ChemComm

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Engineered 3D microporous gelatin scaffolds to study cell migration†

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Here we present a facile method to fabricate microporous hydrogel scaffolds that can be functionalized with a chemokine gradient. These scaffolds allow studying cellular responses in a 3D environment.

Cell migration is a key step during physiological processes such as tissue repair, inflammatory responses and embryonic development,[1,2] as well as during pathological processes such as metastasis of cancer cells.[3] Although some cells migrate randomly, others migrate towards a gradient of chemokines and other chemoattractant molecules secreted by several cell types at specific tissues in the body to form either a soluble gradient or a solid-phase gradient that results from chemo-attractant binding to extracellular matrix (ECM) components.[4] To influence cellular migration it is crucial to understand the underlying mechanisms behind cell migration. So far, many studies focused on cell migration using 2 dimensional set-ups[5–7] that are an oversimplification of a complex reality.[8] 3D structures offer a more realistic representation of the micro-environment of living tissues and allow evaluating both cell translocation and matrix remodelling.[9] To study *in vitro* the three-dimensional response of cells to biomolecules such as chemokines, a more realistic mimic of the ECM implies, among other features, an interconnected microporous network that can be functionalized with a chemoattractant gradient that is stable over a prolonged period.

In this paper we present an all-in-one system whereby a 3D matrix is functionalized with a chemoattractant gradient as well as seeded with cells to study 3D cell migration. Using platelet-derived growth factor-BB (PDGF-BB) as chemo-attractant and dermal fibroblasts as cell type we demonstrate a gradual chemoattractant deposition onto the micropore surface perpendicular to the global scaffold surface. Subsequently we demonstrate directional migration of fibroblasts to the chemo-attractant gradient in the scaffolds.

Gelatin is a protein obtained from hydrolysed collagen and commonly used as a cell culture substrate since it readily allows cellular adhesion and growth. Depending on the method of hydrolysis,[10] gelatin bearing a net negative or positive charge under physiological conditions is available which offers the opportunity to functionalize the gelatin based scaffolds through electrostatic interaction or through covalent chemistry involving carboxylic acid or primary amine containing peptide moieties. Chemokines commonly bear a net positive charge under physio-logical conditions, allowing them to interact *in vivo* with anionic glycosaminoglycans of the ECM.[11] Therefore, we used gelatin with an overall negative charge in further experiments in view to bind chemokines through electrostatic interaction. However, it should be noted that the system presented in this paper can easily be extended to bind anionic proteins, by using cationic gelatin instead of anionic gelatin.

Concentrated aqueous solutions of poly(vinylpyrrolidone) (PVP; a non-ionic hydrophilic polymer) and gelatin phase separate, forming a water-in-water emulsion, upon mixing.[12] Varying the PVP and gelatin concentration as well as the PVP to gelatin ratio allows us to create a homogeneous emulsion after sonication (Fig. 1A; gelatin was fluorescently labelled with Atto 647). To obtain solid structures at physiological temperature, we synthesized methacrylamide-modified gelatin by reacting methacrylic anhydride with gelatin, forming amide bonds between the carbonyl group of the methacrylic anhydride and primary amine groups of the gelatin. After emulsifying PVP and gelatin, the gelatin's pending methacrylamide moieties were crosslinked through radical polymerization to form a stable 3D network (Fig. S1 (ESI†) gives a schematic overview).
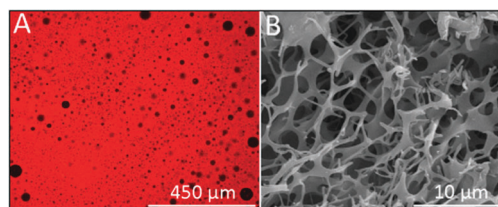
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Fig. 1 (A) Confocal microscopy image of the PVP/gelatin emulsion after sonication. Gelatin was labeled red fluorescent with Atto 647. (B) Scanning electron microscopy images of the microporous 3D gelatin hydrogel.

Note that this crosslinking was required to retain the integrity of the hydrogels at physiological temperature due to the solubility of gelatin above 37 °C. Finally, the PVP was leached out by extensive washing with water and the gelatin hydrogels were snap frozen and lyophilized. Scanning electron microscopy (SEM; Fig. 1B) reveals a 3D structure with interconnected pores within the micrometre range. Such cryogels strongly differ from cryogels that have macropores. These are fabricated by subjecting a gelatin solution to a controlled cooling cycle, inducing phase separation between a gelatin rich phase and ice crystals of several hundreds of micron that will form the pores after lyophilisation. Dubruel and co-workers, including recent work from our group, have demonstrated that cell seeding on top of cryogels readily leads to repopulation of the whole cryogel volume.[13,14] In contrast, we hypothesize that the small pores of the microporous gelatin scaffolds reported in this present paper will prevent cells to spontaneously 'flush' through the 3D matrix.[12] Fig. S2 (ESI†) shows a SEM image of a gelatin-based cryogel, illustrating well the macroporosity that is created using a 'cryo'-approach.[15]

Previously, it has been reported that several cell types infiltrate into 3D scaffolds by creating a path through proteolytic degradation of the hydrogel.[16] To know whether the formation of a polymethacrylamide network, crosslinking the gelatin, has an impact on the biodegradability of the 3D gelatin hydrogels, round shaped gelatin hydrogels (7 mm × 3 mm (diameter × height)) were incubated at 37 °C in aqueous medium containing pronase, a mixture of several proteases capable of cleaving virtually every peptide bound. Optical microscopy images recorded at several time points (Fig. S3, ESI†) show that the hydrogels first reduce in size, likely due to surface erosion and then completely disintegrate through both surface as well as bulk erosion. This demonstrates that the crosslinked gelatin can still be degraded through enzymatic hydrolysis, which paves the road to use these 3D microporous gelatin hydrogels to study cell migration dependent on the secretion of matrix metalloproteinases.

In a subsequent series of experiments we aimed to functionalize the microporous 3D gelatin hydrogels with a bioactive solid phase chemokine gradient. For this purpose platelet derived growth factor-BB (PDGF-BB; 24.3 kDa) was chosen. PDGF-BB is a disulfide-linked homodimer of 2 B-polypeptide chains that might interact with all 3 dimeric combinations of the α- and β-receptor and is involved in cellular activities including chemotaxis and proliferation. For example, attraction of fibroblasts to a wound site mediated through PDGF-BB released by blood platelets is an essential event during the early phase of tissue repair.[17] As PDGF-BB has an isoelectric point of 9.8 it bears a net positive charge at physiological pH and is expected to bind to anionic gelatin hydrogels (isoelectric point of 5) through electrostatic interaction. To gain more insight into this process, we spin coated a gold-coated surface plasmon resonance (SPR) sensor chip with methacrylamide-modified gelatin followed by UV crosslinking to closely mimic the surface of the 3D microporous gelatin hydrogels. Next we investigated the interaction between PDGF-BB in solution (at physiological pH) and gold-supported gelatin hydrogel films. Fig. 2A demonstrates that as soon as PDGF-BB was injected (start injection time = 500 s) through the flow cell, an increase in response units was observed, indicating binding to the gelatin hydrogel film. Since the extent of increase in response units depended on the concentration of the injected solution,
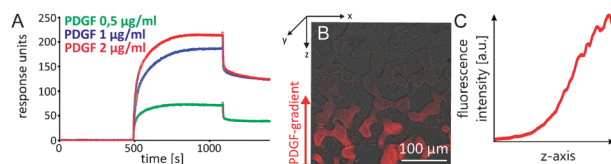

Fig. 2 (A) Interaction between PDGF-BB and gelatin, measured by surface plasmon resonance. (B) Confocal microscopy image of a cross-section showing PDGF-BB (red fluorescence) binding to the 3D microporous gelatin hydrogel. The image is an overlay of the fluorescence and transmission channel. (C) Fluorescence distribution along the Z-axis, calculated as an average of three large areas.

PDGF-BB binding to gelatin is dose-dependent. At the end of the injection ($t = 1100$ s) dissociation took place between loosely bound PDGF-BB and gelatin resulting in a small decrease in the response units. At a concentration of 1 μg ml^{-1} PDGF-BB, saturation of PDGF-BB binding to gelatin was reached since at the end of the injection steps the curves of the response units for 1 μg ml^{-1} and 2 μg ml^{-1} PDGF-BB converged. An extra washing step at $t = 1700$ s (Fig. S4, ESI†) did not result in a further decrease in the response units demonstrating that the binding between PDGF-BB and gelatin was stable. The SPR data demonstrate that PDGF-BB can interact with gelatin indicating that it should be possible to functionalize the 3D microporous gelatin hydrogel with a gradient of PDGF-BB through electrostatic interaction.

To stimulate directional cell migration, we functionalized the 3D microporous gelatin hydrogels with a PDGF-BB solid phase gradient perpendicular to the flat hydrogel surface. Therefore, hydrated disc shaped hydrogels were closely fit into the upper compartment of a transwell system. The lower compartment contained 500 ng ml^{-1} PDGF-BB. Since the gelatin is in excess and no flow was created during the loading procedure, we hypothesize the bound PDGF-BB to exhibit a concentration gradient. To verify the spatial distribution of the adsorbed PDGF-BB, the hydrogel discs were microtomed perpendicular to their flat surface and stained with anti-PDGF-BB, a secondary biotin-labeled antibody and Cy5-streptavidin. The confocal microscopy image in Fig. 2B clearly demonstrates the formation of a PDGF-BB gradient onto the micropores with depth around 200 μm. However, due to the low detection limit of the technique, this gradient might enclose a larger area. The graph in Fig. 2C indicates a fairly linear gradient perpendicular to the hydrogel surface. The amount of PDGF-BB that bound to the microporous hydrogels was measured using radioactive-labeled (*i.e.* iodine-131 (^{131}I)) PDGF-BB and relative to the weight of the gelatin hydrogel a binding of 3.2 ($\pm$0.2) ng PDGF-BB per mg gelatin hydrogel was measured.

Finally, we aimed to assess whether the adsorbed PDGF-BB gradient on the 3D microporous hydrogels was bioactive and could induce cell migration towards this gradient. Under steady state conditions, fibroblasts are non-migratory cells, however chemical as well as mechanical cues may trigger fibroblast migration.[8] Several fibroblast chemoattractants acting in a concentration dependent manner have been identified, including PDGF-BB, TNF-α, IL-4 and IL-13.[18] Dermal fibroblasts were seeded onto the microporous hydrogel discs on the opposite side of the PDGF-BB gradient. After 1, 4 and 8 days of culturing, the discs were microtomed perpendicular to their flat surface.
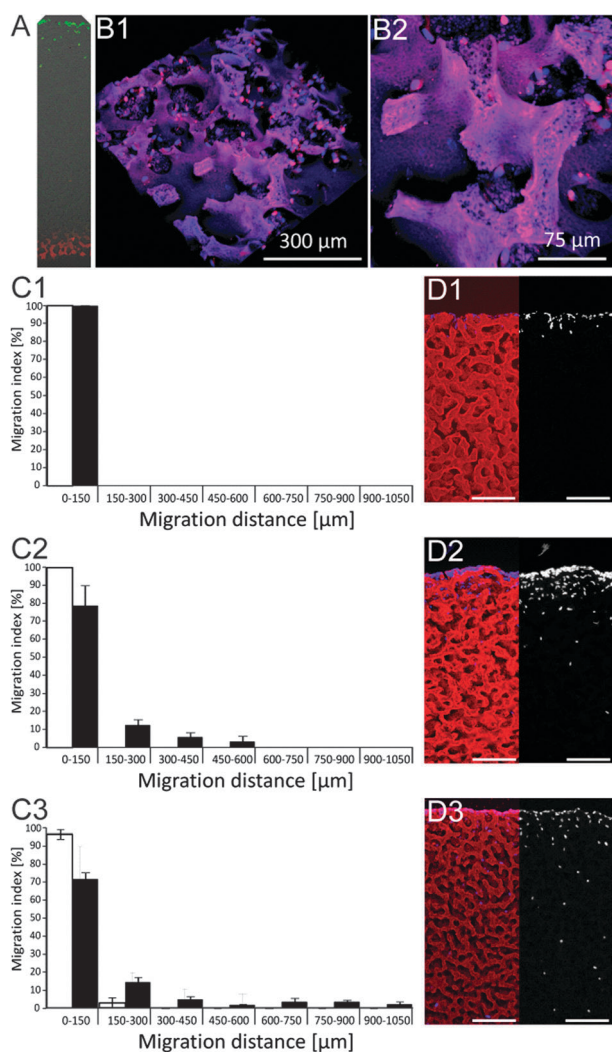
Fig. 3 (A) Fluorescence microscopy image of a cross-section of a gelatin hydrogel stained for PDGF-BB (red color) and fibroblasts (labeled with CellTrackerGreen). (B) 3D two-photon confocal microscopy cross sections at different magnification. Cell nuclei were stained blue fluorescent with DAPI and the purple color of the hydrogel is due to deep red fluorescence from Cy5-labeled poly-ʟ-arginine used to stain the gelatin hydrogel. (C) Migration of fibroblasts seeded on non-functionalized gelatin hydrogels (white bars), or PDGF-BB-functionalized gelatin hydrogels (black—corresponding fluorescence microscopy images). The black and white images solely depict the blue fluorescence channel, allowing a better view of the distribution of cells. Cell nuclei were stained with DAPI, the red fluorescence is due to adsorption of Cy5-labeled poly-ʟ-arginine to gelatin (scale bar = 200 μm).

The fluorescence microscopy images in Fig. 3A, recorded after 1 day of culturing, show the PDGF-BB gradient and the fibroblasts one at each side of the microtomed hydrogel discs. Fig. 3B shows 3D two-photon confocal microscopy cross-sections, visualizing the cells residing both in the pores as well on the hydrogel surface.

Quantitative analysis (Fig. 3C) of fibroblast migration was performed by measuring the migration distance of the cells in PDGF-BB loaded scaffolds and non-PDGF-BB loaded hydrogels as control.

After 1 day incubation, fibroblasts were located in the superficial area (0–150 μm) of both the non-functionalized gelatin hydrogels

as well as of the PDGF-BB-functionalized hydrogels. After 4 days, some fibroblasts were migrated towards deeper located areas (up to 600 μm) of the gelatin hydrogels in response to the PDGF-BB gradient. This migration was absent in the non-functionalized gelatin hydrogels. At day 8, fibroblasts remained in the superficial area of the non-functionalized gelatin hydrogels, while in the PDGF-BB-functionalized samples, fibroblasts had migrated over more than 1 mm towards the PDGF-BB gradient.

To exclude that cell migration was induced by a proliferative effect of PDGF-BB, we seeded non-functionalized hydrogels seeded with fibroblasts and cultured them in cell medium containing similar PDGF-BB concentrations relative to the gelatin scaffold functionalized with the PDGF-BB gradient. After an incubation period of 8 days, fibroblasts resided in the superficial area of the scaffold demonstrating that cell migration in the previous experiment was indeed evoked by the applied PDGF-BB gradient. These findings clearly demonstrate the crucial role of a gradient to allow directional cell migration and highlight an important asset of the microporous gelatin hydrogel scaffold presented in this paper.

In conclusion, we have designed in this paper a synthetic mimic of the extracellular matrix comprising a 3D microporous hydrogel that can easily be functionalized with a chemoattractant gradient. This system holds potential to study cellular responses *in vitro* in a three dimensional environment over prolonged periods to gradients of well defined biomolecules or to complex fluids, *e.g.* obtained from conditioned cell media or specific physiological fluids.

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