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Aligned fibrillar collagen matrices obtained by shear flow deposition

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ABSTRACT

Here we present a new technique to generate surface-bound collagen I fibril matrices with differing structural characteristics. Aligned collagen fibrils were deposited on planar substrates from collagen solutions streaming through a microfluidic channel system. Collagen solution concentration, degree of gelation, shear rate and pre-coating of the substrate were demonstrated to determine the orientation and density of the immobilized fibrils. The obtained matrices were imaged using confocal reflection microscopy and atomic force microscopy. Image analysis techniques were applied to evaluate collagen fibril orientation and coverage. As expected, the degree of collagen fibril orientation increased with increasing flow rates of the solution while the matrix density increased at higher collagen solution concentrations and on hydrophobic polymer pre-coatings. Additionally, length of the immobilized collagen fibrils increased with increasing solution concentration and gelation time.

1. Introduction

Collagen is the most abundant structural protein in mammals. To date, 28 different types of collagens [1] are known to be involved in shaping and maintaining the extracellular matrix (ECM) by forming fibrillar or other large-scale assemblies [2]. Collagen builds tissue-specific architecture that imparts mechanical strength and stability to the ECM and mediates cell attachment, morphology, proliferation and migration [3]. In order to better understand the *in vivo* function of collagen matrices it is of need to develop systems that mimic these structures *in vitro*. In this context it is of great interest to develop methods allowing for the variation and control of the morphology of collagen assemblies *in vitro* to adopt the very versatile structures found in tissues.

Collagen type I is the main collagen found in skin and bone and the major constituent of most ECM variants. Three left-handed supercoiled polypeptide chains (two α1(I) and one α2(I)) in a polyproline-II-like conformation form a right-handed triple helix, the collagen molecule, with a diameter of 1.5 nm and a length of 300 nm [2]. In an entropy-driven self-assembly process termed fibrillogenesis, collagen molecules form fibrils with diameters ranging from 20 nm to 70 nm [2,4]. Turbidimetric analysis of fibril formation *in vitro* showed an initial lag phase followed by a growth phase with a sigmoidal increase in fibril formation rate [5]. It has been suggested that dimer and trimer formation takes place during the lag phase, while rapid lateral aggregation involving five trimers ("microfibril") occurs during the growth phase. Further growth occurs by linear and lateral addition of trimeric units to result in collagen fibril formation [6].

In vivo, collagen fibrils are arranged in complex three-dimensional arrays, often in an aligned manner, to fulfil certain biomechanical functions such as resisting high tensile forces in tendons. Collagens can be found as parallel fibre bundles in tendon and ligaments [7], as concentric waves in bone [8] or as orthogonal lattices in cornea [9]. The spatial organization of collagen fibres *in vivo* is believed to play an important role in directing cell behaviour and fate decisions. For example, it has been reported that fibroblasts align along oriented collagen fibril matrices via contact guidance [10,11]. The direction of cell migration and the distribution of newly synthesized collagen are also determined by the alignment of collagen fibrils [12]. It is thus important that aligned collagen fibril matrices be created *in vitro* to investigate ECM structure and its influence on cell behaviour. However, this is problematic as reconstitution of collagen matrices *in vitro* results in isotropic structural networks containing arbitrarily oriented collagen fibrils [13].

Several approaches have been introduced to reconstitute aligned collagen matrices *in vitro*. Elsdale and Bard presented a technique that involved unidirectional draining of a supporting coverslip during gelation of a collagen solution [14]. Additionally, exposing a gelling collagen solution to a strong magnetic field aligns collagen fibrils due to the diamagnetic properties of collagen molecules [15,16]. Guo and Kaufman [17] also made use of magnetic fields, but did so by adding magnetic beads to the collagen solution then aligning the gelling collagen solution by moving the beads towards their poles. Aligned collagen nanofibre matrices have also

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been produced by electrospinning [18] or use of a mica surface in combination with hydrodynamic flow [19]. Lastly, collagen fibril alignment has been observed in microfluidic channels (<100 μm width) as result of a short initial pressure-driven flow and subsequent static gelation of a collagen solution [20]. Despite the development of these methods [11,15–20], however, the production of aligned collagen matrices still requires complex technical equipment or lacks broad application to cell biology due to the limited size of the area covered or the rather artificial characteristics of "non-native" matrices. Importantly, none of the above-mentioned techniques enables collagen fibril alignment, density and morphology to be varied.

Here we introduce a new technique using a microfluidic system to create well-aligned "native" collagen fibril matrices that cover large areas. This method allows both density and collagen fibril orientation to be controlled by varying the applied solution (gelation time, concentration), hydrodynamic flow (shear rate) and pre-coating of the substrate (hydrophobicity, reactivity, charging).

2. Materials and methods

2.1. Preparation of glass substrates/thin polymer films

For experiments with uncoated glass substrates, freshly cleaned 22 mm × 22 mm glass coverslips (Menzel Gläser, Braunschweig, Germany) were oxidized in a 1:1 mixture of aqueous ammonia solution (Acros Organics, Geel, Belgium) and hydrogen peroxide (Merck). For thin film preparation, cleaned coverslips were functionalized by reaction with 3-aminopropyl-dimethylethoxy-silane (ABCR, Karlsruhe, Germany). Poly(octadecene-*alt*-maleic acid) (POMA; MW = 30,000–50,000, Polysciences, Warrington, PA) and poly(ethylene-*alt*-maleic acid) (PEMA, MW = 125,000, Aldrich, Munich, Germany) were dissolved in tetrahydrofuran (THF, Fluka, Deisenhofen, Germany) in a concentration of 0.08 wt% and 0.15 wt%, respectively, and spin coated (RC 5 Suess Microtec, Garching, Germany) onto the amine-modified coverslips at 4000 rpm for 30 s. Stable covalent binding of the polymer films was achieved by annealing at 120 °C to form imide bonds with the amino-silane on the glass substrate [21]. The hydrophobicity of the surfaces (evaluated by the water contact angle) decreases in the following order: POMA (100°) > PEMA (57°) > Glass (<10°) (previous measurements, see Ref. [21]).

2.2. Collagen solution

Bovine dermal collagen I solution (purified and pepsin-solubilized in 0.012 N HCl, PureCol, Inamed, Milmont Drive, USA) was brought to physiological pH by mixing eight parts of the acidic collagen solution (3.0 mg/ml) with one part of 10-fold concentrated phosphate buffered saline (PBS, Sigma, Steinheim, Germany) and one part 0.1 M NaOH. All components were kept in an ice bath before and after mixing. Appropriate volumes of chilled 1× PBS were added to adjust the final concentration of the collagen solution.

Time-dependent viscosity of the collagen solutions was measured with a rotational rheometer (Physica MCR 300, Anton Paar Germany GmbH, Ostfildern, Germany) using a double concentric cylinder measurement system equipped with a temperature unit that enables heating the sample up to 37 °C. The initially chilled collagen solutions (0.2 mg/ml, 0.4 mg/ml, 0.8 mg/ml) were heated up to 37 °C within 10 min and the viscosity was measured at every minute with an applied shear rate of 40 s^{-1}. Collagen solutions (0.8 mg/ml) showed a slight initial increase in viscosity (2.34–2.37 mPas) in the first 5 min. An increase was also observed for 0.4 mg/ml collagen solutions (1.41–1.44 mPas), however, it had longer time period. The viscosity subsequently decreased, reaching an end value of 1.28 mPas for 0.8 mg/ml and 1.05 mPas for 0.4 mg/ml collagen solutions after 60 min. Viscosity of 0.2 mg/ml collagen solutions showed a slight decrease (1.1–1 mPas) after 60 min (Fig. 1).

2.3. Microfluidic system

The microfluidic system used to deposit aligned collagen fibrils was fabricated by microcasting polydimethylsiloxane (PDMS) and sealed by a coverslip (see above). For detailed information concerning the technology, we refer the reader to Ref. [22]. Two parallel channels of 8 mm length, 1 mm width and 73.6 μm height were generated on one channel plate. In order to improve contact between PDMS and coverslip, additional channels were integrated into the PDMS portion of the channel plate and connected to a vacuum pump. The hydrodynamic flow (flow rate) of the collagen solutions in the micro-channels was controlled by a syringe pump (KDS, Holliston, USA) connected via polyether-ether-ketone (PEEK) tubing (diameter 750 μm, Nordantec, Bremerhaven, Germany) to external valves and to the channel plate.

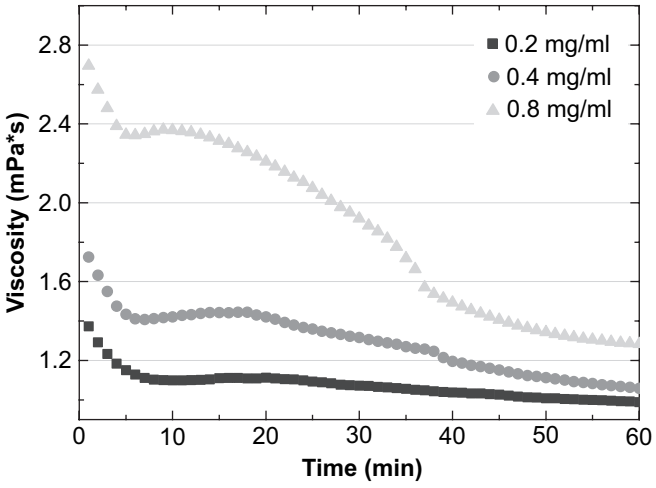

Fig. 1. Time-dependent viscosity of differently concentrated collagen solutions at 37 °C.

2.4. Flow conditions

Experiments were performed under conditions of laminar flow. In all experiments the Reynolds number (as a criterion for laminar flow) was smaller than 1. The following flow rates were applied: 0.45 μl/min, 4 μl/min, and 11 μl/min corresponding to wall shear rates of 8.3 s^{-1}, 73.8 s^{-1}, and 203.1 s^{-1}.

2.5. Deposition of collagen fibrils during fibril formation (Variant A)

The microfluidic system was placed in a CO_2-free incubator (Sanyo, Bensenville, USA) at 37 °C and the syringe pump was kept below 4 °C in a cooled styrofoam box. The collagen solution was prepared at 4 °C and drawn into chilled syringes (Roth, Karlsruhe, Germany), which were quickly installed into the syringe pump to avoid early fibrillogenesis. The streaming process was started immediately by pumping the chilled collagen solution from the cooled storage reservoir through the heated tubing. The length of the tubing leading to the microfluidic channel was varied according to the streaming time of the collagen solution through the heated tubing before entering the channel, defined here as conditioning time. Conditioning was followed by streaming through the microfluidic channel. In all experiments, a deposition time of 1 h was used. The sample was then rinsed with MilliQ and dried under a clean bench.

2.6. Deposition of fully developed collagen fibrils (Variant B)

The collagen solution was prepared at 4 °C in a centrifuge tube (Roth, Karlsruhe, Germany), placed in a CO_2-free incubator (Sanyo, Bensenville, USA) at 37 °C, and allowed to form fibrils for 24 h. The resulting gels were homogenized for 4 min (T-8 Ultra Turrax) and centrifuged (Heraeus, Hanau, Germany) at 1000*g* for 6 min. The supernatant (~2 ml) was drawn into a syringe that was subsequently installed into the syringe pump. The solution was then pumped for 1 h with flow rates of 0.45 μl/min, 4 μl/min, and 11 μl/min through the microfluidic channel. Afterwards the channel was rinsed with PBS for 20 min to flush away loose fibrils and the coated coverslip was removed from the PDMS channel plate. The sample was then rinsed and dried as described above. Experiments were carried out at room temperature.

2.7. Confocal reflection microscopy (CRM)

Confocal reflection microscopy (TCS SP, Leica, Bensheim, Germany) was performed at a wavelength of 488 nm (Ar laser) to visualize the unstained collagen fibrils. CRM pictures of the fibril matrices were obtained using a 40× oil immersion objective as previously described [23].

2.8. Atomic force microscopy (AFM)

Surface topography of the collagen matrices was investigated via intermittent contact scanning force microscopy with a PicoSPM (Molecular Imaging, Phoenix, AZ, United States) using silicon cantilevers (Tap300, BudgetSensors, Bulgaria) with a resonant frequency 300 ± 100 kHz, force constant 40 N/m and tip radius < 10 nm.

2.9. Image analysis to quantify fibril alignment and coverage

The area covered with collagen fibrils and the degree of fibril alignment were quantified with NIH ImageJ 1.37v software. Three samples containing two channels with collagen fibrils were analyzed for each condition at three selected positions of