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Preparation and evaluation of molecularly-defined collagen–elastin–glycosaminoglycan scaffolds for tissue engineering

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Abstract

Extracellular matrix components are valuable building blocks for the preparation of biomaterials involved in tissue engineering, especially if their biological, chemical and physical characteristics can be controlled. In this study, isolated type I collagen fibrils, elastin fibres and chondroitin sulphate (CS) were used for the preparation of molecularly-defined collagen–elastin–glycosaminoglycan scaffolds. A total of 12 different scaffolds were prepared with four different ratios of collagen and elastin (1:9, 1:1, 9:1 and 1:0), with and without chemical crosslinking, and with and without CS. Collagen was essential to fabricate coherent, porous scaffolds. Electron microscopy showed that collagen and elastin physically interacted with each other and that elastin fibres were enveloped by collagen. By carbodiimide-crosslinking, amine groups were coupled to carboxylic groups and CS could be incorporated. More CS could be bound to collagen scaffolds (10%) than to collagen–elastin scaffolds (2.4–8.5% depending on the ratio). The attachment of CS increased the water-binding capacity to up to 65%. Scaffolds with a higher collagen content had a higher tensile strength whereas addition of elastin increased elasticity. Scaffolds were cytocompatible as was established using human myoblast and fibroblast culture systems. It is concluded that molecularly-defined composite scaffolds can be composed from individual, purified, extracellular matrix components. Data are important in the design and application of tailor-made biomaterials for tissue engineering.

Keywords: Collagen; Elastin; Glycosaminoglycans; Biomaterial; Tissue engineering

1. Introduction

Tissue engineering is a field of research, which aims at regenerating tissues and organs. Tissues are basically made up of cells and extracellular matrix. Cells taken out of their context will lose their shape and function. A major goal of tissue engineering is the preparation of a suitable scaffold for cells to proliferate, migrate and differentiate. The scaffold should assist cells to form the desired tissue. To reproducibly prepare bioscaffolds and to study the biological effect of a single component, molecularly-defined scaffolds have to be prepared. In tissues and organs, major extracellular matrix components are collagens, elastin, and glycosaminoglycans (GAGs). Each tissue/organ has its own unique set and content of these biomolecules. Type I collagen is an extracellular matrix protein that is widely used as scaffold material [1]. It provides adhesive properties and tensile strength. Elastin provides elasticity to tissues/organs and is crucial for e.g. blood vessels in order to cope with variations in blood pressure [2]. GAGs are negatively charged polysaccharides with biocharacteristics like hydration of the extracellular matrix [3,4] and binding of effector molecules (e.g. growth factors and cytokines) [5–8]. In contrast to collagen, elastin is rarely used in bioscaffolds and when applied only poorly defined elastin preparations have been used. Aprahamian et al. [9] made a matrix of elastin with fibrin in the presence of type I collagen.

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Rabaud et al. [10] prepared a gel from a collagenous preparation and insoluble elastin. Others used elastin present in isolated blood vessels [11,12] or heart valves [13]. Also, elastin hydrolysates have been added to biomaterials. For example, Singla and Lee [14] prepared glutaraldehyde-crosslinked matrices from soluble α-elastin and collagen to study the calcification rate in a rat model. To our knowledge, highly purified insoluble elastin fibres have never been used to prepare defined scaffolds for tissue engineering.

Here, we report the preparation of defined collagen–elastin–GAG scaffolds. Twelve different scaffolds were prepared using four different ratios of collagen and elastin (1:0; 9:1; 1:1; 1:9), with and without chemical crosslinking, and with and without CS. All scaffolds were characterised biochemically, biomechanically and immunohistologically, and their effect on cell proliferation and differentiation was studied in vitro.

2. Materials and methods

2.1. Materials

Mouse anti-chicken chondroitin sulphate (CS) monoclonal antibody (clone CS-56, C-8035) and FITC-labelled goat anti-mouse IgM (F-9259) were from Sigma (St. Louis, MO, USA). CS A (C-9819) was also from Sigma, and was basically a mixture of 60% chondroitin 4-sulphate and 40% chondroitin 6-sulphate. No other GAGs could be detected using agarose gel electrophoresis with silver staining; also no protein contaminations could be detected as analysed by SDS-PAGE applying 1 mg of material and Coomassie Brilliant Blue staining. Rabbit anti-bovine type I collagen antibody was from Chemicon (Temecula, CA, USA). Alexa 594-labeled goat anti-rabbit IgG was from Molecular Probes (Eugene, OR, USA). Chondroitinase ABC was from Seikagaku (Tokyo, Japan).

2.2. Isolation of collagen and elastin

Insoluble type I collagen was isolated from bovine achilles tendon (deep flexor) using neutral salt and dilute acid extractions as described [15]. Purity of collagen was analysed using SDS-PAGE and amino acid analysis. The collagen preparation was essentially free of other proteins.

Elastin fibres were isolated from equine ligamentum nuchae essentially as described, but with omission of the collagenase digestion [16]. Purity of elastin was assessed using SDS-PAGE, amino acid analysis, and transmission electron microscopy. No impurities could be detected by any of these methods. Microfibrillar components (present in unpurified elastic fibres) were absent.

2.3. Preparation of collagen–elastin–GAG scaffolds

In total, 12 different scaffolds were prepared with different ratios of collagen and elastin, with and without 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide (EDC) crosslinking and with and without CS.

Non-EDC-crosslinked type I collagen scaffolds were prepared as described [15]. Briefly, a 2% (w/v) collagen suspension was prepared in 0.5 M acetic acid (pH 2.5) and shaken at 4°C for 16 h. This suspension was then diluted to 1% (w/v) with ice-cold distilled water, homogenised, and deaerated under vacuum to remove entrapped air bubbles. The collagen suspension was poured into a mould, frozen at −80°C and lyophilised, resulting in porous collagen scaffolds. Non-EDC-crosslinked composite scaffolds of type I collagen and elastin were prepared similarly using suspensions of type I collagen and elastin in a 9:1, 1:1, or 1:9 ratio.

To increase the strength of the scaffolds, chemical crosslinking of collagen and collagen–elastin scaffolds was performed using EDC and *N*-hydroxysuccinimide (NHS) [17]. Scaffolds were incubated for 0.5 h in 20 ml 50 mM 2-morpholinoethane sulphonic acid (MES) (pH 5.5) in the presence of 40% (v/v) ethanol. Subsequently, the scaffolds were crosslinked by immersion in 20 ml 50 mM MES (pH 5.5) containing 33 mM EDC and 6 mM NHS. After reaction for 4 h at 22°C, the scaffolds were washed twice in 0.1 M Na_2HPO_4 (pH 9.1) for 1 h. Finally, the scaffolds were washed with 1 M NaCl and 2 M NaCl for 2 h and 1 day (with six changes of washing solution), respectively, followed by washings with distilled water.

EDC-crosslinking of the scaffolds was also performed in the presence of 2.75% (w/v) CS which results in the covalent attachment of CS to the scaffold.

2.4. Characterisation of the scaffolds

2.4.1. Amine group content

The amine group content of scaffolds was determined spectrophotometrically after reaction with 2,4,6-trinitrobenzene sulphonic acid [18]. The presence of CS did not interfere with this assay.

2.4.2. CS content

The CS content of scaffolds was determined by hexosamine analysis using *p*-dimethylamino-benzaldehyde [15,19]. Scaffolds were hydrolysed with 6 M HCl for 6 h at 105°C. Samples were dried under vacuum and dissolved in distilled water for hexosamine analysis. CS was used as a standard.

2.4.3. Water-binding capacity

Scaffold samples of about 5 mg dry weight were incubated in 3 ml PBS (pH 7.2) at 20°C. After 1 h, the wet weight was determined and the water-binding

capacity calculated using the equation: water-binding capacity = (wet weight − dry weight)/(dry weight).

2.4.4. Scanning electron microscopy (SEM)

Scaffolds were critical point dried using liquid CO_2 with a Polaron E3000 critical point drying apparatus, mounted on stubs and sputtered with an ultrathin layer of gold in a Polaron E5100 SEM coating system. Specimens were studied with a JEOL JSM-6310 SEM apparatus operating at 15 kV.

2.4.5. Transmission electron microscopy (TEM)

Scaffolds were fixed in 2% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 12 h at 4°C, and postfixed with 1% (w/v) osmium tetroxide in 0.1 M phosphate buffer (pH 7.4). After a rinsing period of 3 h, the samples were dehydrated in an ascending series of ethanols and embedded in Epon 812. Ultrathin sections (60 nm) were poststained with lead citrate and uranyl acetate, picked up on formvar-coated grids and examined in a JEOL 1010 electron microscope.

2.4.6. Light microscopy

For conventional histochemical analysis, scaffolds were fixed in 4% (v/v) formaldehyde in phosphate buffer (pH 7.2) and embedded in paraffin. Sections of 5 μm were mounted onto organosilane-coated slides, dewaxed in xylol and hydrated through a descending series of ethanols. Sections were stained for elastin and collagen according to Verhoeff-Van Gieson [20].

2.4.7. Immunofluorescence microscopy

Immunofluorescence microscopy was used to study the distribution of CS in the scaffolds. Scaffolds were hydrated in 0.1 M phosphate buffer (pH 7.2) and frozen in liquid nitrogen. Cryosections of 5 μm were mounted onto organosilane-coated glass slides. After blocking with 1% (w/v) bovine serum albumin (BSA) in PBS (pH 7.2), sections were incubated with mouse anti-chicken CS monoclonal antibody (1:50) and rabbit anti-bovine type I collagen antibody (1:500) for 90 min, washed with PBS, followed by a 1 h incubation with FITC-labelled goat anti-mouse IgM (1:100) and Alexa 594-labeled goat anti-rabbit IgG (1:100). Antibodies were diluted in PBS containing 1% (w/v) BSA. The sections were washed and mounted in mowiol. Elastin was detected on the basis of its autofluorescence using UV optics.

To study if scaffold-bound CS was available for biological interactions, its degradation by chondroitinase ABC was studied. Cryosections of 5 μm were incubated for 30 min in 25 mM Tris-HCl (pH 8.0) + 1 mM magnesium acetate, followed by an incubation with 1 U/ml chondroitinase ABC in the same buffer for 16 h at 37°C. Subsequently, sections were immunostained for CS as described above.

2.4.8. Mechanical properties

Stress–strain analysis of scaffolds was performed by uniaxial measurements using a Zwick Z020 mechanical tester. Scaffolds were hydrated for 24 h in PBS (pH 7.2) and drawn at a speed of 5 mm/min. The elastic modulus was calculated from the inclination of the stress–strain graph; the tensile strength was monitored at rupture of the scaffolds.

2.5. Effects of scaffolds on cells in vitro

The in vitro cytotoxicity of scaffolds was evaluated on the basis of cell morphology, viability, and proliferation. Human myoblasts [21] and human HFL1 lung fibroblasts (ATCC cell line CCL153) were used. All scaffolds were washed with 70% (v/v) ethanol and sterile PBS (pH 7.2).

2.5.1. Proliferation of cells indirectly in contact with scaffolds

To analyse possible release of toxic products by the scaffolds, 4.0×10^4 fibroblasts per well were grown in proliferation medium (10% (v/v) foetal bovine serum, 4 mM glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml) in Dulbecco's modified Eagle medium (DMEM)) on culture plastic (Falcon 3504, Becton Dickinson Labware, NJ, USA). After 24 h, scaffolds (Ø 8 mm) were added onto the cell inserts (Falcon 3492). Cells were allowed to grow for another 48 h. Trypsinised cells were counted using trypan blue in a Bürker counting-chamber. Trypan-blue positive cells indicate non-viable cells. Therefore, only Trypan-blue negative cells were counted. The cell proliferation inhibition index ($CPII_{indirect}$) was calculated according to: $CPII_{indirect} = 100\% - [(\text{number of cells in cultures with scaffold/number of cells in culture without scaffold}) \times 100\%]$ [15]. Cells with no added scaffold will have a $CPII_{indirect}$ of 0%. Means of these data were compared.

2.5.2. Proliferation of cells directly in contact with scaffolds

Fibroblast proliferation was assessed by measuring the mitochondrial dehydrogenase activity using the tetrazolium salt 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulphonate (WST-1) (Boehringer Mannheim, Germany), according to the manufacturer's protocol. Ø 6 mm scaffolds were placed in 96-wells plates. Cells were seeded on the scaffolds (10^4 cells in 200 μl per well) and cultured for 3 days at 37°C/5% CO_2 in proliferation medium (10% (v/v) foetal bovine serum, 4 mM glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml) in DMEM). After the addition of 20 μl WST-1 per well and subsequent incubation for 3 h at 37°C/5% CO_2, the absorption was measured at 490 nm. The mitochondrial dehydrogenase activity of

day 3 cultures was divided by the activity of day 1 cultures. The enzyme activity ratio was expressed as a percentage of the ratio of cells grown on culture plastic. The cell proliferation inhibition index ($CPII_{direct}$) was calculated according to: $CPII_{direct} = 100\% - [(\text{enzyme activity of cells in cultures with scaffold/enzyme activity of cells in culture without scaffold}) \times 100\%]$. Cells grown on culture plastic (no scaffold added) will have a $CPII_{direct}$ of 0%. Means of these data were compared.

2.5.3. Cell morphology

Cell morphology was assessed using SEM. Ø 16 mm scaffolds were placed in 24-well culture dishes and cells were seeded on top. Human fibroblasts were seeded on the airside of the scaffolds with a density of about 10^5 cells per cm^2 in proliferation medium (10% (v/v) foetal bovine serum, 4 mM glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml) in DMEM). Cells were cultured at 37°C and 5% CO_2 for 3 days after which the proliferation medium was changed for differentiation medium which contained 2% (v/v) horse serum, 4 mM glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml) in DMEM. Human myoblasts were cultured on the scaffolds using similar conditions but different media. The proliferation medium contained 4% (v/v) Ultroser G (Sopar-Biochem, Brussels, Belgium), 10% (v/v) rat brain extract, 4 mM glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml) in DMEM. The differentiation medium contained 0.4% (v/v) Ultroser G, 10% (v/v) rat brain extract, 4 mM glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml) in DMEM. After culturing for 14 days, scaffolds were fixed by immersion in 2% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 1 h at 4°C, and prepared for SEM.

3. Results

3.1. Biochemical and biophysical properties of the scaffolds

Table 1 gives an overview of the composition and properties of the 12 scaffolds used in this study.

The amine group content of the pure collagen scaffold was 281 nmol/mg scaffold. The composite collagen–elastin scaffolds contained less NH_2 groups, since elastin contained less amine groups than type I collagen (elastin had 27 ± 2 nmol NH_2 groups/mg elastin). We were not able to construct a stable scaffold made of only elastin, even not after crosslinking.

Collagen–elastin crosslinking using EDC/NHS results in the formation of crosslinks between carboxylic and amine groups, and thus in a reduction of the number of amine groups. Crosslinking efficiency was highest for collagen scaffolds (about 100 nmol amine groups/mg scaffold were utilised upon crosslinking), and smallest for collagen–elastin 1:9 scaffolds (about 30 nmol amine groups/mg scaffold were utilised in the crosslinking process). Under the same conditions, collagen could be crosslinked to a higher extent than collagen–elastin scaffolds, because more amine groups are present. Collagen–elastin crosslinking using EDC/NHS in the presence of CS results, besides the formation of crosslinks between these proteins, in the covalent attachment of CS through its carboxylic groups. Coupling of CS to

Table 1
Biochemical and biomechanical characteristics of collagen–elastin–chondroitin sulphate scaffolds

Scaffold	Crosslinked with EDC/NHS	Amine group content (nmol/mg)	CS content (ng/mg)	Water-binding capacity (# times dry weight)	Tensile strength (kPa)	E-modulus (MPa)
COL	–	281±7	0	20±1	103±15	0.39±0.07
COL	+	185±3	0	20±1	677±191	1.03±0.08
COL-CS[a]	+	186±8	100±4	33±3	520±105	0.97±0.07
COL-EL 9:1	–	256±8	0	19±2	67±6	0.42±0.10
COL-EL 9:1	+	159±4	0	19±1	420±35	0.78±0.07
COL-EL-CS 9:1[b]	+	164±1	85±5	29±4	394±42	0.76±0.13
COL-EL 1:1	–	147±12	0	16±1	63±23	0.24±0.06
COL-EL 1:1	+	87±5	0	16±3	142±8	0.42±0.04
COL-EL-CS 1:1[c]	+	83±2	58±5	21±3	128±10	0.54±0.11
COL-EL 1:9	–	67±7	0	11±1	ND	ND
COL-EL 1:9	+	57±5	0	11±1	ND	ND
COL-EL-CS 1:9[d]	+	35±4	24±2	12±1	ND	ND

COL = collagen; EL = elastin; CS = chondroitin sulphate; ND = not done; scaffolds were too weak to measure tensile strength and E-modulus. Results are mean ± SD of three independent experiments.

[a] 1 g of COL-CS scaffold contains 900 mg collagen and 100 mg CS.

[b] 1 g of COL-EL-CS 9:1 scaffold contains 823 mg collagen, 92 mg elastin, and 85 mg CS.

[c] 1 g of COL-EL-CS 1:1 scaffold contains 471 mg collagen, 471 mg elastin, and 58 mg CS.

[d] 1 g of COL-EL-CS 1:9 scaffold contains 98 mg collagen, 878 mg elastin, and 24 mg CS.

scaffolds generally had no effect on crosslinking efficiency. Since collagen contains more amine groups than elastin, the CS content incorporated into the scaffold is dependent on the collagen–elastin ratio. The content of CS was 10.0% (w/w) for the collagen scaffold, and decreased concomitantly with the collagen content of the collagen–elastin scaffolds.

The coupling of CS to the scaffolds increased the water-binding capacity. This effect was most prominent for the collagen scaffold. In the collagen–elastin scaffolds, less CS was present, and thus the increase in water-binding capacity was less. EDC-crosslinking without CS did not influence the water-binding capacity of the scaffolds.

Regarding the mechanical characteristics of the non-crosslinked scaffolds, the tensile strength of the pure collagen scaffold was the highest (about 100 kPa), and introduction of elastin to the scaffold lowered the tensile strength as well as the E-modulus. Crosslinking of the scaffolds increased the tensile strength and increased the E-modulus. Attachment of CS to a scaffold did not result in a mean change of tensile strength. When manually pulling at a hydrated non-crosslinked scaffold that was glued to a Petri dish, it became clear that scaffolds containing a larger content of elastin could be extended further, and recoiled faster upon release of the force. The collagen and collagen–elastin 9:1 scaffolds could be extended to 125%, the collagen–elastin 1:1 scaffold to 140% and the collagen–elastin 1:9 scaffold to almost 150% (see DivX 5.0 compressed movie at http://www.ncmls.kun.nl/biochemistry/matrix/movies/scaffolds.avi).

3.2. Morphological properties of the scaffolds and distribution of single components

All scaffolds showed a porous structure with pores ranging from 20 to 100 μm. Elastin fibres were much thicker than collagen fibrils and therefore scaffolds containing more elastin had larger pores, since the dry weight/cm^3 was constant for all scaffolds. EDC-crosslinking and coupling of CS to the scaffolds did not alter the porosity. Collagen and elastin were randomly distributed in the scaffolds, although elastin tended to be present in small clusters consisting of up to 20 elastin fibres, especially when a scaffold contained a high amount of elastin (Fig. 1). SEM (Fig. 2) clearly showed thick elastin fibres and thin collagen fibrils. Collagen was also present as lattice-like lamellae, mostly orientated parallel or perpendicular to the surface, but the more elastin was present, the less lamellae were found. SEM indicated that elastin fibres and collagen fibrils physically interact with one another, which was confirmed by TEM (Fig. 3). Collagen connected separate

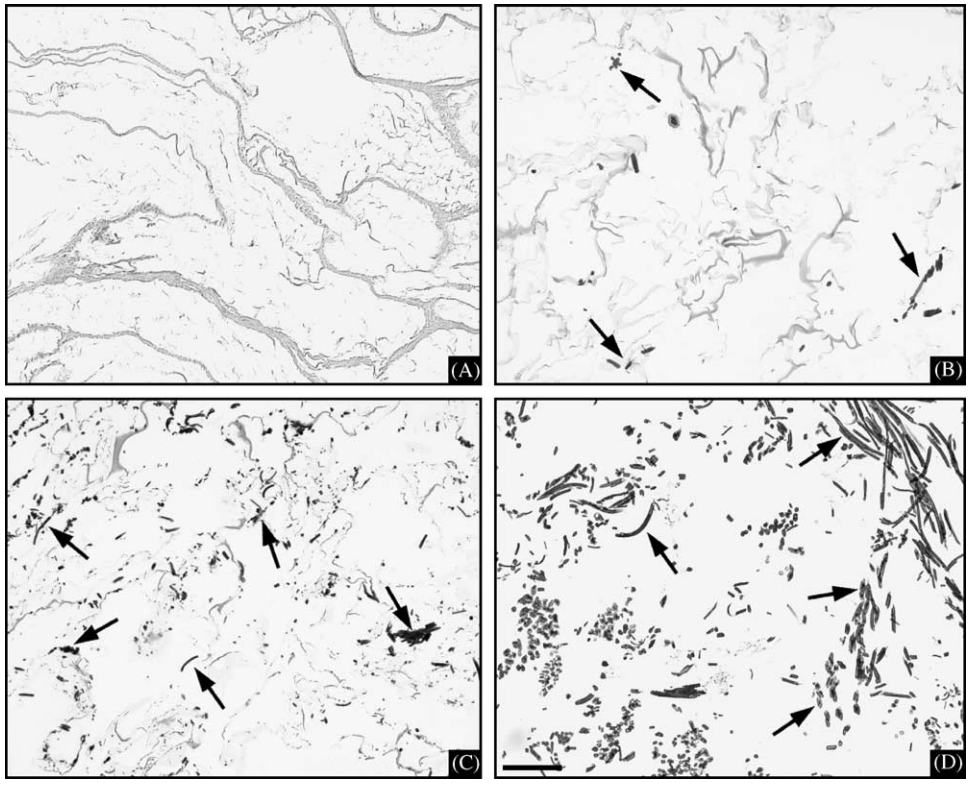

Fig. 1. Verhoeff-Van Gieson staining of a collagen scaffold (A), a 9:1 collagen–elastin scaffold (B), a 1:1 collagen–elastin scaffold (C), and a 1:9 collagen–elastin scaffold (D). Collagen has a grey colour, elastin is black. Arrows indicate elastin fibres. Bar is 100 μm.

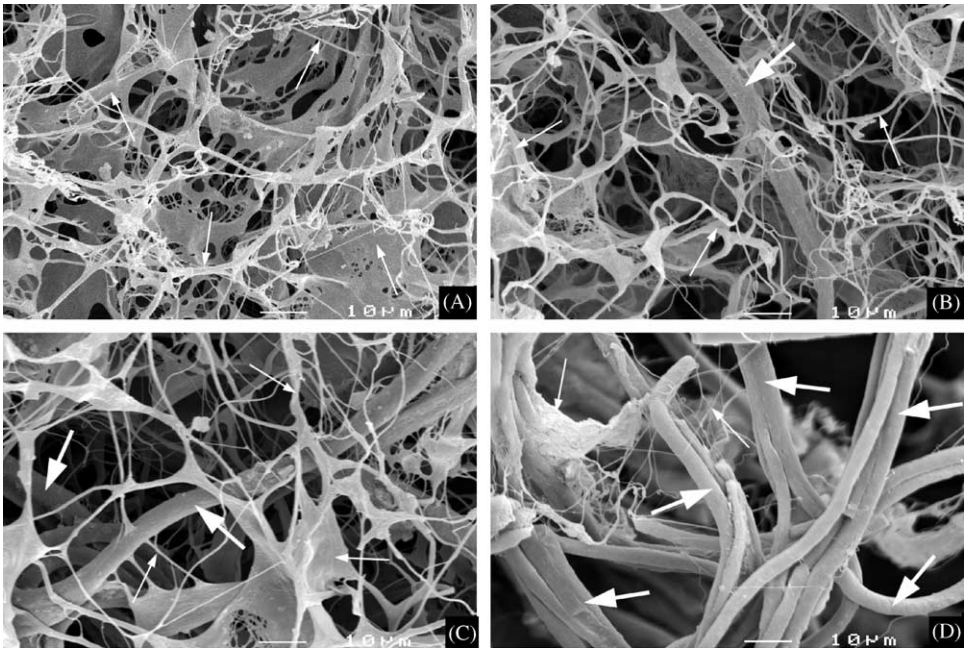

Fig. 2. Scanning electron micrographs of the air side of a collagen scaffold (A), a 9:1 collagen–elastin scaffold (B), a 1:1 collagen–elastin scaffold (C), and a 1:9 collagen–elastin scaffold (D). Collagen is present as fibrils and sheets. Note the interactions between (thick) elastin fibres (large arrows) and collagen fibrils (small arrows). Bar is 10 μm.

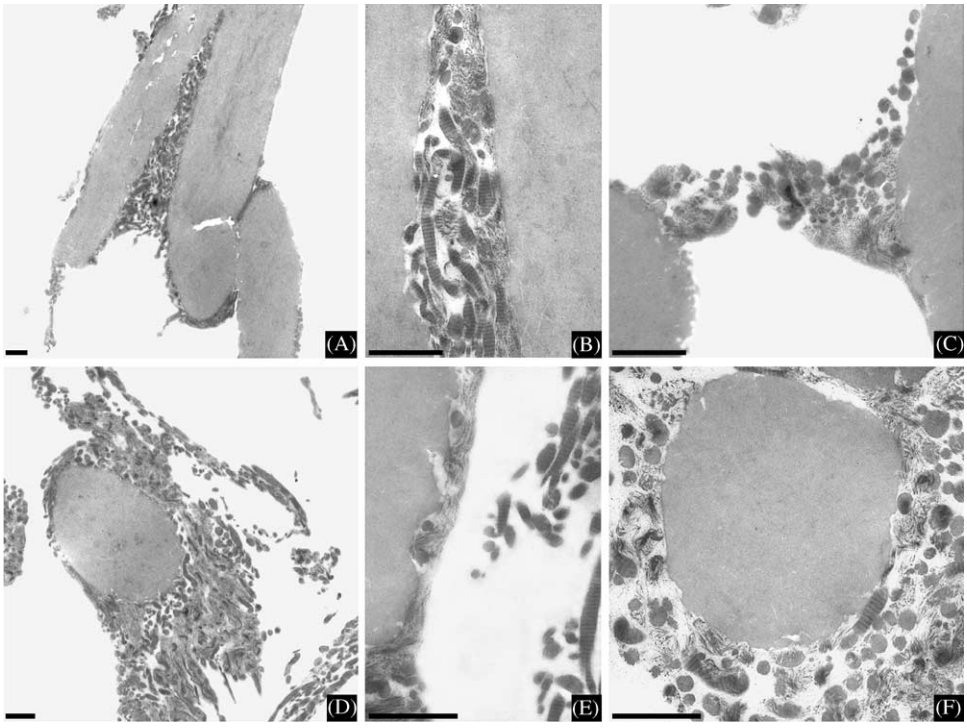

Fig. 3. Transmission electron micrograph of a collagen–elastin 1:1 scaffold at several magnifications. A, B and C show connections between separate elastin fibres by collagen fibrils. D, E and F demonstrate the ensheathment of single elastin fibres by a layer of collagen fibrils that are often frayed at sites of contact with elastin. Large elastin fibres are light grey, while thin collagen fibrils are dark grey. Bar is 1 μm.

elastin fibres, and elastin fibres were ensheathed by a layer of collagen fibrils. In many occasions, the native collagen fibril was frayed into protofibrils at places where it contacted elastin.

Immunofluorescence indicated that CS colocalised primarily with collagen and could be removed from the scaffolds by specific digestion using the enzyme chondroitinase ABC (Fig. 4). This suggests that the CS

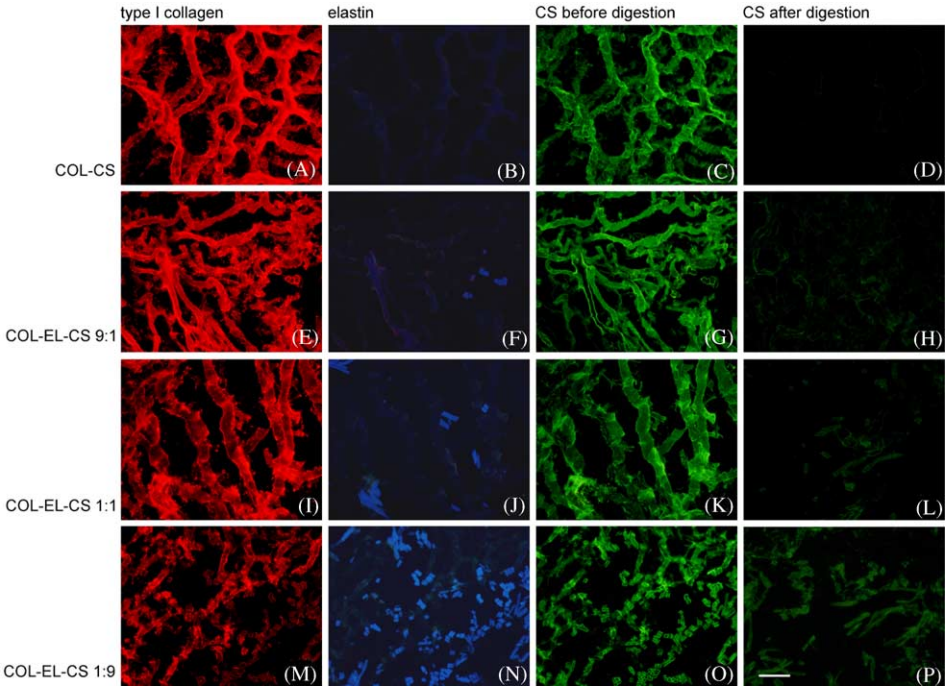

Fig. 4. Localisation of scaffold components by immunofluorescence staining for type I collagen (red), CS (green; before and after chondroitinase ABC digestion), and by UV optics for elastin (blue) of a collagen–CS scaffold (A–D), a 9:1 collagen–elastin–CS scaffold (E–H), a 1:1 collagen–elastin–CS scaffold (I–L), and a 1:9 collagen–elastin–CS scaffold (M–P). Bar is 50 μm.

bound to the scaffolds preserves its natural conformation and remains biologically available (i.e. capable to display its biocharacteristics).

3.3. Cytocompatibility of scaffolds in vitro

3.3.1. Proliferation of cells indirectly or directly in contact with scaffolds

To analyse possible release of toxic products by the scaffolds, fibroblasts were grown indirectly in contact with scaffolds. The values of the indirect cell proliferation inhibition index ($CPII_{indirect}$) did not show negative effects on the proliferation of fibroblasts for all scaffolds (Table 2). The direct cell proliferation inhibition index ($CPII_{direct}$) also did not show effects on the proliferation of fibroblasts for collagen, collagen–elastin 9:1 and 1:1 scaffolds, but a small decrease was found for fibroblast proliferation on collagen–elastin 1:9 scaffolds (Table 2).

Table 2
Results of in vitro cell proliferation of human fibroblasts grown indirectly and directly in contact with scaffolds

Scaffold	Crosslinked with EDC/NHS	$CPII_{indirect}$ [a] (%)	$CPII_{direct}$ [b] (%)
—		0	0
COL	−	−2±10	5±13
COL	+	−1±14	3±6
COL-CS	+	7±7	2±10
COL-EL 9:1	−	2±15	0±2
COL-EL 9:1	+	8±3	7±11
COL-EL-CS 9:1	+	10±14	2±1
COL-EL 1:1	−	15±5	7±5
COL-EL 1:1	+	14±7	7±13
COL-EL-CS 1:1	+	13±1	11±3
COL-EL 1:9	−	8±11	18±14
COL-EL 1:9	+	10±9	15±8
COL-EL-CS 1:9	+	14±2	26±2

Values are mean±SD of three independent experiments.

[a] $CPII_{indirect}$ = indirect cell proliferation inhibition index; $CPII_{indirect}$ of cells grown on culture plastic without scaffold was set to 0%.

[b] $CPII_{direct}$ = direct cell proliferation inhibition index; $CPII_{direct}$ was calculated from mitochondrial dehydrogenase activity using WST-1. $CPII_{direct}$ of cells grown on culture plastic without scaffold was set to 0%.

3.4. Cell morphology

The cell morphology of fibroblasts and myoblasts was studied on the scaffolds after 14 days of culturing. Fibroblasts formed normal spindle-shaped cells on all scaffolds (data not shown). Myoblasts adhered, aligned, and fused to form multinucleated myotubes of several hundreds of μm in length on all scaffolds, except on the collagen–elastin 1:9 scaffolds (Fig. 5). On the latter, cells did not have an elongated form, but a rounder form with small sprout-like structures. This could be caused by a diminished proliferation of myoblasts on the collagen–elastin 1:9 scaffolds, because myoblasts only start to differentiate when cells contact one another.

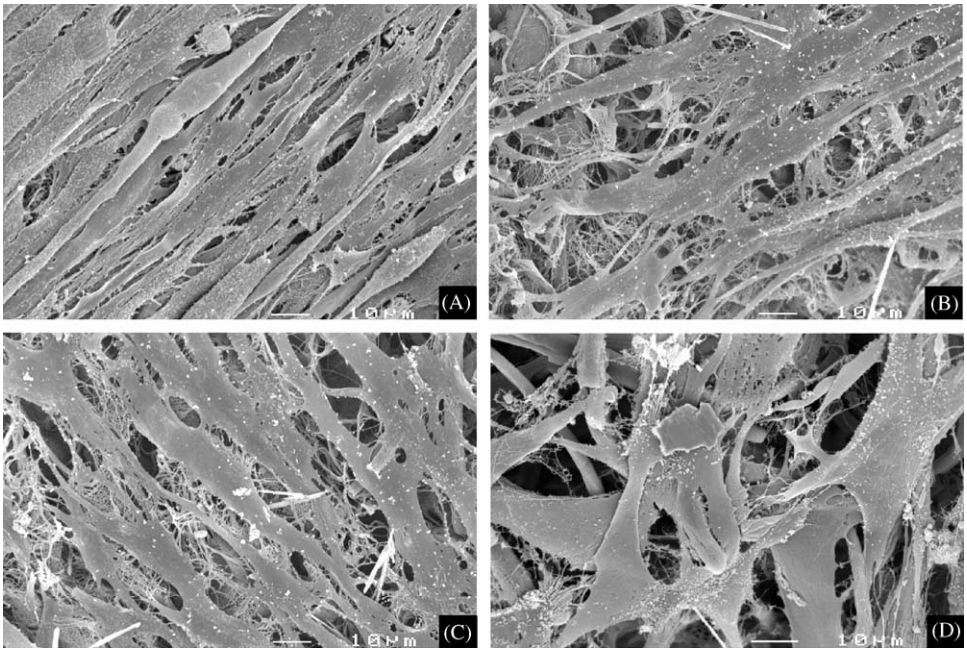

Fig. 5. SEM images of human muscle cells grown on a collagen scaffold (A), a 9:1 collagen–elastin scaffold (B), a 1:1 collagen–elastin scaffold (C), and a 1:9 collagen–elastin scaffold (D). Myoblasts were cultured for 14 days. Myotubes were formed on all scaffolds, except collagen:elastin 1:9 scaffolds. Bar is 10 μm.

Another possibility is that myoblasts do not fully differentiate on an elastin surface. Cells generally followed the direction of the collagen fibrils or elastin fibres of the scaffolds beneath them. No differences of morphology were found between cells cultured on non-EDC-crosslinked and crosslinked scaffolds, or on scaffolds with and without CS.

4. Discussion

For a rational design of scaffolds for tissue engineering, it is essential to study the effect of individual components. To do so, scaffolds have to be designed starting with highly purified molecules and the contribution of each component in the scaffold has to be controlled. This is also crucial when scaffolds are to be constructed which mimic nature's extracellular matrices, and which vary considerably from tissue to tissue. For instance, when making a scaffold for an artery construct, a much larger content of elastin is necessary than when preparing a scaffold for artificial skin. In skin, the ratio collagen:elastin is about 9:1, whereas in an artery this ratio is 1:1 averaging all artery layers, and 1:9 when considering the lamina elastica only [22]. In ligaments, the ratio collagen–elastin is also about 1:9 [22], and in lung about 1:1 [23]. Likewise, the amount (and type) of GAGs, another major scaffold component, varies from matrix to matrix. In cartilage for instance, CS is the main glycosaminoglycan making up 20% of the dry weight. In skin, dermatan sulphate is most abundant (about 1% of the dry weight), whereas in the vitrous body of the eye it is hyaluronate [24]. Given the large heterogeneity in the body's scaffolds, it was the purpose of this study to demonstrate that tailor-made, molecularly-defined scaffolds can be produced from the main components of the extracellular matrix.

We prepared 12 scaffolds with different ratios of highly purified collagen, elastin, and CS, and compared them to each other. With more collagen, the tensile strength of the scaffold was generally higher, whereas increase of elastin increased elasticity. Collagen–elastin 1:1 scaffolds could be extended to about 140% of the original length; collagen–elastin 1:9 scaffolds to almost 150%. Under normal physiological conditions, circumferential stress–strain extensions of blood vessels are about 20% of the original diameter [25]. This extension, caused by differences in blood pressure, can easily be reached with these scaffolds, which also provides the elasticity necessary for coping with changes in blood pressure. Elastin reduced, but CS increased the water-binding capacity. SEM and TEM data indicated that elastin fibres and collagen fibrils interacted with each other. Collagen formed a small layer of fibrils around the elastin fibres, thus incorporating elastin in the scaffolds. Collagen may act as a glue that holds the elastin fibres together. This may explain the inability to construct a stable scaffold of elastin only. Collagen–elastin 1:9 scaffolds are difficult to handle, indicating that a certain amount of collagen is necessary to prepare a coherent scaffold. The inability to prepare scaffolds composed of only elastin could be due to the low

number of amine groups in elastin. The introduction of amine groups (e.g. by a diamine compound) may be a solution for this. In case the amines are too distant from the carboxylic groups in order to be crosslinked, bridging may be accomplished by, e.g. aminocaproic acid. Next to the biochemical composition of the scaffolds, the mechanical properties of the scaffold can be controlled, e.g. by the composition (choice of ECM components and their ratio), the type and extent of chemical crosslinking, and the pore size of the scaffold. The pore sizes can be varied by changing the freezing rate in the manufacturing process. Fast freezing will result in small ice crystals and smaller pores after lyophilisation. The shape of the scaffold can easily be controlled by the mould that is chosen. The water-binding capacity of the scaffolds can be influenced by the attachment of GAGs, as shown with CS. GAGs can also be used as slow-release vehicles for e.g. growth factors [26]. Using specific growth factors, a scaffold may be able to selectively interact with surrounding cells to improve tissue regeneration in vivo.

In conclusion, a variety of biological scaffolds have been prepared with defined biochemical, biomechanical, and morphological characteristics. The methodology applied may be instrumental to produce organ-specific scaffolds for tissue engineering.

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