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Tissue response to poly(ether)urethane-polydimethylsiloxane-fibrin composite scaffolds for controlled delivery of pro-angiogenic growth factors

Paola Losi [a], Enrica Briganti [a], Angela Magera [b], Dario Spiller [a], Chiara Ristori [b], Barbara Battolla [b], Michela Balderi [a], Silvia Kull [a], Alberto Balbarini [b], Rossella Di Stefano [b,1], Giorgio Soldani [a,*,1]

[a] Laboratory for Biomaterials & Graft Technology, Institute of Clinical Physiology, CNR, via Aurelia Sud, 54100 Massa, Italy
[b] Cardiovascular Research Laboratory, Cardiac, Thoracic and Vascular Department, University of Pisa, Pisa, Italy

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ABSTRACT

The development of a scaffold able to mimic the mechanical properties of elastic tissues and to induce local angiogenesis by controlled release of angiogenic growth factors could be applied in the treatment of several ischemic diseases. For this purpose a composite scaffold made of a poly(ether)urethane–polydimethylsiloxane (PEtU–PDMS) semi-interpenetrating polymeric network (semi-IPN) and fibrin loaded growth factors (GFs), such as VEGF and bFGF, was manufactured using spray, phase-inversion technique. To evaluate the contribution of each scaffold component with respect to tissue response and in particular to blood vessel formation, three different scaffold formulations were developed as follows: 1) bare PEtU–PDMS; 2) PEtU–PDMS/Fibrin; and 3) PEtU–PDMS/Fibrin + GFs. Scaffolds were characterized *in vitro* respect to their morphology, VEGF and bFGF release kinetics and bioactivity. The induction of *in vivo* angiogenesis after subcutaneous and ischemic hind limb scaffold implantation in adult Wistar rats was evaluated at 7 and 14 days by immunohistological analysis (IHA), while Laser Doppler Perfusion Imaging (LDPI) was performed in the hind limbs at 0, 3, 7, 10 and 14 days. IHA of subcutaneously implanted samples showed that at 7 and 14 days the PEtU–PDMS/Fibrin + GFs scaffold induced a statistically significant increase in number of capillaries compared to bare PEtU–PDMS scaffold. IHA of ischemic hind limb showed that at 14 days the capillary number induced by PEtU–PDMS/Fibrin + GFs scaffolds was higher than that of PEtU–PDMS/Fibrin scaffolds. Moreover, at both time-points PEtU–PDMS/Fibrin scaffolds induced a significant increase in number of capillaries compared to bare PEtU–PDMS scaffolds. LDPI showed that at 10 and 14 days the ischemic/non-ischemic blood perfusion ratio was significantly greater in the PEtU–PDMS/Fibrin + GFs than in the other scaffolds. In conclusion, this study showed that the semi-IPN composite scaffold acting as a pro-angiogenic GFs delivery system has therapeutic potential for the local treatment of ischemic tissue and wound healing.

1. Introduction

Neovascularization of ischemic cardiac and skeletal muscle may be sufficient to preserve tissue integrity or function and may thus be considered a therapeutic goal. Preclinical and clinical studies have indicated that exogenous administration of angiogenic factors to sites of ischemia, in the heart or the limb, can improve regional blood flow [1–3].

Angiogenesis is a multifactorial process that is regulated by an interplay of a large number of factors. Among the known angiogenic growth factors (GFs), vascular endothelial growth factor (VEGF) has emerged as a central regulator of the angiogenic process under both physiological and pathological conditions [4–6].

Another GF known to enhance angiogenesis is basic fibroblast growth factor (bFGF) that has been successfully used in the treatment of ischemia in limb [7], heart [8] and skin wound healing [9,10].

However, the administration of one GF is not sufficient to create well-developed mature blood vessels. Tumor cells, transfected with VEGF or bFGF, need both of them to form blood vessels [11]. Similar synergistic effects were found by local administration of VEGF and bFGF in a rabbit ischemic hind limb resulting in a higher capillary density and capillary *vs* muscle fiber ratio than either VEGF or bFGF alone [12].

The limitations of VEGF and bFGF use are due to their rapid diffusion and degradation, which cause a low accumulation at wound sites when injected into the body in soluble form [13,14].

* Corresponding author. Tel./fax: +39 0585 4933632.
E-mail address: giorgio.soldani@ifc.cnr.it (G. Soldani).
[1] These authors contributed as co-responsible of the study.

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Delivery systems that release VEGF and bFGF for a long period in a controlled manner could increase their efficacy for angiogenesis in tissue regeneration. One of the most commonly used methods to deliver recombinant GFs in a sustained and site-targeting manner is their incorporation in fibrin matrices [15–17].

Moreover, it has been shown that the addition of heparin in fibrin matrices protects the GFs from rapid clearance, improving the retention of heparin-binding growth factor proteins, such as VEGF and bFGF [7,18,19], resulting in a more gradual and sustained release.

Although fibrin matrices possess excellent biocompatibility [20], biodegradability [21] and cell adhesion [22,23], they lack the adequate mechanical strength [24] required in some clinical applications, such as skin wound healing and cardiac wall regeneration. Therefore, a strategy to provide strength and elasticity to a fibrin matrix could be to combine it with synthetic elastomeric components, such as polyurethanes (PUs) and poly(dimethyl siloxanes) (PDMSs) [commonly referred to as silicones], which have been largely used for biomedical applications. Due to their excellent physical properties, flex life, tensile strength, and ultimate elongation, PUs have often been used for continuously-flexing chronic implants, such as ventricular assist devices, intra-aortic balloons and artificial heart components [25,26]. In recent years, silicone-based elastomers have been used in a wide range of biomedical applications due to their good blood compatibility, low toxicity, good thermal and oxidative stability, low modulus and anti-adhesive properties [27,28]

Our laboratory previously developed a versatile elastomeric formulation that, in the form of a semi-interpenetrating polymeric network (semi-IPN), combines in one step the already-proven good properties of the aforementioned PUs and silicone materials [29]. The PEtU–PDMS semi-IPN has been tested in preclinical applications in form of small-diameter vascular grafts [30] and for the coating of abdominal meshes [31] with relevant results regarding graft patency and the absence of adhesion formation, respectively.

Based on these findings, a novel composite scaffold constituted by a fibrin layer that acts as delivery vehicle for VEGF and bFGF, and a PEtU–PDMS layer that provides mechanical resistance and handling, has been developed and tested *in vitro* [32]. The composite scaffold was manufactured by spray, phase-inversion technique, depositing simultaneously the PEtU–PDMS material and a thrombin solution over a rotating mandrel, then the deposited material was reacted with different fibrinogen concentration to form a fibrin network onto the synthetic layer.

The purpose of this *in vivo* study was to investigate the angiogenic therapeutic potential of a composite scaffold obtained with a fibrinogen solution concentration of 20 mg/ml that has already shown *in vitro* the ability to sustain a prolonged growth factor release rate [32]. Tissue response was evaluated by implanting the composite scaffold in the dorsal subcutis and ischemic hind limbs of adult Wistar rats.

2. Materials and methods

2.1. Materials

The medical-grade, aromatic PEtU (Estane® 5714) was purchased from Lubrizol Advanced Materials, Inc. (Cleveland, OH, USA) and the diacetoxy silyl terminated (tetraacetoxy functional) PDMS from United Chemical Technologies, Inc. (Bristol, PA, USA). The PEtU–PDMS material containing 30% of PDMS was synthesized according to the previously described protocol [33].

Fibrinogen and thrombin were purified from human plasma by the supplier (KEDRION S.p.A., Castelvecchio Pascoli, Lucca, Italy). Human lyophilized fibrinogen was dissolved in 1% L-Arginine and L-Lysine at 100 mg/ml. Human lyophilized thrombin was dissolved in 275 mM $CaCl_2$ in H_2Od at 500 U/ml.

Heparin sodium from porcine intestinal mucosa was purchased from Sigma–Aldrich (S. Louis, MO, USA) and dissolved in H_2O (500 μg/ml). Human recombinant $VEGF_{165}$ and bFGF were purchased from R&D Systems (Minneapolis, MN, USA) and dissolved in H_2O (100 μg/ml).

2.2. Scaffold preparation

Three different scaffolds were prepared as follows: scaffolds were made of PEtU–PDMS material only (called bare PEtU–PDMS), PEtU–PDMS material and fibrin (called PEtU–PDMS/Fibrin) and PEtU–PDMS material and fibrin containing VEGF and bFGF (called PEtU–PDMS/Fibrin + GFs).

Bare PEtU–PDMS scaffold was manufactured in the form of membrane, by the above-mentioned spray, phase-inversion technique [34], in a clean room. Briefly, the manufacturing process consisted of two phases: in the first phase, a 2.5% PEtU–PDMS solution and H_2Od were employed; in the second phase, a 1% PEtU–PDMS solution and H_2Od were sprayed. The bare PEtU–PDMS scaffold was punched to obtain round samples of 1 cm^2 area which were stored in physiological solution at room temperature and protected from light.

PEtU–PDMS/Fibrin scaffold was manufactured as described above, using in the second phase a 25 U/ml thrombin solution instead of H_2Od. Then, the scaffold was punched to obtain round samples of 1 cm^2 area and placed in the bottom of a 24-well culture plate. Finally, fibrinogen solution (20 mg/ml) was added to each well (600 μl/well).

PEtU–PDMS/Fibrin + GFs scaffold was obtained by non-covalent incorporation of VEGF (200 ng/scaffold), bFGF (200 ng/scaffold) and heparin (5 μg/scaffold) to fibrinogen solution (20 mg/ml).

For PEtU–PDMS/Fibrin and PEtU–PDMS/Fibrin + GFs scaffolds, the complete polymerization reaction into fibrin was achieved after an overnight incubation at 37 °C.

2.3. Scaffold structural characterization

2.3.1. Morphological analysis

The morphological analysis of PEtU–PDMS scaffold surface was performed upon staining with a Sudan Black B solution (0.3% w/v in absolute ethanol, Carlo Erba, Milan, Italy) and subsequent stereo-microscopical observation (SZH10 microscope, Olympus Optical Co., Tokyo, Japan). Representative images of sample surface at 60× original magnification were acquired by a video camera (KY-F32, JVC, Milan, Italy) connected to the microscope.

Analysis of the fibrin layer was performed after staining with Ponceau red dye. In brief, samples were dipped in a staining solution (0.5% w/v in 1% acetic acid) for 5 min at room temperature and, afterwards, rinsed in distilled water to remove the excess dye. Moreover, the fibrin layer thickness was measured by a digital micrometer.

2.3.2. Peeling test

Fibrin layer adhesion onto the synthetic surface of all composite scaffolds was qualitatively verified by elevating sample edge with forceps and by observing the presence of synthetic material below the fibrin layer.

2.3.3. Scanning electron microscopy (SEM)

The nanostructure of PEtU–PDMS/Fibrin scaffolds was observed using a scanning electron microscope (SEM, Jeol 5600, Jeol Italia, Milan, Italy) after gold–palladium metallization by a sputter coater system (Sputter coater S150B, Edwards, Irvine, CA). SEM microphotographs were taken at 4000× and 20,000× magnifications with a 20 kV acceleration voltage. The images acquired at 20,000× were analyzed by a computerized image analysis system (Axiovision Rel 4.6, Carl Zeiss, Jena, Germany) to quantitatively determine fibrin fibers' mean diameter; six random measurements were performed for each image.

2.3.4. Histological analysis

PEtU–PDMS/Fibrin + GFs scaffold was snap-frozen in chilled OCT in an isopentane bath cooled with liquid nitrogen. Then the sample was stored at −80 °C and cryostat cut in 7 μm-thick sections. The histological sections were fixed in formalin for a few minutes and stained with hematoxylin and eosin.

2.4. Determination of VEGF and bFGF release

The kinetic of growth factor release from PEtU–PDMS/Fibrin + GFs scaffolds was determined. The scaffold sample of 1 cm^2 area was fixed by a sterile Teflon® ring at the bottom of a 24-well plate and incubated with 1 ml of PBS under continuous agitation in a horizontal shaker (40 rpm) at 37 °C.

Daily for the first week and at day 14, the supernatant was collected and replenished with an equal volume of fresh buffer. The supernatants were immediately frozen at −80 °C until measurement. The amounts of VEGF and bFGF were determined by enzyme-linked immunosorbent assay (ELISA) kit (Quantikine®, R&D Systems Europe, Abingdon, UK). Briefly, 1:50 diluted samples were added to 96-well plates coated with monoclonal antibodies against VEGF and bFGF and incubated for 2 h at r.t. Then, horseradish peroxidase-linked monoclonal antibodies specific for VEGF or bFGF were added to each well and incubated for 2 h at r.t. Afterwards, the substrate (tetramethylbenzidine) was added and incubated for 25 min. The enzyme reaction was stopped by adding an acidic solution. The absorbance of the samples was read at 450 nm using a microplate reader (SpectraFluor Plus, TECAN Austria