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Fibrillar collagen assembled in the presence of glycosaminoglycans to constitute bioartificial stem cell niches *in vitro*

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Fibrillar collagen was reconstituted from mixtures of monomeric tropocollagen and heparin or hyaluronic acid, respectively. Turbidity measurements were utilized to follow the fibrillar assembly and demonstrated the influence of the concentration of the glycosaminoglycan on the maximum optical densities. Thin film coatings of maleic anhydride copolymers were utilized for the covalent immobilization of the fibrillar assemblies to solid supports. Quantification of surface-bound collagen was accomplished by ellipsometry and HPLC-based amino acid analysis indicating that less collagen was immobilized in the presence of the glycosaminoglycans. SEM and AFM revealed various sizes and shapes of the immobilized fibrillar assemblies if collagen fibrils were prepared in the presence of heparin or hyaluronic acid. Human hematopoietic stem cells (HSCs) were cultivated on the surface-bound collagen fibrils and the migration of adherent cells was studied by time-lapse microscopy. Migration rates on fibrillar structures were significantly lower then on tropocollagen indicating a more intimate contact of HSCs to the fibrillar substrates.

1. Introduction

Human hematopoietic stem cells (HSCs) localized in niches of the bone marrow have the ability both of self-renewal and differentiation into all blood cell lineages. Homing, proliferation and differentiation of HSCs are triggered by interactions with stromal cells, proteins and glycosaminoglycans of the extracellular matrix (ECM), and growth factors present on stromal cells or bound to ECM [1]. While collagen and other adhesion proteins are mainly thought to mediate cell adhesion, glycosaminoglycans efficiently support the presentation of growth factors through specific binding sites.

Protocols for the coating of cell culture substrates often make use of monomeric collagen I. However, the monomeric tropocollagen forms only thin layers which do not resemble the functional meshwork of fibrillar collagen. Therefore, procedures were developed to reconstitute native-type fibrils from tropocollagen solutions *in vitro* [2]. These structures can be endowed with other components of extracellular matrix and thus establish three-dimensional meshworks providing tissue-mimetic environments for adherent cells [3].

The stability of collagen coatings to polymer surfaces can be enhanced by the activation of surface carboxy groups by water-soluble carbodiimide and subsequent collagen immobilization [4], by cross-linking of collagen to polycaprolactone membranes using glutaraldehyde [5] or by photochemical immobilization to polystyrene cell-culture plates [6]. Cross-linking was further used for the covalent attachement of glycosaminoglycans as heparin [7] and hyaluronic acid [8] to collagenous scaffolds. However, irradiation and chemical reactions can affect the polymer carrier materials as well as the proteins and glycosaminoglycans themselves. Therefore, there is a need for immobilization methods preventing alterations of the involved biomacromolecules.

We describe herein the deposition of fibrillar collagen assemblies to reactive polymer substrates, which mediate the covalent attachment of proteins and thus enhance the stability of the immobilized collagen assemblies. The method requires no wet chemical treatment or irradiation. In contrast to previous approaches described for the complexation or immobilization of glycosaminoglycans to collagen [7, 9] heparin and hyaluronic acid were added to solutions of monomeric collagen to induce an electrostatically driven complexation and subsequently fibrillogenesis was initiated during the

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immobilization of the biopolymers onto the polymer coated carriers. The migration of adherent human hematopoietic stem cells (HSCs) was analyzed to obtain a first measure of the interaction of cells with the matrix coatings.

2. Materials and methods

2.1. Preparation of thin polymer films

For the preparation of stable thin films of poly(octadecene *alt* maleic anhydride) (PO-MA) (Polysciences Inc., Warrington, PA), a copolymer solution in THF (Fluka, Deisenhofen, Germany) was spin-coated (RC5, Suess Microtec, Garching) onto cleaned and aminosilanized SiO_2-surfaces (silicon wafers or glass coverslips) prepared according to the procedure described elsewhere [10].

2.2. Reconstitution and immobilization of fibrillar collagen assemblies

Formation of collagen I fibrils performed in the presence of varying concentrations of the glycosaminoglycans (GAGs) heparin and hyaluronic acid was accomplished by a previously described procedure [11]. Briefly, an acidic solution of monomeric collagen (Vitrogen, Cohesion Technologies, Palo Alto, CA) was mixed with different concentrations of heparin and hyaluronic acid in PBS (all from Sigma, Steinheim, Germany), respectively, at 4 °C. Adjustment of pH and ion strength was accomplished by addition of 10 fold concentrated PBS and 0.1 M sodium hydroxide. An increase in temperature resulted in the initiation of fibrillogensis. Fibril formation at 37 °C was followed by turbidity measurements at 313 nm using a Specord S10 (Carl Zeiss, Jena, Germany). For the deposition and immobilization of fibrillar assemblies fibril formation was initiated in the presence of PO-MA-coated cell culture carriers and the resulting gel bulk phase was removed after indicated times leaving a thin collagen layer on the substrate.

2.3. Protein quantification, ellipsometry and assessment of surface topography

For protein quantification only well-defined areas of 2 cm^2 were coated with collagen using home-built immobilization chambers. Quantification of immobilized collagen was performed by acidic hydrolysis and subsequent HPLC analysis as described elsewhere [12]. Briefly, substrates were subjected to vapour hydrolysis using 6 M HCl at 110 °C for 24 h and subsequently neutralized. The extraction of hydrolyzed amino acids from the surface was accomplished by repeated rinsing with definite volumes of 50 mM sodium acetate buffer at pH 6.8. The released amino acids were chromatographically separated after pre-column derivatization with ortho-phthalaldehyde on a Zorbax SB-C18 column (4.6 $\times$ 150 mm, 3.5 μm, Agilent Technologies, Böblingen, Germany) using an Agilent 1100 LC-system (Agilent Technologies, Böblingen, Germany) with fluorescence detection.

The thickness values of the deposited collagen layers were determined by ellipsometry using the single-wavelength device (632 nm) ELX-02 (DRE Dr. Riss Ellipsometer Bau GmbH, Ratzeburg, Germany). Refractive indices were determined to 1.5037 for the PO-MA film and 1.6035 for the dried collagen layer, respectively, and kept invariant for the comparison of samples series.

The surface pattern of deposited collagen layers was analyzed by atomic force microscopy (AFM) as well as by scanning electron microscopy (SEM). AFM images (Bioscope, Digital Instruments, Darmstadt, Germany) were obtained from air dried samples. Furthermore, the dried samples were gold coated with a sputter coater (SCD 050, BAL-TEC, Schalksmühle, Germany) and examined by means of a scanning electron microscope (XL 30 ESEM FEG, FEI-Philips, Eindhoven, Netherlands).

2.4. Cell culture and time lapse microscopy

Hematopoietic stem cells were isolated from cord blood using immunomagnetic selection (CD34 Progenitor Cell Isolation Kit, Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were incubated in CellGro medium (CellGenix, Freiburg, Germany) supplemented with 300 ng/ml FLT3-L and 50 ng/ml IL-3 (both R&D Systems, Mannheim, Germany), and 300 ng/ml SCF (CellSystems, St. Katharinen, Germany). After 4 days migration of cells was monitored with a Zeiss Cell Observer (Carl Zeiss Oberkochen, Germany), a special controlled incubator mounted on a heated scanning optical microscope table. The microscope was connected to a digital camera (Zeiss AxioCam Colour). Migration was monitored for 90 min and images were taken every 30 s and subsequently analyzed with the software AxioVision (Zeiss).

3. Results and discussion

Formation of fibrillar collagen in the presence of heparin and hyaluronic acid was followed in the bulk volume by turbidity measurements. 4 h after initiation of the fibril formation graded optical densities were observed for the different preparations (Fig. 1). Increasing portions of heparin and hyaluronic acid caused a slight decline in the maximum optical densities compared to pure collagen solutions so that the fibril formation was obviously affected by both polysaccharides. The differences in turbidity values of fibrillar collagen preparations have to be ascribed to different amounts of fibrils, to varied fibril diameters or to both.

Planar cell culture carriers coated with thin films of poly(octadecene *alt* maleic anhydride) (PO-MA) were utilized for the immobilization of the reconstituted ECM structures. The reactive polymer film was covalently linked to glass slides or silicon wafers which had been modified before with aminopropyldimethylmonoethoxysilane to generate surface-bound amine-groups [10]. The coating of polymer-modified substrates with collagen-GAG assemblies was accomplished by the exposure of the slides or wafers to a mixture of dissolved collagen and heparin or hyaluronic

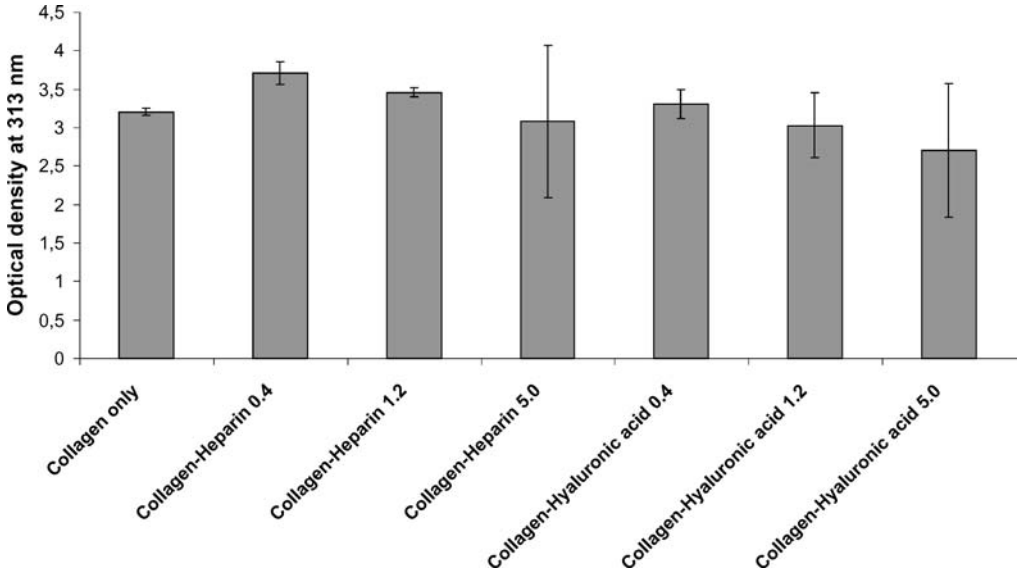

Figure 1 Turbidity measurements of fibrillar collagen formed in the presence of heparin and hyaluronic acid. Initial concentration of the nonfibrillar collagen solution was 1.2 mg/ml. Concentrations of the GAGs were 0.4, 1.2, and 5.0 mg/ml, respectively. Measurements were performed 4 h after initiation of fibrillogenesis.

acid at different concentrations and subsequent initiation of fibrillogenesis. The covalent immobilization was accomplished by the reaction of maleic anhydride groups with lysine side chains of collagen. The immobilized assemblies were characterized with respect to the deposited amount of protein using ellipsometry and acidic hydrolysis/HPLC-based amino acid analysis, respectively (Table I). In comparison to pure fibrillar collagen that provided layers of about 80 nm thickness the deposition of mixtures of fibrillar

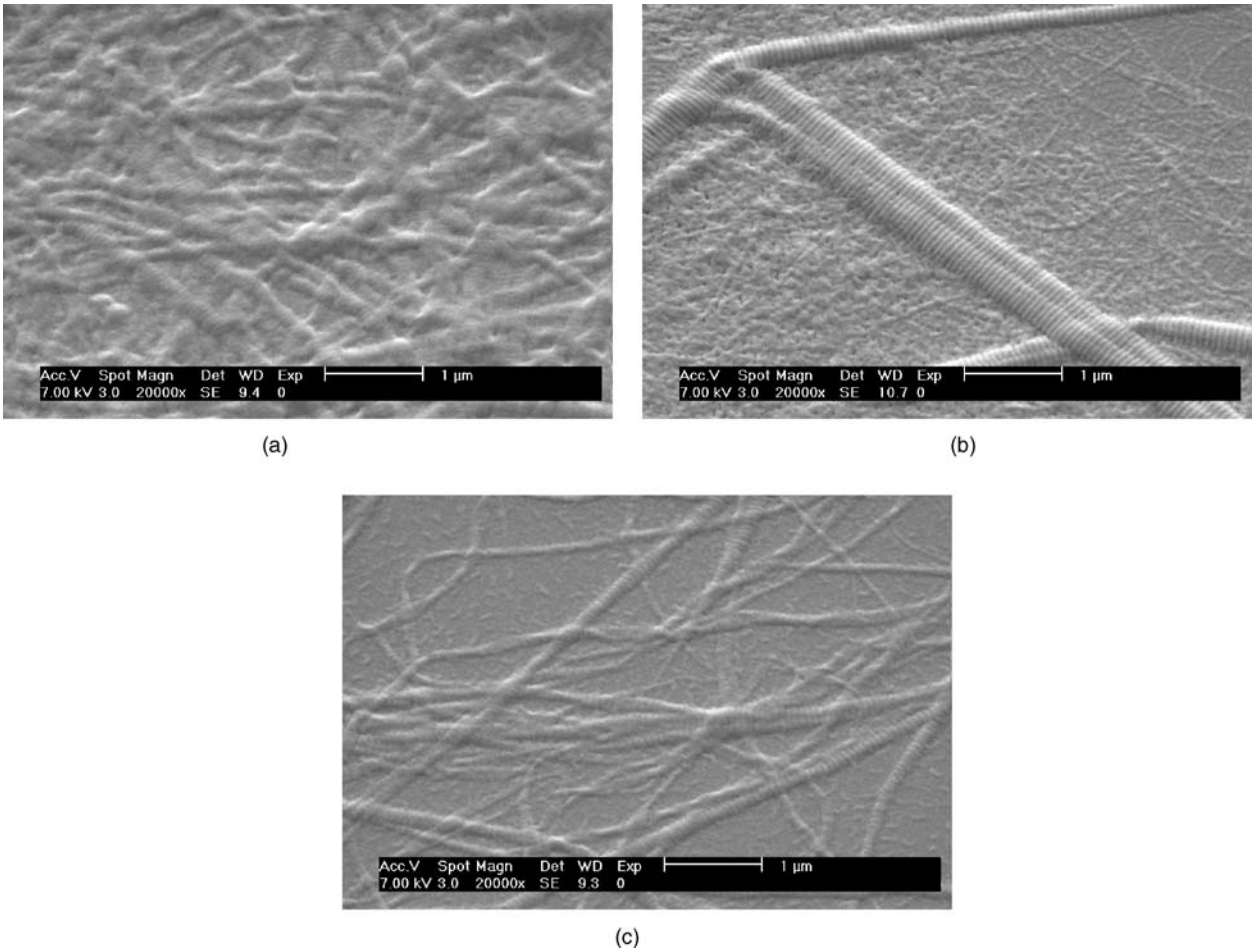

Figure 2 SEM images of collagen (a), collagen-heparin (b), and collagen-hyaluronic acid assemblies (c) immobilized to PO-MA coated surfaces. Initial collagen concentration was 1.2 mg/ml, GAG concentrations were 0.4 mg/ml. Fibrillogenesis was terminated by displacement of the bulk gel phase after 90 min.

TABLE I Immobilization of collagen-GAG conjugates onto PO-MA coated wafers. Fibrillogenesis and immobilization of fibrillar collagen (1.2 mg/ml) was performed for 2 h at 37 °C in the presence of heparin and hyaluronic acid (0.1, 0.4, 1.2 and 5.0 mg/ml, respectively). Thickness of the layers was ellipsometrically determined using the refractive indices of 1.5037 for the PO-MA film and 1.6035 for the dried collagen layer. The collagen amount was quantified by acidic hydrolysis and subsequent HPLC analysis. Table shows one exemplary result

Immobilized assemblies	Thickness of layer (nm)	Collagen amount ($\mu g/cm^2$)
Collagen	78.8	10.8
Collagen/Heparin 0.1 mg/ml	n.d.	4.7
Collagen/Heparin 0.4 mg/ml	15.2	2.5
Collagen/Heparin 1.2 mg/ml	26.1	2.3
Collagen/Heparin 5.0 mg/ml	34.4	3.2
Collagen/Hyaluronic acid 0.1 mg/ml	n.d.	2.5
Collagen/Hyaluronic acid 0.4 mg/ml	n.d.	3.2
Collagen/Hyaluronic acid 1.2 mg/ml	13.7	1.9
Collagen/Hyaluronic acid 5.0 mg/ml	29.6	3.2

collagen and GAGs resulted in noticeable thinner layers in the range between 14 and 32 nm (for fixed, pre-set refractive index values). The quantification of surface bound collagen by amino acid analysis after acidic hydrolysis confirmed the trends obtained by the ellipsometric measurements. Significantly lower amounts of collagen were found on the polymer coated surfaces where collagen fibrils were immobilized in the presence of GAGs.

Furthermore, the topography of immobilized collagen-GAG assemblies was studied using SEM and AFM (Figs 2 and 3). The images showed different surface patterns compared to meshworks of pure collagen and significant differences in shape and dimensions of the fibrils depending on the GAGs were found. Whereas in the presence of heparin wide and straight fibers were formed hyaluronic acid caused the formation of twisted fibrils. Additionally, many small fibrils were detected in the spacings between the large fibrils and the deposited layers appeared smooth and thinner in comparison to the pure collagen layers. However, the surface pattern of the immobilized collagen-GAG assemblies did not represent the volume phase composition of the formed collagen gels regarding fibril numbers and sizes since gels which were desiccated on planar surfaces revealed quite different surface structures. More large and elongated fibrils were found in desiccated collagen gels formed in the presence of heparin and hyaluronic acid (data not shown). Thus, fibrils attached to the polymer coated surfaces did not reflect the meshwork characteristics of the fibrils in the gel bulk phase. These observations confirmed the results of ellipsometry and the quantification of surface-bound collagen. Obviously, the binding of the negatively charged GAGs to collagen caused by ionic interactions as described for chondroitin-6-sulfate [13] and via specific ligand-binding sites as specified for heparin [14] may partially occupy the positively charged side chains of lysine which therefore cannot serve for the attachment of collagen to the PO-MA films. Furthermore, the presence of GAGs in collagen gels most probably resulted in a weaker cohesion of the fibril meshworks and therefore a reduced amount of fibrils remained on the polymer-coated surface after removal of the gel volume phase.

Cell migration studies were performed utilizing time-lapse microscopy in order to analyze cell-material contacts. For that purpose, hematopoietic stem cells isolated from umbilical cord blood were incubated with various collagen substrates. After 4 days of cultivation, non-adherent cells were removed and the migration of the remaining adherent cells was monitored with a time-lapse microscope for 60 min. In each experiment 10 to 32 cells were assessed. The highest migration rates were observed on non-fibrillar tropocollagen (Fig. 4). In contrast, migration rates on fibrillar collagen were reduced, indicating a stronger interaction of cells with the fibrillar collagen. Interestingly, the migration rates on surface-bound collagen-hyaluronic acid and collagen-heparin assemblies were again slower compared to pure collagen fibrils. One explanation for the reduced motility of the cells on gels containing hyaluronic acid could be the high expression of the adhesion molecule CD44, the receptor for hyaluronic acid, on HSCs, which plays an important role in homing and mobilization of HSCs [15].

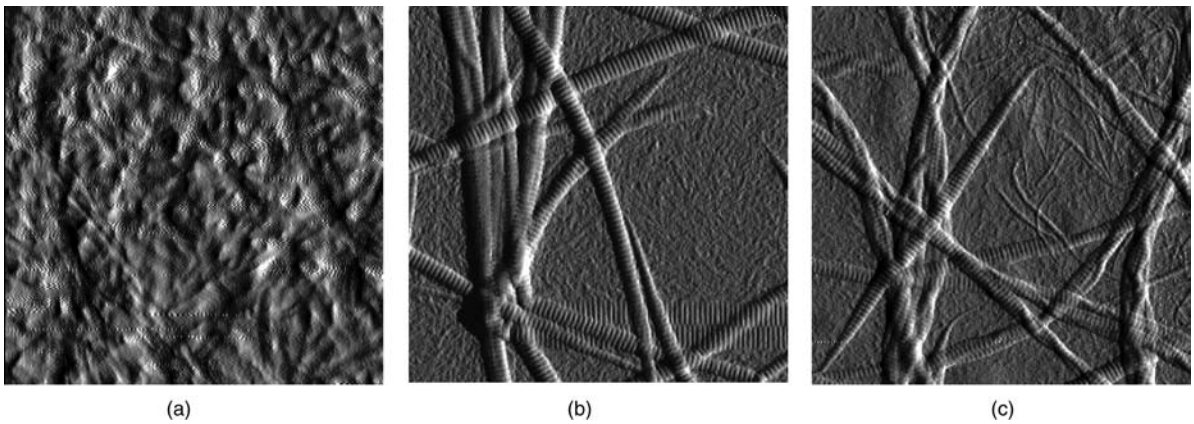

Figure 3 AFM images of collagen (a), and collagen-heparin (b), and collagen-hyaluronic acid (c) assemblies immobilized to PO-MA coated surfaces. Initial collagen concentration was 1.2 mg/ml, concentrations of the GAGs were 5 mg/ml. The collagen gel resulting from fibrillogenesis was removed after 90 min. Image size is 5 μm.

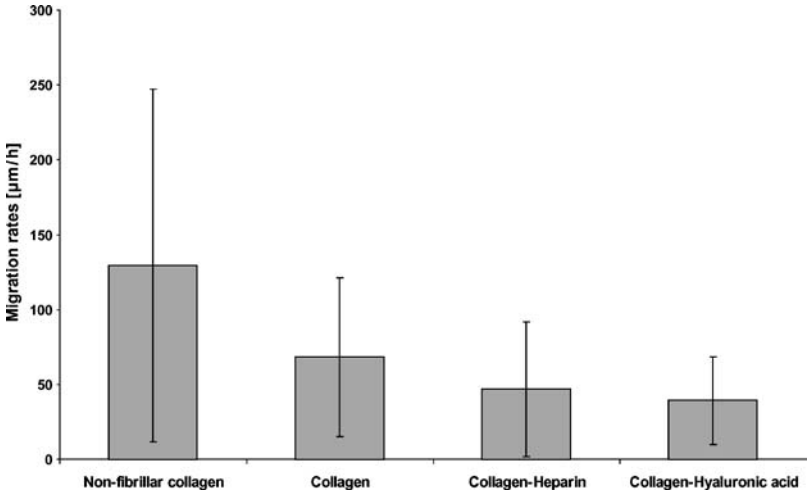

Figure 4 Migration rates of HSCs isolated from cord blood on various collagen samples. Migration was assessed with a Zeiss Cell-Observer over a time period of 60 min. Concentration of GAGs in the fibril forming solution was 0.4 mg/ml.

4. Conclusions

The self-assembly of collagen was analyzed by turbidity measurements in solutions containing varied concentrations of collagen and GAGs indicating an influence of the presence of heparin and hyaluronic acid on the maximum optical densities. Assemblies of collagen and the GAGs heparin and hyaluronic acid were further utilized for the functionalization of solid surfaces coated with reactive polymer films. Quantification of surface-bound collagen by HPLC-based amino acid analysis and ellipsometry as well as surface topographic analysis revealed differences of collagen layers with regard to immobilized amounts, layer thicknesses, and pattern of deposited fibrils depending on the GAG and its deployed concentration. Migration of adherent HSCs on top of the different variants of biopolymer matrices revealed a dependence of both composition and surface pattern of the respective fibrillar assemblies.

Altogether, the introduced method for the immobilization of biopolymer assemblies via *in situ* attachment onto reactive polymer films provides a simple and efficient means for the functionalization of cell culture carriers and biomedical devices. As a major advantage, the technique does not require wet chemical treatments or irradiation and, therefore, conserves structural and functional characteristics of the biopolymers and the carrier materials.

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