

Biochemical and Biophysical Aspects of Collagen Nanostructure in the Extracellular Matrix

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Summary

ECM is composed of different collagenous and non-collagenous proteins. Collagen nanofibers play a dominant role in maintaining the biological and structural integrity of various tissues and organs, including bone, skin, tendon, blood vessels, and cartilage. Artificial collagen nanofibers are increasingly significant in numerous tissue engineering applications and seem to be ideal scaffolds for cell growth and proliferation. The modern tissue engineering task is to develop three-dimensional scaffolds of appropriate biological and biomechanical properties, at the same time mimicking the natural extracellular matrix and promoting tissue regeneration. Furthermore, it should be biodegradable, bioresorbable and non-inflammatory, should provide sufficient nutrient supply and have appropriate viscoelasticity and strength. Attributed to collagen features mentioned above, collagen fibers represent an obvious appropriate material for tissue engineering scaffolds. The aim of this minireview is, besides encapsulation of the basic biochemical and biophysical properties of collagen, to summarize the most promising modern methods and technologies for production of collagen nanofibers and scaffolds for artificial tissue development.

Key words

Collagen • Extracellular matrix • Nanofibers • Biomechanical properties • Scaffold

The extracellular matrix (ECM) plays a substantial role in both prenatal and postnatal development of organs and in rebuilding of organs during many pathological situations (Pelouch *et al.* 1995,

Adamcova *et al.* 2003, Simko *et al.* 2005, Vanhoutte *et al.* 2006). Its biochemical and biophysical properties are responsible for integrity of individual cells, migration, adhesion, nutrition and differentiation of cells,

angiogenesis and formation of intracellular contacts. Moreover, ECM forms contact sites between (cardio)myocytes and fibroblasts both in skeletal and myocardial muscles and participates also in the migration of some specified cells as well as in proteolysis (via apoptosis). Therefore, ECM plays an active role in both physiological and pathophysiological remodelations (Herget *et al.* 1996, Kolar *et al.* 1998).

ECM composition and biochemistry

ECM is composed of collagenous (different collagen types) as well as non-collagenous proteins, such as elastin, fibronectin, laminin or entactin. Most of them are glycosylated with sugar content ranging between 5 and 10 %. There is also a significant content of proteoglycans (e.g., perlecan) in ECM (Pelouch *et al.* 1993, Pelouch *et al.* 1995). The synthesis of those proteins is predominantly under the effect of many growth factors, catecholamines and cytokines. Their degradation, on the other hand, is due to the effect of metalloproteinases (MMPs) – enzymes containing Zn^{2+} and they need Ca^{2+} ions for their activation). The family of MMPs could be divided into a few groups (e.g. collagenases, gelatinases) – they either cleave the molecule of different collagen types or other ECM proteins into very precisely defined fragments or MMPs splitting off only previously formed fragments; for details see Kukacka *et al.* (2005), Janssens and Lijnen (2006), Vanhoutte *et al.* (2006). The turnover all ECM proteins is very slow.

The principal structural elements of ECM are, however, formed by collagens. They play a dominant role in maintaining the biological and structural integrity of various tissues and organs, including bone, skin, muscle, tendon, blood vessels, and cartilage. Collagens are a family of closely related but distinct extracellular matrix proteins. Collagen type I usually consists of three coiled subunits: two $\alpha 1(I)$ and one $\alpha 2(I)$ chains forming fibrils of 50 nm in diameter; collagen type II is composed of three identical $\alpha 1(II)$ chains forming fibrils less than 80 nm in diameter; collagen type III fibrils are formed from three $\alpha 1(III)$ chains resulting in diverse fibril diameters between 30 and 130 nm (Bowlin 2002, Matthews *et al.* 2002, Matthews *et al.* 2003). The important role of collagens in maintaining the extracellular structure resulted in numerous tissue engineering applications, employing collagen as an ideal scaffold or matrix for cell growth and proliferation (Yang *et al.* 2004).

Family of collagens

The family of collagens is mainly composed of fibrillar collagens, collagens with globular domains and other collagen structures. A total of 44 genes for 26 collagen types were described up to now; they are divided according to their supramolecular structure into 9 groups; most collagens are associated either with different proteins of ECM or with a another collagen type. The primary sequence of amino acids is characteristic: -GLY-X-Y-; every third amino acid is glycine, in position X there are different amino acids; however, proline is typical of this position, in the Y position there are different amino acids but approximately every seventh position of Y is occupied by hydroxyproline. Three α -chains form the polypeptide structure of each collagen type: they are either identical (homotrimer) or only two chains are the same (two times $\alpha 1$) and the last chain is different ($\alpha 2$) = heterotrimer. Alpha-chains are characteristic for each collagen type: the chains are numbered according to the respective collagen type.

The structure of three major collagen types is given as examples: $[\alpha 1(III)]_3$ is the structure of homotrimer in collagen III, $[\alpha 1(I)_2 \alpha 2(I)]$ is the structure of heterotrimer in collagen I,

$[\alpha 1(I)]_3$ is the structure of homotrimer in collagen I. Both heterotrimer of collagen I (up to 80 %) and homotrimer of collagen III (up to 10 %) are the major collagen types in adult tissues of different organs (e.g. muscle, skin, bone). During embryonic life, a relatively high amount of collagen III, together with homotrimer of collagen I, was detected in different organs (Pakkanen *et al.* 2003).

Biosynthesis of collagen molecules is a stepwise procedure; it starts in the endoplasmic reticulum where different posttranslational reactions occur. The first step is hydroxylation; it is the effect of different enzymes: both prolyl-4-hydroxylase and prolyl-3-hydroxylase (formation of hydroxyproline - HYP) and lysyl-hydroxylase (formation of hydroxylysine - HYL). The second step is N-glycosylation (transfer of either glucose or galactose to some HYL) via transferase mechanisms; these steps prepare three collagen chains for the procollagen structure. Finally, both N- and C-terminal proteinases remove the sequences from both collagen ends. Furthermore, there comes the association of three molecules of collagen of appropriated propeptides. Then the new structure is transferred into the extracellular compartment. Moreover, covalent cross-links arise among different both inter- and intramolecular chains of

collagens; it is due to the effect of enzyme: lysyloxidase (Pelouch *et al.* 1993, Maki and Kivirikko 2001, Myllyharju and Kivirikko 2004).

The high stability of collagen molecules is caused by the presence of an -OH group in HYP on the pyrrolidine ring; however, it is not due to hydrogen bridges or to the water net around the molecules. Oxygen in -OH group stabilizes the peptide bond despite the inductive effect and so higher thermal stability of three-stranded polypeptide units; the evidence for it was provided by experiments where several 4-HYP were exchanged for 4-fluoroproline (Holmgren *et al.* 1998). Fibrillar collagens are synthesized from their procollagen; carboxy- and amino- terminal propeptides are cleaved.

Prolyl-4-hydroxylase (P4H) (EC 1.14.11.2) is the key enzyme of collagen synthesis: it catalyzes hydroxylation of proline on 4-HYP. There are two isoforms of this P4H: the first one is localized in luminal ER, the second one in either the cytosol or in the nuclear compartment where it hydrolyzes HIF (hypoxia-inducible factor); PH4 is a tetramer ($\alpha_2\beta_2$) – the α -subunit has three isoforms (I, II, III; however, I is dominant), the β -subunit is identical with protein disulfide isomerase (PDI, EC 5.3.4.1). Hydroxylation of proline to hydroxyproline by P4H occurs in the presence of oxygen only. Moreover, decarboxylation of 2-oxoglutarate requires both Fe^{2+} and ascorbate and the minimum sequence of *-X-Pro-Gly-*.

Lysylhydroxylase (EC 1.14.11.4) is a protein tightly bound to the ER membrane; three isoforms of the enzyme are responsible for hydroxylation of some lysine to hydroxylysine (HYL – in both collagen and other ECM proteins). This procedure is the same as was described above and the minimum sequence for enzyme action is *-X-Lys-Gly-*; the function of HYL is both stabilization of intramolecular cross-linking and of the newly formed -OH groups; these groups can bind monosaccharides (galactose or glucosylgalactose). Hydroxylated and glycosylated chains are organized into triads, using the same mechanism of disulfide bridges in the presence of PDI as was described for the β -subunit of P4H. The functional reason is still a matter of speculations but the formation of ECM fibrils and specific properties of basal membrane occurring in collagen IV are known to be due to the sugar moiety (Rautavuoma *et al.* 2002).

Other ECM proteins and cytoskeletal structures

Fibronectin is localized in both the extracellular matrix of different cells and extracellular fluid (blood

plasma). Its dimer is composed of two subunits bound together in the C-terminal parts by disulfide bridges; various amounts of sugar contamination were found in different organs (Kim *et al.* 1999, Sharma *et al.* 1999). Fibronectin has an important role in the organization of ECM. *Laminins*, the most important non-collagenous glycoproteins in ECM, are composed of three different chains connected by disulfide bridges. They participate in both cell adhesion and differentiation. Laminins, often bound to collagen type IV, are responsible for both selective filtration of different metabolites and connections between the cell and the ECM (Paulsson *et al.* 1991, Nomizu *et al.* 1994, Kim *et al.* 1999). *Perlecan* is a typical proteoglycan of basal membranes; the structure of this protein is responsible for cell adhesion and regulation of cell growth (Murdoch *et al.* 1992). There are many other ECM proteins, such as *desmin*. Desmin is responsible for the structural properties of ECM in different muscles and for connection of neighboring sarcomeres. Then there are *tubulin*, *vinculin*, *vimentin*, *dystrofin*, *actin*, *talin* or *spectrin*. Their physiological and pathological roles remain still not fully understood (Heling *et al.* 2000).

Collagen nanostructure tissue engineering aspects

Collagen fibers seem to be crucial for tissue engineering also from the biophysical point of view. They transmit forces, dissipate energy, prevent premature mechanical failure and provide biological signals to adjacent cells that regulate functional responses (Huang *et al.* 2001). Moreover, collagen is resorbable, it has high water affinity, low antigenicity, very good cell compatibility and ability to promote tissue regeneration. The most abundant collagens in the ECM are collagen types I, II and III. Other substances, such as proteoglycans, glycosaminoglycan and various minerals, are important components of ECM (Teo *et al.* 2006).

The modern tissue engineering task is to develop three-dimensional scaffolds of appropriate biological and biomechanical properties, at the same time mimicking the natural ECM and promoting tissue regeneration. The scaffold should permit cell adhesion, infiltration, and proliferation for ECM synthesis. Furthermore, it should be biodegradable, bioresorbable and non-inflammatory, should provide sufficient nutrient supply and have appropriate viscoelasticity and strength. Attributed to collagen features mentioned above, collagen fibers represent an obvious appropriate material for tissue engineering scaffolds.

Electrospinning, a suitable technique for the production of small-diameter fibers. It has been developed in the first half of the 20th century (Formhals 1932, Taylor 1969). Recently, Reneker and coworkers have investigated the process in more detail (Reneker and Chun 1996, Reneker *et al.* 2000). It uses an electrical field to control the formation and deposition of polymer fibers on a target surface. It is remarkably efficient, rapid and inexpensive (Matthews *et al.* 2002). A polymer solution is injected through a thin needle opposite to a collecting target. At a critical voltage, the charge imbalance overcomes the surface tension of the polymer solution, forming an electrically charged jet. Within the electric field, the jet is directed toward the grounded target, the solvent evaporates, the jet becomes thinner via its whipping and fibers are formed. On the grounded target, the single fibers are deposited as a non-woven highly porous mesh (Bowlin 2002, Buttafoco *et al.* 2006). Placing a rotating collector between the needle and the grounded plate allows deposition of the fibers on the collector with a certain alignment. Fiber orientation involves proper interaction with the cellular environment as well as strength and function of the engineered tissue (Deitzel *et al.* 2001, Xu *et al.* 2004, Zhong *et al.* 2006). Synthetic non-biodegradable polymers, biodegradable polymers, natural polymers, composites and even ceramic precursors can be electrospun to form nanofibers.

During the last decade a lot of effort has been spent to enhance electrospinning productivity via the development of so-called needle-less methods. Needle-less electrospinning concerns self-organization of polymeric jets on free liquid surfaces and is a result of electrohydrodynamics on the surface. Electrohydrodynamic analysis reveals the development of unstable surface waves, depending on the strength of the external electrostatic field. Although physically similar, theoretical considerations show substantial differences between needle and needle-less electrospinning. The dependence of critical field strength on the polymeric solution surface tension, self-organization of jets and the fact that jetting density in the instance of needle-less electrospinning depends on the field strength value, are notable. The needle-less electrospinning is supported with various electric field strength concentrators. Thus, the polymeric materials are spread on cylindrical surfaces (Jirsak *et al.* 2005) or on spikes of magnetic liquids (Yarin and Zussman 2004).

The electrospinning process has the potential to produce collagen fibrils that closely mimic and at some

point may even fully reproduce the structural and biological properties of the natural fiber. *In vitro* and *in vivo* experiments demonstrate that electrospun scaffolds hold great potential for tissue engineering applications.

Fiber size was shown to be crucial for proper interaction of the scaffold with cell and tissue formation (i.e. for cell morphology, adhesion, migration, proliferation, and differentiation). The wide range of possible fiber diameters permits a large variation in the production of tissue engineering scaffolds via electrospinning (Bowlin 2002). The possibility to produce meshes with a high surface area makes electrospinning an ideal procedure for scaffold design in tissue engineering (Li *et al.* 2002). Furthermore, the architecture of the produced non-woven meshes is similar to extracellular matrices (Xu *et al.* 2004). Nanofibrous scaffolds are highly porous. The pore size, much smaller than the normal cell size, inhibits cell migration. In spite of this fact, the results indicate the capacity of nanofibrous meshes to infiltrate cells (Zhang *et al.* 2005). The cells entering into the matrix through amoeboid movement to migrate through the pores can push the surrounding fibers aside to expand the pore. The dynamic architecture of the fibers allows the cells to adjust according to the pore size and grow into the nanofiber matrices. In spite of producing typical electrospun fibers in the form of two-dimensional non-woven meshes, three-dimensional scaffolds consisting of aligned fibers and tubular scaffolds can be made. Synthetic materials with superior mechanical strength lack the cell-recognition signal (Kim and Mooney 1998); scaffold constructed from naturally occurring proteins in the ECM, such as collagen, allows much better infiltration of cells into the scaffold. For many tissue engineering applications nanofiber modifications are necessary to achieve the required scaffold properties. Polymer blending, co-electrospinning, multilayering and mixing for nanofiber production or cross-linking, surface modifications and coating of the scaffold can improve the stability and biocompatibility.

Many surface modifications were used to modify the synthetic polymer and to bring ECM components onto the scaffold surface. Collagen-coated poly(L-lactic acid)-*co*-poly(ϵ -caprolactone) nanofiber mesh with mechanical properties suitable for vascular graft enhanced endothelialization and preserved phenotype of human coronary artery endothelial cells, as shown by increased spreading, cell viability, attachment and phenotypic maintenance (He *et al.* 2005a, b).

Blended nanomaterials possess the potential of adding various ingredients (e.g. growth factors) according to cell-type requirements. Blended nanoscaffold, prepared from collagen with chondroitin sulfate, exhibited after cross-linking excellent biocompatibility when seeded with rabbit conjunctiva fibroblasts. Combination of nanofibrous collagen-glycosaminoglycan scaffold prepared from natural ECM components seems to mimic closely the ECM and thus has a great potential in tissue engineering applications (Zhong *et al.* 2005).

In multilayering electrospinning, after electrospinning the first component, the second polymer is sequentially electrospun on the same target producing a multilayered meshes with hierarchically ordered layers made from particular fibers. For example, a trilayered electrospun mesh, composed from type I collagen, styrenatedgelatin, and segmented polyurethane were prepared; a bilayered tubular construct composed of a thick segmented polyurethane microfiber mesh as an outer layer and a thin type I collagen nanofiber mesh as an inner layer was fabricated (Kidoaki *et al.* 2005).

Different polymers are simultaneously electrospun from different syringes under special conditions in the mixing electrospinning. The produced fibers are mixed on the same collector, resulting in the formation of a mixed fiber mesh, e.g. mixed electrospun-fiber mesh composed of segmented polyurethane and polyethylene oxide (Kidoaki *et al.* 2005). Scaffolds can better mimic the extracellular matrix and thus promote tissue regeneration.

The aligned nanofibrous collagen scaffold with distinct fiber alignment fabricated using a rotational wheel collector was used to evaluate the alignment effect on cell orientation. Collagen nanofibers were cross-linked in glutaraldehyde vapor and seeded with rabbit conjunctiva fibroblasts. Decreased cell adhesion but increased proliferation were found on aligned collagen scaffold when compared with random collagen. Most cells on aligned scaffold adhere and elongate themselves along the nanofiber alignment direction, while the random scaffold provided cell spread in all directions. The elongated proliferation pattern of the cells coincides with the native tissue cell morphology (Zhong *et al.* 2006).

Collagen types I, II and III were successfully electrospun (Buttafoco *et al.* 2006, Casper *et al.* 2007). The fiber diameter and structural properties produced by electrospinning depend on the collagen type, tissue origin and concentration (Matthews *et al.* 2002, Matthews *et al.*

2003). Optimizing conditions for calf-skin type I collagen produced a matrix composed of 100-nm fibers that exhibited the 67-nm banding pattern that is characteristic of native collagen (Matthews *et al.* 2002). Because of the solubility of collagen fibers, different methods of fiber cross-linking are used to stabilize fibrous scaffolds (Kidoaki *et al.* 2005, Li *et al.* 2005, Buttafoco *et al.* 2006, Casper *et al.* 2007). Some applications of collagen nanofibrous scaffolds are mentioned below.

Cartilage grafts. Cartilage is predominantly composed of collagen type II. Matthews *et al.* (2003) report electrospun chicken sternal cartilage collagen type II to form fibers 0.11–1.8 μm in diameter. The scaffold fixed in glutaraldehyde vapor support cell growth, human articular chondrocytes can freely penetrate the matrix and are readily infiltrated.

A significant difference was identified between the noncross-linked and the cross-linked scaffolds when comparing scaffold thickness and fiber diameter (Shields *et al.* 2004). The electrospun noncross-linked scaffolds were stiffer but were not able to attain as high a tensile strength as native cartilage. Vapor-glutaraldehyde cross-linked scaffolds seeded with human articular chondrocytes revealed the potential of the scaffold for articular cartilage repair.

Three-dimensional nanofibrous matrices from poly-L-lactic acid and poly(ϵ -CBZ-lysine) (carbo-benzyloxy-protected L-lysine) with free NH_2 groups for the covalent binding of recombinant collagen II variants were prepared and human chondrocyte attachment to collagen type II with differently deleted D periods was analyzed (Fertala *et al.* 2001).

Skin grafts. With collagen type I nanofibers cross-linked by glutaraldehyde vapor, a relatively low adhesion of human oral keratinocytes was observed (Rho *et al.* 2006). ECM protein coating of this matrix, specifically treatment with type I collagen or laminin, promoted cell adhesion and spreading of cells as a consequence of the great surface area available for cell attachment and of the restoration of biological and structural properties of natural ECM proteins. Polycaprolactone (PCL) and collagen represent a favorable matrix for preparing a dermal substitute for engineering skin. PCL nanofiber membrane coated with collagen shows good growth, proliferation and migration inside the matrix of human dermal fibroblasts (Venugopal and Ramakrishna 2005). Venugopal *et al.* successfully used blended collagen type I and PCL nanofibers for culture of human dermal fibroblasts (Venugopal *et al.*

2006). Matrices promoted cell adhesion, proliferation and spreading and have the potential for skin defect and burn wound healing. Two different methods of nanofiber coating were investigated for human dermal fibroblast skin graft preparation – coaxial electrospinning with collagen type I as shell material and wrapped PLC core component, and post-coating by immersing the PLC nanofibrous scaffold into collagen solution (Zhang *et al.* 2005). Coaxial electrospinning resulted in individually surface-coated nanofibers, while a rough surface coating was accomplished by soaking nanofibers in a coating medium. Individually collagen-coated nanofibers resembled the natural ECM rather than the rough collagen coating and encouraged human dermal fibroblast migration into the scaffold and cell proliferation.

Cardiovascular grafts. In tissue engineering of blood vessels, collagen constructs have a limited applicability due to lack of structural integrity when subjected to intraluminal physiological pressure. Electrospinning of blended solutions of collagen and elastin results in materials of high porosity, surface area and fibers with extraordinary mechanical properties. Furthermore, electrospinning of solutions separately can be utilized for the production of multilayered scaffolds with controlled morphology and/or mechanical properties (Buttafoco *et al.* 2006). Besides the required biomechanical properties, cell proliferation and adhesion parameters, vascular grafts should have anti-coagulant activity until the endothelial cell lining is fully achieved.

Vascular graft scaffolds have been fabricated using electrospun polymer blends of collagen type I, elastin and poly(D,L-lactide-*co*-glycolide) with a length of 12 cm and a thickness of 1 mm. The scaffolds possess tissue composition and mechanical properties similar to native vessels. The electrospun vessel matrix is biocompatible for bovine endothelial and smooth muscle cells, seeded separately or on either side, as well as *in vivo* subcutaneously implanted in mice (Stitzel *et al.* 2006). The equimolar copolyester poly(L-lactide-*co*- ϵ -caprolactone) (PLCL) was co-electrospun with collagen type I. The tensile strength was decreased with increasing collagen content. Human umbilical vein endothelial cells were highly elongated and well spread on the fibrous matrices containing 5 and 10 % collagen, while round or restricted spread cells were observed on the 30–50 % collagen-blended PLCL scaffolds (Kwon and Matsuda 2005). On the other hand, collagen-blended PLCL (70:30) nanofibers supported endothelialization – enhanced the viability, spreading, and attachment of

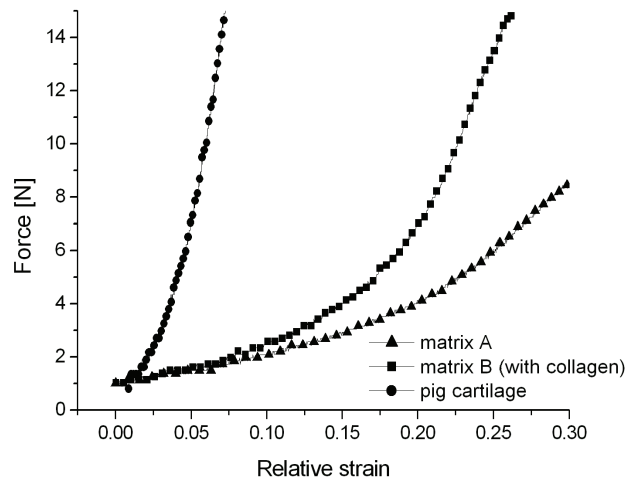


Fig. 1. Loading diagrams of native pig joint cartilage and fibrin-glue-based matrices A and B, where matrix B contains 9 % of collagen in volume.

human coronary artery endothelial cells and preserved the phenotype (He *et al.* 2005a, b). Homogeneous fibers were prepared from calf skin collagen type I and elastin solution with addition of poly(ethylene oxide) and NaCl. On scaffolds, cross-linking with N-(3-dimethylamino-propyl)-N'-ethylcarbodiimide hydrochloride and N-hydroxysuccinimide, smooth muscle cells were successfully cultured and a confluent layer of cells was observed after 2 weeks on the surface (Buttafoco *et al.* 2006).

Collagen in native tissue seems to be a key element for the cartilage structural network. Collagen fibers provide the cartilage with its tensile stiffness and strength. On the other hand, collagen fibers show only a little resistance to compression or shear. Different orientation of collagen fibrils, however, can significantly influence the mechanical properties of individual native cartilage layers (cartilage anisotropy). Random orientation of collagen fibers implies a lower tensile modulus than their parallel orientation. Though the role of proteoglycans is reported to be crucial for compressive properties of cartilage tissue, involvement of collagen is undoubtedly important as well. Different chemical composition and different techniques of scaffold preparation can significantly modify the biomechanical properties of the scaffold and, consequently, after cell seeding also of artificial tissue.

The essential role of collagen on cartilage biomechanics is presented in Fig. 1. There were three samples tested: native cadaveric pig joint cartilage and two different fibrin matrices. The matrices were formed from fibrin glue based on Tissuecol® Kit, one of them

(matrix B) containing 9 % of collagen in volume. Mechanical properties were tested according to Varga *et al* (2007); the resulting mean force-displacement curves are shown in Fig. 1. The data clearly demonstrate the basic role of collagen for biomechanical properties of fibrin gel structures which are very commonly used for tissue engineering. When collagen was added to fibrin matrix, overall sample stiffness was increased. Collagen fibers formed internal structure of higher porosity which resulted in less linear course of loading curve (more resembling the properties of viscoelastic material). It should be stated that the presence of collagen in fibrin gel shifted biomechanical properties of the scaffold closer to the native tissue. While the fibrin matrix showed a relatively low value of derivation of the force/strain dependence (Fig. 1, line A) compared to the much higher value of the derivation for the native cartilage from pig knee (Fig. 1, line C), simple presence of collagen can significantly improve the biomechanical properties of the fibrin gel and “shift” these properties toward the properties of native tissue (Fig. 1, line B).

Appropriate determination of biomechanical properties of artificial tissue and scaffolds is, therefore, one of the crucial steps in tissue engineering. Testing of native or artificial tissue for biomechanical properties is not easy from several points of view. First of all, we have mostly to deal with a very tiny sample which is rather

difficult to examine. Second, in spite of several methods that have been already employed for the determination of biomechanical properties (e.g. Young modulus), there are rather large drawbacks which still need to be overcome to establish a quick and reliable detection method.

Most common approaches for material mechanical properties testing employ commercially available testing machines. Though these devices usually offer a limited interval of applicable loading rates and face difficulties with attaching and subsequent testing of humid and slippery samples of limited size, they are still most frequently used for the evaluation of cartilage tissue biomechanical properties. Some alternatives have been proposed to yield more comprehensive knowledge about cartilage mechanics, e.g. nanoindentation (Hu *et al.* 2001), ultrasonic (Saarakkala *et al.* 2004) or dynamic impact loading (Repo and Finlay 1977).

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References

- ADAMCOVA M, PELOUCH V, GERSL V, KAPLANOVA J, MAZUROVA Y, SIMUNEK T, KLIMTOVA I, HRDINA R: Protein profiling in daunorubicin-induced cardiomyopathy. *Gen Physiol Biophys* **22**: 411-419, 2003.
- BOWLIN GL: Electrospinning Collagen Scaffolds. *Sci Med* **8**: 292-293, 2002.
- BUTTAFOCO L, KOLKMAN NG, ENGBERS-BUIJTENHUIJS P, POOT AA, DIJKSTRA PJ, VERMES I, FEIJEN J: Electrospinning of collagen and elastin for tissue engineering applications. *Biomaterials* **27**: 724-734, 2006.
- CASPER CL, YANG W, FARACH-CARSON MC, RABOLT JF: Coating Electrospun Collagen and Gelatin Fibers with Perlecan Domain I for Increased Growth Factor Binding. *Biomacromolecules*, 2007.
- DEITZEL JM, KLEINMEYER J, HARRIS D, TAN NCB: The effect of processing variables on the morphology of electrospun nanofibers and textiles. *Polymer* **42**: 261-272, 2001.
- FERTALA A, HAN WB, KO FK: Mapping critical sites in collagen II for rational design of gene-engineered proteins for cell-supporting materials. *J Biomed Mater Res* **57**: 48-58, 2001.
- FORMHALS A: *Improvements in or relating to Processes and Apparatus for the Production of Artificial Filaments*. United Kingdom patent no. 364, 780; 1932.
- HE W, MA Z, YONG T, TEO WE, RAMAKRISHNA S: Fabrication of collagen-coated biodegradable polymer nanofiber mesh and its potential for endothelial cells growth. *Biomaterials* **26**: 7606-7615, 2005a.
- HE W, YONG T, TEO WE, MA Z, RAMAKRISHNA S: Fabrication and endothelialization of collagen-blended biodegradable polymer nanofibers: potential vascular graft for blood vessel tissue engineering. *Tissue Eng* **11**: 1574-1588, 2005b.

- HELING A, ZIMMERMANN R, KOSTIN S, MAENO Y, HEIN S, DEVAUX B, BAUER E, KLOVEKORN WP, SCHLEPPER M, SCHAPER W, SCHAPER J: Increased expression of cytoskeletal, linkage, and extracellular proteins in failing human myocardium. *Circ Res* **86**: 846-853, 2000.
- HERGET J, PELOUCH V, KOLAR F, OSTADAL B: The inhibition of angiotensin converting enzyme attenuates the effects of chronic hypoxia on pulmonary blood vessels in the rat. *Physiol Res* **45**: 221-226, 1996.
- HOLMGREN SK, TAYLOR KM, BRETSCHER LE, RAINES RT: Code for collagen's stability deciphered. *Nature* **392**: 666-667, 1998.
- HU K, RADHAKRISHNAN P, PATEL RV, MAO JJ: Regional structural and viscoelastic properties of fibrocartilage upon dynamic nanoindentation of the articular condyle. *J Struct Biol* **136**: 46-52, 2001.
- HUANG L, APKARIAN RP, CHAIKOF EL: High-resolution analysis of engineered type I collagen nanofibers by electron microscopy. *Scanning* **23**: 372-375, 2001.
- JANSSENS S, LIJNEN HR: What has been learned about the cardiovascular effects of matrix metalloproteinases from mouse models? *Cardiovasc Res* **69**: 585-594, 2006.
- JIRSAK O, SANETRNÍK F, LUKAS D, KOTEK V, MARTINOVA L, CHALOUPEK J: *A Method of Nanofibres Production from A Polymer Solution Using Electrostatic Spinning and A Device for Carrying out The Method*. WO2005024101; 2005.
- KIDOAKI S, KWON IK, MATSUDA T: Mesoscopic spatial designs of nano- and microfiber meshes for tissue-engineering matrix and scaffold based on newly devised multilayering and mixing electrospinning techniques. *Biomaterials* **26**: 37-46, 2005.
- KIM BS, MOONEY DJ: Development of biocompatible synthetic extracellular matrices for tissue engineering. *Trends Biotechnol* **16**: 224-230, 1998.
- KIM H, YOON CS, KIM H, RAH B: Expression of extracellular matrix components fibronectin and laminin in the human fetal heart. *Cell Struct Funct* **24**: 19-26, 1999.
- KOLAR F, PAPOUSEK F, PELOUCH V, OSTADAL B, RAKUSAN K: Pressure overload induced in newborn rats: effects on left ventricular growth, morphology, and function. *Pediatr Res* **43**: 521-526, 1998.
- KUKACKA J, PRUSA R, KOTASKA K, PELOUCH V: Matrix metalloproteinases and their function in myocardium. *Biomed. Pap. Med. Fac. Univ. Olomouc* **149**: 225-236, 2005.
- KWON IK, MATSUDA T: Co-electrospun nanofiber fabrics of poly(L-lactide-co-epsilon-caprolactone) with type I collagen or heparin. *Biomacromolecules* **6**: 2096-2105, 2005.
- LI M, MONDRINOS MJ, GANDHI MR, KO FK, WEISS AS, LELKES PI: Electrospun protein fibers as matrices for tissue engineering. *Biomaterials* **26**: 5999-6008, 2005.
- LI WJ, LAURENCIN CT, CATERSON EJ, TUAN RS, KO FK: Electrospun nanofibrous structure: a novel scaffold for tissue engineering. *J Biomed Mater Res* **60**: 613-621, 2002.
- MAKI JM, KIVIRIKKO KI: Cloning and characterization of a fourth human lysyl oxidase isoenzyme. *Biochem J* **355**: 381-387, 2001.
- MATTHEWS JA, BOLAND ED, WNEK GE, SIMPSON DG, BOWLIN GL: Electrospinning of Collagen Type II: A Feasibility Study. *Journal of Bioactive and Compatible Polymers* **18**: 125-134, 2003.
- MATTHEWS JA, WNEK GE, SIMPSON DG, BOWLIN GL: Electrospinning of collagen nanofibers. *Biomacromolecules* **3**: 232-238, 2002.
- MURDOCH AD, DODGE GR, COHEN I, TUAN RS, IOZZO RV: Primary structure of the human heparan sulfate proteoglycan from basement membrane (HSPG2/perlecan). A chimeric molecule with multiple domains homologous to the low density lipoprotein receptor, laminin, neural cell adhesion molecules, and epidermal growth factor. *J Biol Chem* **267**: 8544-8557, 1992.
- MYLLYHARJU J, KIVIRIKKO KI: Collagens, modifying enzymes and their mutations in humans, flies and worms. *Trends Genet* **20**: 33-43, 2004.
- NOMIZU M, OTAKA A, UTANI A, ROLLER PP, YAMADA Y: Assembly of synthetic laminin peptides into a triple-stranded coiled-coil structure. *J Biol Chem* **269**: 30386-30392, 1994.
- PAKKANEN O, HAMALAINEN ER, KIVIRIKKO KI, MYLLYHARJU J: Assembly of stable human type I and III collagen molecules from hydroxylated recombinant chains in the yeast *Pichia pastoris*. Effect of an engineered C-terminal oligomerization domain foldon. *J Biol Chem* **278**: 32478-32483, 2003.

- PAULSSON M, SALADIN K, ENGVALL E: Structure of laminin variants. The 300-kDa chains of murine and bovine heart laminin are related to the human placenta merosin heavy chain and replace the α chain in some laminin variants. *J Biol Chem* **266**: 17545-17551, 1991.
- PELOUCH V, DIXON IM, GOLFMAN L, BEAMISH RE, DHALLA NS: Role of extracellular matrix proteins in heart function. *Mol Cell Biochem* **129**: 101-120, 1993.
- PELOUCH V, MILEROVA M, OSTADAL B, HUCIN B, SAMANEK M: Differences between atrial and ventricular protein profiling in children with congenital heart disease. *Mol Cell Biochem* **147**: 43-49, 1995.
- RAUTAVUOMA K, TAKALUOMA K, PASSOJA K, PIRSKANEN A, KVIST AP, KIVIRIKKO KI, MYLLYHARJU J: Characterization of three fragments that constitute the monomers of the human lysyl hydroxylase isoenzymes 1-3. The 30-kDa N-terminal fragment is not required for lysyl hydroxylase activity. *J Biol Chem* **277**: 23084-23091, 2002.
- RENEKER DH, CHUN I: Nanometre diameter fibres of polymer, produced by electrospinning. *Nanotechnology* **7**: 216-223, 1996.
- RENEKER DH, YARIN AL, FONG H, KOOMBHONGSE S: Bending instability of electrically charged liquid jets of polymer solutions in electrospinning. *Journal of Applied Physics* **87**: 4531-4547, 2000.
- REPO RU, FINLAY JB: Survival of articular cartilage after controlled impact. *J Bone Joint Surg Am* **59**: 1068-1076, 1977.
- RHO KS, JEONG L, LEE G, SEO BM, PARK YJ, HONG SD, ROH S, CHO JJ, PARK WH, MIN BM: Electrospinning of collagen nanofibers: effects on the behavior of normal human keratinocytes and early-stage wound healing. *Biomaterials* **27**: 1452-1461, 2006.
- SAARAKKALA S, KORHONEN RK, LAASANEN MS, TOYRAS J, RIEPPO J, JURVELIN JS: Mechano-acoustic determination of Young's modulus of articular cartilage. *Biorheology* **41**: 167-179, 2004.
- SHARMA A, ASKARI JA, HUMPHRIES MJ, JONES EY, STUART DI: Crystal structure of a heparin- and integrin-binding segment of human fibronectin. *EMBO J* **18**: 1468-1479, 1999.
- SHIELDS KJ, BECKMAN MJ, BOWLIN GL, WAYNE JS: Mechanical properties and cellular proliferation of electrospun collagen type II. *Tissue Eng* **10**: 1510-1517, 2004.
- SIMKO F, PELOUCH V, TOROK J, LUPTAK I, MATUSKOVA J, PECHANOVA O, BABAL P: Protein remodeling of the heart ventricles in hereditary hypertriglyceridemic rat: effect of ACE-inhibition. *J Biomed Sci* **12**: 103-111, 2005.
- STITZEL J, LIU J, LEE SJ, KOMURA M, BERRY J, SOKER S, LIM G, VAN DYKE M, CZERW R, YOO JJ, ATALA A: Controlled fabrication of a biological vascular substitute. *Biomaterials* **27**: 1088-1094, 2006.
- TAYLOR G: Electrically Driven Jets. *Proceedings of the Royal Society of London Series a-Mathematical and Physical Sciences* **313**: 453, 1969.
- TEO WE, HE W, RAMAKRISHNA S: Electrospun scaffold tailored for tissue-specific extracellular matrix. *Biotechnol J* **1**: 918-929, 2006.
- VANHOUTTE D, SCHELLINGS M, PINTO Y, HEYMANS S: Relevance of matrix metalloproteinases and their inhibitors after myocardial infarction: a temporal and spatial window. *Cardiovasc Res* **69**: 604-613, 2006.
- VARGA F, DRŽÍK M, HANDL M, CHLPIK J, KOS P, FILOVÁ E, RAMPICHOVÁ M, NEČAS A, TRČ T, AMLER E: Biomechanical characterization of cartilages by a novel approach of blunt impact testing. *Physiol Res* **56** (Suppl 1): S61-S68, 2007 (This issue).
- VENUGOPAL J, RAMAKRISHNA S: Biocompatible nanofiber matrices for the engineering of a dermal substitute for skin regeneration. *Tissue Eng* **11**: 847-854, 2005.
- VENUGOPAL JR, ZHANG Y, RAMAKRISHNA S: In vitro culture of human dermal fibroblasts on electrospun polycaprolactone collagen nanofibrous membrane. *Artif Organs* **30**: 440-446, 2006.
- XU CY, INAI R, KOTAKI M, RAMAKRISHNA S: Aligned biodegradable nanofibrous structure: a potential scaffold for blood vessel engineering. *Biomaterials* **25**: 877-886, 2004.
- YANG C, HILLAS PJ, BAEZ JA, NOKELAINEN M, BALAN J, TANG J, SPIRO R, POLAREK JW: The application of recombinant human collagen in tissue engineering. *BioDrugs* **18**: 103-119, 2004.
- YARIN AL, ZUSSMAN E: Upward needleless electrospinning of multiple nanofibers. *Polymer* **45**: 2977-2980, 2004.

-
- ZHANG YZ, VENUGOPAL J, HUANG ZM, LIM CT, RAMAKRISHNA S: Characterization of the surface biocompatibility of the electrospun PCL-collagen nanofibers using fibroblasts. *Biomacromolecules* **6**: 2583-2589, 2005.
- ZHONG S, TEO WE, ZHU X, BEUERMAN R, RAMAKRISHNA S, YUNG LY: Formation of collagen-glycosaminoglycan blended nanofibrous scaffolds and their biological properties. *Biomacromolecules* **6**: 2998-3004, 2005.
- ZHONG S, TEO WE, ZHU X, BEUERMAN RW, RAMAKRISHNA S, YUNG LY: An aligned nanofibrous collagen scaffold by electrospinning and its effects on in vitro fibroblast culture. *J Biomed Mater Res A* **79**: 456-463, 2006.
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