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Introducing chemical functionality in Fmoc-peptide gels for cell culture ☆

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Abstract

Aromatic short peptide derivatives, i.e. peptides modified with aromatic groups such as 9-fluorenylmethoxycarbonyl (Fmoc), can self-assemble into self-supporting hydrogels. These hydrogels have some similarities to extracellular matrices due to their high hydration, relative stiffness and nanofibrous architecture. We previously demonstrated that Fmoc-diphenylalanine (Fmoc-F_2) provides a suitable matrix for two-dimensional (2D) or three-dimensional (3D) culture of primary bovine chondrocytes. In this paper we investigate whether the introduction of chemical functionality, such as NH_2, COOH or OH, enhances compatibility with different cell types. A series of hydrogel compositions consisting of combinations of Fmoc-F_2 and *n*-protected Fmoc amino acids, lysine (K, with side chain R = $(CH_2)_4NH_2$), glutamic acid (D, with side chain R = CH_2COOH), and serine (S, with side chain R = CH_2OH) were studied. All compositions produced fibrous scaffolds with fibre diameters in the range of 32–65 nm as assessed by cryo-scanning electron microscopy and atomic force microscopy. Fourier transform infrared spectroscopy analysis suggested that peptide segments adopt a predominantly anti-parallel β-sheet conformation. Oscillatory rheology results show that all four hydrogels have mechanical profiles of soft viscoelastic materials with elastic moduli dependent on the chemical composition, ranging from 502 Pa (Fmoc-F_2/D) to 21.2 KPa (Fmoc-F_2). All gels supported the viability of bovine chondrocytes as assessed by a live–dead staining assay. Fmoc-F_2/S and Fmoc-F_2/D hydrogels in addition supported viability for human dermal fibroblasts (HDF) while Fmoc-F_2/S hydrogel was the only gel type that supported viability for all three cell types tested. Fmoc-F_2/S was therefore investigated further by studying cell proliferation, cytoskeletal organization and histological analysis in 2D culture. In addition, the Fmoc-F_2/S gel was shown to support retention of cell morphology in 3D culture of bovine chondrocytes. These results demonstrate that introduction of chemical functionality into Fmoc-peptide scaffolds may provide gels with tunable chemical and mechanical properties for in vitro cell culture.

Keywords: Hydrogels; Cell culture; Self-assembly; Biomolecule; Peptides

1. Introduction

Hydrogel scaffolds for in vitro cell culture, either from natural or synthetic building blocks, are used in a wide variety of disciplines including drug discovery, cell biology and oncology. Ideally a hydrogel scaffold would encompass the following characteristics: (i) ease of handling at 37 °C and physiological pH, (ii) rapid and reproducible gelation under

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mild conditions, (iii) mechanical properties that resemble those of natural tissue, (iv) gel uniformity at the nano, micro and macroscopic levels, (v) compatibility with long term culture, (vi) possibility to formulate gels to match cell type, (vii) optical transparency for straightforward analysis of results. We are interested in developing hydrogels for in vitro cell culture that meet the above criteria, building on our previously reported results on 2D and 3D culture of bovine chondrocytes with hydrogels obtained via self-assembly of 9-fluorenylmethoxycarbonyl-diphenylalanine (Fmoc-F_2) [1].

It has been recognized since the early 1990s that peptide-based self-assembled hydrogels provide useful cell scaffolds for cell culture and tissue regeneration [2]. Peptides and their derivatives are attractive in this regard, as they mimic certain aspects of natural extracellular matrices, such as their nanofibrous architecture and high hydration [3]. A number of successful studies leave no doubt about the feasibility of using peptide-based hydrogels as cell scaffolds for a range of cell types [4–7]. Various researchers have demonstrated that very short peptide sequences, containing just two or three amino acids with aromatic functionality (either provided by amino acid side chains, or by appended aromatic ligands) can form self-supporting nanostructured hydrogels [1,8–12]. In these systems, aromatic interactions as well as hydrogen bonding play key roles [13]. Variations in chemical structure (i.e. changes in amino acid sequence) have significant effects on the properties of the resulting gels [14].

We hypothesized that aromatic short peptide derivatives may provide suitable building blocks for development of cell culture matrices. Indeed, we found that bovine chondrocytes cultured in Fmoc-F_2 and mixed compositions containing 1:1 ratios of Fmoc-F_2 and Fmoc-G_2 (diglycine) as well as Fmoc-F_2 and Fmoc-K (lysine) maintained their metabolic activity for up to 7 days as evidenced by MTT assay [1]. In independent work, Gazit and co-workers simultaneously reported that Fmoc-F_2 hydrogels support cell culture of Chinese hamster ovary cells [11]. This was more recently followed by a study by Liebmann et al., using astrocytes, MDCK and COS 7 cells [15].

It is well known that cell adhesion and proliferation of various cell types can be enhanced (or reduced) by modification of hydrogel surfaces with simple chemical functionality, including amine (NH_2), carboxyl (COOH), and hydroxyl (OH) [16,17]. Similarly, self-assembled monolayers have been used to demonstrate cell-specific responses to chemical functionality on 2D surfaces [18–20]. We hypothesized that by the introduction of building blocks with chemical functionality, it may be possible to create Fmoc-F_2-based hydrogels that are matched with the requirements of a specific cell type.

In addition to chemical functionality, it is well known that mechanical properties of gel scaffolds can affect the behaviour of cells [21,22]. Materials with shear moduli (G') of around 100 Pa are thought to be compatible with culture of soft tissue cells such as neurons and certain epithelial cells. Chondrocytes exhibit increased growth and proliferation markers on stiffer gels with compliance similar to hard cartilage with shear moduli between 10 and 100 KPa. Moduli within the 100 Pa–10 KPa range have been observed for gels based on aromatic short peptide derivatives [9–13] and there may be opportunities to exploit this in directing cell behaviour.

The objectives of this work are therefore to: (a) develop gels formed from a range of compositions of Fmoc-F_2 hydrogels (Fmoc-F_2 and Fmoc-F_2/X where X = Fmoc-lysine (K), Fmoc-serine (S), Fmoc-glutamic acid (D)) at physiological conditions; (b) develop a method to form reproducible gels without having to rely on pH change [1] or dissolution from an organic solvent [11,15]; (c) investigate how different types of cells (bovine chondrocytes, 3T3 fibroblasts and human dermal fibroblasts) respond to these hydrogel scaffolds.

2. Materials and methods

2.1. Materials

Fmoc-F_2 and Fmoc-amino acids (K, S, and D) were purchased from Bachem Ltd., UK. Cell culture inserts for 12-well multiwell plates with 1.0 μm pore size were purchased from Greiner bio-one, UK.

2.2. Formation of the Fmoc hydrogels

Four Fmoc peptide compositions were investigated as shown in Fig. 1a. Fmoc-F_2 and Fmoc-X powders were sterilized under UV light for 30 min prior to use. Fmoc-F_2 and Fmoc-amino acid solutions were prepared separately in glass vials by suspending the powders to a concentration of 20 mmol l^{-1} in sterile H_2O and 0.5 M sterile NaOH. NaOH was added dropwise until the powders were dissolved and a clear solution was formed. During this time samples were placed in a sonicator and vortexed interchangeably. Two solutions were then mixed in varying molar ratios for 2D and 3D culture. The pH of the solutions was checked (7.5–8.0) and 300 μl of each solution transferred to cell culture inserts in 12-well plates. A volume of 1300 μl of standard culture medium (DMEM) (Gibco-Invitrogen) containing 10% fetal calf serum and antibiotics was added to each well (outside the insert) and incubated for 1 h at 37 °C in a humidified atmosphere with 5% CO_2 to allow gelation (Fig. 1c). Following gelation the medium surrounding the inserts was replaced and 200 μl of medium gently added to the surface of the gels. Following overnight culture the pH of the gel stabilized at around pH 7.5 (±0.5). Medium was removed from hydrogels that were used for spectroscopic, microscopic, mechanical characterization.

2.3. Cryo-scanning electron microscopy (Cryo-SEM)

Cryo-SEM was performed using a Philips XL30 ESEM-FG equipped with an Oxford Instrument Alto 25000 cryo

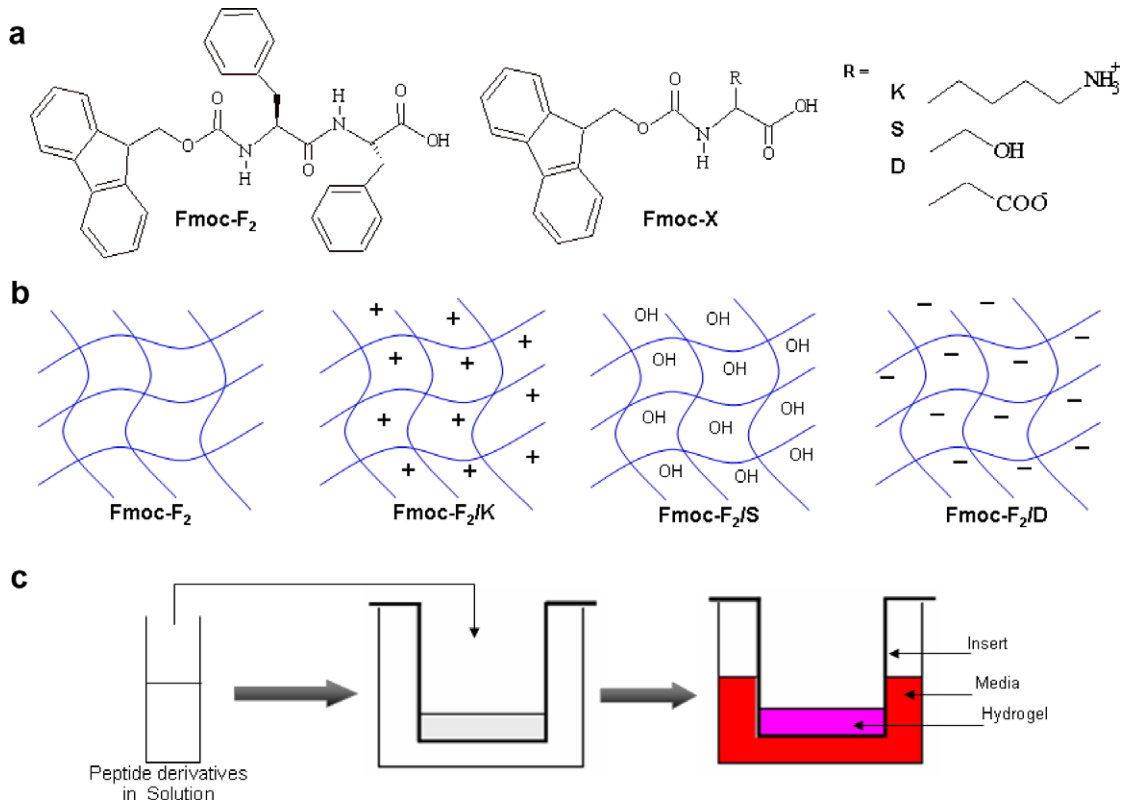

Fig. 1. Preparation of hydrogels. (a) Chemical structures of the four hydrogel systems studied; (b) a representation of the four structures; and (c) a schematic of hydrogel formation in cell culture inserts.

transfer system. Samples of each of the hydrogels were slam-frozen in liquid nitrogen slush in transfer rods before being transferred to the cryostat chamber. The temperature in the chamber was maintained at −150 °C. Using the attachment provided, the top rivet was flicked off and the resulting fractured surface was etched prior to coating with gold. The coated sample was then transferred to the microscope chamber maintained at −150 °C. Images were captured with the microscope operating under high vacuum and with an accelerating voltage of 5 kV. Image J software was used to measure the fibre diameters (http://rsb.info.nih.gov/ij/).

2.4. Atomic force microscopy (AFM)

Imaging was performed using a Veeco Multimode Scanning Probe Microscope with an "E" scanner and a Nanoscope IIIa controller. Muscovite mica and metal AFM support stubs were obtained from Agar Scientific (Stansted, Essex, UK). Olympus high-aspect ratio etched silicon probes with spring constant of 42 N m^{-1} were obtained from Veeco Instruments S.A.S. (Dourdan, France). Hundred microliters of peptide derivative solution, prepared using the standard procedure described above (the sample was diluted with water in 1:1 ratio), was placed on a trimmed, freshly cleaved mica sheet attached to an AFM support stub. The sample was allowed to air dry (for fibril deposition) in a dust-free environment overnight before being washed with 1 ml of distilled water. It was then allowed to air dry again, prior to imaging. Samples were imaged by intermittent contact mode in air. Cantilever oscillation varied between 300 and 350 kHz while the drive amplitude was determined by the Nanoscope software. Height images (5 μm^2) were captured at a scan rate of 1.49 Hz. The set point was adjusted to just below the point at which tip–sample interaction was lost and all images were captured at a relative humidity of <35%. WSxM v3.0 software was used to measure the fibre diameters (www.nanotec.es).

2.5. Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR)

FTIR spectra were obtained from a Nicolet 5700 ATR-FTIR apparatus. The smart orbit accessory was selected to test reflection spectra between 7800 and 370 cm^{-1} for 32 scans at a resolution of 4 cm^{-1}. Hydrogels were transferred onto the crystal for analysis. A background spectrum was subtracted from all the samples' spectra.

2.6. Rheology

To verify the mechanical properties of the resulting hydrogels, dynamic frequency sweep experiments were carried out on a strain-controlled rheometer (Bohlin C-CVO) using a cone-plate geometry (40 mm diameter, 4°). The

experiments were performed at 25 °C and this temperature was controlled throughout the experiment using an integrated electrical heater. Extra precautions were taken to minimize solvent evaporation and to keep the sample hydrated: a solvent trap was used and the atmosphere within was kept saturated. To ensure the measurements were made in the linear viscoelastic regime, an amplitude sweep was performed and the results showed no variation in G' and G'' up to a strain of 1%. The dynamic modulus of the hydrogel was measured as a frequency function, where the frequency sweeps were carried out between 0.1 and 100 Hz. The measurements were repeated at least three times to ensure reproducibility.

2.7. Cell culture

Bovine chondrocytes were isolated from cartilage of the proximal side of the metacarpalphalangeal joint obtained courtesy of Higginshaw abattoir (UK). Mouse Swiss Albino Embryo Fibroblast cells (3T3) were purchased from European Cell Culture (No. 85022108). Human dermal fibroblasts (HDFs) were received from Cascade Biologics. Chondrocytes were cultured in DMEM containing 1000 mg l^{-1} D-glucose, Glutamax™, 10% FBS, 1% penicillin and streptomycin (antibiotics) and 25 mg l^{-1} ascorbic acid. 3T3 and HDF cells were cultured in DMEM containing 1000 mg l^{-1} D-glucose, Glutamax™, 10% FBS, 1% penicillin and streptomycin.

2.8. 2D/3D culture

For 2D cell culture, after overnight incubation of the hydrogel, the cell medium on the surface and around the insert was removed. The medium in the well was replaced with fresh medium. The medium on the surface of the gel was refilled with 100 μl cell suspension (1×10^5 cells) along with 200 μl of new medium. The fresh medium was mixed with the cell suspension using a pipette to even out the cell distribution on the surface.

In 3D culture, 300 μl of the mixed peptide solution was transferred to an insert and mixed with 100 μl of cell suspension (1×10^5 cells). Cell medium (1300 μl) was then added to the well and incubated for 1 h under humidified atmosphere of 5% CO_2 at 37 °C for gelation to occur. After this time, another 200 μl of fresh medium was added on top of the gel surface. In both culture methods, the old medium was replaced every 24 h (in the first 2 days) and subsequently after every 48 h.

2.9. Live–dead staining

A fluorescent live–dead staining assay (Invitrogen) was used to visualize the proportion of viable and non-viable cells present in the hydrogels at each time point. Gels were incubated at room temperature for 20 min in a solution of 4 μM ethidium homodimer-1 (EthD-1) and 2 μM calcein AM in PBS. The labelled cells were then viewed under a Nikon Eclipse E600 fluorescence microscope and images captured using Lucia software. Viable cells were stained with calcein (green), while non-viable cells were stained with EthD-1 (red). For quantitative analysis, a total of 200 cells were counted from each sample over three randomly chosen areas and the live and dead cell counts were recorded.

2.10. LDH assay

A lactate dehydrogenase assay kit (Promega) was used to quantify the number of viable cells in each gel. At each time point, medium was removed from the wells, the gels transferred to microcentrifuge tubes and 500 μl fresh medium added. Samples then underwent three freeze–thaw cycles conducted to lyse the cells. The tubes were centrifuged at 250*g* for 5 min and 50 μl were transferred to wells of a 96-well plate and 50 μl of substrate mix was added. Plates were incubated at room temperature. After 30 min 50 μl of stop solution containing acetic acid was added to terminate the reaction. Absorbance at 490 nm was read in triplicate using a microplate reader and the average value normalized to a standard curve of known numbers of monolayer cultured cells. Cell-free gels were also used as negative controls and background absorbance subtracted from absorbance readings of test samples.

2.11. Cytoskeleton staining

Following hydrogel culture, cell-seeded gels were rinsed in PBS and fixed in 4% paraformaldehyde for 20 min at room temperature. Following fixation the cells were washed twice with PBS before being permeabilized using 0.5% Triton X-100 in PBS for 5 min at 4 °C. After being washed twice with PBS buffer, non-specific staining was blocked using 1% bovine serum albumin (BSA) in PBS for 30 min at room temperature. The samples were then incubated with FITC-conjugated Phalloidin (10 μg ml^{-1}) for 20 min at 4 °C, followed by staining with DAPI (10 μg ml^{-1}) for 2 min at 4 °C. The samples were then mounted onto glass slides with Prolong Gold anti-fade reagent (Invitrogen) and images captured using a Nikon Eclipse E600 fluorescence microscope.

2.12. Histology

To investigate cell distribution and morphology in 2D and 3D culture, gels were fixed in 4% paraformaldehyde at 4 °C overnight then processed to paraffin wax. Five micron sections were cut, mounted onto glass slides and stained with haematoxylin and eosin (H&E) using standard protocols. Haematoxylin stained cell nuclei blue, while eosin counterstained the cytoplasm and extracellular matrix red/pink. All slides were visualized using a Leica RMDB microscope and images captured using a digital camera and Bioquant Nova image analysis system.

2.13. Statistical analysis

To analyse statistically significant differences in the mean live cell counts measured in live–dead staining and LDH assay, analysis of variance (ANOVA) test was performed. One-way ANOVA tests provide F statistics to indicate whether any differences in the mean cell numbers measured are statistically significant. A significant F is indicative of a significant difference in cell numbers where the probability is less than 0.05 ($P < 0.05$) between three time points. The statistical software package SPSS version 15 was used to perform this analysis.

3. Results and discussion

Formation of hydrogels from Fmoc-peptide derivatives is possible using a number of different routes, including dilution from solvents [11,15], application of a sequential pH change [1], or enzymatic (reversed) hydrolysis of non-gelling precursors [23,24]. For applications in cell culture it is attractive for gelation to occur upon dilution into cell culture media [6,25]. This approach has not yet been demonstrated for self-assembling hydrogels based on Fmoc-peptides. A first objective was therefore to develop a method of hydrogel formation in which self-assembly and hydrogelation are triggered by the addition of a standard cell culture media (DMEM) to aqueous peptide solutions. This was achieved by diffusion of media through the semi-permeable membrane of cell culture inserts. This approach allowed formation of hydrogels from Fmoc-F_2 and the three composition gels Fmoc-F_2/X (where X is any of the amino acids S, D, and K; Fig. 1) solutions at a physiological pH between 7.5 and 8. All hydrogels formed within 1 h and had a water content of over 99% (w/w). Cell culture media adjusted the pH of the hydrogel while the media provides nutrients required for cell culture. UV spectroscopic analysis of culture media after 24 h revealed minimal leakage of Fmoc-peptides and amino acids into the solution (0.9% for Fmoc-F_2, 1.4% for Fmoc-F_2/K, 2.1% for Fmoc-F_2/S, and 2.6% for Fmoc-F_2/D), which is related to the low aqueous solubility of the Fmoc group.

Cryo-SEM (Fig. 2A) and AFM (Fig. 2B) were used to investigate the fibrous morphologies of these materials. Micrographs revealed that these four hydrogels posses similar fibrous architecture (Fmoc-F_2/K shown, micrographs for other structures are provided in Supplementary information). In all samples, the individual fibres are highly entangled and form densely packed three-dimensional fibre networks. The following fibre thicknesses were observed: 54 ± 19 nm for Fmoc-F_2, 41 ± 17 nm for Fmoc-F_2/K, 43 ± 14 nm for Fmoc-F_2/S and 47 ± 11 nm for Fmoc-F_2/D (Cryo-SEM) and 65 ± 9 nm for Fmoc-F_2, 32 ± 6 nm

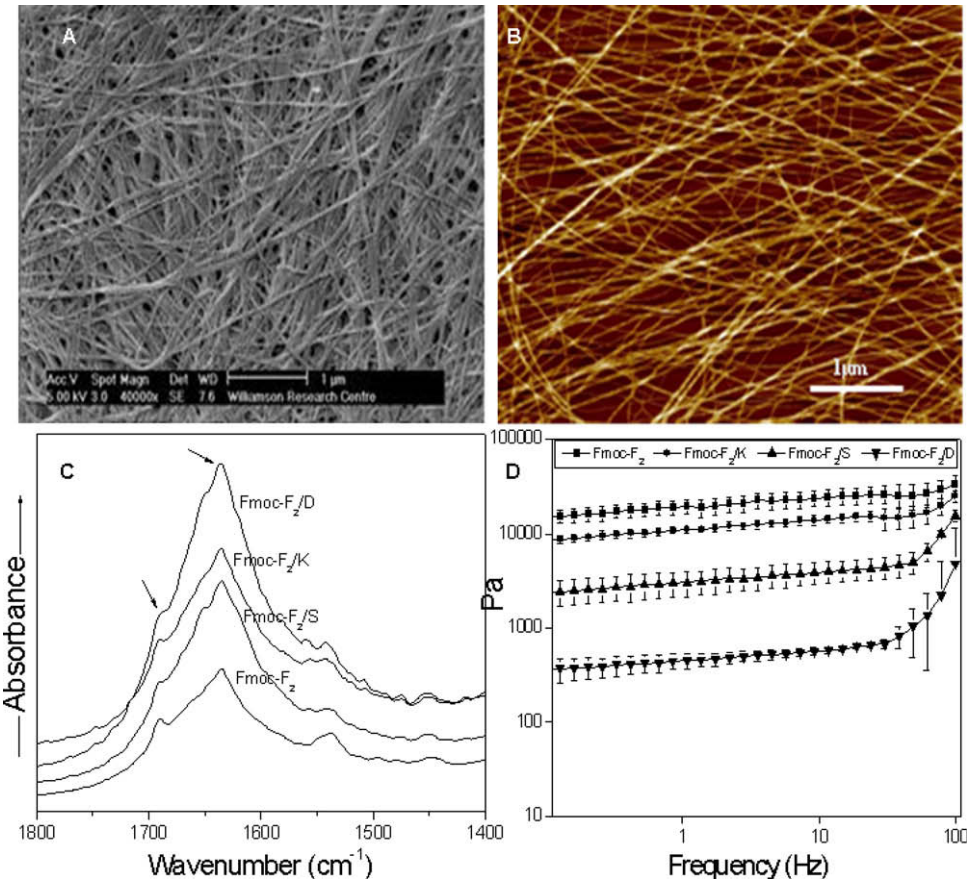

Fig. 2. Characterization of the nanofibrous hydrogel structures. (A) Cryo-SEM image of Fmoc-F_2/K (1:1); (B) AFM image of Fmoc-F_2/K (1:1); (C) ATR-FTIR spectra suggest an anti-parallel β sheet structure for all four structures; and (D) linear viscoelastic spectra for the four samples, 20 mmol l^{-1}, 25 °C.

for Fmoc-F_2/K, 51 ± 5 nm for Fmoc-F_2/S and 58 ± 14 nm for Fmoc-F_2/D (AFM). The two readings for the fibre diameters do not coincide exactly, which may be related to the differences in sample preparation for the two methods (vitrified versus air-dried). Observed differences in thickness show the same order for data obtained by both methods: Fmoc-F_2 > Fmoc-F_2/D > Fmoc-F_2/S > Fmoc-F_2/K.

In the amide I region of the FTIR spectra a distinct peak was observed between 1630 and 1640 cm^{-1} in all samples (Fig. 2C), suggesting that the peptide derivatives within these hydrogels adopt a β-sheet rich structure [26,27]. A secondary medium intensity peak was also observed in all samples around 1690 cm^{-1} suggesting anti-parallel β-sheet arrangements [27–29]. The Fmoc-F_2 components are most likely responsible for these observed interactions [14], with the Fmoc-amino acid components incorporated via π-stacking interactions between the aromatic Fmoc groups and side chain dependent interactions that are electrostatic (D and K) or hydrogen bonding (S) in nature.

Fig. 2D shows the linear viscoelastic responses of the four hydrogels. The average elastic storage modulus values observed over the frequency range of 0.1–100 Hz were 21.2 KPa (Fmoc-F_2), 12.3 KPa (Fmoc-F_2/K) and 3.5 KPa (Fmoc-F_2/S) which highlights the ease with which the modulus of these gels can be tuned by the addition of a second component. Furthermore, the measured elastic storage moduli are similar or up to an order of magnitude higher compared to values reported in the literature for other peptide [11,30] and protein based hydrogels [31]. The storage moduli (G') exceed those of their loss moduli (G'') by a factor of 4.8 (for gel Fmoc-F_2), 4.3 (for gel Fmoc-F_2/K) and 5.1 (for gel Fmoc-F_2/S), indicating that these three materials are true hydrogels with a predominantly elastic component [30,32,33]. This observation is consistent with the highly entangled fibrillar network structure observed by cryo-SEM and AFM (Fig. 2). Interestingly, the Fmoc-F_2/D hydrogel had a significantly lower storage modulus over the same frequency range (average value 502 Pa). We hypothesize that for Fmoc-F_2/K, the electrostatic interactions between terminal carboxylic groups (from Fmoc-F_2) and the side chain amine groups of lysine contribute to hydrogelation via formation of salt bridges, in addition to the backbone hydrogen bonds and the π–π interactions from the Fmoc-F_2. Fmoc-F_2/S may be stabilized through hydrogen bonding of the OH group. Fmoc-F_2/D is significantly weaker, possibly because of electrostatic repulsion due to the presence of additional COO^- groups.

As discussed, materials with shear moduli (G') in the range of 10–100 KPa are known to promote enhanced proliferation in culture of chondrocytes [21,22]. One might therefore expect three of the four gels in this study to have mechanical properties that are matched to chondrocyte cell culture. In addition to proliferation rates, Genes et al. [34] found chondrocyte morphology changes with substrate stiffness. A rounded morphology was observed on weaker substrates compared to more rapid attachment and the development of actin stress fibres on stiffer substrates. The effect of substrate compliance on fibroblasts has also been documented. 3T3 fibroblasts have been shown to have reduced spreading on flexible surfaces compared to rigid surfaces [35]. Wang et al. [36] demonstrated that normal 3T3s (as opposed to transformed 3T3s) responded to and were more sensitive to substrate rigidity. More apoptosis

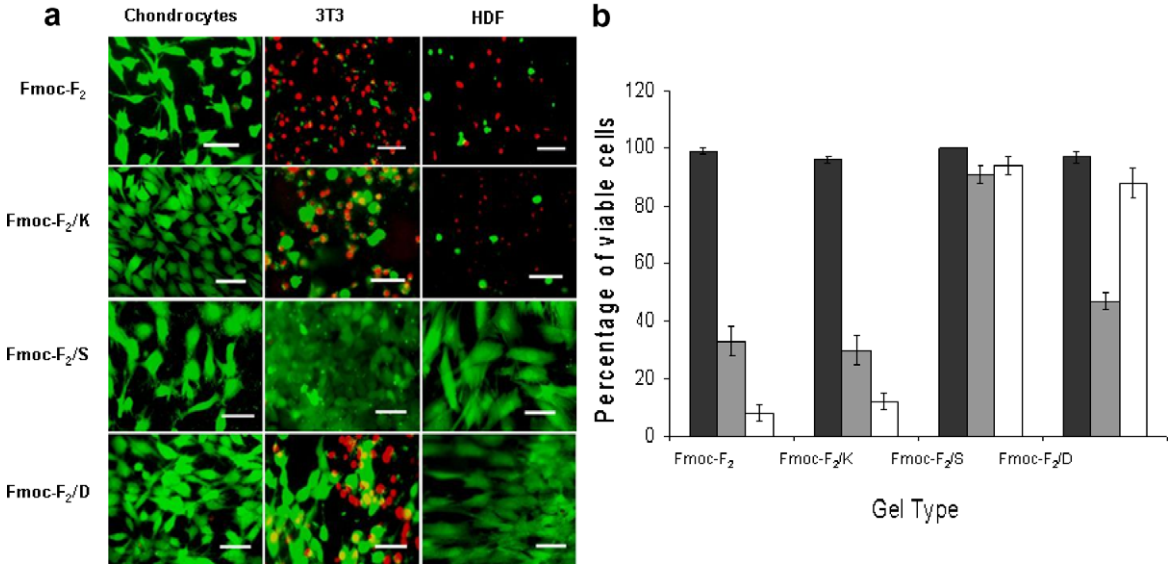

Fig. 3. (a) Comparison of Fmoc-F_2, Fmoc-F_2/K (1:1), Fmoc-F_2/S (1:1), Fmoc-F_2/D (1:1) in relation to 2D culture of chondrocytes, 3T3 fibroblasts and HDFs. Live–dead staining results after 48 h in culture. Green staining indicates live cells, red staining indicates dead cells. Scale bar represents 50 μm. (b) Quantitative analysis of Live/dead staining results, chondrocytes (black), 3T3 (grey) and HDF (white). Values represent means where $n = 5$ with error bars showing the standard deviation. The percentage of viable chondrocytes, 3T3 cell and HDF cells between the four samples measured at day 2 are significantly different ($P < 0.01$ for chondrocytes and $P < 0.001$ for 3T3 cells and HDF cells). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

was observed on flexible substrates compared to stiff for normal 3T3s, with higher proliferation on the stiffer substrates.

Viability of chondrocytes, 3T3 and HDF cells on the four hydrogel surfaces (2D culture) was visually (Fig. 3a) and quantitatively (Fig. 3b) analysed using live–dead staining. The results indicate that all gels tested support chondrocytes at around 100% viability after 48 h with no obvious difference in cell morphology. Fmoc-F_2 or Fmoc-F_2/K show similar cell compatibility which could be attributed to their similar mechanical properties, which satisfied the mechanical criterion suggested for chondrocytes [32]. Fmoc-F_2/S also shows chondrocyte compatibility although its stiffness is just below 10 KPa. Fmoc-F_2/D is a significantly weaker gel and it may be surprising that this gel supports chondrocyte viability. It is likely that chemical functionality plays a role here; one might expect enhanced protein adsorption, as commonly observed on charged surfaces [19].

Fmoc-F_2/S and Fmoc-F_2/D gels also supported higher HDF cell viability, in contrast to Fmoc-F_2 and Fmoc-F_2/K hydrogels. Fmoc-F_2/S gels supported high 3T3 cell viability over the 48 h time course (at around 91%), while in the other three gel types only around 38% of 3T3 cells survived. The observation of better fibroblast compatibility of these lower stiffness gels contrasts observations by Wang et al. [36], and could imply a role for chemical functionality in addition to mechanical properties. The most likely effect of chemical functionality on cell/gel compatibility relates to integrin binding, which is required for cell spreading. Effects of chemical functionality on integrin binding are indirect – charged groups may promote attachment of integrin binding proteins present in culture media such as fibronectin or vitronectin while OH functionality may repel these proteins [19]. Our observations do not show straightforward relationships as those observed on well defined 2D surfaces, with enhanced attachment observed for HDFs on Fmoc-F_2/D but not 3T3s with no cell attachment on

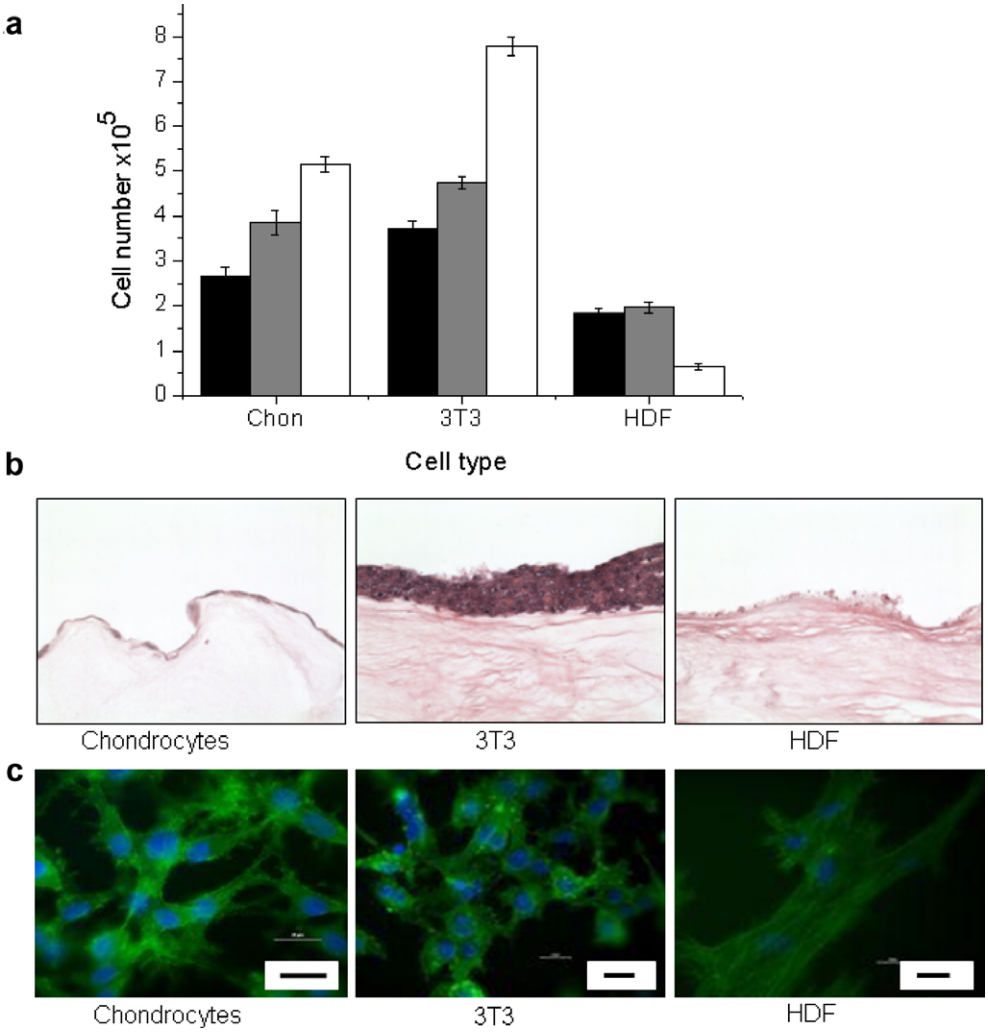

Fig. 4. (a) 2D cell culture on Fmoc-F_2/S (1:1) hydrogels. LDH assay results for day 1 (black), 3 (grey) and 7 (white). (b) Histology (day 7 in culture). (c) F-actin staining for chondrocytes, 3T3 cells and HDF cells (day 3 in culture). Scale bar represents 20 μm. Statistical analysis suggests that although there are no significant differences between the gel surfaces and the cell numbers measured at day 1, there is a statistically significant difference (at least $P < 0.05$) in all three cell types between the gel surfaces measured at day 3 and day 7. The differences are more significant for chondrocytes and 3T3 cells ($P < 0.001$) than for HDF cells ($P < 0.05$).

Fmoc-F_2/K gels. By contrast, the observed compatibility of Fmoc-F_2/S implies that the rules for hard 2D surfaces cannot be applied directly for soft gel surfaces. Indeed, protein adsorption onto gel surfaces is a relatively unexplored area.

The morphological differences observed between the two types of fibroblast on Fmoc-F_2/S reflects their morphology in standard cell culture, with 3T3s adopting a more cuboidal morphology compared to the more spindle-like morphology of HDFs and by possible differences in integrin expression. It is known that in collagen lattices, fibroblasts exert traction via the $\alpha_2\beta_1$ integrin [37] whereas smooth muscle cells do so via the $\alpha_1\beta_1$ integrin [38]. Lee and Loeser [39] propose that non-motile polygonal chondrocytes rearrange their surrounding extracellular matrix via their β_1 integrins.

As the Fmoc-F_2/S hydrogel was most broadly compatible with different cell types, it was studied further. An LDH cell viability assay was performed over a 7 day time course to quantify the metabolic activity of chondrocytes, 3T3 fibroblasts and HDF cells growing on the surface (Fig. 4a). Chondrocytes demonstrated around a 5-fold increase in viable cell number over the 7 days, while 3T3 fibroblasts demonstrated an 8-fold increase by day 7 suggesting active proliferation of both cell types on Fmoc-F_2/S hydrogels. Conversely HDF viable cell number appeared to decrease slightly over the 7 days, although the decrease was not significant. The results suggest that HDFs do not actively proliferate on Fmoc-F_2/S hydrogels.

Histological staining of gel sections (Fig. 4b) showed a multi-layer of 3T3 cells on the surface of Fmoc-F_2/S hydrogels. Chondrocytes demonstrated a flattened morphology similar to that seen in conventional monolayer cultures and appeared to have proliferated to cover the majority of the gel surface by day 7. A number of HDF cells adhered to the surface of the gels, but a proportion of these cells displayed a ragged morphology suggesting cell death was occurring and this concurred with the LDH assay data.

Cell attachment and spreading by all three cell types on the surface of the Fmoc-F_2/S hydrogels by day 3 was further confirmed by fluorescence microscopy for cytoskeletal F-actin. The images in Fig. 4c demonstrate that all three cell types have an organized cytoskeleton (green) surrounding their nuclei (blue). While chondrocytes and 3T3 fibroblasts still appear to have a more stellate appearance (after day 1, images are not shown), the majority of the HDF cells have adopted a classic elongated, "fibroblastic" morphology seen in conventional monolayer cultures. The fluorescence images at day 3 show that, over time, the majority of chondrocytes lost their spherical appearance and the cells adopted a more elongated shape. These observations could be related to de-differentiation of chondrocytes on monolayer culture. The possibility of an extended morphology for the chondrocytes growing on monolayer cultures in general is well documented [40,41]. The general appearances for HDF cells and 3T3 cells remained unchanged at day 3 in culture.

Comparison of 2D and 3D culture of chondrocytes in Fmoc-F_2/S hydrogels demonstrated differences in cell morphology and cytoskeletal organization between the two culture systems. Both 2D and 3D culture promotes chondrocyte proliferation over time (Fig. 5a); the rate of increase is higher for 2D culture than for 3D culture samples. Light microscopy images (Fig. 5b) demonstrated that chondrocytes in 2D culture had a more elongated, fibroblastic morphology consistent with monolayer culture, while those in 3D demonstrated a classic rounded morphology more similar to that displayed by chondrocytes in vivo. Similarly, the cytoskeletal organization present in the 2D cultured chondrocytes was lost in 3D culture (see Fig. 5c) and these data again are similar to that described in current 2D and 3D culture systems [42]. Although our initial cell viability, morphology and proliferation data suggests that these gels have potential for cell culture in both 2D and 3D, further work is required to look at longer culture times, matrix deposition and gene expression.

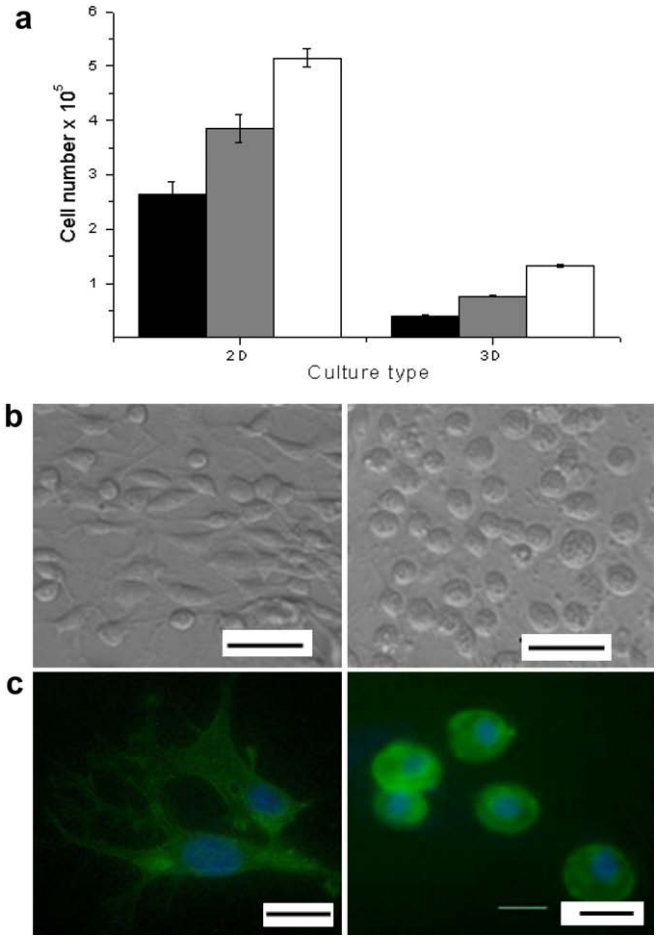

Fig. 5. 2D vs. 3D culture of chondrocytes in Fmoc-F_2/S hydrogel (in 2D Fmoc-F_2/S 1:1 ratio and 3D Fmoc-F_2/S 2:1 ratio. (a) LDH Assay results for day 1 (black), 3 (grey) and 7 (white). (b) The light microscope images suggest a spread morphology for 2D culture of chondrocytes but a round morphology in 3D culture (day 3). Scale bar represents 50 μm. (c) F-actin staining further confirms the morphological changes to chondrocytes in 2D and 3D culture (day 3). Scale bar represents 20 μm.

4. Conclusion

Introduction of chemical functionality into Fmoc-peptide gels may be achieved by the addition of Fmoc-protected amino acids with varying side groups. This approach results in the formation of viscoelastic, nanofibrous hydrogels with predominantly anti-parallel β-sheet conformation whereby mechanical properties were governed by the chemical composition of the gel. Cell culture analysis showed that the resulting scaffolds, Fmoc-F_2, Fmoc-F_2/S, Fmoc-F_2/K and Fmoc-F_2/D differ significantly in their compatibility with cell culture of bovine chondrocytes, mouse 3T3 fibroblasts and human dermal fibroblasts. The results suggest that introduction of chemical functionality or changes in mechanical stiffness into Fmoc-peptide scaffolds allows for straightforward formulation of cell scaffolds that may be matched to different cell types. At this stage it is not possible to disentangle the relative importance of mechanical properties and chemical functionalities on controlling cell behaviour in Fmoc-peptide gels, however it is clear that both are important and can be varied by formulation. These results provide a further step towards development of Fmoc-peptide gels as 3D in vitro cell culture systems.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.actbio.2009.01.006.

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