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Physical characterization of vascular grafts cultured in a bioreactor

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Abstract

Tubular scaffolds of collagen and elastin (weight ratio 1:1) with interconnected pores were prepared by freeze drying and crosslinked with *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) in the presence or absence of a Jeffamine spacer (poly(propylene glycol)-bis-(2-aminopropyl ether), J230). The crosslinked and uncrosslinked matrices had porosities of 90% and average pore sizes of 131–151 μm. Smooth muscle cells (SMC) were cultured in the crosslinked and uncrosslinked tubular scaffolds under pulsatile flow conditions (mean flow rate 9.6 ml/min, 120 beats/min, pressure 80–120 mmHg). All the constructs could withstand cyclic mechanical strain in the absence of any mechanical support without cracking or suffering permanent deformation. After 7 d, SMC were homogeneously distributed throughout the uncrosslinked and EDC/NHS crosslinked constructs, whereas hardly any cell was observed on the luminal side of J230/EDC/NHS crosslinked matrices. Considering the better mechanical performance of EDC/NHS crosslinked matrices compared to non-crosslinked constructs after 7 d of culture, SMC were dynamically cultured in the former scaffolds for 14 d. During this period, the high strain stiffness of the constructs increased more than two-fold to 38 ± 2 kPa, whereas the low strain stiffness doubled to 8 ± 2 kPa. The yield stress and yield strain were 30 ± 10 kPa and 120 ± 20%, respectively. SMC were homogeneously distributed throughout the EDC/NHS crosslinked collagen/elastin constructs and collagen fibres tended to orient in the circumferential direction.

Keywords: Collagen; Crosslinking; Elastin; Smooth muscle cells; Vascular grafts

1. Introduction

The development of a functional small-diameter vascular graft has long been a "holy grail". Although some successful solutions are available for large-diameter vessels (inner diameter > 6 mm) [1–3], the occurrence of thrombosis is still the main problem encountered in small-diameter blood vessel reconstructions. In order to develop a suitable small-diameter vascular prosthesis, research has been directed to tissue-engineered constructs. Up to now, either synthetic or natural occurring materials have been used as scaffolds in attempts to engineer a vascular graft. Biodegradable synthetic materials have been used, because they are relatively easy to process and often match the required mechanical properties [4,5]. Many polylactide- or polyglycolide-based scaffolds have thus been produced [4,6–9], but achieving the necessary compliance, cell adhesion, proliferation and matrix synthesis is still a challenge [10].

The use of polymers present in the wall of natural blood vessels may obviate some of these problems, especially in terms of cell adhesion. However, tissue-engineered collagen-based vascular grafts matching the desired mechanical properties, especially in terms of compliance and burst strength, have not been developed yet [11–13]. In non-compliant grafts, the transmission of pulsatile wave energy to the downstream vasculature is hampered.

Our aim is to develop a functional tissue-engineered small-diameter blood vessel based on polymers present in the vascular wall, extracellular matrix components and human cells. In this study, insoluble collagen type I from

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bovine Achilles tendon and insoluble elastin from horse neck ligament were used to prepare tubular scaffolds. In nature, collagen is one of the main components of blood vessels. Collagen fibres are essential to maintain the structural integrity of arteries [14]. Incorporation of elastin in collagenous vessels, can result in enhanced elasticity and compliance [15]. At physiological pressures, elastin prevents vascular creep [16]. In previous work, we have shown that after 20 cycles up to 10% of strain, non-porous scaffolds containing both collagen and elastin have a degree of strain recovery of $70 \pm 5\%$, much higher than that recorded for collagen ($42 \pm 6\%$) under the same conditions [17]. Moreover, elastin is involved in the control of smooth muscle cell (SMC) function [18]. The ability of SMC to contract and relax is essential to ensure maintenance of the vascular tone [19].

In this study, porous tubular scaffolds of collagen and elastin were prepared by freeze drying and successively stabilized by crosslinking with a water-soluble carbodiimide, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS), in the presence or absence of a Jeffamine spacer (poly (propylene glycol)-bis-(2-aminopropyl ether), J230). After morphological and mechanical characterization, scaffolds were cultured with SMC for different time periods in a pulsatile flow bioreactor. Scaffolds cultured under static conditions were used as control. The effects of dynamic vs. static culture conditions on the morphology and mechanical properties of the resulting constructs were monitored.

2. Materials and methods

All materials and chemicals were purchased from Sigma & Aldrich (Zwijndrecht, The Netherlands) unless otherwise stated.

2.1. Preparation of tubular porous structures

Insoluble collagen type I derived from bovine Achilles tendon (University of Nijmegen, The Netherlands; purification procedure as in [20]) and insoluble elastin derived from equine ligamentum nuchae (University of Nijmegen, The Netherlands; purification procedure as in [21]) were swollen in 0.25 M acetic acid in order to obtain a suspension containing 1% w/v of each of the components. The resulting suspension was homogenized first with a Philips Blender for 4 min and then for 15 min at 4 °C with an Ultra-Turrax T25 (IKA Labortechnik, Staufen, Germany). The suspension was degassed at 0.1 mbar (Edwards, oil vacuum pump), poured in glass tubular moulds having an outer diameter of 6 ± 1 mm and an inner diameter of 3 mm and then frozen at −18 °C. The cooling rate was not controlled. After freezing, samples were freeze-dried for 24 h.

2.2. Crosslinking

Freeze-dried collagen/elastin tubes were crosslinked with a water-soluble carbodiimide in the presence or absence of a Jeffamine spacer (J230). Ethanol/water (40% v/v) was used as solvent in all the reactions.

Carboxylic acid groups of collagen and elastin were activated by *N*-(3-dimethylaminopropyl)-*N*′-EDC in the presence of NHS. This combination limits the occurrence of secondary reactions and induces crosslinking with free primary amine groups [22]. Tubular freeze-dried scaffolds (OD = 6 ± 1 mm, ID = 3 mm, length = 4 cm) were incubated (215 ml g^{-1} of sample) for 30 min at room temperature in a 2-morpholinoethane sulfonic acid (MES) buffer (0.05 M, pH 5.5) using ethanol/water (40% v/v) as solvent. Crosslinking was carried out in the same MES buffer containing 2.3 g EDC and 0.56 g NHS per gram of collagen/elastin (molar ratio of EDC/NHS = 2.5). The reaction was performed for 2 h at room temperature. After removal of the crosslinking solution, samples were incubated for 2 h in 0.1 M sodium phosphate (pH = 7.4). A further washing step was performed with ethanol/water (40% v/v). The total incubation time was 2 h, during which ethanol/water was refreshed every 30 min. Finally the samples were rinsed for 10 min with demineralized MilliQ water. The crosslinked matrices were frozen at −18 °C, and subsequently freeze-dried for 24 h.

Crosslinking reactions in the presence of poly(propylene glycol)-bis-(2-aminopropyl ether) (J230) were performed by first incubating the freeze-dried scaffolds for 30 min in a MES buffer (0.05 M, pH 5.5 in ethanol/water (40% v/v)) containing 0.062 M J230 at room temperature. Subsequently, 5.75 g EDC and 1.38 g NHS were added per gram of collagen/elastin and crosslinking was performed overnight at room temperature (molar ratio of J230:EDC:NHS = 2.1:1:0.4). The samples were rinsed with sodium phosphate (0.1 M, pH 7.4) and ethanol/water (40% v/v) and then freeze-dried as described above.

2.3. Smooth muscle cell culture

SMC were isolated from human umbilical veins by a collagenase digestion method and subsequently characterized as previously described [23]. Sub-confluent SMC cultures (passage 5–9) were harvested by trypsinization (2 min, 0.125% trypsin/0.05% EDTA) after which cells were resuspended in culture medium (Dulbecco's modified Eagle's medium containing 10% v/v human serum, 10% v/v foetal bovine serum, 50 U/ml penicillin and 50 μg/ml streptomycin). Cells were counted with a Bürker hemacytometer and seeded by means of a filtration seeding procedure in crosslinked and uncrosslinked collagen/elastin scaffolds (length 4 cm, ID 3 mm, OD ca. 6 mm) as described in [24]. Briefly, a tubular scaffold was cannulated at both ends with stainless-steel tubes and mounted in a glass flow chamber. After disinfection of the scaffold with 70% ethanol and overnight incubation in culture medium, 10^7 SMC in 20 ml culture medium were infused into the lumen of the scaffold from both ends simultaneously using two syringes. In this way, the cells were filtered through the porous wall of the scaffold. The compartment of the glass flow chamber outside the scaffold was completely filled with culture medium, after which the chamber was placed in an incubator (37 °C and 5% CO_2) and rotated 90° around the longitudinal axis every 30–60 min during the first 2.5 h to promote homogeneous cell adhesion in the scaffold.

After an additional incubation period of 24 h without rotation, four chambers each containing a seeded scaffold were mounted in a bioreactor operating at 37 °C and 5% CO_2 [24]. The parallel chambers were connected through the stainless-steel connectors and silicon rubber tubing (Watson Marlow, Brussels, Belgium, 3.2 mm ID × 6.4 mm OD) to a fluid reservoir containing culture medium. A pulsatile flow was generated by a peristaltic pump (Watson Marlow Sci-Q-323) placed proximal to the vessels. A pressure of 100 mmHg (compressed air) was applied to the culture medium reservoir and regulated by an electronically controlled Venturi valve (T5200-50, Fairchild Company, Winston-Salem, NC, USA). Pulsatile flow was gradually increased from 30 to 120 beats/min after 3 d of culture, yielding a volumetric flow rate of 9.6 ml/min in each vessel and pressures in the range of 80–120 mmHg at the proximal connections of the flow chambers. The cells were dynamically cultured for 1, 3, 7 or 14 d in culture medium, which was refreshed every 2 d. SMC-seeded scaffolds cultured under static conditions for the same time periods were used as controls.

2.4. Morphology

The morphology of the porous tubular scaffolds before and after SMC culture was studied by SEM (Leo Gemini 1550 FEG-SEM apparatus). Freeze-dried non-cultured scaffolds were analysed without further