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Functional porous hydrogels to study angiogenesis under the effect of controlled release of vascular endothelial growth factor

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ABSTRACT

Angiogenesis occurs through a cascade of events controlled by complex multiple signals that are orchestrated according to specific spatial patterns and temporal sequences. Vascularization is a central issue in most tissue engineering applications. However, only a better insight into spatio-temporal signal presentation can help in controlling and guiding angiogenesis in vivo. To this end, versatile and accessible material platforms are required in order to study angiogenic events in a systematic way. In this work we report a three-dimensional porous polyethylene glycol (PEG) diacrylate hydrogel bioactivated with heparin that is able to deliver vascular endothelial growth factor (VEGF) in a sustained and controlled manner. The efficiency of the material has been tested both in vitro and in vivo. In particular, the VEGF released from the hydrogel induces cell proliferation when tested on HUVECs, retains its bioactivity up to 21 days, as demonstrated by Matrigel assay, and, when implanted on a chorion allantoic membrane, the hydrogel shows superior angiogenic potential in stimulating new vessel formation compared with unfunctionalized hydrogels. Moreover, in the light of potential tissue regeneration studies, the proposed hydrogel has been modified with adhesion peptides (RGD) to enable cell colonization. The porous hydrogel reported here can be used as a valid tool to characterize angiogenesis, and, possibly, other biological processes, in different experimental set-ups.

1. Introduction

Recovering the physiological functions of degenerated or injured tissues is one of the most critical aspects in tissue engineering and regenerative medicine [1,2]. Vascular integration between the implant and the surrounding tissues is key to achieving this goal. This requires the rapid formation of new capillaries to supply oxygen and the necessary nutrients and to remove waste products from cells [3,4]. In this field angiogenesis, i.e. blood vessel formation from pre-existing ones, plays a pivotal role. It is a very complex phenomenon which is regulated by several biochemical and biophysical factors. Several issues must be addressed in the promotion of angiogenesis in biological matrices such as porous scaffolds and hydrogels. Among these, tight control of the dose and temporal evolution of bioactive signals is fundamental to guide and direct proper cell functions [5]. In particular, for functional angiogenesis pore dimension and the spatial arrangement of bioactive molecules within the matrix play a critical role in blood vessel formation in vitro and vessel invasion in vivo. It is known that the minimum porosity required to regenerate blood vessel is generally considered to be 30–40 μm [6], in order to enable the transport of metabolic components and the induction of endothelial cell invasion. Furthermore, signals presented by the extracellular matrix (ECM), such as soluble macromolecules (e.g. growth factors (GF), chemokines, and cytokines) and insoluble factors (e.g. ECM proteins, glycoaminoglycans, and proteoglycans), also play a major role in tissue regeneration. Accordingly, angiogenic processes are guided by various growth factors whose spatial and temporal presentation is strictly regulated by various ECM components. For example, heparin molecules are known to bind various angiogenic growth factors, such as for vascular endothelial growth factor (VEGF), *basic fibroblast growth factor* (bFGF), and transforming growth factor *beta* (TGFβ), through non-covalent and reversible interactions [7]. Such an interplay brings two major benefits: first, bound GFs are less prone to degradation; second, the spatial arrangement of heparin molecules and their binding affinity for GFs provides cells with directional and temporal cues which guide and direct the process of new vessel formation. Cells are very sensitive to both the local concentration of VEGF and to the way it is delivered. High doses of VEGF elicit an evident tissue response, but generally lead to dysfunctional growth. Indeed, undesired

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vascularization at non-target sites, tumor growth at locations away from the implant, hypotension, and edema [8] have been reported. Only VEGF that is delivered in adequate doses and in a sustained manner leads to de novo generation of functional vessel networks [9].

Recapitulating the complex angiogenic events within polymeric matrices requires the development of novel chemical and material processing strategies to obtain fine control of the spatial arrangement of pro-angiogenic signals along with the temporal evolution of their concentration. Functionalization of hydrogels has proved to be a very versatile strategy to deliver or present bioactive proteins. Hydrogels possess a high water content, tunable mechanical properties and controllable degradation rates. With respect to angiogenesis, dextran [10], alginate [11], and hyaluronan hydrogels [12] have been used as factor delivery vehicles demonstrating superior capacities in promoting new vessel formation.

Recently delivery of VEGF by heparin-functionalized PEG scaffolds has revealed efficient revascularization [13]. However, irrespective of the scaffold composition, great care must be taken when defining the materials, structural properties and processing conditions, since these may have adverse effects on growth factor bioactivity. In fact, such drawbacks might end up in not enabling the control of the angiogenetic process, and thus not allowing definition of the optimal biochemical and biophysical conditions necessary to promote and sustain new vessel formation. To this end, simple and tunable material platforms are required to study vessel formation in a systematic way.

To overcome these limitations we have developed a material model to study VEGF delivery from a three-dimensional (3-D) porous matrix, the angiogenetic potency at large loading capacity, the retention of biological activity and the controlled sustained release of VEGF from a heparin functionalized hydrogel [3,5]. Heparin was cross-linked to PEG by UV irradiation to fabricate a porous 3-D hydrogel through a foaming process. The resultant matrix was characterized by a highly porous and interconnected pore structure. In vitro and in vivo experiments were performed to assess the angiogenic potential of the released VEGF. The release kinetics of the loaded VEGF were quantified and the bioactivity of the released factor evaluated by means of mitogenic induction and capillary tube formation assay in vitro and on chick chorioallantoic membrane (CAM) in vivo.

2. Materials and methods

2.1. Synthesis of poly(ethylene glycol) diacrylate and methacrylation of heparin

Poly(ethylene glycol) diacrylate (PEGDA) was synthesized by dissolving PEG (M_W 3400) in methylene chloride. Triethylamine (TEA) at 30% molar excess was added drop-wise, and the solution mixed under nitrogen for 15 min. Acryloyl chloride at 20% molar excess was mixed with methylene chloride and added drop-wise to the PEG/TEA solution, and the final mixture stirred overnight at room temperature. The product was precipitated in ice cold diethyl ether, filtered, and dried in a dessicator. After drying the PEGDA was redissolved in deionized H_2O and dialyzed (Spectrum, 1000 M_W cut-off) over 12 h with two distilled water exchanges. ^1H NMR analysis (in chloroform-d_3, Cambridge Isotopes) of the PEGDA revealed an average of 90% acrylation. Five hundred milligram heparin (M_W 18 kDa) and a twofold molar excess of N-(3-aminopropyl) methacrylamide hydrochloride (APMMA) relative to the –COOH groups in heparin were dissolved 15 ml of deionized water. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and hydroxybenzotriazole (HOBt) were used as activation agents for the –COOH groups of heparin (Fig. 1). They were dissolved in 2 ml of a 1:1 mixture of dimethysulfoxide (DMSO)/water and added into the above solution. The molar ratio EDC:HOBt was 1:2 with respect to 1 mol of heparin. The conjugation reaction was carried out for 8 h with stirring. The APMMA-derivatized heparin was dialyzed against deionized water for 4 h (Spectra/Por, M_W cut-off 1000) and then freeze dried. Percent modification of heparin by APMMA was determined by analyzing the ^1H NMR spectra using a Varian Unity Inova spectrometer operating at 500 MHz. The degree of substitution of heparin with APMMA was calculated by comparing the relative peak intensity ratio between two protons of the vinyl group in APMMA (=C$\underline{H}_2$, 5.5 and 5.8 p.p.m.) with the methane proton of the 2-O-sulfated iduronic acid unit in heparin (–C$\underline{H}$, 5.1 p.p.m.). The degree of substitution was ~19%. The chemical shift (δ) was measured in p.p.m. using 3-(trimethylsily) propionic-2,2,3,3-d_4 acid sodium salt as an internal reference.

2.2. Three-dimensional porous scaffold preparation

Hydrogel porous structures were produced by matching the polymer photo-cross-linking reaction with a foaming process. Therefore, hydrogel synthesis is summarized as follows: (1) preparation of the starting polymer solution; (2) addition and mixing of the foaming agent; (3) photo-cross-linking of the resulting foam. PEG (M_W 3400 Da, Shearwater Polymers) and PEG bioactivated with heparin (Hp-PEG) (weight ratio PEG to Hp 5:1) were used for hydrogel preparation. Hydrogel disks were fabricated starting either from a solution containing 10 wt.% PEG monomer or from a solution containing 10 wt.% PEG monomer with 5 mg methacrylated heparin in phosphate-buffered saline (PBS). Then acrylic acid (25 μl ml^{-1}), Pluronic F127 as a surfactant, (4 mg ml^{-1}), $NaHCO_3$ (40 mg ml^{-1}) as a foaming agent, and 2,2-dimethoxy-2-phenylacetophenon (Sigma–Aldrich, St. Louis, MO), at a final concentration of 3 wt.% in N-vinylpyrrolidone, were added to the polymer solution. The viscous foam was solidified upon exposure to a UV light source at a wavelength of 365 nm and wattage of 30 mW cm^{-2} for 2 min. Hydrogels were extensively washed in bi-distilled water to remove unreacted chemicals.

2.3. Morphological and biochemical characterization of 3-D hydrogels

The samples were placed in a conventional incubator for cell culture for at least 24 h. Under these conditions the hydrogels were sufficiently moist to retain their initial shape and allow gold sputtering. The latter was done in order to perform scanning electron microscopy (SEM) examinations with a Leica S440 microscope. Mercury intrusion porosimeter was used to measure the average pore size of porous the hydrogels in the low pressure regime pressure range (0.1–400 kPa) with a Pascal 140 porosimeter (Thermo Finnigan). In particular, three samples were freeze dried prior to porosimetry testing. Reconstruction of the pore size distribution was done under the assumption of a cylindrical pore shape.

The ability of the hydrogel to take up water was assessed by evaluating the swelling ratio (Q). This was considered to be the ratio of the volume of swollen hydrogels to the volume of dry samples. Cylindrical PEG or Hp-PEG hydrogels were soaked in excess PBS for 24 h in an incubator at 37 °C. The height and diameter of the swollen hydrogels were measured with a digital caliper. The samples were dried in a oven at 37 °C for 24 h, after which measurements were taken.

The unreacted heparin in the supernatant was analyzed for total heparin content using the dimethylmethylene blue (DMMB) dye method as desorbed in Taylor and Jeffree [14]. The absorbance at 530 nm was measured immediately after the reaction. The heparin concentration was determined by comparing the absorbance values with the calibration curve.