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Directing collagen fibers using counter-rotating cone extrusion

Henk R. Hoogenkamp [a,*], Gert-Jan Bakker [b], Louis Wolf [b], Patricia Suurs [c], Bertus Dunnewind [c], Shai Barbut [d], Peter Friedl [b,e,f], Toin H. van Kuppevelt [a], Willeke F. Daamen [a]

[a] Department of Biochemistry 280, RIMLS, Radboud University Medical Center, PO Box 9101, 6500 HB Nijmegen, Netherlands
[b] Department of Cell Biology 283, RIMLS, Radboud University Medical Center, PO Box 9101, 6500 HB Nijmegen, Netherlands
[c] Marel Townsend Further Processing, Handelstraat 3, 5831 AV Boxmeer, Netherlands
[d] Department of Food Science, University of Guelph, 88 McGilvray Street, Guelph, ON N1G 2W1, Canada
[e] David H. Koch Center for Applied Research of Genitourinary Cancers, Department of Genitourinary Medical Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA
[f] Cancer Genomics Center Netherlands,[1] HP Stratenum 3.217, Universiteitsweg 100, 3584 CG Utrecht, Netherlands

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ABSTRACT

The bio-inspired engineering of tissue equivalents should take into account anisotropic morphology and the mechanical properties of the extracellular matrix. This especially applies to collagen fibrils, which have various, but highly defined, orientations throughout tissues and organs. There are several methods available to control the alignment of soluble collagen monomers, but the options to direct native insoluble collagen fibers are limited. Here we apply a controlled counter-rotating cone extrusion technology to engineer tubular collagen constructs with defined anisotropy. Driven by diverging inner and outer cone rotation speeds, collagen fibrils from bovine skin were extruded and precipitated onto mandrels as tubes with oriented fibers and bundles, as examined by second harmonic generation microscopy and quantitative image analysis. A clear correlation was found whereby the direction and extent of collagen fiber alignment during extrusion were a function of the shear forces caused by a combination of the cone rotation and flow direction. A gradual change in the fiber direction, spanning +50 to −40°, was observed throughout the sections of the sample, with an average decrease ranging from 2.3 to 2.6° every 10 μm. By varying the cone speeds, the collagen constructs showed differences in elasticity and toughness, spanning 900–2000 kPa and 19–35 mJ, respectively. Rotational extrusion presents an enabling technology to create and control the (an)isotropic architecture of collagen constructs for application in tissue engineering and regenerative medicine.

1. Introduction

1.1. Heterogeneity of the extracellular matrix in vivo

Biomaterials that can mimic the target extracellular matrix (ECM) morphology and mechanical properties are crucial for the success of tissue engineering and regenerative medicine strategies [1]. Tissue structure is generally heterogeneous, and frequently displays the anisotropic organization of the cells and the surrounding ECM. Fibrillar collagen, the main component of the ECM, which has a major impact on the mechanical properties of tissues, shows remarkable variability of fibrillar organization, density and alignment [2,3]. The architecture of collagen fibers of e.g. the skin (basket-weave orientation) and tendon (parallel oriented) are highly defined, as is the fiber orientation in organs such as bladder, artery, cartilage and bone [4–9]. Controlling the collagen fiber organization upon scaffold construction is critical for achieving a controlled impact on matrix-influenced complex cell functions, including proliferation, differentiation and migration [10–13]. The importance of ECM geometry on cell function is illustrated by the orientation of cell-derived ECM deposition, which follows the orientation of the pre-existing template [14].

1.2. Bio-inspired generation of collagen films

Few approaches allow the generation of anisotropic fibrillar collagen constructs from native collagen fibers and/or fibrils due to the insoluble nature of collagen. Scaffolds prepared from native collagen fibrils rather than from soluble collagen molecules may be preferential since collagen fibrils harboring native cross-links

* Corresponding author. Tel.: +31 24 361 4272.
E-mail address: Henk.Hoogenkamp@Radboudumc.nl (H.R. Hoogenkamp).
[1] www.cancergenomics.nl.

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and molecular alignment (quarter staggered array) are the structural components that cells encounter in the body. Techniques based on weaving, casting and/or freezing are the only enabling technologies that can produce constructs consisting of insoluble collagen for tissue engineering and regenerative medicine applications [14,15]. These techniques allow the use of strong and physiological collagenous fibers/fibrils, but are limited by the control over the construct morphology. In contrast, a high degree of control has been demonstrated for a wide range of approaches by aligning soluble collagen molecules, including electrospinning [16,17], magnetic patterning [18], printing [19], dip-pen (nano)lithography [20], shear flow patterning [21], microfluidic shear flow channels [22], salt and/or particulate leaching [23], electrical gradient [24] and others [25]. These techniques exploit the orientational control of collagen monomers during self-assembly to form aligned collagen fibrillar structures [26,27]. Constructs produced from monomeric collagen allow for high precision and standardization, and further allow for the seeding of cells before the polymerization of the gel into its final form [27]. Pre-seeded collagen gels are amenable for engineering of several tissues with defined collagen architecture like blood vessels and heart valves [26–28]. However, despite promising results, constructs based on monomeric collagen suffer from non-physiological small fibril dimensions and poor mechanical strength [3].

1.3. Extrusion technology

Largely unknown to the tissue engineering and regenerative medicine community is the utilization of extrusion techniques to produce materials for soft tissue replacement. Extrusion is defined as "the act or process of shaping a material by forcing through a die" [29]. This has been applied in the biomedical material industry to produce all sorts of synthetic polymer threads or tubing, e.g. sutures, slings and silicon tubing. Its application to collagen is limited to making collagen fibrils, by extruding either monomeric collagen solution or fibrillar collagen through a circular die into a fibril-forming solution to produce a collagen monofilament which can be further processed by braiding, knitted and/or bundling into a strand, fabric or large bundle [30–32]. Two groups reported the use of a commercially available extruded fibrillar insoluble collagen film with undefined fiber orientation for in vitro cell differentiation, treatment of burn wounds and myocardial infarction with varying results [33–37]. Collagen extrusion technology using either one rotating cone or independently counter-rotating cones to influence collagen alignment originates from the packaging and meat industry, where it was designed to generate edible food casings from e.g. bovine or porcine skin-derived collagen fibers (see Fig. 1A and B) [38]. By using different types of extruders, defined fiber alignment, including linear or interwoven "crisscross" collagen fiber orientation, can be achieved [39]. In this study, we applied state-of-the-art controlled fiber alignment extrusion technology, using counter-rotating cones, to develop a tubular collagen construct with defined morphology and fiber architecture.

2. Materials and methods

2.1. Tissue preparation

Porcine tissues (Achilles tendon, left subclavian artery and skin) were purchased from a local slaughterhouse and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 24 h at 4 °C. The samples were sliced with a vibratome (500 μm) [40].

2.2. Collagen source

A 4% (w/v) collagen fiber suspension, or gel (Unicoll™813, Barentz Raw Materials, Hoofddorp, Netherlands), swollen in 1.0% (w/v) acetic acid and 1.5% (w/v) lactic acid (final pH 3), purified from bovine hide splits was used [39,41]. In general, bovine hides are alkaline-dehaired (usually using 6–10% w/v calcium hydroxide) and the corium layer is split from the hide parts used for leather production (performed by tanneries). Subsequently, the corium layers are decalcified, ground into smaller particles, swollen in acid and finally homogenized under high pressure to produce an insoluble fibrous collagen dispersion. This dispersion is easily precipitated by the removal of water by using for example high concentration salts [39].

2.3. Film extrusion

The collagen gel was mixed using a vacuum mixer (VM60A, Stephan, Hameln, Germany) for 30 min at 4 °C and subsequently loaded into the extrusion system [42]. The coextrusion system (see Fig. 1C) consists of a piston stuffer (FA30 TOP, Handtmann, Biberach, Germany), followed by a metering pump, extrusion head and pneumatic piston (all performed in a refrigerated conditions at 4 °C, Marel Townsend Further Processing, Boxmeer, Netherlands). Briefly, the piston stuffer applies a constant collagen pressure (5 bar) to the metering pump (55 rpm), which subsequently supplies the extrusion head (continuously cooled to 4 °C) with a 700 g collagen suspension per min. The interchangeable extrusion head (Fig. 1D) was a 24 mm (inner Ø) extrusion head with independently counter-rotating cones. The collagen traveled from the pump through the rotating extrusion head, in between the inner and outer cone (Fig. 1E), and was finally extruded through a 350 μm circular die. The collagen was deposited on a Ø 23 mm mandrel, which was forced out by a pneumatic piston at 80 mm s^{-1} in order to keep a constant traveling speed. After extrusion, the collagen-coated mandrels were placed in 6 M NaCl to set the collagen into a film. The films were stored in 6 M NaCl until further testing commenced. At least 20 mandrels were extruded, from which two films for texture analysis and one sample for microscopy were randomly taken per mandrel. The collagen films were maintained orthoptically with defined longitudinal and transversal directions up until second harmonic generation imaging (as described in Section 2.5).

2.4. Mechanical characterization

The collagen films were removed from the mandrels and cut into 150 mm (l) × 60 mm (w) rectangles, whilst retaining the original extrusion orientation in the lengthwise (l) direction. To facilitate reproducible tensile strength testing, a 5 mm notch on each side in the middle of the length of the film was made using a scalpel and the film was subsequently stretched to failure to assess its mechanical characteristics (TA.XT Plus texture analyzer, Stable Micro Systems, Surrey, UK). The films were clamped between tensile grips and subjected to tensile analysis (test speed: 40 mm s^{-1}; trigger type: button; 100 mm intra-clamp spacing; $n = 15$). The resulting tensile profiles were analyzed for maximum force (N), the slope (the average between 20 and 80% of the maximum force, corrected for film thickness to yield kPa) and the area under the curve (total work to rupture until maximum force, in mJ) using eXponent software (Stable Micro Systems). Films that tore at the site of the tensile grips were excluded from analysis. The thickness was assessed using a texture analyzer equipped with a Ø 5 mm stainless steel cylindrical probe (test mode; compression, test