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Fabrication of electrospun silk fibroin scaffolds coated with graphene oxide and reduced graphene for applications in biomedicine

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ABSTRACT

Silk fibroin and graphene are both promising biomaterials described in the bibliography. Hybrid scaffolds combining their properties could be attractive for tissue engineering applications. In this work, a new methodology to produce electrospun fibroin scaffolds coated with graphene materials is provided. The mechanical, electrical and electrochemical properties of the materials attained were characterised. The fibre diameters were measured (from 3.9 to 5.2 μm). The samples coated with reduced graphene were electronic conductors and electroactive in liquid electrolytes, showing maximum oxidation and reduction (around −0.4 V peak). The chronoamperometric responses showed a reduction shoulder, pointing to the entrance of balancing cations from the solution by nucleation–relaxation: the reaction induced structural changes in the graphene. In order to check the biocompatibility of the materials, they were seeded with L929 fibroblasts. The excellent biocompatibility of silk fibroin meshes was maintained after coating with graphene, being the proliferation results equal in all the treatments 7 days after the seeding (Tukey, $p > 0.05$).

The conductive and electroactive properties of meshes coated with reduced graphene allow the potential application of local electric fields or local ionic currents to cell cultures, biological interfaces or animal models without host response.

1. Introduction

Life involves chemical reactions and electrical (ionic) currents. The use of materials to support cell proliferation for tissue restoration requires excellent biocompatibility, and strong electrical and ionic interactions with cells during proliferation. So, in addition to biocompatibility, materials for biological uses must be electronic conductors and electroactive in aqueous electrolytes. Their electroactivity will allow the storage and delivery of anions or cations, and volume variations — in a continuous way or by pulses during cell proliferation. It is expected that, by using suitable materials and ions, the simultaneous presence of electric fields, mechanical effects and ionic currents can allow some, until now inexistent, mechanical and chemical control of cell growth. This electroactivity must occur at low overpotentials, inside the potential window of the aqueous electrolyte and — as far as possible — of the water electrolysis (oxygen evolution or hydrogen evolution potential).

Electrical stimulation of cells and tissues is a well-known technology used to improve their different biological functions. Some cellular types, namely neurons, cardiomyocytes and skeletal muscle cells, are electroactive and respond directly to electrical impulses. Other biological processes show an indirect positive response. As a consequence, there is a permanent demand for improved biomaterials and scaffolds designed to carry electrical stimulation to the cells growing on them. This function has been traditionally accomplished by conducting polymers (CPs), a special class of polymeric materials with electronic and ionic conductivity [1]. Their conductivity arises from the presence of conjugated double bonds along the backbone of an otherwise insulated structure. The CPs perform well in a variety of approaches in the tissue engineering field [2,3,4]. However, they present some limitations, such as the brittleness of the materials.

The field of electroconductive biomaterials has been enriched significantly after the introduction of a material with exceptional electroconducting properties, graphene. This is a carbon-based material consisting of a sheet of two-dimensional, single-layer sp2 hybridised carbon atoms in a honeycomb lattice configuration [5]. The singularity of this molecular structure confers graphene with outstanding properties in the field of physics. It has high electrical and thermal

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conductivities and very-high mechanical strength, as well as excellent optical properties. Thus, graphene is being developed in a considerable diversity of applications, in diverse fields such as energy conversion [6] biosensing [7], imaging [8] and the fabrication of many kinds of electronic, optical and energy storage devices [5,9]. In addition, graphene is also biocompatible [10] and it is apt for diverse biomedical applications [11,12].

Interestingly, graphene surfaces have a good affinity with cells. Graphene is a biocompatible material, as are CPs, but it also has a stimulatory effect on cell proliferation. Numerous reports have noted the optimal adhesion and proliferation of diverse type of cells in plastic or glass plates coated with graphene or graphene oxide (GO) [13,14]. A notable aspect is that the chemical nature of graphene seems to act also on the differentiation of stem cells, raising the possibility of favouring this process by modifying the chemical configuration or surface of this material. This effect is well documented in the differentiation of neural stem cells to neurons [15,16,17], mesenchymal stem cells to osteoblasts [18,19], periodontal ligament stem cells to osteoblasts [20] and iPSC to different cellular lineages [21]. This evidence shows the considerable potential of graphene as a component in tissue engineering scaffolds [22].

Under the usual terms, graphene is grouped as a complete family of carbonaceous nanomaterials with very-different oxidation states, obtained by different methods and showing different physical formats. Two main configurations of graphene have been described for tissue engineering applications. One is a carbon monolayer placed on copper foil by carbon vapour deposition (CVD) that, after etching of the metal, can be transferred to different types of bi-dimensional substrates. [18]. By extension of this concept, the monolayer can be deposited on a Ni foam that, after etching, produces a tri-dimensional graphene foam. [17]. Alternatively, graphene can be exfoliated from graphite, by a very-oxidative chemical treatment that yields GO; this takes the form of nanoflakes, with a variable number of layers and many carboxylate and epoxy groups, which are not electroconductive. This reactivity allows the formation of stable, colloidal aqueous solutions of GO that can be reduced again, with a variety of reducing agents, to yield graphene — which thus recovers some of its original properties, especially the electroconductivity, and can be used to coat diverse surfaces [5,9].

However, most of the experiments performed to date to illustrate the behaviour of cells on graphene surfaces used glass or plastic surfaces covered by graphene monolayers, or papers made of GO flakes recovered by filtration of an aqueous solution. These constructs are scarcely implantable, or have a minimal mechanical strength, reducing their applicability in a biological context. As a consequence, the need arises to develop composite materials in which graphene could provide excellent electroconductive properties, while a polymeric filler could give tridimensionality and mechanical strength to the construct. Many different types of composites exist and are being developed around graphene [23]. One of the most-interesting polymers that could be combined with graphene is silk fibroin (SF). In addition to being an excellent and well-known biocompatible biomaterial in itself [24], fibroin is a protein with a secondary molecular structure in the form of a beta-sheet that combines well with graphene, producing hybrid film formats [25,26] that sustain well the growth of cells [27]. However, there are other configurations of fibroin scaffolds that could be improved after combination with graphene. One of these is a non-woven nanofibre mat made by electrospinning. As these mats are good biomimetic structures with an extracellular matrix (ECM), they are very apt for cellular growth and have been extensively tested [28,29]. Previous experiments have attempted to provide electroconductivity to electrospun fibroin mats by coating them with polypyrrol, obtaining good conductivity and an improvement of mechanical properties while allowing an adequate proliferation rate of fibroblasts and mesenchymal stem cells [30].

Here we study the electrochemical and mechanical behaviour of SF meshes coated with graphene materials, as well as the biological performance after seeding them with fibroblasts, to evaluate the possibilities for their use as supporting electroactive materials for cell culture under controlled electric fields and ionic currents.

2. Material and methods

2.1. Silk fibroin processing

Cocoons of *Bombyx mori*, obtained from silkworms reared in the sericulture facilities of the IMIDA (Murcia, Spain), were chopped into 4 or 5 pieces and boiled in 0.02 M Na_2CO_3 for 30 min, to remove the glue-like sericin proteins. Then, the raw SF was rinsed thoroughly with water and dried at room temperature for 3 days. The extracted SF was dissolved in 9.3 M LiBr (Acros Organics) for 3 h at 60 °C, to generate a 20 wt.% solution that was dialysed against distilled water for 3 days (Snakeskin Dialysis Tubing 3.5 KDa MWCO, Thermo Scientific), with eight total water changes (at 4 °C). The resultant 6–7 wt.% SF solution was concentrated by dialysis against 30 wt.% PEG (11,000 Da) for 24 h, to obtain 19–20 wt.% SF solutions that were used for the electrospinning experiments [31].

2.2. Electrospinning and post-treatment of the mats

The electrospinning system used in these experiments followed the design described in detail by Jose et al. [32]. It consisted of an insulated cabinet, which housed an electrically-charged spinneret protruding through a focal plate, a syringe pump and a grounded, circular metallic collector. The collector surface (200 cm^2) was covered with aluminium foil. The electrospinning conditions were adjusted so that the Taylor cone was stable. A voltage of +21.5 kV was applied to the capillary tube and −1.5 kV to the collector, the distance between the tip of the tube and the collector was adjusted to 42 cm and the selected injection rate of the polymer solution was 3 mL h^{-1}. In all cases, 3 mL of 19–20 wt.% SF solution were electrospun onto the same surface.

The environmental conditions were recorded during the experiments, the average temperature and relative humidity being 23–24 °C and 30–40%, respectively.

After fabrication, the electrospun meshes were annealed by immersion in a bath of absolute methanol for 45 min, to induce a structural transition from an amorphous (random coil) to a β-sheet conformation. The electrospun meshes were placed between two pieces of filter paper, to facilitate drying for 24 h. This technique was employed to prevent the mats from curling or folding.

2.3. Graphene oxide coating and in situ reduction

The SF electrospun meshes were coated with graphene oxide (GO) by means of immersion in 50 mL of aqueous suspensions of GO (GRAPHENEA, San Sebastian, Spain) at concentrations of 1, 2, 3 and 4 mg mL^{-1}, in order to probe the optimal concentration to make the mat conductive. Five cycles of immersion (20 min each) in these aqueous suspensions of GO were performed, alternating them with drying periods of 24 h. During the drying periods the mats were placed between two pieces of filter paper in order to prevent them from curling or folding.

After this, some of the electrospun meshes were immersed in an aqueous solution of ascorbic acid (20 mM) for 3 h at 70 °C, following the method proposed by Fernández-Merino et al. [33], to reduce the GO. The reduced graphene (RG) incorporated in this way into the SF fibres conferred electroconductivity to the mats.

Henceforth, the SF mats coated with different amounts of GO will be referred to as SF/GO1, SF/GO2, SF/GO3 and SF/GO4, for the mats immersed in GO suspensions at 1, 2, 3 and 4 mg mL^{-1}, respectively. The corresponding mats with RG incorporated into their fibres will be referred to as SF/RG1, SF/RG2, SF/RG3 and SF/RG4, respectively.

The SF mats without incorporated GO were used as negative controls and were subjected to five steps of immersion in distilled water