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Electrospinning of Hyperbranched Poly-L-Lysine/Polyaniline Nanofibers for Application in Cardiac Tissue Engineering

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Electrospun polyaniline nanofibers are one of the most promising materials for cardiac tissue engineering due to their tunable electroactive properties. Moreover, the biocompatibility of polyaniline nanofibes can be improved by grafting of adhesive peptides during the synthesis. In this paper, we describe the biocompatible properties and cardiomyocytes proliferation on polyaniline electrospun nanofibers modified by hyperbranched poly-L-lysine dendrimers (HPLys). The microstructure characterization of the HPLys/polyaniline nanofibers was carried out by scanning electron microscopy (SEM). It was observed that the application of electrical current stimulates the differentiation of cardiac cells cultured on the nanofiber scaffolds. Both electroactivity and biocompatibility of the HPLys based nanofibers suggest the use this material for culture of cardiac cells and opens the possibility of using this material as a biocompatible electroactive 3-D matrix in cardiac tissue engineering.

Keywords: Tissue engineering, hyperbranched poly-l-lysine, polyaniline nanotubes, conductive electrospun nanofibers

1 Introduction

During the past thirty years, cardiovascular diseases (CVDs) have increased substantially in many developing countries. The coronary artery disease, known as myocardial infarction, is the most common manifestation of CVD, contributing to a great extent to the mortality rate. Current projections based on epidemiologic studies estimate that the global mortality rates from coronary artery disease will double between 1990 and 2020 (1, 2).

It is well known that acute myocardial infarction (MI) causes irreversible loss of cardiomyocytes (CM) and endothelial cells compromising the cardiac function of patients. The MI results from a biphasic ischemia/reperfusion injury to the heart which begins with the cardiomyocyte death by apoptosis (3–4). As consequence of MI a large number of fibroblasts appear on the site of injury, leading to collagen deposition and formation of extensive areas of fibrosis. A large number of fibroblasts appear at the lesion site, leading to collagen deposition and formation of extensive areas of fibrosis in a process called healing. Although the blood flow can be restored in areas of fibrosis does not contribute to global myocardial performance due to loss of the contractile cells. The adult human heart is unable to self-regenerate to a significant degree, and a fibrous non-contractile scar tissue is formed in the area of myocardial infarction. The non-contractile fibrous scar tissue does not effectively conduct the electrical wave front, reducing heart contractile efficiency. A promising technique for the restoration of cardiac function after myocardial infarction seems to be the replacement of damaged heart tissue with new myocardium cells, conveniently grown on synthetic 3-D scaffolds (5, 6). The latter has motivated several research groups, aiming at developing 3-D scaffolds for cardiac cell transplantation, based on natural and synthetic polymers (7, 8).

The electrospinning process has recently emerged as a useful tool for generating 3-D scaffolds with very high porosity, from a variety of synthetic and natural polymers for tissue engineering applications (9). This simple and low cost technique has been applied to the fabrication of nanoscale fibers from a wide range of polymers (10).

Because of its similarity to biological tissues and biocompatible properties, hydrogels, have been frequently used in combination with conductive polymers to obtain nanofibers for cardiac tissue engineering by electrospinning (11). The use of electrospun nanofibers composed of

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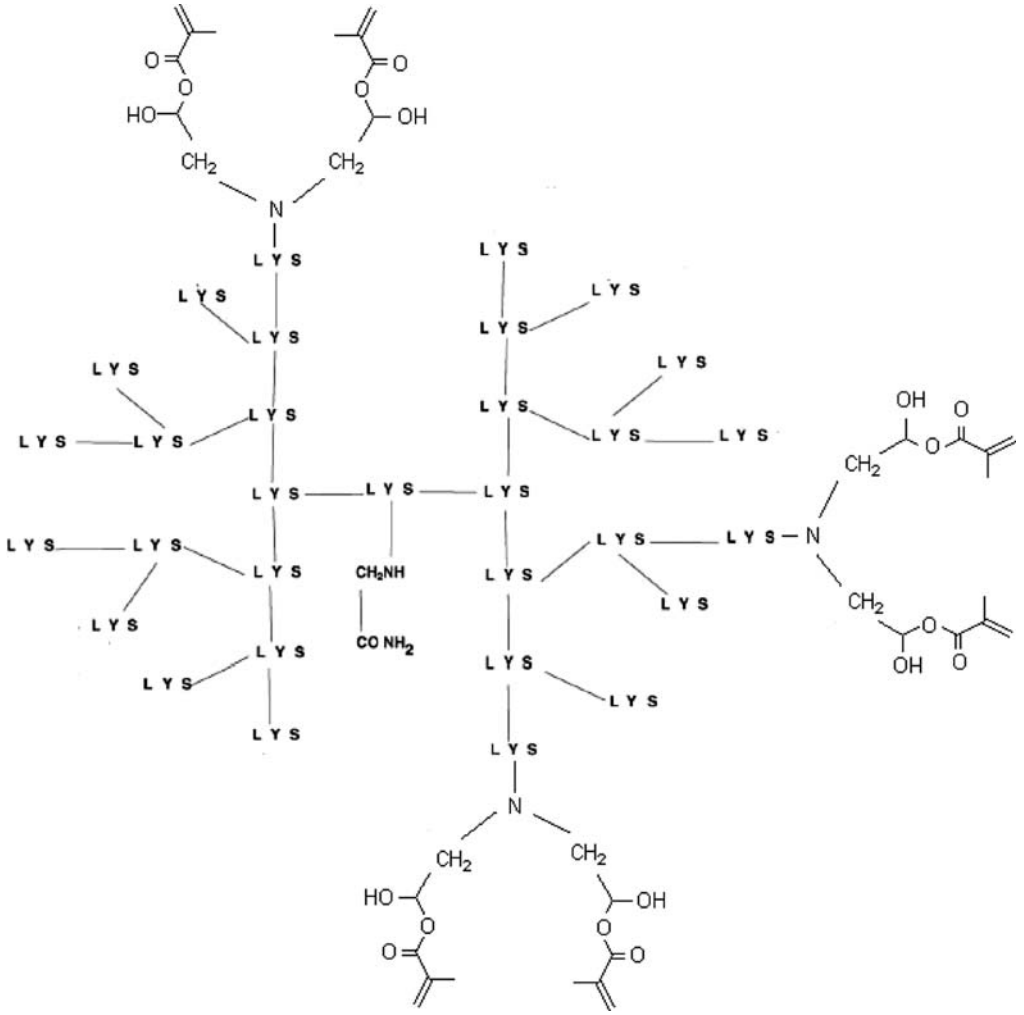

Fig. 1. Illustrative structure of epoxidized HPLys after reaction with GMA.

natural or synthetic biodegradable hydrogels has received attention for use as scaffolds in cardiac tissue engineering due to their elastic properties, similar to the heart tissues (12). The use of polyaniline (PANI), in particular, is due to the evidence that cell functions such as attachment, proliferation, migration, and differentiation, could be modulated through electrical stimulation (13). The three-dimensional interconnected pore networks of electrospun nanofibers generate structures that resemble native extra-cellular matrix (ECM) elements, improving cell attachment and growth, and regulating cell differentiation (14). Due to their low shrinkage, ability to promote cell adhesion and good biocompatible properties, hyperbranched poly-L-lysines (HPLys) are suitable for use in the development of 3-D scaffolds for cardiac tissue engineering (15). HPLys hydrogels may act as cardiovascular adhesives, via their swelling ability, promoting cardiomyocytes infiltration through its mesh structure. Although electrospinning is a widespread method of producing tissue engineering scaffolds, little is known about the biological properties of scaffolds based on the hyperbranched polylysine/polyaniline nanotubes electrospun fibers. Motivated by the development of cardiac tissue engineering based on electrically active electrospun nanofibers, we report the preparation of electrospun HPLys nanofibers containing polyaniline in the form of nanotubes (PANINTs). We investigated the *in vitro* biological properties of HPLys-PANINTs electrospun nanofibers and its suitability for application as 3-D scaffolds cardiac tissue engineering.

2 Experimental

HPLys (Fig. 1) was synthesized from appropriately protected lysine using the Fmoc solid phase peptide synthetic methods (16). The HPLys was purified by HPLC and its molecular weight was confirmed by matrix assisted laser desorption ionization (MALDI) mass spectrometry. An HPLys with molecular weight of 8,950 g/mol and hydrodynamic diameter of ca. 4.5 nm was obtained. Epoxi groups were introduced in HPLys after reaction with glycidyl methacrylate (GMA) to promote the covalent crosslinking

of the HPLys chains and hydrogel formation. An HPLys with GMA degree substitution of 10% (w/w) was attained in the derivatization reaction. Polyaniline was obtained in the form of nanotubes (PANINTs) via electrochemical synthesis, according to our previous publication (17).

Nanofiber scaffolds were prepared by electrospinning from 10% w/v HPLys-PANINTs solution in 50:50 v/v dichloromethane (DCM) and dimethylformamide (DMF). Initially, an adequate concentration of HPLys was magnetically stirred in DMF:DCM solution at room temperature (25°C) until r complete dissolution. Different amounts of PANINTs were dispersed ultrasonically in HPLy solution until a homogeneous dispersion was obtained. The amount of polyaniline nanotubes in the electrospun nanofiber varied from 0.5% to 5% (w/w), since this range provided a good dispersion of the conducting polymer in the HPLy solution.

Electrospinning was carried out with a flow rate of 20 mL/h using 15–20 kV applied to the needle tip (outside diameter = 0.91 mm) with the grounded collector placed at 20 cm from the tip. A 10.2 × 10.2 cm, 0.5 mm thick nanofiber sheet was deposited onto an aluminum foil. Surface morphologies of the electrospun scaffolds were investigated using scanning electron microscopy (SEM, Phillips XL 30).

The swelling of crosslinked HPLys - PANINTs membrane was carried out upon immersing the scaffold in phosphate saline buffer (PBS) 0.1 M and pH 7.2 solution at 37°C for 24 h. The swelling ratio (Q) was gravimetrically estimated, defined as the ratio between the weight of the gel at time t (W_t) and its initial weight (W_o).

For comparison, HPLys-PANINTs composites were also processed in the form of thin, cast films. The homogeneous dispersions of HPLy-PANINTs in DMF/DCM were cast onto siliconized Petri dishes, and the solvent was evaporated at 60°C for 48 h. The thickness of both the electrospun HPLys-PANINTs fibers sheet and the cast films was controlled between 20 and 30 μm.

The potential use of the HPLys- PANINTs scaffolds for cardiac regeneration was evaluated *in vitro* with cardiomyocite (CM) primary cell culture extracted from the ventricular portion of hearts of 2–4 day old Sprague-Dawley rats, according to published protocols (18). The myocytes were plated on designed scaffolds and matrices were placed in medium M 199 supplemented with 2% of fetal bovine serum at 37°C, 5% CO_2 and 95% humidity. The MC's cell proliferations were measured by fluorescence (18, 19).

Electrical stimulation was carried out according to adapted methodology previously described in the literature (20). Briefly, 3×10^5 cells were plated on wall-coated (5 μg.mL^{-1}) 200 cm^2 tissue culture flasks, to which an electrostimulation device using parallel graphite electrodes was attached. Electrical stimulation of the cells started 8 h after plating upon applying a voltage varying from 10 to 40 V as 0.5 Hz, 5 ms pulses. Cells were analyzed after 1–96 h of stimulation.

The cytotoxicity assay was performed by incorporating HPLys - PANINTs extracts to a CHO cell culture on a Petri plate (15 × 60 mm^2). The positive and negative controls were 0.02 vol% phenol solution and ultra-high molecular weight polyethylene (UHMWPE), respectively. Details on the extract preparation may be found in reference (21, 22). The cytotoxicity potential of the material was expressed by an index of cytotoxicity, IC_{50} (%), which represents the concentration of the extract that suppresses the formation of cell colonies by 50% in comparison to the control.

2.1 Statistical Analysis

All the experiments were performed in triplicate. Data are reported as means ± standard deviation. One-way ANOVA was performed, followed by multiple pair-wise comparison procedure (Tukey test). Significance was at the 0.05 level.

3 Results and Discussion

The ability of mimicking the extracellular matrix (ECM) is crucial for artificial ECM fabrication. The reason of using synthesized nanofibers is because cells can attach and organize well around fibers with diameters smaller than the cells diameter [23, 24]. The morphology of the electrospun HPLys-PANINTs nanofibers is shown in Figure 2. A very homogeneous fibrous scaffold with fiber diameters in the range of 69–80 nm may be observed for the optimized PANINTs concentration of 1.5% w/w. It is important to note that the diameter of the nanofibers shown in Figure 2

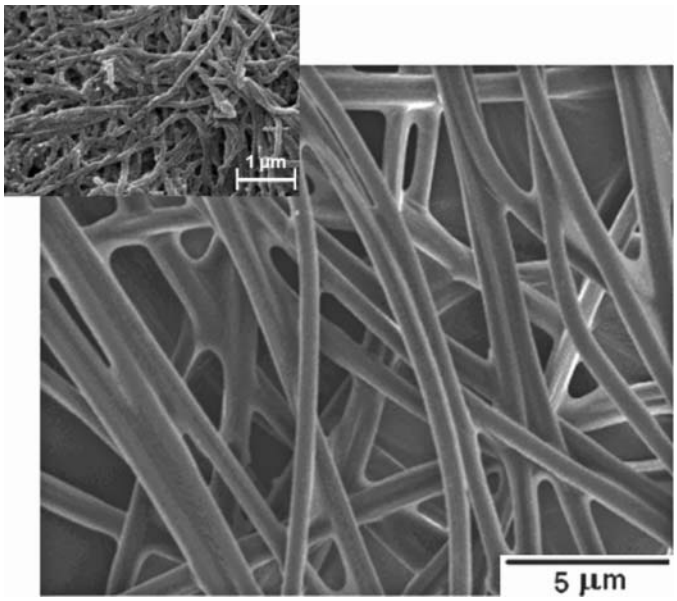

Fig. 2. SEM micrograph of the HPLy-PANINTs electrospun fibers at a PANINTs concentration of 1.5% w/w. The inset in the upper left shows the micrograph of the PANI nanotubes used for HPLys-PANINTs nanofibers fabrication.

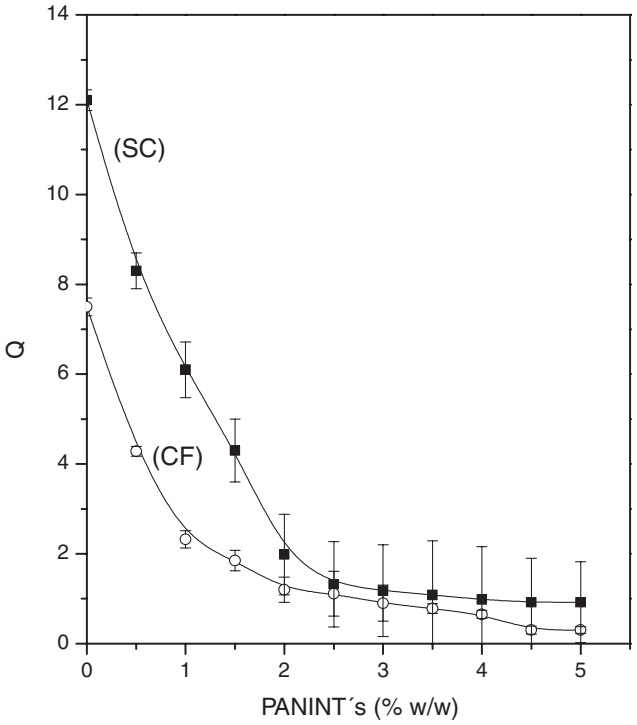

Fig. 3. Swelling behavior of the HPLys-PANINTs electronspun fibers. SC (■) and CF (○) are fibrous scaffold and casting HPLys-PANINTs, respectively.

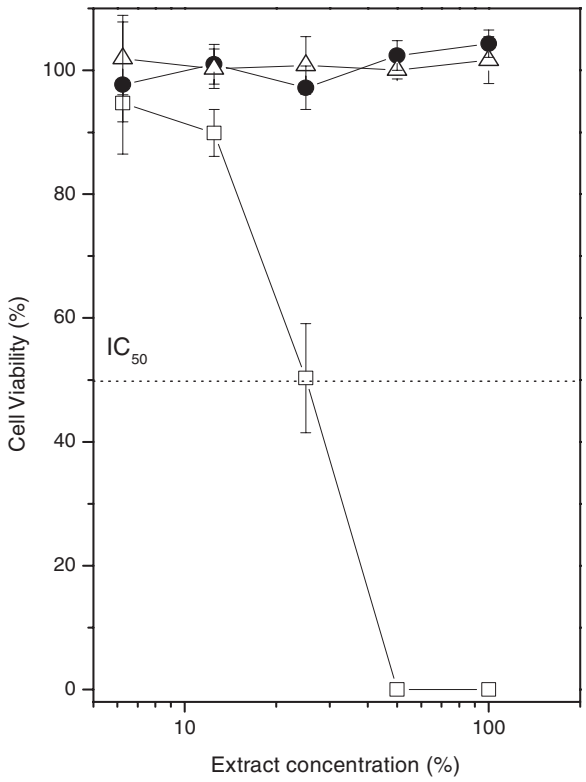

Fig. 4. Cytotoxicity of the HPLys-PANINTs electrospun nanofibers. Negative control (•) and positive control (□) against Chinese hamster ovary (CHO) cells (Δ). PANINTs concentration: 1.5% w/w.

are in the same range of the collagen, the major component of the extracellular matrixes, which has a fibrous structure varying in diameter from 50 to 500 nm (25, 26). Thinner fibers may be advantageous since the high surface-volume ratio allows the exchange of nutrients and can encourage cell growth, migration, adhesion and differentiation (10, 27). Diameter and homogeneity of the nanofibers can be controlled by the charge density and the conductivity of the solution used for electrospinning (10, 28). Other parameters such as polymer concentration, flow rate, electric current, and temperature may also be taken into account. For example, Li et al. reported that the diameter of the fibers based on PANI-incorporated gelatin decreased from 800 to 60 nm upon increasing the PANi concentration from 0 to 5.3% w/w (29).

Due to their low shrinkage, ability to promote cell adhesion and good biocompatibility, HPLys may offer innovative solutions to address tissue engineering challenges, such as the design of novel 3-D scaffolds for cardiac tissue engineering. HPLys hydrogels may act as bioadhesives, via their swelling ability, promoting cardiac cells infiltration through their dendritic structure. Figure 3 shows the swelling behavior of the HPLys-based electrospun nanofibers as a function of amount of PANINTs. It was observed that HPLys fibers swelled less upon increasing PANINTs concentration, indicating the formation of a rigid network, and consequently the occurrence of more intermolecular cross links. The high swelling ratio observed for the fibrous HPLys-PANINTs scaffold may be due to the very high surface area, as well as the high porosity of electrospun fibers that promote a better water interaction, in comparison to the casting HPLy- PANINTs films.

The *in vitro* HPLys-PANINTs cytotoxicity results are shown in Figure 4. No toxic effects could be observed on the CHO cells, neither in terms of cell viability reduction nor inhibition of cell growth. The biocompatibility of the polyaniline-based nanofibers modified by HPLys for cardiac myoblasts, as shown here, is in agreement with other systems using polyaniline modified by peptides (26).

Conjugated polymers have been used as "intelligent" scaffolds for culture of neuronal and muscular cells. Kotwal et al., investigated the application of electrical stimulation in PC-12 cells cultured on polypyrrole (PPy) based matrixes. The electrical stimulation of the PPY matrix enhances the nerve cell function facilitating the regeneration of damaged or degenerated nerves (30). The potential use of the HPLys-PANINTs nanofibers as electroactive scaffolding materials for MC's tissue was evaluated *in vitro* and the results are shown in Figure 5. The results show that HPLys-PANINTs nanofibers promote a better proliferation and differentiation of MCs cells, in comparison to the cast HPLys-PANINTs films. According to the literature, a possible explanation for such effect is that electrical stimulation changes protein adsorption onto electroactive HPLys-PANINTs , as a result of changes in the local electrical fields exhibited by extracellular matrix (30, 31).

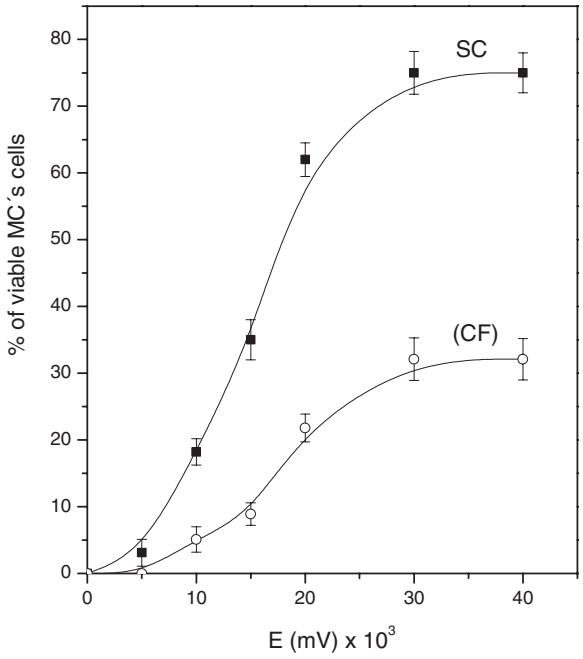

Fig. 5. Effects of electrostimulation on cell growth after 72 h. SC (■) and CF (○) are fibrous scaffold and cast HPLys-PANINTs films, respectively. (PANINTs concentration in the nanofiber was 1.5% w/w).

4 Conclusions

A very homogeneous fibrous scaffold with fibers diameters in the range of 69–80 nm was obtained for the optimized PANINT concentration of 1.5% w/w in the electrospun fibers. The HPLys-PANINT nanofibers did not induce toxic effects on the CHO cells showing high cell viability, promoting a better proliferation of MC's cells (comparing with films of HPLys-PANINTs obtained by casting). The biocompatibility of HPLys- PANINTs makes this material suitable for cardiac tissue engineering. Both electroactive and biocompatible characteristics of electrospun HPLys-PANINTs nanofibers observed in this paper demonstrate their potential for the culture of cardiac cells and open the possibility of using this material as an electroactive scaffold in cardiac tissue engineering.

Acknowledgments

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