL) after 1, 24, and 48 h, respectively.

DISCUSSION

Polymer composites are increasingly evaluated for biomedical applications in hopes to marry the positive effects of the materials and overcome their independent shortcomings. In the present study, the effect of HA reinforcement on the PET microfibers was investigated on L929 fibroblasts viability and proliferation, as well as RAW 264.7 macrophages activation were investigated. Although PET/HA microfibers are being developed for orthopedic applications, both the indirect and direct contact assays were carried out with L929 fibroblasts due to their high sensitivity.[25] Also the choice of L929 fibroblasts was due to their model properties that can reliably determine the general biocompatibility of novel material and screen them for future *in vitro* and *in vivo* experiments.[26] The effect of polymer composite fibers and their extracts on RAW 264.7 macrophages were also studied because macrophages are the principal cells found in the pseudo-membranous tissue formed around hip implants at revision surgery.[27] This study is the first one to look at the biocompatibility of the novel fiber composites.

The effect of extractable products of polymers on their biological environment is important in determining the biocompatibility of promising biomaterials. In this work, the well-known MTT assay was used to quantify the short-term effects of the extractable products on the viability of L929 fibroblasts. Results showed that the extracts from the polymer fibers without HA and the polymer fibers reinforced with HA had no effect on the cell viability, which suggests the biocompatibility of the extracts. However, it is not impossible that the conditions of the present study (250 rpm agitation for 24 h at 37°C) may be insufficient to generate the same levels of extractables or leachables products than in an *in vivo* environment with mechanical constraint. For example, as a potential composite coating material for total hip replace-

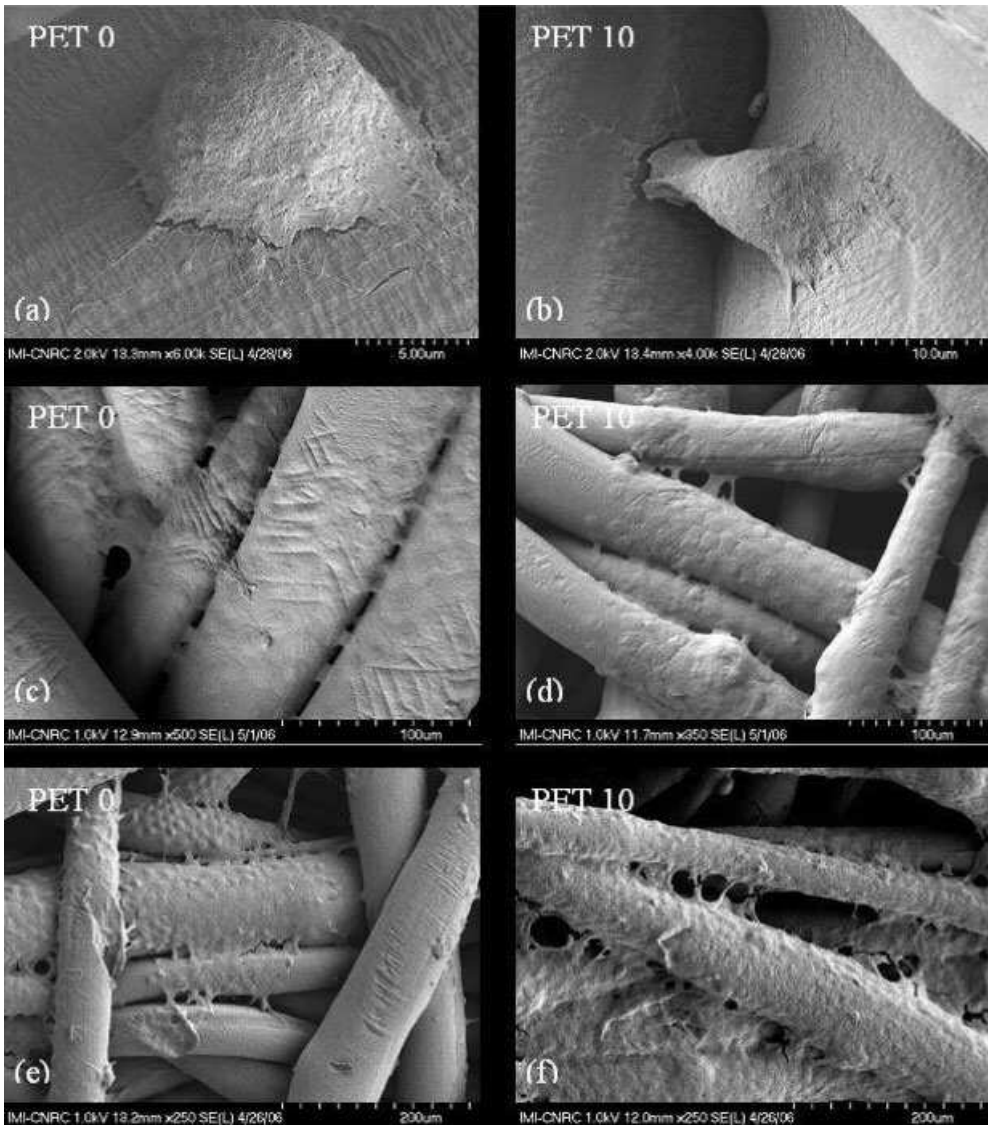

Figure 5. FEG-SEM images of L929 fibroblasts cells seeded on fiber scaffolds. L929 fibroblasts were cultured for 1 day on (a) PET0 and (b) PET10, for 7 days on (c) PET0 and (d) PET10, and for 14 days on (e) PET0 and (f) PET10.

ment prosthesis, the effect of wear debris on cell viability and activity should be determined in the future. The size, shape, and exact composition of these putative particles are not yet known. It should not be forgotten that the polymer fibers reinforced with 10% HA, the main focus of this study as they will potentially have the highest bioactivity, presented a minimal cellular viability of 105% at the three different time points (24, 48, and 72 h), which amply demonstrates an acceptable biocompatibility of the extracts under the conditions used in the present study.

As the ultimate application of the fibers used in the present study would be a bone fixation material for total hip replacement prosthesis surfaces, the inflammatory responses due to extractable products was also quantified by looking at the release of TNF-α by RAW 264.7 macrophages. The present experiments demonstrated that macrophages cultured with PET/HA composite fibers extracts released more TNF-α than control cells. The variations in TNF-α release following incubation with the material extracts cannot be qualified as a dose-response to the initial percentage of HA present in the materials. This could be due to the fiber composite nature (polymeric/ceramic) that may release degradation products such as undesired additives (processing aids) or impurities or/and the surface exposed HA. The extracts chemical nature is under further chemical characterization and will be published elsewhere. Furthermore, to the authors' knowledge, no studies examining the TNF-α levels released following *in vitro* incubation of cells and HA composites materials extracts have been reported. However, the stimulation of TNF-α is comparable to other materials of orthopedic interest, such as ceramic

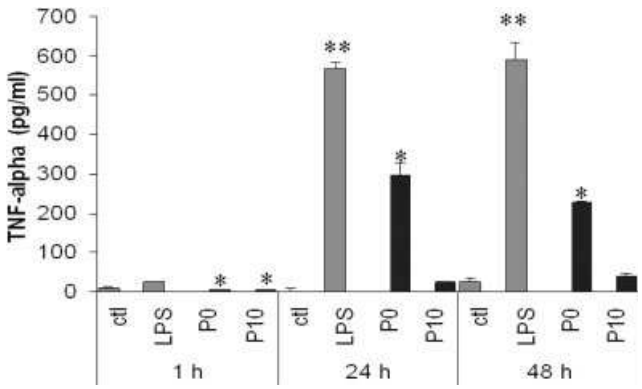

Figure 6. Effect of fiber scaffolds on TNF-α release. Raw 264.7 macrophages were seeded on PET0 and PET10 fiber scaffolds for 1–48 h. Supplemented DMEM was used as a negative control while 10 μg/mL of lipopolysaccharide (LPS) was used as a positive control. Results are the mean ± standard deviation of three experiments.

particles[28] and with metal ions.[29] Additionally, the stimulation is less important than observed with ultra-high molecular weight polyethylene particles,[29] also of orthopedic interest. The effect of PET/HA fiber leachables on TNF-α release is also very low compared to positive LPS control cultured macrophages. Taken together, these results suggest that extracts from PET/HA fibers had comparable inflammatory to different materials of orthopedic interest. This is supported by optical microscopic analyses of RAW 264.7 macrophages in contact with PET fiber extracts that remained round without the formation of foreign body giant cell (results not shown).

The inflammatory responses due to the initial interactions between PET0 and PET10 scaffolds and RAW 264.7 macrophages were also quantified by looking at the release of TNF-α. The interaction between macrophages and HA charged orthopedic implants is an important parameter since macrophages can produce a variety of inflammatory factors, such as cytokines and prostaglandins, that are known to stimulate inflammation and osteoclastic bone resorption.[30] Several factors modulate the activation of macrophages and mediate the production of cytokines. Among these factors, surface physical and chemical properties and adhesion specific signals are believed to have important roles.[20] Concerning HA reinforced composites, contradictory results in terms of cytokines production are presented in literature. Ninomiya et al.[31] reported that HA enhanced the production of TNF-α by human fibroblasts *in vitro*. Marques et al.[20] on the other hand found that the addition of HA resulted in a significant reduction of those inflammatory cytokines, by monocytes and lymphocytes *in vitro*. Furthermore, Huang et al.[23] found no correlation between the TNF-α release and the concentration of nanoHA crystals added to *in vitro* macrophage culture. In the present study, the presence of HA in the fiber scaffolds significantly decreased the TNF-α release as compared to the pure PET fibers scaffolds. Finally, the release of TNF-α in PET10 fibers and macrophages coculture was not significantly greater than the negative control, indicating that PET10 fibers may not trigger a severe inflammatory response.

The indirect cytotoxicity contact tests were complemented with direct contact tests. This allows evaluating the cell-material contact arrangements dictated by material surface features and properties, which may lead to differences in cytotoxicity. The direct contact proliferation assay showed that L929 fibroblasts remained viable for at least 14 days on PET/HA fibers. It also showed that the metabolic activity, associated with cell number, increased proportionally with the amount of HA in the composite fibers, up to 7 days. This trend in the increased cell viability relatively to the HA amount may be explained by the followings: (a) the increased surface roughness of the composite fibers with higher HA concentrations, as the HA nanocrystals tend to aggregate on the surface of the fibers (Fig. 1), (b) the chemical exposure of the HA nanocrystals aggregates, or (c) both possibilities. Although a conclusive mechanism of cell adhesion is not yet established, the shape and surface texture of an implant are important factors determining the cell-material contact and influencing cell proliferation.[13] Other studies have also reported that cell adhesion and proliferation of different types of cells onto various surfaces depend on the material surface characteristics like surface charge, wettability and most importantly topography. More specifically, it has been reported that there is a favored cell attachment on roughened surfaces.[32]

However, increased fibroblast proliferation proportional to the HA dosage was not observed after 7 days in culture, as at day 7 cellular viability on PET0 and PET10 fibers approximately level off, suggesting that the number of fibroblasts is similar on both scaffolds. The direct contact Alamar Blue results beyond day 7 cannot be extrapolated to *in vivo* conditions; they for example contradict the SEM analysis, which demonstrates a more important proliferation of L929 fibroblasts on PET10 than on PET0 fibers, especially beyond day 7. These contradicting results can be explained by the fact that as cells grow in culture their metabolic activity maintains a reductive environment in the surrounding culture medium, while growth inhibition produces an oxidative environment.[33] In this test, metabolic activity (reduction) causes color change of the Alamar Blue indicator from nonfluoresent (blue) to fluorescent (red). As cell growth is inhibited by the physical constraints of the tissue culture well beyond day 7 on the PET10 fibers, the surrounding culture medium starts being oxidized as well as reduced leading to an underestima-

tion of the cell metabolic activity and the cell number by the Alamar Blue assay. It should not be forgotten that the direct contact assay performed in this work demonstrated that the three-dimensional form of the PET10 composite fibers did not present any toxicity, reduced cell adhesion, or delayed proliferation rate and therefore can be considered as a potential biomaterial.

It is well known that the initial interaction between biomaterials and cells is mediated by a previously absorbed layer of proteins resultant from cell culture medium *in vitro*.[34] In the present study, a new variable factor is introduced in the outer layer of the PET composite fibers formed of HA nano- and micro-crystals (aggregated nanocrystals). In this context, based on the surface modifications induced by the addition of the HA nanocrystals, it seems that proteins are differently absorbed on the composites and consequently the cells interactions with the different composites modified. This fact may not only affect the pattern of adhesion of fibroblasts cells to the material, but also the reorganization in their cytoskeleton.[35] In this regard, the hypothesis of the present *in vitro* study was that the novel composites fibers, PET10, would be highly biocompatible in that it will be a suitable substrate for adhesion and cell-matrix interactions to support cell growth and differentiation, and organization of cells to form a specific tissue.[36] This hypothesis is further supported by the SEM results, which revealed that at day 1 the fibroblasts seeded on the PET10 fibers have a more spread and flattened morphology than those seeded on the PET0 fibers, indicating a stronger cellular adhesion. By the end of the second week, the degree of colonization of the PET10 fibers appeared higher and denser as compared to the PET0. This is coherent with studies demonstrating that small modifications in the composition and texture of the surfaces of materials can have an impact on the subsequent host-implant interactions. Although more conclusive studies are needed to characterize the extracellular-like matrix formed, the results suggest that the novel composites fibers would be able to support three-dimensional proliferation as they sustain adhesion, growth, healthy cell morphology, and migration upon fibroblast culturing. Further studies with cell lines that have the potential to differentiate into the osteoblast phenotype are necessary in order to evaluate PET10 composite fibers potential as bioactive scaffolds.

CONCLUSION

The effects of HA nanocrystals dosage in PET polymer based fibers were investigated on L929 fibroblasts and RAW 264.7 macrophages. The results of cell behavior on the nanocomposite fibers showed that throughout the time points, L929 fibroblasts proliferated well as monolayer cultures, which is expected to have a final outcome on the support of new tissue formation at the interface. It was also possible to demonstrate that the 3D structures with high HA content PET fibers have low inflammation effect on RAW 264.7 macrophages. Overall, these results strongly support the biocompatibility of the PET10 nanocomposite fibers.

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