The interplay between physical and chemical properties of protein films affects their bioactivity.

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Abstract

While mechanical properties, roughness and receptor molecule expression have all been shown to influence the cellular reactivity of collagen-based biomaterials, their relative contribution, in a given system remains unclear. Here, we study films containing combinations of collagen, gelatin and soluble and insoluble elastin, crosslinking of which results in altered film stiffness and roughness. Collagen and gelatin have similar amino acid sequences, but altered cell binding sites. We studied cell response with both C2C12 myoblast cells, (which possess RGD-recognising integrins $\alpha_V\beta_3$ and $\alpha_5\beta_1$) and C2C12-α2+ cells (which, in addition, express the collagen-binding integrin $\alpha_2\beta_1$) to establish the effect of altering the available binding sites on cell adhesion and spreading on films. Systematically altering the composition, crosslinking and cell type, allows us to deconvolute the effects of physical parameters and available binding sites on the cell reactivity of films in this system. Collagen-based films were rougher and stiffer and supported lower cell surface coverage than gelatin-based films. Additionally, C2C12-α2+ cells showed preferential attachment to collagen-based films compared with C2C12 cells, but no significant difference was seen using gelatin-based films. The cell count and surface coverage was found to decrease significantly on all films after crosslinking (Coll XL coverage = 2−6%, Gel XL coverage = 20−32%), but cell area and aspect ratio on collagen films was affected to a greater extent than on gelatin films. The results show that, in this system, the composition, and more significantly, crosslinking, of films affects the cell reactivity to a greater extent than their stiffness or roughness.

1. Introduction

Intense research into the effect of substrate stiffness on cell adhesion and spreading has lead to the postulation that cells respond to the stiffness of the matrix on which they are seeded, and that these mechanical properties have a significant effect on the ability of cells to proliferate and grow *in vitro* [1-4]. Additionally, the cellular response can depend on the specific cell type, seeding density, and the available adhesion receptor binding motifs on the material substrate [5,6]. It has been unclear to what extent the ability of cells to interact with biomaterials is influenced by force-induced signalling as well as signalling induced by other parameters such as roughness, composition and receptor interaction [5,7,8].

Our research has been focused on the effect of altering the composition and crosslinking on the physical properties and cell activity of thin films. Thin films are useful in studying the cell-scale properties of 3D matrix systems: their composition and crosslinking can be systematically modified so that they can be used to quickly indicate the suitability of biomaterials for specific applications [5,8]. We have studied collagen and gelatin, with additions of 10% soluble or insoluble elastin, to compare the effect of altering the protein composition on the physical properties and cell reactivity of films. Collagen and gelatin have similar amino acid sequences, but altered cell binding sites; cell-binding motifs in triple-helical collagen interact with cells via specific integrins, notably $\alpha_2\beta_1$ [9]. The type I collagen motifs that bind with this universal collagen-binding integrin take the form GXOGER (using standard single-letter amino acid nomenclature, where G, E and R are glycine, glutamate, and arginine, and O = hydroxyproline), and X is one of several possible amino acids e.g. phenylalanine in the high affinity sequence GFOGER[10]. Heat-denaturation of collagen, to produce gelatin, destroys the ordered three-dimensional triple-helical structure which disrupts the binding sites for collagen-binding integrins, leaving, after cooling, a mixture of mis-aligned triple helices, peptide fragments and random coils, unable to fully assemble and form ordered fibrils [5,10-11]. Denaturation exposes RGD-containing cell-recognition motifs (where D is aspartate) that, although present, remain cryptic in the native collagen helix, where they are less able to engage integrins, for example, $\alpha_V\beta_3$ and $\alpha_5\beta_1$. Combinations of collagen, gelatin and elastin are likely to result in altered physical properties of films such as stiffness: collagen typically provides tissues with strength and stiffness whereas elastin provides elasticity and the ability to store energy [12-14]. In combining collagen, gelatin and elastin we expect to be able to investigate the relationship between composition, physical properties and cell activity of biomaterial films.

Our experiment is also designed to test the effect of carbodiimide crosslinking on stiffness, strength and surface roughness, as well as the ability of cells to adhere to and spread on these films. These crosslinks typically form between free primary amino groups (on lysine residues) and carboxylate anions (on glutamate or aspartate residues) and are therefore likely to affect the availability of cell-

binding sites. In systematically altering the composition and crosslinking we have a simple, but effective, way to deconvolute the effects of physical parameters and chemistry (available binding sites) on the cell activity of films. Additionally, the myoblast cell line selected allows a comparison to be made between the interaction of collagen-based films with cells that express a collagen-binding integrin (C2C12-α2+ expressing $\alpha_2\beta_1$) and a parent cell line not expressing this collagen receptor (C2C12 expressing RGD-recognising integrins $\alpha_V\beta_3$ and $\alpha_5\beta_1$, but no collagen-binding integrins). It may be expected that by altering the available binding sites (by changing the base protein from collagen to gelatin) the ability of the different cell lines to adhere to and spread on films will also be affected [15-17].

Films were prepared and characterised in terms of their physical properties and cellular interactions. Scanning Electron Microscopy and Atomic Force Microscopy (AFM) were used to characterise the film microstructure and surface roughness, while the Young's modulus was used to assess the effect of composition and crosslinking on mechanical properties. All films were seeded with mouse myoblast cells and cultured for three days. Calculation of the cell surface coverage as well as morphological analysis (aspect ratio and area) of the cells was performed. The specific objective of this study was to investigate to what extent the cell activity of films was affected by physical properties (stiffness and roughness) and/or chemical properties (composition and crosslinking).

2. Materials and Methods

2.1 Film Fabrication

In total, seven different collagen-based films were prepared by drying a suspension of protein on either Teflon sheets or in 24-well plates, and are detailed in Table 1. Briefly, a suspension containing 0.5% (w/v) collagen (bovine dermal type I, DevroMedical) with or without insoluble or soluble elastin (both bovine neck ligament, Sigma-Aldrich) was swollen overnight in 0.05 M acetic acid at 4 ± 2°C. The resulting suspension was homogenised on ice for 10 min at 9,500 rpm using an Ultra-Turrax VD125 (VWR International Ltd, UK). Air bubbles were removed from the suspension by centrifuging at 2,500 rpm for 5 min (Hermle Z300, Labortechnik, Germany).

Gelatin-based slurries could not be prepared by homogenisation. Dissolution of the powder required heating and homogenisation at high temperatures resulted in the formation of a foam. However, the solution began to gel at temperatures below approximately 15°C, so homogenisation could not be carried out at low temperatures either. Gelatin-based solutions, 0.5% (w/v), were instead prepared by dissolving gelatin (bovine skin type B, Sigma-Aldrich) with or without insoluble or soluble elastin in 0.05 M acetic acid at 37–45ºC with stirring for 1 h. The solutions were then cooled to room temperature with stirring. The mixed collagen-gelatin 1:1 suspension was prepared by mixing a gelatin solution with a collagen slurry (both 0.5% w/v), before centrifuging.

The protein suspensions were then dried overnight on a Teflon sheet at room temperature in a laminar flow hood. This process produces a thin film of protein, with an average thickness of 15 μm.

2.2 Chemical Crosslinking

After drying, crosslinking was carried out using 1.150 g 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) and 0.276 g N-hydroxy-succinimide catalyst (NHS, both Sigma-Aldrich) per gram of film as previously described [18]. The crosslinking solution was prepared in 75% ethanol/water (v/v) [19,20]) and the samples were crosslinked for 2 h. After crosslinking the films were washed twice in 75% ethanol, followed by three times in deionised water for 30 min each. The crosslinked films were allowed to dry overnight in a fume hood.

2.2.1 Films for AFM

Films for AFM were dried directly onto glass cover-slips and crosslinked in situ.

2.2.2 Films for Cell Culture

To produce films for cell culture, slurries 0.5% (w/v) were added to 24-well tissue culture wells (400 μl per well) and dried in a tissue culture hood overnight. Films were then crosslinked in situ.

2.3 Determination of Primary Amine Group Content and Degree of Crosslinking

The degree of crosslinking can be estimated from the residual amount of free amine groups of (non-) crosslinked samples. The concentration of free primary amine groups ($-NH_2$) present in the initial non crosslinked and the crosslinked films was determined using a 2, 4, 6-trinitrobenzenesulfonic acid (TNBS) assay with a protocol similar to those reported by Sashidar *et al.* and Ofner *et al.* [21,22]. To each sample (1-3 mg) 0.5 ml of a 4% (w/v) NaHCO3 solution and 0.5 ml of a freshly prepared solution of 0.05% (w/v) TNBS was added. After reaction for 2 h at 40°C, 1.5 ml of 6 M HC1 was added and the samples were hydrolyzed at 60°C for 90 min. The reaction mixture was diluted with distilled water (2.5 ml), cooled to room temperature and the absorbance at 320 nm (A_{320}) was measured using a Fluostar Optima spectrophotometer. Controls (blank samples) were prepared using the same procedure, except that HCl was added prior to the TNBS solution. The absorbance of the blank samples was subtracted from each sample absorbance. The absorbance was correlated to the concentration of free amino groups using a calibration curve obtained with glycine in an aqueous $NaHCO_3$ solution (0.1 mg/ml), where the relationship between absorbance and concentration of primary amino groups was found to be linear.

The experiment was repeated three times and the average, along with the standard error, was calculated. The non-crosslinked sample was assumed to contain 100% of the available free amine groups and this value was used to calculate the percentage of free amine groups consumed during the crosslinking treatment i.e. the degree of crosslinking.

2.4 Atomic Force Microscopy (AFM)

The AFM apparatus (Dimension 3100) was operated in light tapping mode, using standard tapping probes (RTSEP Silicon AFM tips, Veeco: resonant frequency 200-400 Hz, spring constant 20-40 N/m) under ambient conditions. All image analysis was carried out using WSxM software [23]. Root mean square (RMS) roughness (R_q) was measured for five different locations on each film: image size 50 x 50 μm, scan rate of 0.2 Hz and 256 samples/line. Images were first flattened (offset) and then the

average RMS roughness of four 20 x 20 μm squares (randomly distributed within the image) was measured.

2.5 Mechanical Testing

Uniaxial tensile testing was used to determine the effect of composition and crosslinking on the modulus of the films. Mechanical testing of films was carried out using a Hounsfield tester equipped with a 5 N load cell and specially designed testing apparatus with a submerged film-gripping system. Film samples used for mechanical testing were first cut into rectangular strips, 7 mm wide by 45–70 mm long ($n = 14$ per group), using a scalpel. Samples were pre-hydrated in water for 30 min before measuring the thickness at three different areas; testing was then carried out in a bath of water. Samples were clamped in a horizontal position, gauge length 10 mm, and testing was conducted at a constant extension rate of 6 mm min^{-1}. All samples were stretched until failure and data was used to plot a stress-strain curve. The tensile modulus (E) was defined as the slope of a tangent to the stress-strain curve at 5 % strain, 20 % strain and high values of strain (where the maximum gradient was reached).

Hydration of non-crosslinked films made them difficult to handle and significantly weaker. Tensile testing could only be performed on non-crosslinked collagen film as all other compositions were too weak to handle when hydrated.

2.6 Cell Culture

C2C12 and C2C12-α2+ mouse myoblast cells were a gift from Prof D. Gullberg, University of Bergen, Norway, where C2C12 is the parental cell line and C2C12-α2+ is a stably transfected cell line (with the human integrin α_2 subunit). Cells were cultured in T−75 flasks (nunc) using Dulbeccos Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% FBS (Invitrogen), 1% L-glutamine and 2% penicillin/streptomycin (Sigma). Before seeding, cells (passage 2-3) were detached using trypsin (Invitrogen) and spun down to a pellet. All films were rinsed three times with sterile phosphate buffered saline (pH 7.4, Invitrogen) and pre-incubated with DMEM for 30 min at 37°C.

A study was conducted using C2C12-α2+ cells marked using CellTracker Green (CMFDA, Invitrogen) to enable measurement of the degree of cell spreading and surface coverage. Cells were re-suspended in 1 ml CellTracker stock (10 μM) made up in DMEM and incubated with rocking for 60 min at 37°C. The suspension was then centrifuged at 403 g for 5 min (Geneflow) and washed in DMEM. This was repeated and cells were then suspended in supplemented DMEM for use in culture. Cells were then seeded on films in 24-well plates, in triplicate, at a density of 2000 cells/well in 800 μl DMEM and placed in an incubator for 72 h at 37ºC under 5% CO_2. After 3 d cells were imaged as detailed in the next section.

To compare the interaction of films with specific integrins both C2C12-α2+ and C2C12 cells were used. The films were seeded with either C2C12-α2+ or C2C12 cells, in triplicate, at a density of 1000 cells/well in 800 μl DMEM and then placed in an incubator for 72 h at 37 ºC under 5% CO_2.

2.7 Hoechst-PI Assay

Cells were visualised for counting and morphometry using the stains Hoechst 33258 and propidium iodide (PI) (Sigma-Aldrich). A working solution of the dyes was prepared, and after the specified incubation time, the working solution was added to each well to give a final concentration of 4μg/ml Hoechst and 1μg/ml PI. This was incubated for 15 min at 37ºC and the fluorescence was then measured on two channels (Hoechst: excitation 355nm; emission 461nm and PI: excitation 535nm; emission 617nm) with an additional channel for CellTracker fluorescence of the time point study (excitation 492 nm; emission 517nm) using a fluorescence spectrophotometer (AF6000 LASAF confocal microscope). Four images were taken from the centre of each well and analysed using ImageJ software (NIH, USA) for live and dead cell count, cell area, aspect ratio and surface coverage.

2.8 Comparative Study

To determine to what extent physical properties, chemical crosslinking or composition effected the cell activity of films, data from physical characterisation was plotted against the data obtained from

the cell culture work, with the results being grouped according to their predominant composition and crosslink status i.e. collagen/gelatin, crosslinked/non-crosslinked.

2.9 Statistical Analysis

Results are expressed in the figures as mean ± standard error. Student's t-test was used to compare the modulus of films and two-way analysis of variance was used to evaluate the effects of crosslinking and composition on the cellular attachment. Statistically significant differences at a significance level of $p \leq 0.05$ were tested between non-crosslinked and crosslinked samples of the same composition (denoted by *), and between samples of different compositions (annotations explained in figure captions).

3. Results

3.1 Free amino group content

The reaction of TNBS with the primary amino groups in the proteins is used to determine the number of free amino groups in the film samples (Table 2) and subsequently calculated the degree of crosslinking. The degree of crosslinking is the difference between the chemically determined number of uncrosslinked amino groups before and after crosslinking. In determining the degree of crosslinking, it has been assumed that each lost amine group participates in one cross-link.

Non-crosslinked gelatin has a higher number of free amine groups compared with collagen samples (23×10^{-5} mol/g compared to 14×10^{-5} mol/g). The addition of elastin increases the number of free amine groups in collagen samples (but not significantly in gelatin samples). After crosslinking, the free amino group content of all samples decreased significantly, suggesting that the crosslinking procedure is successful. The value of free amine groups in all samples after crosslinking is $4-9 \times 10^{-5}$ mol/g.

3.2 Effect of Composition on Surface Structure AFM

Atomic Force Microscopy was used to compare the surface structure and properties of films of different compositions and crosslinking. The surface roughness was quantified by AFM measurements where the average change in surface height (h) and RMS roughness (R_q) of the collagen-based films was significantly greater than that of gelatin films (Figure 1 and 2, Coll nX h = 3.2 μm, R_q = 0.4176 ± 0.035 μm; Gel nX h = 266 nm, R_q = 7.9 ± 1.5 nm). Figure 1 shows clearly that the addition of insoluble elastin to the films resulted in a two-fold increase in the height and roughness of collagen (Coll-IE nX h = 6.3 μm, R_q = 0.94 ± 0.28 μm) and over a twenty-fold increase in the height and roughness of gelatin (Gel-IE nX h = 5.7 μm, R_q = 0.97 ± 0.30 μm). Insoluble elastin fibre bundles could be clearly seen in AFM images (Figure 3), and had a larger diameter (of 7.77 ± 1.07 μm) compared with collagen and gelatin fibre bundles (diameter 0.82 ± 0.16 μm and 0.18 ± 0.28 μm respectively). The addition of soluble elastin, which is a powder, reduced the height and roughness of collagen films (Coll-SE nX h = 1.6 μm, R_q = 0.29 ± 0.02μm), but there was no significant difference between surface characteristics of gelatin films and those containing soluble elastin (Figure 1). The height and RMS roughness of mixed collagen-gelatin films was intermediary between the pure films (h = 727 nm, R = 147 ± 30.5 nm).

The effect of crosslinking was to reduce the roughness of the film surface (except for films containing insoluble elastin, Figure 1 and 2). There was no change in fibril bundle size or the periodic banding pattern after crosslinking.

3.3 Effect of Composition and Crosslinking on Mechanical Properties

All films showed J-shaped stress-strain curves, typical of biomaterials (Figure 3a). Crosslinking of collagen using EDAC and NHS was found to increase both the strength at failure and the Young's modulus (Coll nX σ_f = 0.54 MPa, E = 5.66 MPa; Coll XL σ_f = 11 MPa, E = 31 MPa, Figure 3b). All other non-crosslinked films were too weak to be tested in tension.

The addition of both types of elastin reduces the stiffness of collagen by about a third (E = 15-20 MPa), as does the addition of gelatin to produce the mixed collagen-gelatin films. Gelatin has reduced mechanical properties compared with collagen (Gel XL σ_f = 1 MPa, E = 15 MPa) and the addition of insoluble elastin, which did not mix well with the gelatin, resulted in a decrease in stiffness compared to pure gelatin films (E = 6 MPa), whereas adding soluble elastin retained the mechanical properties of the gelatin film. The combination of gelatin and collagen resulted in a film with stiffness most similar to that of pure gelatin films (E = 16 MPa).

3.4 Effect of Composition and Crosslinking on Cell Number, Surface Coverage and Area

Figure 4 show immunofluorescence images of cells seeded on collagen and gelatin films both non- and crosslinked. It is apparent that the cell number and surface coverage is highest on non-crosslinked gelatin-based films, with a lower surface coverage on collagen films. Additionally the process of crosslinking causes a reduction in cell number and surface coverage on films of both compositions (Figure 4 c and d). Further analysis of these images using ImageJ software enabled live cell count, dead cell count, surface coverage, cell area and aspect ratio to be determined and are detailed in Figure 5. The number of live cells on the films (Figure 5a) was found to vary most significantly with predominant composition and crosslinking i.e. collagen-based films compared with gelatin-based films and non-/crosslinked, in agreement with prior research conducted in our group [24]. The composition was seen to significantly affect the number of live cells of both cell lines: C2C12-α2+ and C2C12 cells (Figure 6) showed a higher cell count when seeded on gelatin films compared with collagen films. α2-positive cells also showed an increased cell count on collagen films compared with C2C12 cells, but there was no significant difference in the cell count of the different cell lines seeded on gelatin-containing films (Figure 5a and 6). This result is shown in more detail in Figure 7: a linear relationship between the cell counts of both cell lines was established on all films, with gelatin-based films showing direct correlation between the counts of both the parent and transfected cell. However, the α2-positive cells show a higher cell count on collagen-based films compared with the parent cell. Coll-Gel films behaved more like collagen films in terms of their cell interactions.

Importantly, regardless of the cell line used, there was an overall reduction in live cell number and surface coverage on all crosslinked films compared with non-crosslinked films (Figure 5a and e, Figure 6). The number of dead cells was also found to be higher on crosslinked films compared to non-crosslinked films (Figure 5c). This result is an agreement with recent work that showed a lower cell count on EDAC crosslinked collagen compared to a control (non-crosslinked) [25]. The degree of cell spreading was quantified by the cell area and aspect ratio (Figure 5 b and d). Crosslinking caused a decrease in cell spreading on collagen-containing films, with cells tending to a more rounded morphology (aspect ratio 1), but there was no difference seen in the spreading of C2C12-α2+ cells seeded on non-/crosslinked gelatin films.

3.5 Comparative Study

In plotting data from physical characterisation and cell culture studies together we could determine the relationship between composition, crosslinking, physical properties and cell adhesion/spreading and thus establish if there was one dominant factor or a synergistic effect in determining the cell activity of films (Figure 8 and 9). Cell number, cell area or aspect ratio was plotted against film stiffness, roughness or degree of crosslinking. The percentage Δ(cell count) is the difference between cell counts on crosslinked and non-crosslinked films; a large positive or negative value indicates a significant difference between the cell counts after crosslinking. Data was colour-coded according to its predominant composition and crosslink status and relationships between physical properties and cell interactions were determined.

The majority of data was clustered in groups determined by the film composition or crosslink status and not by physical properties. The data spread horizontally in groups according to the predominant component of the film and crosslink status. Cell count, surface coverage, cell area and aspect ratio were all independent of film roughness or stiffness for both C2C12-α2+ and C2C12 cell lines (only cell count data is shown for C2C12 cells). Crosslinking was found to reduce the C2C12-α2+ cell count by 50-90% on collagen films and 20−80% on gelatin films, but by a similar amount on both composition films for C2C12 cells of 80−90% (Figure 9).

Cell area and aspect ratio were independent of mechanical properties or surface roughness, but instead the effects were dominated by the composition and crosslinking. The cell area and aspect ratio of cells seeded on collagen films showed high sensitivity to crosslinking (Δ(cell aspect ratio) and Δ(cell area) both 30−70%) whereas the spreading of cells seeded on gelatin films was less affected by crosslinking (zero average change in aspect ratio or area after crosslinking).

4. Discussion

The aim of this study was to investigate to what extent the cell activity of films was affected by physical properties and/or chemical properties (composition and crosslinking). The affect of altering the composition and crosslinking was to provide a selection of films with altered physical properties and cell binding sites. Although crosslinking imparts mechanical stiffness and reduces the surface roughness of all composition films, it has a negative effect on the cell activity of films. It has been suggested that altering the stiffness alone can change the ability of cells to interact with biomaterials [1-4]. However, we have shown that, in this system, composition is more important than stiffness or roughness in determining cell activity, with crosslinking having an effect over and above this; in line with other studies that suggest it is a combination of factors that determine the cell response [5,6].

In this study we wished to establish the effect of composition and crosslinking on the physical properties of films, and then look to see if these physical properties determined cell activity. AFM images displayed in Figure 2 showed that collagen-based films were significantly rougher than gelatin-based films (Coll nX $R_q = 0.4176 \pm 0.035$ μm; Gel nX $R_q = 7.9 \pm 1.5$ nm), with the roughest films containing insoluble elastin, with a roughness of 0.94 ± 0.28 μm and 0.97 ± 0.30 μm for collagen-insoluble elastin films and gelatin-insoluble elastin films respectively (Figure 1). The insoluble elastin fibre bundles were significantly larger than the other proteins in the films and thus gave rise to this increased roughness; the measured roughness of the surrounding film, not including the elastin fibres was equivalent to that of those films not containing elastin. Soluble elastin decreased the roughness of collagen films and may act to 'fill' gaps between fibrils, thus producing a smoother

surface, whereas there was no significant difference in surface roughness of Gel-SE films since the proteins are initially both powders and produce a homogeneous surface. Crosslinking reduced the overall surface roughness of films, which may be as a result of a contraction of the fibre structure due to the formation of inter-fibre bonds.

The crosslinking process also provided strength and mechanical stability to the films (Coll nX E = 5.66 MPa and Coll XL E = 31 MPa, Figure 3). The addition of elastin reduces the stiffness due to the reduction in the total collagen content. Gelatin lacks those long fibres that give rise to the stiffness in collagen, and thus the mechanical properties are lowered compared with collagen (Gel XL E = 15 MPa). The insoluble elastin fibres did not interact well with the gelatin and are likely to have acted like defects in the otherwise smooth film, decreasing the stiffness, whereas the soluble elastin, which appears to have produced a single-phase material with the gelatin, gave rise to a film with similar physical properties to those of pure gelatin.

Our main objective was to determine whether physical properties dominate in determining the cell activity of films or whether other factors such as composition and crosslinking are as important. Previous research has looked at the variation of stiffness and its effect on cell adhesion and spreading, and some studies have suggested that there may be an interplay between adhesion receptors and stress fibres in determining the cellular response matrix on which they are seeded [2,4,6,7]. The comparative study we have produced has helped to separate out the most important factors in determining cell activity of biomaterial films. We have shown that composition and, more importantly, crosslinking are the dominant factors in determining cell adhesion and spreading in this system.

On comparing the degree of crosslinking, roughness and stiffness of films with the cell count, area and aspect ratio it is apparent that composition and crosslinking play a greater role than the physical properties of films in determining the cell activity (Figure 8 and 9). Cell count is greater on gelatin-based films, suggesting an increased number of adhesion receptors (RGD sequences) compared with collagen (Figure 5). Additionally, C2C12-α2+ cell count was higher than C2C12 cell count on

collagen films, but there was no significant difference on gelatin films (Figure 6 and 7). Since C2C12 cells do not contain collagen-binding integrins it is expected that the transfected cell line, C2C12-α2+ (that do express the collagen-binding integrin $\alpha_2\beta_1$), will show increased binding to collagen-containing films. These α2-positive cells were indeed shown to be able to bind more to collagen-based films than the parent cell line [5,10,15,17]. There was no significant difference in binding of the α2-positive cells to gelatin compared with the parent cell (similar cell count shown for both cell lines, Figure 6), since both cells express the same RGD-binding integrins [7,10].

Most significantly, the results indicate that crosslinking has a detrimental affect on the ability of both C2C12 and C2C12-α2+ cells to attach to and proliferate on films of all compositions (Figure 8 and 9). C2C12-α2+ cell count and surface coverage was reduced by 50−90% on crosslinked collagen-based films and 20−90% on crosslinked gelatin based films compared to non-crosslinked films. The C2C12 cell count was also reduced by 80−90% on both collagen- and gelatin-based films, indicating the similar sensitivities of both base proteins to crosslinking for C2C12 binding; due to similar binding mechanism available on both films (RGD sequences).

Crosslinking of films composed of collagen had a greater effect on the ability of cells to spread than on gelatin-based films. The absolute cell area and aspect ratio was reduced on crosslinked collagen films, with the cell aspect ratio indicating a more rounded cell after interaction with crosslinked films (Figure 5). However, cells on gelatin-based films showed a similar degree of spreading regardless of the film crosslink status. Cell proliferation and hence cell count and surface coverage appears to be detrimentally affected by crosslinking of both collagen and gelatin films, whereas cell spreading is only reduced by crosslinking of collagen-based films. Binding to the GXOGER sequences may be more sensitive to crosslinking than binding to RGD sequences, however cell-signalling from both sequences (to encourage cell proliferation) is similarly affected. We may hypothesise that this is due to the crosslinking process resulting in a re-organisation of the collagen fibrils and rearrangement of the cell binding sites, restricting their availability and thus resulting in the observed reduced cell

spreading for all crosslinked collagen films. This implies a possible role for collagen receptors in the spreading and elongation of cells.

The cell activity of different composition films may be affected by a combination of cell receptor sites, stiffness and roughness of the films. It appears that the composition is more important than roughness, stiffness or other mechanical properties in determining the cell activity of films in this system. The influence of crosslinking was then found to be significantly greater than the physical properties or indeed the composition, which may be as a result of altered stiffness, roughness or reduced cell binding sites.

5. Conclusion

This research has shown that changing the composition and crosslinking not only affects the physical properties, such as surface roughness and mechanical stiffness, but also plays a great role in determining the cell activity of the films. The mother myoblast cell line binds to available RGD sequences in both collagen and gelatin, and the transfected cell line shows increased binding to collagen where additional interactions via the integrin $\alpha_2\beta_1$ and collagen-binding motifs can occur. As hypothesised, cell activity is not simply affected by physical properties but is highly dependent on the composition and crosslink status of the films. In fact, we have shown that the composition, and more significantly, crosslinking, of films affects the cell activity to a greater extent than their stiffness or roughness.

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Figures

Table 1. Film abbreviations.

Table 2. Number of moles of free amine groups per gram of film (x 10^5) in non- and crosslinked films as determined by the TNBS assay, and calculated degree of crosslinking. Results are mean ± standard error measurement (n = 3).

Figure 1. Surface properties of films of different composition and crosslink status. Route mean squared roughness (R_q, μm) of films (nX non-crosslinked, XL crosslinked) measured using AFM on dry samples. Note: * indicates statistically significant difference in comparison to non-crosslinked sample of same composition, ■ indicates statistically significant difference to Gelatin-based samples of equivalent composition, Δ indicates significant difference to all other samples not containing insoluble elastin for statistical significance of $p \leq 0.05$ in Student's *t*-test. The error bars indicate the standard error of the mean (n = 5).

Figure 2. Effect of film composition and crosslinking on surface roughness and height as measured by AFM topography images of films (nX non-crosslinked, XL crosslinked). Images are representative of the film as a whole, showing regions of mixed composition where appropriate (Coll-IE and Gel-IE). (a) Coll nX, (b) Coll-IE nX, (c) Coll-SE nX, (d) Gel nX, (e) Gel-IE nX, (f) Gel-SE nX, (g) Coll-Gel nX, (h) Coll XL. X-Y scale 50 μm, Z maximum height.

Figure 3. Mechanical properties of films of different composition and crosslink status. (a) Comparison of representative curves of films having different composition and crosslink status (crosslinked collagen, collagen-gelatin and gelatin, and non-crosslinked collagen). (b) Tensile modulus of films of different composition and crosslink status at different strains (5%, 20% and high strain). Analyses have been performed at room temperature on fully hydrated samples (after swelling in water for 30 min). Non-crosslinked films were weak when hydrated and it was only possible to test Coll nX. Note: * indicates statistically significant difference in comparison to non-crosslinked sample

of same composition, ■ indicates statistically significant difference to Coll XL, Δ indicates statistically significant difference to Gel XL for statistical significance of $p \leq 0.05$ in Student's *t*-test. The error bars indicate the standard error of the mean (n = 14).

Figure 4. Immunofluorescence images showing staining of cell nuclei (Hoechst) and cytoplasm (CellTracker) of C2C12-α2+ cells seeded for 72 h on films of different composition. (a) Coll nX, (b) Gel nX, (c) Coll XL and (d) Gel XL. Scale bar is 100 μm.

Figure 5. Effect of film composition and crosslinking on (a/c) live/dead cell count, (e) surface coverage, (b) cell area (μm^2) and (d) aspect ratio for C2C12-α2+ cells after 72 h culture. Note: * indicates statistically significant difference in comparison to non-crosslinked sample of same composition, ■ indicates statistically significant difference between collagen-based and gelatin-based films for statistical significance of $p \leq 0.05$ in two-way ANOVA. The error bars indicate the standard error of the mean.

Figure 6. Effect of film composition and crosslinking on cell count of C2C12 cells after 72 h culture. Note: * indicates statistically significant difference in comparison to non-crosslinked sample of same composition, ■ indicates statistically significant difference between cell lines (C2C12 and C2C12-α2+, shown in figure 4a) for statistical significance of $p \leq 0.05$ in two-way ANOVA. The error bars indicate the standard error of the mean.

Figure 7. Comparative study: effect of composition and crosslinking on cell count of C2C12 and C2C12-α2+ cell lines. Trendlines are for all collagen (circles, orange) or all gelatin (triangles, blue) samples. Coll-Gel samples are plotted separately as black squares. The error bars indicate the standard error of the mean.

Figure 8. Comparative study: effect of composition, crosslinking (g-i) and physical properties (roughness a-c, stiffness d-f) on live cell count (left column of graphs), cell area (central column of

graphs) and aspect ratio (right column of graphs) of C2C12-α2+ cells. Samples are grouped according to their 'predominant' composition and crosslink status (Coll nX orange circles, Coll XL red cirlces, Coll-Gel nX black squares, Coll-Gel XL grey squares, Gel nX blue triangles, Gel XL green triangles). The error bars indicate the standard error of the mean.

Figure 9. Comparative study: effect of composition, crosslinking (c) and physical properties (roughness a, stiffness b) on live cell count of C2C12 cells. Samples are grouped according to their 'predominant' composition and crosslink status (Coll nX orange circles, Coll XL red cirlces, Coll-Gel nX black squares, Coll-Gel XL grey squares, Gel nX blue triangles, Gel XL green triangles). The error bars indicate the standard error of the mean.

Figures

Table 1.

Composition	Abbreviation Non-Cross-linked	Abbreviation Cross-linked
Collagen	Coll nX	Coll XL
Collagen + insoluble elastin (ratio 9:1)	Coll-IE nX	Coll-IE XL
Collagen + soluble elastin (ratio 9:1)	Coll-SE nX	Coll-SE XL
Gelatin	Gel nX	Gel XL
Gelatin + insoluble elastin (ratio 9:1)	Gel-IE nX	Gel-IE XL
Gelatin + soluble elastin (ratio 9:1)	Gel-SE nX	Gel-SE XL
Collagen + Gelatin (ratio 1:1)	Coll-Gel nX	Coll-Gel XL

Table 2.

	Uncross-linked	Cross-linked	Degree of Cross-linking
Coll	14 ± 2.8	7.3 ± 1.4	0.49
Coll-IE	16 ± 9.1	8.6 ± 3.6	0.46
Coll-SE	24 ± 1.8	7.83 ± 2.2	0.67
Gel	23 ± 14	4.2 ± 2.3	0.82
Gel-IE	21 ± 8.2	7.9 ± 0.9	0.61
Gel-SE	24 ± 15	4.5 ± 0.20	0.82
Coll-Gel	25 ± 5.8	8.7 ± 1.9	0.66

Figure 1.

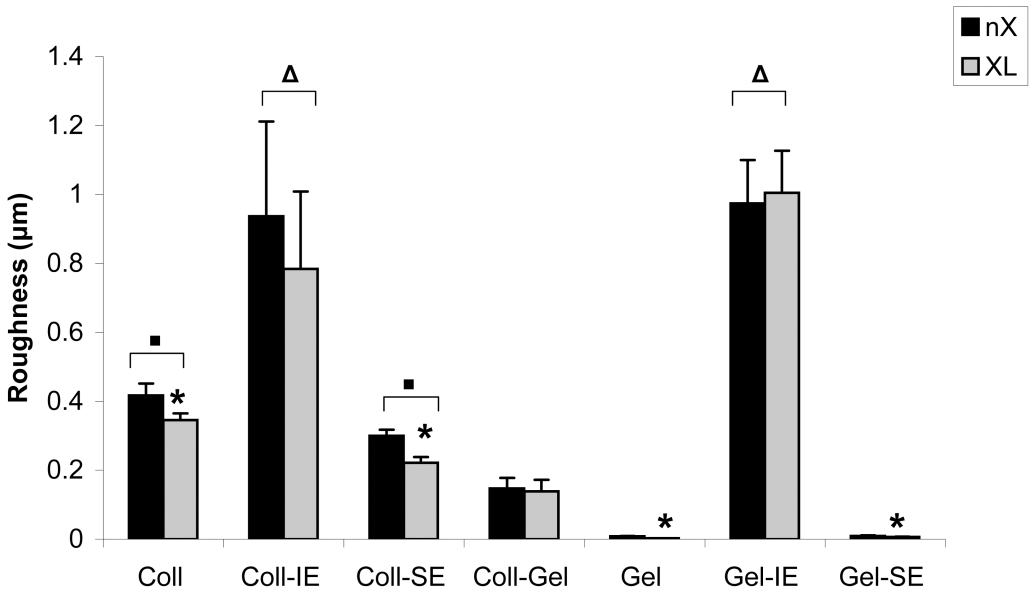


Figure 2.

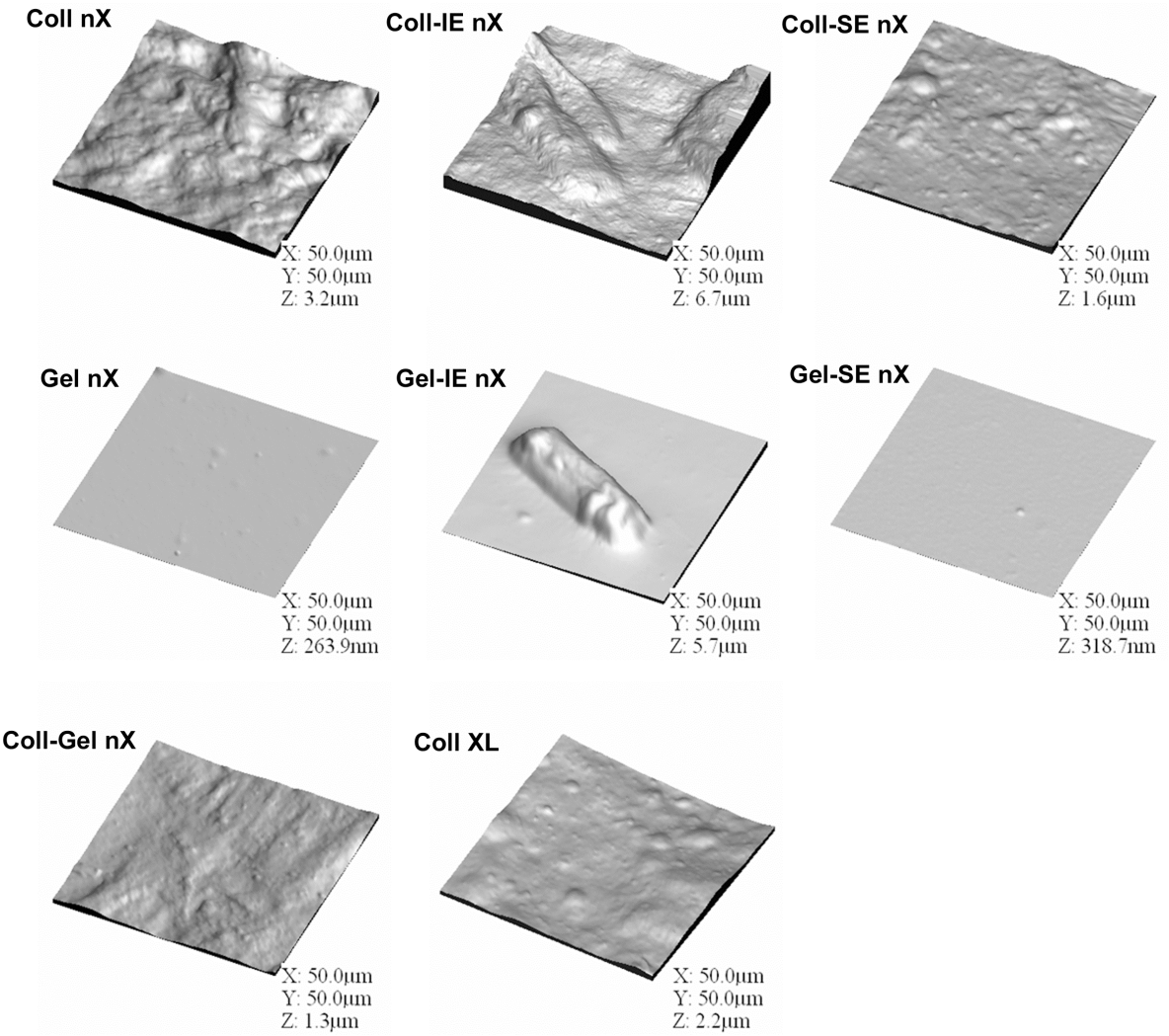

Coll nX
X: 50.0µm
Y: 50.0µm
Z: 3.2µm
Coll-IE nX
X: 50.0µm
Y: 50.0µm
Z: 6.7µm
Coll-SE nX
X: 50.0µm
Y: 50.0µm
Z: 1.6µm
Gel nX
X: 50.0µm
Y: 50.0µm
Z: 263.9nm
Gel-IE nX
X: 50.0µm
Y: 50.0µm
Z: 5.7µm
Gel-SE nX
X: 50.0µm
Y: 50.0µm
Z: 318.7nm
Coll-Gel nX
X: 50.0µm
Y: 50.0µm
Z: 1.3µm
Coll XL
X: 50.0µm
Y: 50.0µm
Z: 2.2µm

Figure 3.

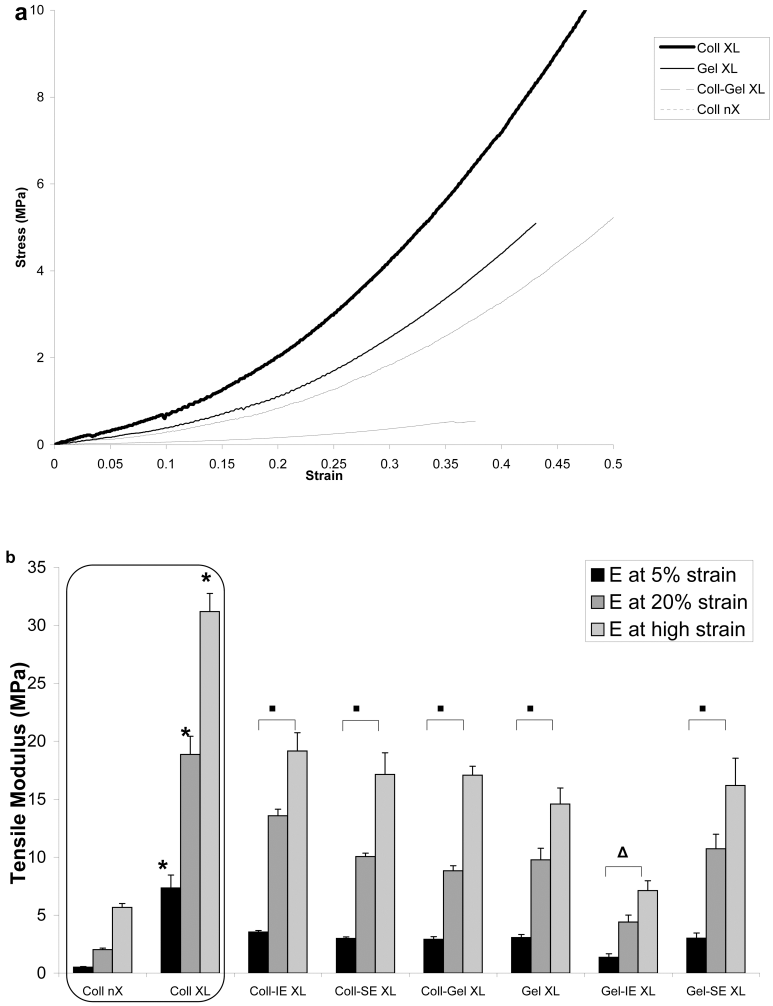
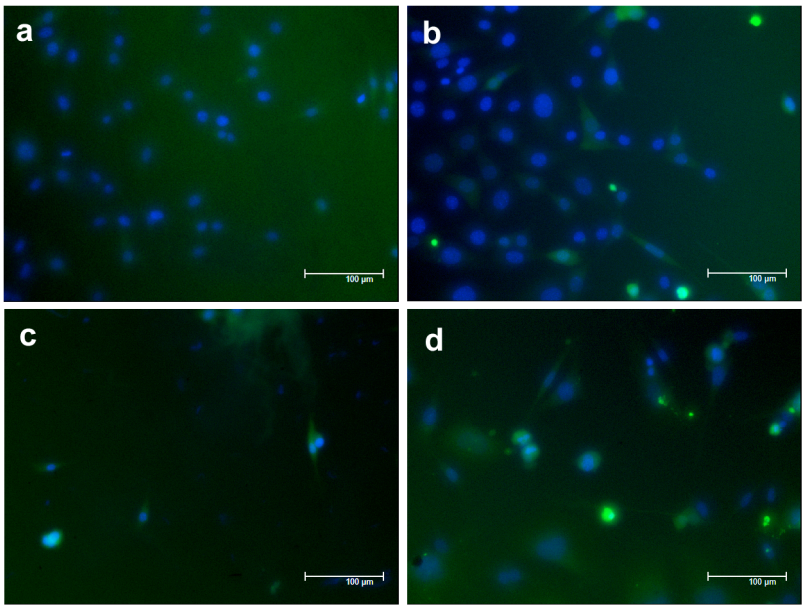


Figure 4.



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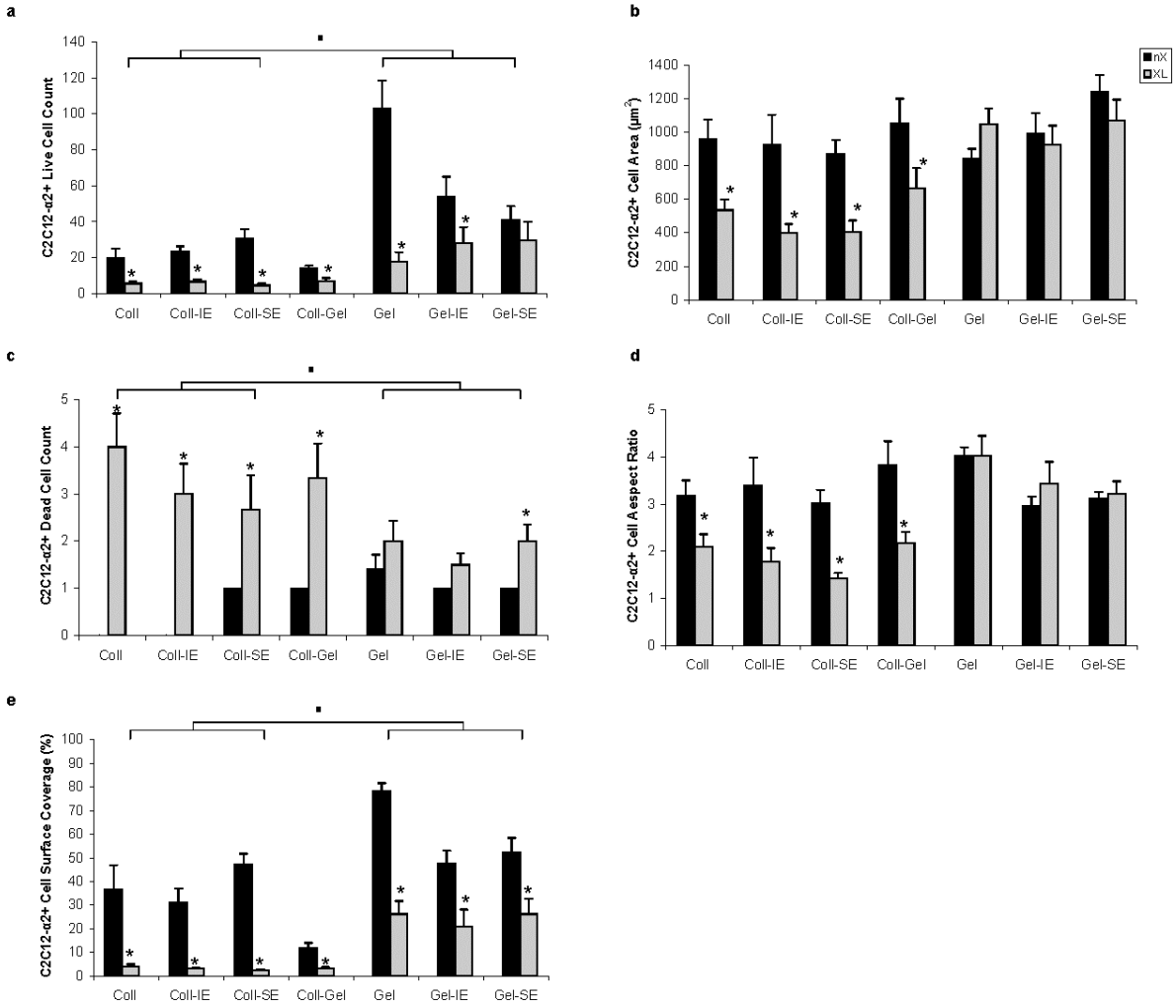

a
C2C12-α2+ Live Cell Count
b
C2C12-α2+ Cell Area (μm²)
nX
XL
c
C2C12-α2+ Dead Cell Count
d
C2C12-α2+ Cell Aespect Ratio
e
C2C12-α2+ Cell Surface Coverage (%)
Coll
Coll-IE
Coll-SE
Coll-Gel
Gel
Gel-IE
Gel-SE

Figure 6.

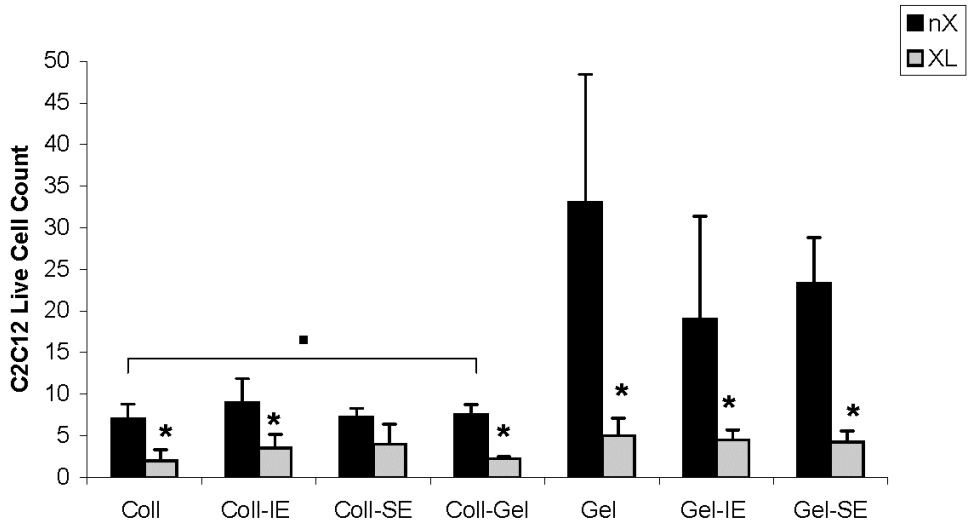


Figure 7.

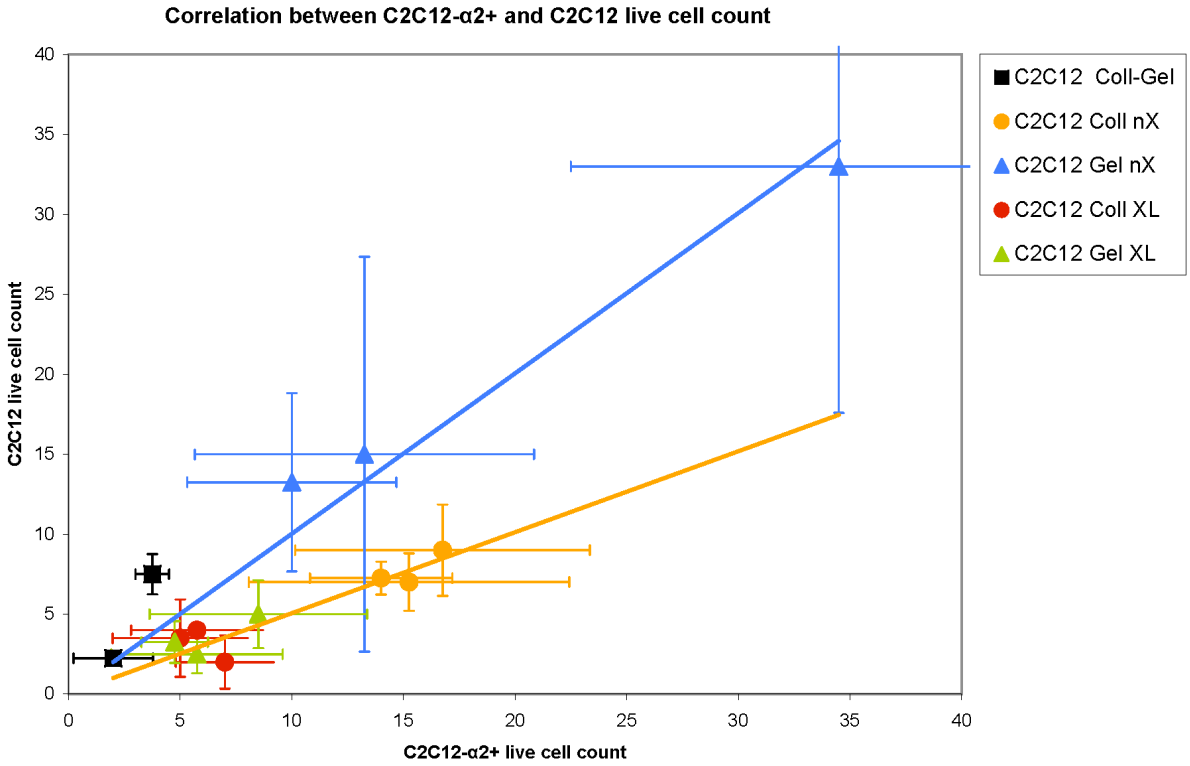


Figure 8.

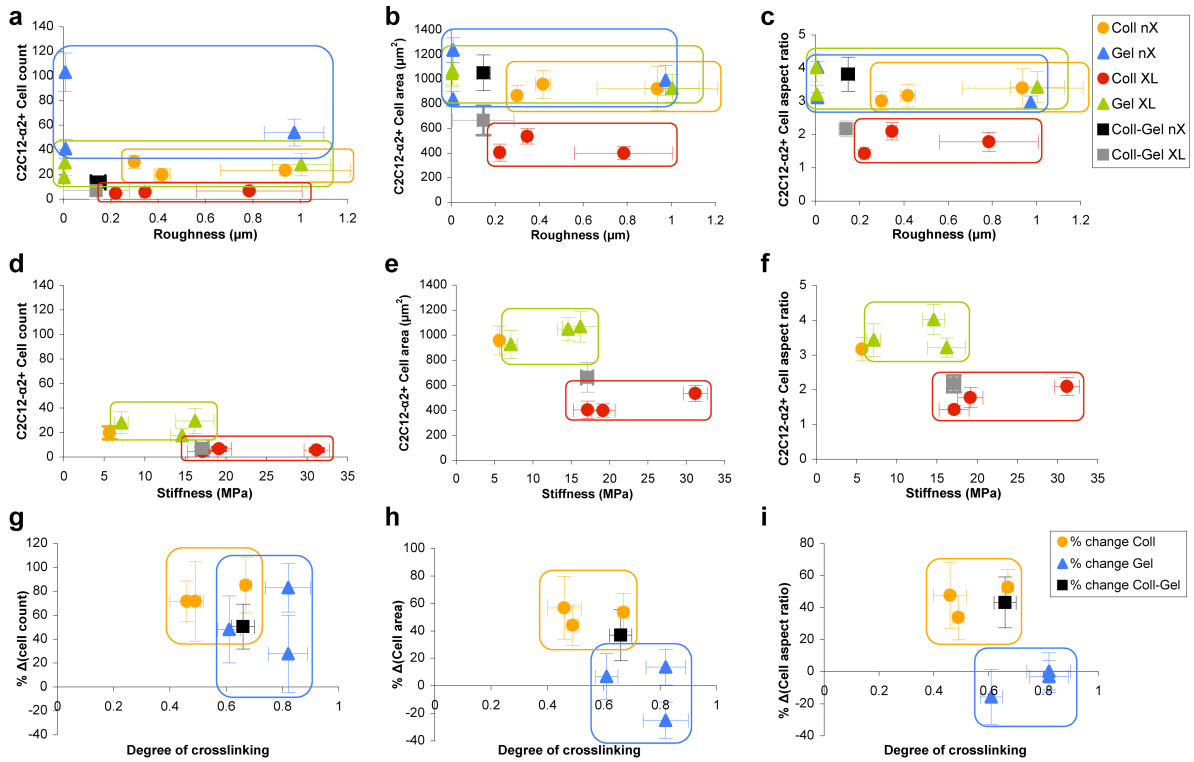
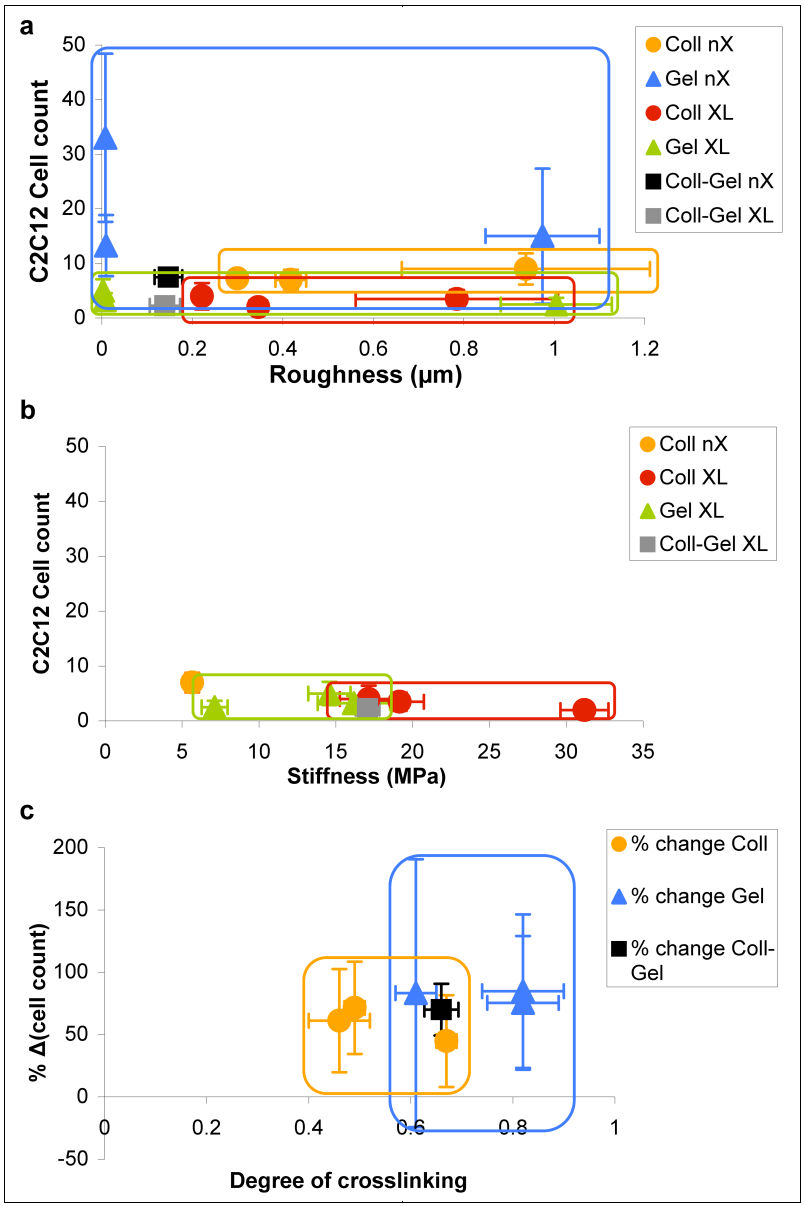

a
C2C12-α2+ Cell count
Roughness (μm)
b
C2C12-α2+ Cell area (μm²)
Roughness (μm)
c
C2C12-α2+ Cell aspect ratio
Roughness (μm)
Coll nX
Gel nX
Coll XL
Gel XL
Coll-Gel nX
Coll-Gel XL
d
C2C12-α2+ Cell count
Stiffness (MPa)
e
C2C12-α2+ Cell area (μm²)
Stiffness (MPa)
f
C2C12-α2+ Cell aspect ratio
Stiffness (MPa)
g
% Δ(cell count)
Degree of crosslinking
h
% Δ(Cell area)
Degree of crosslinking
i
% Δ(Cell aspect ratio)
Degree of crosslinking
% change Coll
% change Gel
% change Coll-Gel

Figure 9.


a
C2C12 Cell count
Roughness (μm)
Coll nX
Gel nX
Coll XL
Gel XL
Coll-Gel nX
Coll-Gel XL
b
C2C12 Cell count
Stiffness (MPa)
Coll nX
Coll XL
Gel XL
Coll-Gel XL
c
% Δ(cell count)
Degree of crosslinking
% change Coll
% change Gel
% change Coll-Gel