The effect of matrix characteristics on fibroblast proliferation in 3D gels

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Abstract

Engineering synthetic hydrogels on a molecular basis to introduce natural features that are important in instructing cell behavior is becoming increasingly crucial in biomaterial-based approaches for regenerative medicine and in cell biology to study cell–matrix interactions in three-dimensions (3D). Here, we used collagen gels and exploited the design flexibility of the biological, biochemical and physical characteristics offered by a PEG-based hydrogel system to systematically study the effect of specific extracellular microenvironments on the behavior of primary human fibroblasts in 3D. We firstly found that the proliferation profiles of fibroblasts from different patients cultured within collagen gels (3D) differed significantly from their behavior observed on tissue culture plastic (2D). Furthermore, using the biomimetic PEG-based matrix we showed that cell proliferation in 3D could be selectively manipulated via alteration of the gel characteristics. In particular, this study revealed that, in spite of matrix sensitivity to proteases (e.g. MMP) and the presence of cell-integrin binding sites, at high stiffness (elastic modulus, G′ >1200 Pa) the matrix acts as a barrier for cells cultured in 3D. Finally, a comparison between the biomimetic PEG-based and collagen gels indicated that differences in their viscoelastic behaviours, determined by the nature of network structures and cross-links, may influence the mechanism(s) cells employ to remodel their 3D extracellular microenvironment. In conclusion, these studies highlight that for proliferation in 3D, compared to 2D, cells require strategies to overcome the physical impediment posed by the matrix. We also demonstrate that by exploiting the design flexibility of the characteristics offered by these biomimetic hydrogels, it is possible to separately investigate complex aspects characterizing the cell–matrix interactions in 3D; this has the potential to have great impact in regenerative medicine, as well as in cell biology and cancer research.

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1. Introduction

To date, naturally-derived matrices represent the gold standard in biomaterials-based approaches to tissue regeneration (e.g. collagen, fibrin and de-cellularised scaffolds) and also for matrix-based 3D cell culture models (e.g. Matrigel™ and collagen). This is primarily because of their physiological properties [1–3]. However, due to the intrinsic features of their components (e.g. purified ECM macromolecules), naturally-derived materials possess limited design flexibility, in terms of being able to modulate their characteristics, batch-to-batch variability and poor handling characteristics [3,4]. As a consequence, these drawbacks may substantially reduce not only their adaptability to a wide range of the therapeutic applications, but also impact on reproducibility of experimental outcomes and on comparative studies when used as 3D cell culture models. Traditionally, synthetic materials lack specific biological signals, such as cell recognition or activation sites [3,5]. However, recent advances in biomaterials science have enabled the development of synthetic provisional matrices that mimic key features of the natural ECM, thereby providing functionalities that are critical in establishing physiological cell–matrix interactions [6–8].

Over the last decade an increased range of biomaterial technology platforms has been developed with the ability to incorporate key features of the natural ECM in synthetic matrices. These approaches mainly utilize chemical (e.g. Michael-type conjugate addition [9–13], photopolymerization [14–16], enzyme-catalysed [17–20] or physical (e.g. self-assembling peptide [21–23]) cross-linking reactions that occur in mild and near-physiological conditions, thereby allowing material formation in the presence of cells [7,24]. For example, incorporation of biologically active molecules
(e.g. cell-binding sites and growth factors), together with modifications that render these matrices susceptible to cell-activated proteases (e.g. MMPs and plasmin) has been demonstrated to be essential for mimicking key characteristics of native ECM to promote and facilitate tissue regeneration [9–11,25–27]. These emerging approaches in biomaterials science and regenerative medicine have been also considered as useful tools in cell biology, in particular in cancer research, as they provide tunable and reproducible 3D cell culture models to study complex interplays between cells and their extracellular microenvironment [8,28–30]. This has been especially driven by the increasing evidence that cells cultured in 3D, compared to those grown in 2D, exhibit functional characteristics that are more similar to their in vivo behavior [28,31,32].

To highlight the potential of artificial matrices to investigate complex cell–matrix interactions important in regenerative medicine, as well as in fundamental cell biology, we present here a comprehensive and systematic investigation of the influence of key natural ECM characteristics on cell behavior in 3D. Additionally, we also compared the behavior of cells cultured within synthetic matrices versus those cultured in collagen gels. For these investigations, we have exploited the factor XIII (FXIII)-catalysed cross-linking mechanism of fibrinogen during natural fibrin coagulation to form artificial hydrogels from peptide-functionalized multiarm-PEG [19,20]. These synthetic hydrogel materials allow modular tailoring of their biological and biochemical functions, as well as enable easy and independent modification of their physicochemical characteristics. As previously demonstrated, desired biomolecules (e.g. the RGD cell-adhesion motif and growth factors) can be specifically and stably incorporated into the hydrogel network during matrix formation and by means of the same enzymatic reaction [19,20] (Fig. 1).

Furthermore, matrix proteolytic degradability can be modulated by incorporation of protease substrates with different sensitivities within the hydrogel network [20].

### 2. Materials and methods

#### 2.1. Vinyl sulfone functionalization of 8-arm-PEG-OH

Functionalization of multiarm-PEG (8-arm-PEG-OH, Mn ~ 40,000 kDa, Nektar, Huntsville, AL, USA) with vinyl sulfone groups (8-arm-PEG-VS) was performed as previously described [33,34]. 1H NMR (CDCl₃) was used to confirm the reaction product and determine the extent of end group conversion, which was approx. 90%. 8-arm-PEG-VS: δ = 3.4–3.7 ppm (PEG backbone), 6.0–6.1 ppm (CH₂ – CH–SO₂, d, 2H), 6.3–6.4 ppm (CH₂–CH–SO₂, d, 2H), 6.7–6.9 ppm (CH₂ – CH–SO₂, dd, 2H). The yield of the functionalized product was approximately 88%.

#### 2.2. Precursor design and preparation for FXIII-cross-linked PEG-based hydrogels: 8-PEG-Gln and 8-PEG-MMP-lys or 8-PEG-noMMP-lys

Hydrogel precursors were produced as previously described [19,20]. Briefly, peptides (NeoMP S.A., Strasbourg, France) containing complementary substrates for FXIII-catalysed cross-linking (with key residues for cross-linking indicated in bold: NQEQVSPFRCQ–NH₂ (TG-Gln, Mn = 1358.5 g/mol, purity HPLC > 95%) or Ac-PFGCYPGK–NH₂ (TG-MMP-lys, Mn = 1717.2 g/mol, purity HPLC > 90%), were coupled to 8-arm-PEG-VS (prepared as described above) via Michael-type conjugate addition between vinyl sulfone groups of end-functionalized PEG and thiol of peptide cystein residues (indicated in italic), yielding the hydrogel precursors 8-PEG-Gln and 8-PEG-MMP-lys, respectively (Fig. 1). After the coupling reaction the solutions were dialysed (MWCO: 10000, PIERCE) extensively against ultra pure ddH₂O and were subsequently freeze-dried (Christ Alpha 2–4, Germany). The TG-MMP-lys peptide also included a matrix metalloproteinase (MMP) substrate [35] (underlined, i indicates cleavage position) to render the final hydrogels susceptible to proteolytic degradation [9]. To form MMP-insensitive hydrogels the precursor 8-PEG-MMP-lys was replaced by 8-PEG-noMMP-lys, in which the MMP-degradable

![Figure 1](image_url)

**Fig. 1.** Schematic of material precursor preparation and FXIII-catalysed hydrogel formation. Peptide-functionalized branched-PEG macromers, 8-PEG-Gln and 8-PEG-MMP-lys or 8-PEG-noMMP-lys, are used to form proteolytically sensitive or insensitive hydrogels via FXIII-catalysed reaction. Simultaneously, by means of the same reaction, biomolecules (e.g. the cell-integrin binding peptides, TG-Gln-RGD) can be stably incorporated within the hydrogel network to obtain multifunctional biomimetic matrices.
substrate was substituted by the MMP-insensitive sequence -DGQGICAF- (9,35) (TG-
moMMP-Lys: Ac-FRGGDGQICAFRGFCG-NH2, Mw = 1639.4 g/mol, purity HPLC = 95%).

2.3. FXIII-catalysed PEG-based hydrogel formation

Conditions for hydrogel formation have been optimized previously [19]. Briefly, gels (Fig. 1) were formed by FXIII-catalysed cross-linking of stoichiometrically balanced 8-PEG-Gln and 8-PEG-MMP-Lys (for MMP-sensitive gels) or 8-PEG-moMMP-
lys (for MMP-insensitive gels), produced as described above, in Tris-buffer (TBS, 50 mOsm, pH 7.6) containing 50 mM calcium chloride and 10.7 U/mL of FXIII (kindly donated by Baxter, Austria). For example, to produce 120 μL of 5% w/v hydrogel solutions the following solutions were used: 43.17 μL (8-PEG-MMP-Lys, 67.64 mg/mL), 45.83 μL (8-PEG-MMP-Lys or 8-PEG-moMMP-Lys, 67.20 mg/mL), 19 μL sparse volume (for the incorporation of RGD- or RGD-ptide and/or of encapsulation of cells; or Tris-Buffer for blank gels), 6 μL calcium chloride (1 x) and 6 μL of active FXIII (213.5 U/mL, activated as described previously [19]). For hydrogels with lower polymer dry mass-% (w/v), the polymer solutions were diluted accordingly. RGD- or RGD-func-
tionalized hydrogels (50 μL) were produced by addition of the peptides NQEQVSPL-
GRGDSPG-NH2 or NQEQVSPHLRGDGSPG-NH2, respectively (TG-Gln-RGD- or TG-Gln-RDG for blank gels), 6 μL calcium chloride (1 x) and 6 μL of active FXIII (213.5
U/mL, activated as described previously [19]).

For hydrogels with lower polymer dry mass-% (w/v), the polymer solutions were diluted accordingly. RGD- or RGD-functionalized hydrogels (50 μL) were produced by addition of the peptides NQEQVSPL-GRGDSPG-NH2 or NQEQVSPHLRGDGSPG-NH2, respectively (TG-Gln-RGD- or TG-Gln-RDG for blank gels), 6 μL calcium chloride (1 x) and 6 μL of active FXIII (213.5 U/mL, activated as described previously [19]).

2.4. Formation of Collagen-I hydrogels

A single batch of rat tail collagen type I solution (Collagen-I, BD Biosciences) was used to produce 3.12 mg/mL collagen gel. For example, to prepare a 100 μL volume of gel the following solutions were used: 76 μL of collagen-I stock solution (4 mg/mL), 1.7 μL sodium hydroxide (1 x), 8.45 μL 10% PBS (Tablets, Oxoid, England) leaving 13.85 μL of sparse volume (e.g. for the encapsulation of cells). The pH was controlled with a paper pH-indicator and adjusted by varying the volume of the sodium hydroxide solution and sparse volume [36]. Hydrogels were formed at 37 °C and in a 5% CO2 humidified atmosphere for 35 min.

2.5. Hydrogel mechanical tests

Blank PEG-based hydrogels with 1.5%, 2% and 2.5% w/v polymer dry mass were prepared as described above by dilution of concentrated precursor solutions. Liquid drops (53 μL volume) for the PEG-based hydrogels were sandwiched between two hydrophobic glass microscopy slides (coated with SigmaCote, Sigma, USA) with 1.2 mm thick spacers and were gelled at 37 °C and 5% CO2 humidified atmosphere for 35 min. After swelling in PBS, gel discs of 6 mm in diameter were produced using a biopsy punch and were then stored in the same buffer prior to mechanical measurements. Collagen gel discs (200 μL, 3.1 mg/mL) were prepared as described in the previous section and were subsequently swollen in PBS.

Rheological measurements were performed using an Advanced Rheometric Expansion System (TA Instruments, version 6). PEG-based or collagen hydrogel discs were placed between the two parallel plates of the rheometer and compressed up to 80% of their original height to avoid slipping. Strain sweeps at constant frequencies were conducted in order to confirm that measurements were performed within the linear viscoelastic behaviour range of the hydrogels. Elastic shear modulus (G′) was recorded at constant strains (10–15% for the PEG-gels and 3% for the collagen gels) as a function of the frequency (1–100 rad/s). The value of G′ for each swollen disc sample was calculated as the average of the G′ values measured between 1 and 10 rad/s (Supplementary Fig. 3). All measurements were conducted at room temperature (23 °C).

2.6. Isolation and culture of human primary fibroblasts

Human dermal fibroblasts (HDF) were isolated from skin surgical discards from consenting patients undergoing breast reductions or abdominoplastics at the St. Andrews and Wesley Hospitals, Brisbane, Australia (Ethic approval ID 3865H issued by Human Research Ethics Committee for QUT and the above Hospitals). Briefly, the skin biopsies were digested in 2.5% Trypsin (GIBCO) at 4 °C overnight. After removing the epidermis the fibroblasts were isolated from the dermis by overnight digestion of the chopped dermal pieces in DMEM containing 0.05% collagenase A (GIBCO) and 100 μL at 37 °C and in a 5% CO2 humidified atmosphere. After centrifugation (2000 rpm for 10 min) of the digested products the pellet containing the fibroblasts was resuspended and the cells were subsequently cultured in flasks under standard cell culture conditions (culture media: DMEM containing 5% FBS, 1% antibiotic-antimycotic and 1% L-Glutamine; 37 °C and 5% CO2 humidified atmos-
phere). Fibroblasts were passaged using 0.05% tryp-EDTA (GIBCO) and were used in experiments at passages 5 to 10.

2.7. Encapsulation and culture of human primary fibroblasts within hydrogels

In both PEG-based and collagen gels, fibroblasts were added to the hydrogel precursor solutions through the “sparse volume” as the last component in the mix (2.5 x 105 cells/mL gel, if not otherwise specified). Immediately thereafter and prior to cross-linking, 20 μL of the hydrogel precursor solutions were sandwiched between two sterile and hydrophobic glass microscopy slides (previously treated with SigmaCote, Sigma, USA) with 1.5 mm spacers. After polymerization at 37 °C and in a 5% CO2 atmosphere for 35 min the 20 μL droplet gels, were removed from between the two glass slides and were then immersed in cell culture media. The media was replaced weekly (if not specified otherwise), and the collected conditioned media was stored frozen (–80 °C) prior to further analysis.

For inhibition studies the cell culture media was supplemented with 20 μM of GM6001 (Biomatrat) MMP-inhibitor (Cheminco, Teneula, Canada). The inhibitory media for the 3D studies was present between day 7 to day 14 in the culture of the degradable PEG-based gels with 1.5% and 2% w/v polymer dry mass and day 2 to day 7 for 0.3% collagen gels. After removing the media with the inhibitor, the gels were washed twice in fresh cell culture media. The inhibitory media for the 2D cell culture was present between day 2 to day 6. After removing the inhibitory media, the cell layers were washed with PBS before adding fresh cell culture media.

2.8. Staining and confocal laser scanning microscopy

For examination of cell morphology, fibroblasts cultured within the different hydrogels discs produced as described in the previous sections, were stained for F-actin and nuclei. After the completion of the cell culture experiments the samples were washed in PBS and subsequently fixed and permeabilized with 4% para-
formaldehyde PBS solution (PFA, Sigma Aldrich, Germany) containing 0.25% Triton X-100 (Merck, Germany) at room temperature (RT) for 30 min. Samples were subsequently washed with 0.1% glycine in PBS, followed by repeated washing in PBS. Cytoskeletal F-actin fibres were stained for 1 h with 0.8 units/mL rhodamine-
labelled phallolidin (Molecular Probes, Oregon, USA) in PBS containing 1% bovine serum albumin (BSA, Calbiochem, Canada) for 1 h at RT and protected from light. Cell nuclei were then stained with 2.5 μg/mL 4,6-diamidino-2-phenylindole (DAPI). Molecular Probes, USA) for 50 min. After staining and prior to fluorescence microscopy, the samples were washed extensively with PBS. Confocal laser scanning microscopy (CLSM) images of cells cultured within the hydrogels were recorded using Leica’s SP5 system (Leica Microsystems, Germany). Z-series of approximately 100 equidistant x-y-z scans at 2 μm intervals were acquired and projected onto a single plane using the software provided by the SP5 system.

2.9. Cell proliferation

For 2D fibroblast culture the cells were seeded in 24-well plates at 500 cells/well, and for 3D cultures the fibroblasts were encapsulated and cultured within either collagen-I or PEG-based hydrogel matrices as described in the previous sections. At different time points the samples were washed with PBS and the tissue culture plates (for 2D studies) and hydrogel discs (for 3D cell culture experiments) were frozen at −80 °C.

The CQY昆T™ cell proliferation assay kit (Molecular Probes, USA) was used according to manufacturer’s instruction to measure the total DNA content of the samples harvested at different time points. Briefly, after thawing the samples were digested with 0.5 mg/mL Proteinase K (Invitrogen, USA) diluted in phosphate buffered saline (PBS, 20 mg/mL). The cell suspension was then washed with PBS. DNA was extracted from the cell suspension and DNA content was measured using a POLARstar OPTIMA reader (software: FLUOstar OPTIMA; ExBMG Labtech GmbH, Germany) at the emission wavelength λem of 520 nm (excitation at λex = 480 nm).

The samples and DNA standards were measured in this assay in triplicate. For the 3D culture samples the fluorescence signals obtained from blank hydrogels (i.e. without cells, cultured and processed similarly to those with cells) were used as background values (blanks). The proliferation data were expressed as n-fold DNA increase, representing the ratios between the DNA content per well (in 2D prolif-
eration studies) or per gel (in 3D proliferation experiments) measured at different time points and the amount of DNA in cells after 6 h after seeding (for 2D cultures) or per gel at day 1 (for 3D cultures).

2.10. Gelatin zymography

Conditioned media collected from the cell culture studies were analysed for MMP-2 and MMP-9 activity and compared to MMP-2 and MMP-9 standards (MMP-2, active, human, Calbiochem, Canada; MMP-9, monomer, human neutrophil, Cal-
biochem, Canada; both diluted in 50 mM tri-Base, 50 mM NaCl, 10 mM CaCl2, 0.05% Brij-35). Briefly, loading buffer (5 x, 0.5% bromophenole blue, 50% glycerol, 0.5 x Tris-Base, pH 6.8, 10% SDS) was mixed with the conditioned media samples; and these were then loaded onto freshly prepared 4% stacking/10% resolving poly-
acylamide gels containing 1 mg/mL porcine gelatin (Sigma, Germany) at 3.5 μg total protein per lane (total protein content in condition media samples was determined...
using the Better Bradford™ Assay Kit, Pierce, USA). Electrophoresis was carried out using a Mini PROTEAN Tetra cell-System (BioRad, China) and using a Tris–glycine running buffer (25 mM Tris-Base, 246 mM glycine, 0.1% Sodium dodecyl sulphate; pH 6.8) under non-reducing conditions at 150 V on ice until the dye front ran off the gel. Following electrophoresis the gels were washed in 2.5% Triton X-100 (Merck, Germany) for 30 min and again for 60 min to remove SDS. The samples were incubated in incubation buffer (50 mM Tris-Base, 10 mM CaCl2, 50 mM NaCl; pH 7.6) at 37 °C for 2 h on a shaker. The gels were then stained with 0.25% Coomassie Brilliant Blue R (Sigma Aldrich, Germany) in 45% Methanol, 10% Acetic acid and finally were destained with destaining solution (40% Methanol, 10% Acetic acid) [37,38]. Gelatinolytic activity in the samples was visualised by clear bands in the stained gels and compared to molecular weight and standards.

2.11. Statistics

Statistical analyses were performed using full-factorial univariate analyses of variance (ANOVA). Data are expressed as mean ± standard deviation (SD). Probability (P) values <0.05 were considered as significant.

3. Results and discussion

3.1. The importance of investigating cell–biomaterial interactions in 3D

Preliminary experiments were performed to investigate the proliferative capacity of dermal fibroblasts obtained from three different patients on conventional tissue culture plastic-ware (2D) and within the collagen gels (3D). In 2D there was no significant difference in the proliferation of cells between the three patients (Supplementary Fig. 1A). However, when cultured in 3D within collagen gels, the fibroblasts from patients 1 and 2 proliferated similarly, but their proliferation rates were statistically different compared to that found with fibroblasts from patient 3 (Supplementary Fig. 1B).

These differences between fibroblasts cultured in 2D and 3D are not surprising and are in agreement with an increasing body of evidence suggesting that the behaviour of cells cultured in 2D vs. 3D may differ significantly [8,28,31,32]. For example, it has been shown that some phenotypical differences between normal and malignant epithelial breast cells, as well as fundamental cell–matrix interactions, could only be observed and studied in 3D cell cultures [28,39,40]. Consequently, these and our data suggest that cell culture experiments carried out in 2D may not be sufficiently selective to dissect differences between cell types or cells harvested from different patients. The additional dimensionality of stimuli that the cells are experiencing in 3D vs. 2D, which is mainly characterized by a different spatial distribution of cell-adhesion sites and the presence of a physical barrier, may significantly influence integrin ligation, cell contraction, signalling from the outside to the inside of the cells, and ultimately cell behaviour [8,31,32]. For example, fibroblasts in 2D cultures normally exhibit a flat morphology with dorsal-ventral polarity and large lamellipodia [32,41]; and when they migrate and proliferate they do not experience major physical barriers [42]. In contrast, when encapsulated in a 3D matrix, the fibroblasts re-acquire a natural spindle-shaped morphology [32,41] and need to overcome impediments posed by the surrounding environment in order to be able to proliferate and migrate [42]. Accordingly, cells in 3D vs. 2D are not only exposed to different stimuli, but also require different mechanisms to interact with and to overcome their extracellular microenvironment [8,42,43].

In light of these observations, and in view of the fact that tissue regeneration processes as well as most cell-ECM interactions in vivo occur in 3D, with cells embedded within a constantly changing and evolving extracellular microenvironment, our studies focused on a systematic investigation of the influence of specific hydrogel characteristics on fibroblasts cultured within differently functionalized materials. In particular, cell morphology and proliferation in 3D were investigated systematically as a function of matrix-incorporated adhesion ligands, matrix proteolytic degradability and mechanical properties. The fibroblasts with the least proliferative capacity in 3D (patient 1) were used in the studies reported here.

3.2. Fibroblast proliferation in 3D as a function of matrix characteristics

Preliminary studies were performed to investigate the biochemical (Supplementary Fig. 2) and physicochemical (Supplementary Fig. 3) characteristics of the FXIII-cross-linked PEG-gels. Hydrogels with different biochemical characteristics were obtained by incorporation of specific peptide substrates in the hydrogel network with selected sensitivities to MMP proteolytic degradation [34,44] as shown in Supplementary Fig. 2 and previously described [9,19,20,26]. Physicochemical characteristics of MMP-sensitive and insensitive FXIII-cross-linked hydrogels were subsequently modified by variation of the polymer dry-mass concentrations (2.5%; 2% and 1.5% w/v) as demonstrated with rheological and swelling measurements depicted in Supplementary Fig. 3A and B. At constant dry-mass concentrations the elastic shear moduli of the MMP-sensitive and insensitive PEG-hydrogels were similar, indicating that these hydrogels differed exclusively in their biochemical properties (Supplementary Fig. 3B; Table 1).

3.2.1. Matrix biological characteristics: the influence of cell-integrin binding sites

Covalent incorporation of cell-adhesion motifs containing at least the three amino acid peptide RGD within hydrogels (e.g. PEG-NQEQVSPL-RGD(Gly-Arg-Asp) NH2, TG- Glu-Glu-RGD) and equipped with the FXIII substrate on its N-terminus [46] (italics) could be stably incorporated within FXIII-cross-linked PEG-based hydrogels; and this occurred in a one pot reaction simultaneously with material formation (Figs. 1 and 2a).

Human dermal fibroblasts encapsulated within MMP-degradable gels (2% w/v) containing the RGD peptide (50 μM) adopted a typical spindle-shaped morphology and commenced creating interconnected multicellular networks during a cell culture period of 14 days (Fig. 2b). In contrast, fibroblasts cultured in control hydrogels, either with incorporated RGD-peptide (NQEOVSVL-GRDGSPG-NH2, TG-Gln-RGD, 50 μM) or control treatments, i.e. without incorporated cell-binding sites, predominantly remained round, showing some sporadic and limited cell spreading. Similar to the spreading behaviour, cell proliferation was exclusively observed in hydrogels functionalized with the cell-adhesion motif RGD (Fig. 2c). In contrast, the DNA content was observed to decrease in all the control gel conditions over time, indicating that minimal or no cell proliferation occurred (Fig. 2c, Table 1). Inhibition of both cell spreading and proliferation within RGD-functionalized hydrogels were also observed by addition of soluble cRGD-peptides in cell culture media, suggesting that soluble cRGD-peptides were competing with the hydrogel-incorporated RGD-motifs for binding to the cell surface integrins [11,14].

These outcomes highlight the versatility and efficacy offered by this hydrogel technology platform in selectively engineering their biological characteristics. As shown in similar studies, the biological functionality of these hydrogels can be further expanded to include other chemically [46–48] and recombinantly-derived [49] multi-functional ECM-protein domains and growth factors [19,50] to satisfy the increasing demand of tuneable cell instructive materials.
for tissue regenerative applications or in cell biology to investigate specific cell–matrix interactions.

3.2.2. Biochemical characteristics: the importance of matrix proteolytic remodelling

In pathological (e.g. during wound healing and cancer progression) or normal conditions cells normally secrete and activate proteolytic enzymes (e.g. MMPs) in order to invade or remodel their extracellular microenvironment [51]. Accordingly, proteolytic sensitivity, together with the incorporated cell-adhesion sites discussed in the previous section, represents a further key feature that regulates and influences interactions between cells and their extracellular microenvironment.

By exploiting the possibility of tailoring the biochemical characteristics of these FXIII-cross-linked hydrogels (Suppl. Fig. 2), we investigated the influence of the proteolytic sensitivity of the

![Fig. 2. Incorporation of cell-adhesion motifs (RGD) in biomimetic hydrogels influences the morphology and proliferation of fibroblasts cultured in 3D. (A) Schematic of cross-linked gels with incorporated peptides. (B) Confocal laser scanning micrographs of HDF cultured within differently functionalized MMP-sensitive PEG-based hydrogels (2% w/v) at day 14. Cells were double-stained with DAPI (nuclei, blue) and rhodamine-labelled phalloidin (f-actin filaments, red). Fibroblasts within the RGD-functionalized hydrogels (50 μM) spread and started forming a 3D cell network. In contrast, cells remained mostly round in the control gels. (C) Cell proliferation rate within the MMP-degradable PEG-gels (2% w/v) increased only in gels with RGD-peptides incorporated. In contrast, the number of cells in the different control hydrogel conditions remained constant or decreased with culture time. (n = 15 for RGD; n = 3 for RGD + soluble cRGD; n = 6 for RGD and without peptide, ±SD, * = statistically different with P < 0.05, ** = statistically not different).](#)
matrices on fibroblast proliferation within RGD-functionalized MMP-sensitive or insensitive hydrogels with similar mechanical properties (Fig. 3). In both 2% and 1.5% w/v hydrogels, the fold change in DNA content, representing cell proliferation, was significantly higher in MMP-degradable gels compared to that measured in the corresponding MMP non-degradable hydrogels (3A and B, Table 1). Additionally, MMP-inhibition studies confirmed that proteolytic remodelig of the matrix was crucial for cell proliferation in 3D. In both MMP-sensitive PEG-gels, the cell proliferation rate was significantly decreased in the presence of the MMP-inhibitor (GM6001, 20 μM) between day 7 and 14 and was similar to that observed within the MMP-insensitive gels. After the removal of the MMP-inhibitor from the cell culture medium, cell proliferation was restored (Fig. 3A and B). This indicated that the inhibitor GM6001 did not irreversibly affect cells cultured within the biomimetic PEG–hydrogels.

The natural ability of fibroblasts to remodel their extracellular microenvironment by expressing/activating proteolytic enzymes (e.g. MMPs) was also assessed by gelatin zymography of conditioned media collected weekly during cell culture (Fig. 3C). Zymography analysis of conditioned media collected from the different cell culture conditions clearly showed the presence of pro-MMP-2 running at a Mw of approximately 72 kDa [52]; and pro-MMP-2 activity was observed to increase with the presence of cells cultured in the different gels (Fig. 3C). In contrast, bands of pro-MMP-9, running at a Mw of about 92 kDa [52], were detected at very low levels on the zymograms (data not shown), and their intensities did not vary in conditioned media harvested from gels with or without cells (data not shown). This indicated that pro-MMP-9, in contrast to pro-MMP-2, was most likely provided exclusively by the serum present in the cell culture media. These zymography studies revealed that cells cultured within these hydrogels were producing and secreting pro-MMP-2. However, neither active MMP-2 nor other MMPs (e.g. pro– or active MMP-9) could be detected using this method.

These results clearly show that the proteolytic machinery of cells is required for remodelling of the extracellular microenvironment in order to create sufficient space for cell proliferation and the formation of an interconnected 3D cellular network within these biomimetic hydrogel matrices (Suppl. Fig. 4). A similar dependency on cell-mediated proteolytic degradation of the hydrogel network has previously been observed for cell migration in 3D [9,11,53], as well as in vivo for bone regeneration applications, with similar biomimetic hydrogels [9–11,26]. Interestingly, and in agreement with previous investigations [20,25,53], our results also suggest that matrix degradation by proteolysis is most likely confined and localized in the vicinity of the cells [51]. In contrast to our biochemical experiments with soluble active MMP-1 (Suppl. Fig. 2), during long-term cell cultures (21 days) all PEG-based gel conditions remained stable without any observable changes in matrix shape or volume. These observations are also in line with the zymography analysis indicating no detectable activity of soluble MMPs in the culture media. Consequently, hydrogel degradation occurred most likely through cell-secreted pro-MMPs, whose activation and proteolytic activity were associated with cell membrane-bound proteins and proteases [54–56]. Alternatively, or concomitantly, MT1-MMPs have also been shown to play a central role in pericellular ECM proteolysis [57,58].

3.2.3. Physicochemical properties: the influence of matrix stiffness

Fibroblast proliferation and morphology in 3D were also investigated as a function of matrix stiffness while maintaining constant biological (RGD-functionalization of hydrogels, 50 μM) and biochemical (MMP-sensitive or insensitive) characteristics in the

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**Fig. 3.** Matrix proteolytic sensitivity influences cell proliferation in 3D. Within MMP-sensitive or insensitive hydrogels (Fig. 2A) produced with (A) 2% and (B) 1.5% w/v polymer dry mass and functionalized with the cell-adhesion motif RGD (50 μM). MMP-inhibitor (GM6001, 20 μM) was added to the cell culture media of MMP-sensitive gels between days 7 and 14. During the inhibition period cell growth rate was reduced to levels similar to the proliferation rate of cells within MMP-insensitive gels (n = 9–15 without inhibitor, n = 4 with inhibitor, ± SD, * = statistically different with P < 0.05, ** = statistically not different). (C) Gelatin–zymograms of conditioned media harvested after 2 and 3 weeks (with weekly media change) from different hydrogel cell cultures (2% MMP-sensitive, RGD-functionalized hydrogels). All lanes were loaded with 3.5 μg total protein as determined with Bradford-Assay.
hydrogels. Matrices with different shear moduli (G') were obtained by modulation of their polymer dry-mass content as shown in Supplementary Fig. 3B.

Proliferation of HDF encapsulated in the MMP-degradable PEG-gels increased with decreasing material stiffness (Fig. 4, Table 1). In soft PEG-gels (1.5% w/v) the influence of matrix degradability on cell proliferation was already observable after 7 days in culture (Fig. 4A) and became more pronounced in stiffer gels (2% w/v) only after 14 (Fig. 4B) and 21 days (Fig. 4C). In contrast, cell proliferation within MMP-degradable gels with the highest elastic modulus tested, remained low and did not differ from that measured in MMP-insensitive gels with similar mechanical properties. This suggests that at high stiffness, matrix degradability was no longer influencing cell proliferation in 3D, and also suggests that cell-mediated proteolytic activity of the cells is not sufficient to overcome the physical barrier imposed by the highly dense network. Within MMP-insensitive hydrogels the proliferation of fibroblasts was in general observed to increase slightly over time. In addition, proliferation did not vary significantly across the entire range of mechanical properties investigated, except in the soft gels (1.5% w/v) after 21 days (Fig. 4C). This increase in proliferation within the soft 1.5% MMP-insensitive PEG-hydrogels after 21 days may in part be explained by the presence of defects in the gel network that allowed migration and proliferation of cells without proteolytic activity [9,11]. Alternatively, non-specific degradation or the involvement of other proteases not investigated in this study may contribute to slow and local degradation of the hydrogel network, thereby allowing cell proliferation [9,11]. Both phenomena are more likely to occur in gels with lower rather than with higher polymer dry-mass concentration [9,11].

Similar to cell proliferation, cell spreading and their ability to form interconnected multicellular 3D networks was generally observed to be more pronounced within hydrogels with low G' (Fig. 5, Suppl. Fig. 4). After 21 days the fibroblasts formed dense interconnected multicellular networks within the 1.5% and 2%, but not the 2.5% degradable gels. In the MMP-insensitive gels the cells remained essentially round, however, they exhibited a more spread morphology as the initial mechanical properties of the hydrogels decreased, indicating their ability to interact with the RGD-motifs incorporated in the gel network.

Of note, the 3D studies we report here show an opposite trend to that reported previously for cells in 2D on material surfaces. These prior reports indicate that cell proliferation [13,59,60] and spreading [61,62] increase with increasing material stiffness. We suggest that the physical barrier and confinement posed by the hydrogel network in 3D, in contrast to 2D, may underpin these differences. Our observations in this regard are also in agreement with the results described in the previous section, which demonstrated the requirement of cells to remodel their pericellular extracellular environment in order to spread and proliferate.
Similarly, 3D cell migration within biomimetic matrices has previously been shown to follow a comparable profile to our own data. Thus cell migration was shown to be a function of hydrogel physicochemical properties [9,11] and therefore substantial differences to migration in 2D may also be attributed to the biophysical hindrance caused by the matrix in 3D [42]. Accordingly, the higher the mechanical moduli $G_0$, the denser the matrix surrounding the cells, and the more difficult it is for the fibroblasts to create enough space to spread, proliferate and migrate. This is also in accordance with some recent studies reporting that lack of degradability and highly cross-linked hydrogel matrices result in poor cell spreading and low viability in 3D, despite the presence of the cell-binding RGD-motif [60,63]. These represent additional examples of major differences that distinguish cell behaviours and responses in 2D vs. 3D, as well as emphasize some limitations in translating outcomes observed in 2D to the 3D context [31,32,64].

Fig. 5. Morphology of fibroblasts within differently functionalized PEG-based matrices and in collagen gels. In MMP-sensitive PEG-based hydrogels cell spreading decreased with increasing polymer dry-mass concentration (increasing mechanical properties) and the fibroblasts were able to create interconnected multicellular networks within 1.5% and 2% w/v gels. Compared to MMP-sensitive gels, reduced cell spreading was observed within MMP-insensitive PEG-gels over the range of mechanical properties investigated. Fibroblasts encapsulated in collagen gels started to spread soon after cell seeding and formed a dense cell network.
These data demonstrated that through use of these FXIII-cross-linked hydrogels, in contrast to naturally-derived matrices, it is possible to selectively investigate the influence of mechanical characteristics on cell responses in 3D independently from the matrix's biological and biochemical properties. Our outcomes also reveal that hydrogels with higher stiffness impair and act as a barrier for cell proliferation in 3D. This suggests that mechanical properties of therapeutic provisional matrices need to be adapted accordingly in order to elicit the desired cell responses in 3D (e.g. cell viability, proliferation, matrix invasion).

3.3. The dependence of matrix response to cell activities on gel viscoelastic characteristics

Collagen-based biomaterials represent to date the gold standard biomaterials in tissue regeneration applications [1,2], hence they were used here for comparison with the biomimetic PEG-based gels. Fibroblast proliferation in the collagen gels increased quite quickly within the first week. However, it did not vary between day 14 and 21 (Fig. 4, Supplementary Fig. 1B). In addition, fibroblasts encapsulated within collagen gels exhibited a higher degree of cell spreading and the formation of interconnected multicellular networks at earlier time points, compared to the PEG-based matrices (Fig. 5). Subsequently, as a consequence of collagen gel shrinkage (Suppl. Fig. 5), the cells were observed to form a dense network and stopped proliferating, most likely because of lack of space and cell-cell contact inhibition (Figs. 4 and 5) [65].

Collagen gel contraction has been previously reported in a number of studies [66-69]. Interestingly, we found that matrix shrinking was exclusively observed in collagen gels, but not in the different PEG-gels. This could be due to the lower mechanical properties of collagen gels, expressed as G’ modulus, compared to the PEG-gels (Table 1). However, the initial elastic modulus of the collagen gels did not differ considerably from that measured in the 1.5% PEG-gels (MMP-degradable) and the latter did not shrink, even though they contained roughly 3 times the amount of cells than observed in the collagen gels (Fig. 4C, Table 1). In light of these results we hypothesize that not only the elastic modulus, but also the molecular characteristics of these hydrogels, in particular their different cross-linking mechanisms (i.e. covalent cross-linking for the PEG and physical cross-linking for the collagen gels) and the resulting viscoelastic behaviours, may also influence the ability of cells to contract the gels.

The covalently cross-linked PEG-gels behaved like a pure elastic material (Suppl. Fig. 3A). In contrast, the physically cross-linked collagen gels generally displayed a viscoelastic behaviour, characterized by G’ being dependant on testing frequency and relatively higher G” and phase angle δ compared to the PEG-gels (Suppl. Fig. 3A) [53,70]. These rheological characteristics indicate that a portion of the collagen G’ results from entanglement of collagen molecules that may normally slide freely on each other, but in the situation tested they may act as cross-links with increasing test frequencies due to their inability to relax [70]. As a consequence, fibroblasts residing inside the physically cross-linked collagen gels, in contrast to those within the covalently formed PEG-gels, may easily displace and dislocate these physical bonds and cause macroscopic material contraction. These observations are supported by a recent study that correlated microscopic collagen gel shrinkage with the spatial rearrangement and dislocation of collagen fibres during fibroblast-driven remodelling of collagen gels in 3D [68]. Furthermore, matrix contraction has been also reported in 3D culture of fibroblasts within other physically cross-linked gels (formed from self-assembled peptides) that had higher elastic moduli (G’) than reported here, but showed similar viscoelastic behaviour as observed in the collagen gels [23]. This further indicates that viscoelastic characteristics of hydrogel matrices resulting from different cross-linking mechanisms and molecular arrangements may have a profound influence on how materials respond to cell stimuli, i.e. on the processes cells employ to remodel their extracellular environment (e.g. physical vs. biochemical driven remodelling), and vice-versa [71]. The comparison of the hydrogels reported herein suggests that at least within the first three weeks in cell culture, remodelling of the biomimetic PEG-based gels appears to be mainly driven by biochemical processes. In contrast, the data from the collagen gels suggest that this initially occurs predominantly by physical rearrangement of the collagen fibres and subsequently most likely also through proteolytic-driven mechanisms.

4. Conclusion

The tissue regeneration process, as well as most cell-ECM interactions in vivo, occur in 3D environments, with the cells normally embedded within an ECM that is constantly changing and remodelled by the cells themselves. By using PEG-based hydrogels that form via the natural FXIII-catalysed cross-linking mechanism, we systematically investigated and quantitatively showed how the proliferation of fibroblasts cultured within these matrices (in 3D) was influenced by the single characteristics of the hydrogels. In particular, despite the presence of integrin binding sites and the proteolytic degradability, we showed that at higher stiffness the matrix acts as a physical barrier for cells in 3D gels, impeding their proliferation and migration. Interestingly, a comparison between the biomimetic PEG-based and collagen gels indicated that differences in their viscoelastic behaviours, determined by the nature of network structures and cross-links, influence the mechanism(s) cells employ to remodel their 3D extracellular microenvironment. Furthermore, our results also indicate that these synthetic hydrogel matrices, in contrast to naturally-derived materials, may be adapted to mimic desired extracellular microenvironments and to incorporate target therapeutic proteins. This provides great potential for these matrices not only in regenerative medicine applications, but also as 3D cell culture models, for example in cell biology and in cancer research to study fundamental questions related to complex cell-matrix interactions.

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Appendix. Supplementary material

The supplementary data associated with this article can be found in the on-line version at doi:10.1016/j.biomaterials.2010.07.046.

Appendix

Figures with essential color discrimination. Figs. 1–3 in this article are difficult to interpret in black and white. The full color images can be found in the on-line version, at doi:10.1016/j.biomaterials.2010.07.046.

References


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