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Injectable composites of loose microfibers and gelatin with improved interfacial interaction for soft tissue engineering

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ABSTRACT

Poly(L-lactic acid) (PLLA) microfibers were surface functionalized by graft photopolymerization of 2-hydroxyethyl methacrylate (HEMA) onto the fiber surface. Grafted fibers were easily dispersed in enzymatically gelling tyramine-substituted gelatin, forming a homogeneous dispersion without hindering subsequent gelatin crosslinking. The obtained injectable hydrogels showed improved mechanical properties compared to analogues based on non-modified fibers. The composite with 1% (w/v) of surface modified fibers had a three-fold higher shear storage modulus (535.2 ± 90 Pa) than pure gelatin (184.9 ± 32 Pa) while no significant increase was observed in the case of non-grafted fiber composites. Moreover, PHEMA grafting on PLLA fibers did not compromise cell viability and proliferation within the hydrogel. The new injectable hydrogels offer improved potential as substrates for the regeneration of soft tissues.

1. Introduction

The principal characteristic of soft tissues, e.g. myocardium, blood vessels, skeletal muscle, adipose tissue and cartilage, is their high hydration, which confers a three dimensional environment to cells that absorbs external forces for proper cell stimulation [1]. Consequently, hydrogels have been proposed as the most appropriate type of scaffolds for their regeneration. Natural hydrogels are materials with a comparatively low compression modulus, ranging from 1 to 40 kPa [2–6], which can be improved by combining the hydrogel with stronger materials in ways not compromising nutrient diffusivity and load absorption. Examples found in the literature include blending hydrogels with fibers, textiles or microparticles, impregnating the pores of stiff scaffolds with hydrogels and building multi-layered structures [7–13].

Recent publications (see, for instance [14–16]) highlight the use of injectable hydrogels in soft tissue engineering, not only because they are applied to the injured tissue with minimally invasive surgery, but also because they perfectly adopt the shape of the defect. Their integration with the host tissue in some cases may even be so good that they crosslink with extracellular matrix fibers [17,18], enhancing the transmission of loads from the host tissue to the implanted hydrogel. Reinforcing fibers should be incorporated into the hydrogel solution prior to crosslinking, so as not to compromise the injectability of the hydrogel in the tissue defect. The fibers must hence be produced in the form of loose or short microfibers. In the latter case, both the adhesion between the fiber and the matrix and the fiber aspect ratio –i.e., the ratio between its length and diameter– are especially relevant. Moreover, some micromechanical events (such as variations in stress distribution along the fiber-matrix interphase) and end effects also play a significant role [19,20].

So far, only a few attempts have been made to produce loose nano/microfibers for their later dispersion in an injectable hydrogel. One example is the work of Hsieh et al. where poly(3-

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caprolactone-co-D,L-lactide) and collagen short fibers were obtained by sonicating their electrospinning meshes [21].

We recently [22] presented two methods for the preparation of loose poly(L-lactic acid) (PLLA) microfibers for their incorporation in an injectable gelatin matrix. Although surface plasma treatment of fibers allowed homogeneous dispersion of fibers in the hydrogel precursor solution, minimum or even null reinforcement was observed due to the later agglomeration and low interaction between the hydrophilic gelatin matrix and the hydrophobic fibers.

In our previous work, PLLA was selected for its high biocompatibility [23] and processability [24]. However, in comparison to gelatin and other natural hydrogels it has a low degradation rate, mainly due to its hydrophobicity and crystallinity. It also lacks reactive side-chain groups for the grafting of bioactive moieties to modulate their response. These recurrent drawbacks have motivated different studies on surface modification of PLLA [25]. Surface engineering can be as simple as coating by adsorption [26], entrapment of biomacromolecules [27], plasma treatment [28] or the use of migratory additives [29]. It can also take more complicated forms, such as the formation of covalent bonds between the PLLA and the functional groups (i. e. chemical conjugation [30] or photografting [21–34]). A carefully chosen strategy for hydrophilic surface modification of PLLA loose fibers would improve gelatin-fiber interaction, and suppress agglomeration, without compromising the mechanical performance and hence provide a more functional matrix than the previously demonstrated plasma-modified fiber composites [22]. Holloway et al. recently showed that grafting poly(vinyl alcohol) (PVA) onto ultra-high-molecular-weight polyethylene improved the interaction with the PVA matrix, although their work was on non-injectable hydrogels [35], where non loose or short fibers are needed.

Our aim was to prepare injectable PLLA loose fiber reinforced gelatin hydrogel composites with enhanced mechanical properties by utilizing a purposely developed strategy for HEMA graft polymerization onto the PLLA fibers before matrix dispersion. PHEMA grafting was selected for its high hydrophilicity, widely proved biocompatibility, and applicability in the biomedical field [36–41]. Although most of the studies focused on its ophthalmic use and drug delivery potential [40–43], it has also been proposed as a substrate for neural regeneration [44], such as a coating on biosensors [45] or in the manufacture of artificial skin patches [46]. Our composites are a combination of three kinds of macromolecules with different *in vivo* degradation rates. Gelatin is needed in the first stages of regeneration as agglutinative of the fibers and to retain encapsulated cells. The mechanical properties of this construct should be high, and it is precisely in this first stage of regeneration when the role of the PHEMA grafting is important. In few days, cells will secrete their own extracellular matrix (ECM) and this new matrix will play the role of gelatin that is already degraded or is highly degraded. As this first regenerated matrix is still not mature, remaining PLLA fibers confer the mechanical support to the new ECM. Later, as the tissue will mature and more ECM will be secreted, fibers can slowly degrade, not compromising the mechanical stability of the defect. Although PHEMA is not a biodegradable polymer, our system has only short and few grafting chains acting as compatibilisers between the filler and matrix. These chains will be released together with degraded chains from PLLA and eliminated by macrophages phagocytosis [47] causing no adverse effects.

2. Materials and methods

2.1. Materials

Dioxane (extra pure, stabilized with 2.5 ppm of 2,6-Di-tert-butyl-4-methylphenol (BHT)) and sodium hydroxide (pellets, reagent grade, ACS, ISO) were purchased from Scharlab, Barcelona, Spain. Poly(L-lactic acid) (PLLA) Ingeo 4042D (number average molecular weight of 200,000 g/mol, 92% L-lactide and 8% D-lactide units [48]) was supplied by Natureworks LLC, Savage, MN, USA. Gelatin from porcine skin (gel strength 300, type A, with 80 mmol COOH/100 g gelatin), hydrogen peroxide solution (30% w/w in H_2O, with stabilizer), peroxidase from horseradish type I (HRP), tyramine hydrochloride (≥98%) (TA), 2-(N-morpholino) ethanesulfonic acid (>99%) (MES), N-hydroxysuccinimide (98%) (NHS), 4-(2-hydroxyethyl)piperazine-1-ethanesulphonic acid (HEPES), calcium chloride (technical grade −4 to +30 mesh), potassium phosphate monobasic, 2-hydroxyethyl methacrylate (HEMA) (97%), sodium phosphate dibasic (≥99%, ACS Reagent), benzophenone (ReagentPlus® 99%) (BP) and dialysis tubing (12400 MWCO) were provided by Sigma–Aldrich, Germany. N-(3-dimethylaminopropyl)-N′-ethylcarbodiimidehydrochloride (EDC) was provided by Iris Biotech GmbH, Marktredwitz, Germany. Ethanol (96% vol GPR Rectapur®) and silicon oil 47v50 (Rhodorsil®) were purchased from VWR International, France. Chloroform (D, 99,9%) and deuterium oxide (D, 99,9%) were provided by Cambridge Isotope Laboratories Inc., MA, USA. Sodium chloride and potassium chloride GR were provided by Merck KGaA, Darmstadt, Germany. Potassium dihydrogen phosphate (analytical reagent, buffer substance) was supplied by Riedel-de Häen GmbH, Seelze, Germany.

Dulbecco's Phosphate Buffered Saline (DPBS) solution was prepared with 0.2 g/L potassium phosphate monobasic, 0.2 g/L potassium chloride, 8 g/L sodium chloride and 1.15 g/L sodium phosphate dibasic.

Krebs Ringer Buffer (KRB) solution was prepared with 115 mM sodium chloride, 5 mM potassium chloride, 1 mM calcium chloride, 1 mM potassium dihydrogen phosphate and 25 mM HEPES.

2.2. Loose PLLA microfibers preparation

Loose PLLA fibers were obtained from a 5% (w/v) PLLA solution in dioxane. Aliquots of the PLLA solution (5 mL) were injected at a rate of 34 mL/min using a syringe pump through a 0.9 mm needle into highly agitated cold ethanol at −20 °C. The projected solution was stirred at 15,000 rpm using a IKA T25 digital ULTRA-TURRAX mixer (Germany) for a total time of 2 min. For complete elimination of the dioxane, the fibers were washed repeatedly with ethanol, which was later changed for water drop by drop and under stirring to avoid fiber agglomeration. Water dispersed fibers were sieved through nylon meshes of different pore dimensions, collecting the fibers between 80 and 30 μm, which were finally lyophilized.

2.3. PHEMA grafting to loose PLLA microfibers

HEMA was graft polymerized onto PLLA microfibers in a two-step procedure.

Firstly, the PLLA microfibers were activated by dispersing 40 mg fibers in 20 mL ethanol containing 5% (w/v) BP. This fiber dispersion was placed into a pyrex tube and submitted to an orbital shaking for 5 min and then irradiated for 20 min with an UV-lamp (Osram Ultra Vitalux, 300 W) of 280–320 nm wavelength and 38 mW/cm^2 output intensity. The dispersion container was covered with a quartz plate to prevent ethanol evaporation and allow penetration of UV light. The distance between the UV lamp and the fiber dispersion was 14 cm. The activated fibers were washed with ethanol two times and then the ethanol was substituted by water drop by drop. Next, 2 mg/mL of the activated fibers (PLLA-BP) was dispersed in a 20% (v/v) HEMA/ethanol solution and homogenized