Application of PDLLA/carbon nanotubes/nHAp scaffolds for bone regeneration

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Abstract: We evaluated the biological application of polymeric Poly (DL-Lactide acid)(PDLLA) membranes with the incorporation of carbon nanotubes and nanohydroxyapatite developed by two methods; (a) nHAp1 by electrodeposition and (b) nHAp2 by immersion in simulated body fluid (SBF). We evaluated the cytotoxicity and bone regeneration using human osteoblasts bone defect in the calvaria of mice, respectively. Our results shows that both materials developed are biocompatible but PDLLA/VAMWCNT-O2/nHAp2 showed faster degradation than the PDLLA and complete bone regeneration within 4 months of implantation.

Keywords: PDLLA, Hydroxyapatite, Carbon nanotube, bone regeneration.

Introduction

Poly (DL-Lactide acid)(PDLLA) is a biocompatible and biodegradable polymer with a fast and controllable degradation time [1-3]. Several studies have investigated how to improve the physical and chemical characteristics of the polymer, as well as interaction with the biological medium and promote osteoconductivity [4-5]. The dispersion of nanoparticles of carbon nanotube and nanohydroxyapatite has been target of research in order to formulate a material that mimics bone.

Here, we presented the preliminary *in vitro* and *in vivo* studies using novel polymeric PDLLA membranes with incorporated carbon nanotubes and nanohydroxyapatite nanoparticles.

Materials and methods

Production of porous PDLLA/VAMWCNT-O2/nHAp membranes

PDLLA/VAMWCNT-O_2/nHAp scaffolds with the incorporation of hydroxyapatites synthesized by two different methods, such as: electrodeposition (nHAp1) [6] and soaked in Simulated Body Fluid (SBF) (nHAp2) [7].

After, we dispersed the nanoparticles (nHAp 1 and nHAp2) in 20 mL of chloroform using ultrasound irradiation for two min (1200 $J.mL^{-1}$) maintained at a temperature less than 40°C. After that, we diluted the 1g of PDLLA, 9/04 m/m (Purasorb®; Puracbiochem, Holland) in chloroform solution containing dispersed nanoparticles under constant agitation by 120 min. We inserted the solutions into a square mold with 0.5 mm diameter and dried in ambient temperature for 12 h in slow evaporation in controlled relative humidity, 70-80% and at room temperature. We performed a simple and fast functionalization to obtain superhydrophilicity character due to incorporation of oxygen-containing groups, using a pulsed-DC plasma reactor with an oxygen flow rate of 1 sccm, at a pressure of 85 mTorr, -700 V, at a repetition rate of 20 kHz. We used PDLLA membranes without any incorporated nanoparticles or oxygen plasma treatment as control.

Characterization by scanning electron microscopy

Two Scanning electron microscopes (SEM) were used: (i) model JEOL JSM 5610 VPI, was used forthe magnifications ranging from 100 - 15.000 times and (ii) High resolution SEM (Field emission Gun, FEG-SEM JSM 6330F) was used to magnifications ranging from 10.000 - 100.000 times.

Cytotoxicity assay

Cells were seeded in 24-well plates ($1x10^4$ cells/well). Control groups were established as follows: a negative control (only cell culture media, no cells) and a positive control (cells cultured in regular culture media). Scaffold groups were established as previously described. Scaffolds were put directly in the medium with no contact to the cells, in order that their degradation products were able to induce cytotoxicity. After 5 days, the scaffolds were removed, cells washed in phosphate buffered saline (PBS, Gibco, Carlsbad, USA) and fixed with 10% trichloroacetic acid (1 hour at 4ºC). After washing and drying, cells were stained by a 0,4% sulphorodhamin B solution in 1% acetic acid (SRB, Sigma, St. Louis, USA) for 30 minutes at room temperature. Cells were washed with 1% acetic acid to remove unbound dye and dried. The bound dye was solubilized in 1mM Tris-base (Sigma), shaked and the solution was transferred to a 96-well plate, in order to be read at 570nm. Data were normalized by the positive cell controls.

In vivo study

Adult, male, C57BL/6/JUnib mice (22–28 g body weight, CEMIB/UNICAMP, Campinas, Brazil) were used in this study. This project was approved by the Institutional Ethics Committee for Animal Research at

the University of Campinas— UNICAMP, Brazil (nº3154-1). Rats were anesthetized with an Intraperitoneal injection of 2.5 % (225–240 mg kg-1) Avertin (2,2,2-tribromoetanol).The hair over the calvarium was shaved and cleaned. After the skin was incised, a fullthickness circular critically sized defect (diameter of 0.5 mm) was made in the calvarium using ultrasound equipment CVDent1000 equipped with a sterile CVDENTUS diamond tip under irrigation with sterile normal saline to avoid damage to the bone.

The sterilized nanocomposite disks, 0.5 mm diameter, (separated into groups) were placed into the defects, the surgical site was irrigated with sterile normal saline, and the wound was closed in layers with bio-reabsorbable sutures. There were three mice in each treatment group. Four months after implantation of the disks, mices were sacrificed by Halotano inhalation.

Histology

Block sections of the calvarium containing the disks were harvested from the surgical sites and fixed in 10 % neutral buffered formalin. Specimens were then dehydrated for 12 days with a graded series of alcohols. Tissue sections were stained with Stevenel's blue and Van Gieson'spicrofuchsin.

Results

Figure 1 shows micrographs of the produced PDLLA (Fig. 1a), PDLLA/VAMWCNT-O2/nHAp1 (Fig. 1b), and PDLLA/VAMWCNT-O2/nHAp2 (Fig. 1c). In general, we observed differences in surface morphology between groups after incorporated nanoparticles. Figure 1a illustrates the smaller pores on the surface of PDLLA. The nHAp1 nanoparticles incorporated, formed larger pores with diameter between 12-31µm on the surface (Fig. 1b) and 21-35µm after nHAp2 nanoparticles dispersion in polymer matrix (Fig. 1c), respectively.

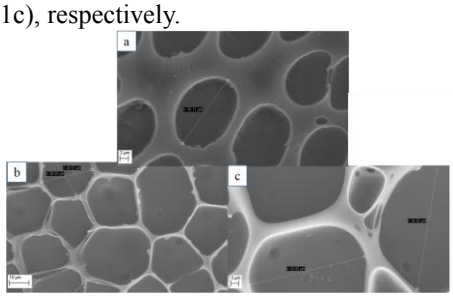

Figure 1: Micrographs of (a) PDLLA, (b) PDLLA/VAMWCNT-O2/nHAp1, and (c) PDLLA/VAMWCNT-O2/nHAp2 membranes.

As observed in figure 2 a, all scaffolds tested presented low cytotoxic, keeping the viability close to that observed for the control group (above 80% of this group readings). Analysis of variance indicated differences between groups (p=0.04113), and the Tukey's post hoc test pointed differences between the cell positive control and the PDLLA/VAMWCNT-O2/nHAp1 group. We do not observe any diferences.

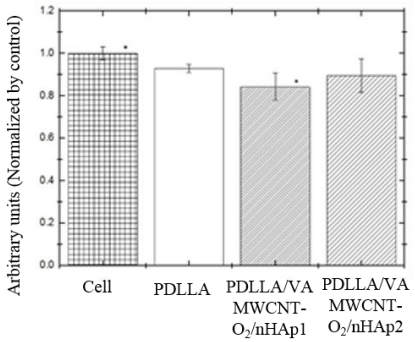

Figure 2: Analysis of cell viability. The osteoblasts were cultivated for 5 days on the nanocomposites.

Figure 3 shows typical histological sections after 4 months of implantation. In general, our data encourage the application of PDLLA/VAMWCNT-O_2/nHAp in the production of scaffolds for bone tissue regeneration, because there are no signs of inflammatory processes in bone tissue. We found in our study that there was not any behavior change in the animals from the treatment group compared to control.

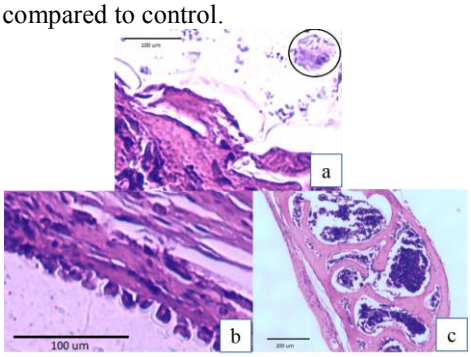

Figure 3: Photomicrograph of histological slides identifying bone regeneration after four months of implant in groups; Bone regeneration: (a) PDLLA; Details of macrophages in circle. (b) PDLLA/VAMWCNT-O_2/nHAp1; Interface region between the polymer and bone (*), which resembles the Howship's lacunae formed by osteoclasts. (c) PDLLA/VAMWCNT-O_2/nHAp2.

In the PDLLA control group (Fig.3 a), the biomaterial was not reabsorbed and the new tissue has disorganized aspect. In addition, there is the presence of macrophages indicating a mild local inflammatory response.

In PDLLA/VAMWCNT-O_2/nHAp2 bioimplant (Fig.3c), we obtained amazing results, complete healing of the defect can observed and newly formed bone is precisely organized and there were delimited marrow space.

Discussion

In general, cell culture analysis for the scaffolds suggested a high level of biocompatibility of all groups,

since viability levels were above 80% when compared to the cell positive control. Viability assessment with SRB is described in the literature [8-9], with advantages that it does not rely on the ability of cells in metabolizing a salt. In fact, it is related to the staining and quantification of cell proteins, in order it gives a direct correlation between the amount of proteins and number of cells.

Some authors reported that the PDLLA/HA osteoconductivity, is related with the HA exposure on the material surface [7]. Deplaine et al. reported that the plasma treatment and HA incorporation into the polymer improves nucleation in SBF and forming a thicker layer of biological apatites. This material when implanted in osteochondral lesions of sheep, promoted the appearance of osteoid many similar mature bone [10].

Hasegawa et al., implanted PDLLA/HA scaffolds in the femoral intercondylar notch, with a time of 26 weeks occurred an increase in bone formation and PDLLA/HA showed more fast degradation than the HA control. The lower crystallinity of HA dispersed in the polymer can be contribute to this result [11]. The degradation time of the amorphous PLLA or PDLLA , occurs on average at 1 year [11]. Our results showed that the biomimetic apatite by immersion in SBF when dispersed in the matrix PDLLA contributes to accelerate polymer degradation, the PDLLA/VANCNT-O2/nHAp2 was degraded completely in 4 months.

Conclusion

Our *in vitro* results showed that the nanocomposites simultaneously promoted bioactivity and non-cytotoxic. *In vivo* study showed that the PDLLA/VAMWCNT-O_2/nHAp2 membranes mimic the immature bone and induced bone remodeling. The PDLLA/VAMWNT-O_2/nHAp honeycomb films presented superior properties, morphology and porosity. These findings indicate surface improvement and the applicability of this new nanobiomaterial as ‘‘scaffold’’ for bone regenerative medicine.

Acknowledgment

The authors thank the Fundacao de Amparo a Pesquisa do Estado de Sao Paulo (2011/17877-7), (2011/20345-7), (2012/02159-4), CAPES and FVE for financial support, and to everyone from Laboratory of Biomedical Nanotechnology for all their support in the procedures. Thanks to Purac that kindly provide the PDLLA. Thanks to José Evaldo Corat by collaboration. Special thanks to Priscila Leite for scanning electron microscopy images.

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