Article:
Yang, L., Yaseen, M., Zhao, X. et al. (6 more authors) (2015) Gelatin modified ultrathin silk fibroin films for enhanced proliferation of cells. Biomedical Materials, 10 (2). ISSN 1748-6041

https://doi.org/10.1088/1748-6041/10/2/025003

Gelatin Modified Ultrathin Silk Fibroin Films for Enhanced Proliferation of Cells

Luyuan Yang[1], Fang Pan[1], Xiubo Zhao[2], Yuming Wang[3], Paul Coffey[1], Mohammed Yaseen[1], Hai Xu[3], John Webster[4], and Jian R. Lu[1]*

[1]Biological Physics Laboratory, School of Physics and Astronomy, University of Manchester, Schuster Building, Manchester M13 9PL, UK

[2]Department of Chemical and Biological Engineering, University of Sheffield, Mappin Street, Sheffield, S1 3JD, UK

[3]Centre for Bioengineering and Biotechnology, China University of Petroleum (East China), 66 Changjiang West Road, Qingdao 266580, China

[4]ISIS Neutron Facility, Rutherford Appleton Laboratory, STFC, Chilton, Didcot, Oxon OX11 0QX, UK

*To whom correspondence should be addressed (email: j.lu@manchester.ac.uk)

Abstract

Silk fibroin (SF) was modified by mixing with gelatin (G) and films coated with different mixtures of SF and G were used to explore if mixing of G in SF could enhance the surface biocompatibility of the natural SF as cell growth biomaterials. Ultrathin films were coated from aqueous SF solutions pre-mixed with different amounts of G. It was found that the stability of SF/G blended films after methanol treatment was greatly improved in physiological conditions compared with pure G films fabricated under similar conditions. G helped smooth the surface morphology of the SF/G films formed. Surface exposed RGD sequences were successfully identified on the SF/G films through specific recognition of an integrin mimicking peptide (bearing the sequence of CWDDGWLC). Cell culture experiments with 3T3 fibroblasts demonstrated that SF/G films with 1.2% ~ 20% (w/w) G gave clear improvement in promoting cell attachment and proliferation compared to pure SF films. Films containing 10% ~ 20% (w/w) of G showed overall cell attachment and growth even superior to the pure G films. Such enhanced cellular responses must result from improvement in film stability arising from SF and in cytocompatibility arising from G. These SF/G blended biomaterials have great potential in tissue engineering and biomedical engineering where physical and biological properties could be manipulated via mixing either as bulk biomaterials or for coating based applications.

Keywords: Silk fibroin; Gelatin; Ultrathin films; Film stability; Biocompatibility; RGD; Cell attachment; Proliferation

1. Introduction

Attachment, spreading and proliferation of anchorage dependent cells are strongly influenced by surface biocompatibility including surface physical, chemical and biological properties such as structural stability, roughness, wettability, charge, chemical groups and cell-binding ligands [1-4]. How to mediate the cell-surface interaction remains a big challenge in cell culture, tissue engineering and biomedical applications. For the purpose of enhanced cellular performance, a cell friendly surface coating material is often utilized to provide suitable and consistent support to a population of cells throughout their life cycle.

Silk fibroin (SF) from the domesticated *Bombyx mori* silkworm is a competitive candidate for such cases not only because it is natural and inexpensive, but also because it has many other unique properties. Earlier studies observed that native SF films had good mechanical strength in the wet state, and high oxygen and drug permeability [5-7]. *In vivo* and *in vitro* experiments also showed that inflammatory responses to SF films were similar to or less than collagen films [8]. The purification process used to extract fibroin from raw silk disrupts the native crystallised β-sheet domains. However, with dehydration treatment (methanol, heat, etc.), these β-sheet structures can be induced and any SF based materials can therefore be reconstituted to be water-insoluble. This is a valuable property for a biomaterial to meet the requirements for long lasting performance in physiological conditions. Although SF on its own was reported to support some mammalian cell growth [9-11], the fact that it lacks specific integrin ligands or cell recognition sequences such as arginine-glycine-aspartic acid (RGD) has limited its range of application. On the other hand, Gelatin (G), which is hydrolysed from collagen, shows excellent cell attachment, proliferation and differentiation properties possibly due to its RGD sequences and other cell binding motifs [12, 13]. It is widely used in pharmaceutical and biomedical fields since it is nontoxic, biodegradable, low cost and non-immunogenic [14]. However, because of its brittleness, high aqueous solubility, low strength and uncontrollable degradation rates, G is rarely used alone [12, 14]. A possible compromise would be to combine these two naturally occurring polymers together for enhanced stability and biocompatibility.

Several previous studies have shown potential for SF/G blends as films, scaffolds and hydrogels for tissue engineering and controlled drug release [13-20]. So far the reported SF/G films have often been cast by drying the mixture on polystyrene plates at a mild temperature overnight or up to several days [14, 18-20]. This generates a relatively thick film with lack of control of the surface properties. In contrast, we have developed a spin coating method that can fabricate ultrathin SF films. The thickness and morphology of the films can be precisely controlled, thus providing quick and reproducible production of films. In this study, the same method was used to fabricate a series of SF/G blended films at the nanometre scale. Their stability, secondary structures, morphology and performance for culturing mouse embryo fibroblasts were investigated. The aim of this work was to find out the optimal ratios of the SF/G blends for enhanced stability and cellular responses as well as to demonstrate the potential for these well controlled ultrathin SF/G films as new biocompatible surface coating materials.

2. Materials and methods

2.1. Materials

Bombyx mori raw silk was donated by a silk reeling manufacturer in Jiangsu Province in China. Gelatin powder from porcine skin (Type A, ~300 g bloom) was obtained from Sigma-Aldrich. All the other chemicals used in this study were purchased from Fisher Scientific or Sigma-Aldrich if not specified.

2.2. Preparation of regenerated silk fibroin solution

Raw silk was degummed in boiling aqueous 0.5% (w/v) Na_2CO_3 solution for 30 minutes, followed by rinsing with distilled water. This degumming process was repeated twice, followed by drying of the degummed silk fibres in a drying cabinet at 37 ˚C for 2 days. The degummed silk was dissolved at 60 ˚C for 4 hours (under mild stir) in 9.3 M LiBr solution (about 16% (w/v)). The resulting solution was then dialysed against Elgastat ultrapure (UHQ) water for approximately 5 days, in 2 kDa molecular weight cutoff dialysis tubing at room temperature. The dialysis water was frequently changed, until the electric conductivity of the dialysis solution matched the pure UHQ water for more than 12 hours. The successful removal of LiBr was also confirmed by X-ray

photoelectron spectroscopy experiments, in which both elements of Li and Br were not observed on the electron spectra. To facilitate longer storage, the solution after dialysis was lyophilised into powder and kept in the fridge at 4 ˚C. The lyophilised powder is readily dissolvable in water for future use.

2.3. Fabrication of SF/G films

Both SF and G powders were dissolved in PBS buffer (pH 7.4) at 4 mg/ml separately and sterile-filtered with 0.2 μm syringe filters as stock solutions. All sample solutions were then made at 4 mg/ml by adding G stock solution into SF stock solution at the contents of 0.625%, 1.25%, 2.5%, 5%, 10% and 20% (w/w). Films were coated on silica <111> wafers (1.2 cm ×1.2 cm) for the stability test and on sterilised glass wafers (diameter: 13 mm) for cell culture with a specially designed spin coater (WS-400-6NPP-LITE; Laurell, USA) running at a speed of 3000 rpm for 20 seconds. Pure and modified SF films were immersed in methanol for 10 minutes to stabilise the films by inducing β-sheet formation. All films were then rinsed by flowing UHQ water for 45 seconds, dried by N_2 and vacuumed overnight.

2.4. Film thickness and stability

A Jobin-Yvon UVISEL phase modulated spectroscopic ellipsometer was used to measure the thickness and stability of the SF/G films. Coated silica wafers were immersed in PBS culture buffer (Sigma-Aldrich) for 8 days at 37 ˚C. Each day the wafers were taken out, dried with N_2 and measured in air. When measurements were finished, the wafers were put back to PBS buffer. The experimental data was analysed by the software DeltaPsi II.

2.5. Secondary structures

The secondary structures of the SF/G films with or without methanol treatment were characterised by a Fourier - transform infrared (FTIR) spectroscopy attached with a special attenuated total reflectance (ATR) cell enabling direct ATR measurement at the solid (ZnSe crystal)/D_2O interface (Nicolet 6700, Thermo Scientific, USA). Each protein film was fabricated by covering the ZnSe crystal surface with the relevant protein solution (mixed or pure solution made in D_2O, 10 mg/ml, pH 7). After 20 min, the solution was removed, followed by methanol treatment to the surface adsorbed film for

10 min. After the methanol removal, the surface was then dried for about 30 min, followed by filling the cell with D_2O. ATR measurements were performed in the transmission mode with a nominal resolution of 4 cm^{-1} and 64 scans for each experiment. The recorded wavenumber ranged from 400 to 4000 cm^{-1}. Background measurement (in the presence of D_2O) was taken before the protein sample was mounted and this background was subtracted from the sample readings. The average film thickness following such treatment was found to be around 10 nm from samples coated on an optically flat silicon wafer measured by spectroscopic ellipsometry. But note that such film preparation could lead to relatively large variations in film thickness and uniformity, though good enough for ATR measurements.

2.6. Surface morphology

Surface analysis of the SF/G films was carried out using an atomic force microscope (AFM) (Nanoscope IIIa, Digital Instruments, USA) in the tapping mode with an oxide-sharpened Si_3N_4 tip mounted on a triangular cantilever with spring constant of 0.58 N/m (specified by the manufacturer). Images were taken in air at room temperature and flattened as required. Average values of roughness were analysed using the software Nanoscope 7.20.

2.7. RGD epitope identification

The RGD motifs on the surface of SF/G films were identified using an integrin mimicking RGD-binding peptide CWDDGWLC-biotin [21, 22] using the Fmoc solid phase peptide synthesis. The binding sites were further visualised either by streptavidin-colloidal gold (10 nm diameter, BBInternational) with silver enhancer (Sigma-Aldrich) or by Alexa Fluor® 488 Streptavidin-colloidal gold (10 nm, Invitrogen) at room temperature. The films were first treated in a blocking buffer (pH=8.6) containing 0.1 mol/L $NaHCO_3$, 0.02% (w/v) NaN_3, and 5% (w/v) bovine serum albumin for 1 hour. Each film was washed five times in wash buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.1% (v/v) Tween-20, pH=7.5). After incubation with the integrin mimicking peptide (0.1 mg/ml in wash buffer, 30 min), the films were washed five times in the wash buffer to remove unbound peptide. Part of the films was treated with Streptavidin-colloidal gold (3 ng/ml in wash buffer, 45 min) and rinsed in wash buffer and enhanced by a silver enhancing kit for 20

minutes. The other films were treated with Alexa Fluor® 488 Streptavidin-colloidal gold (20 μg/ml, 45 min). Finally, all the samples were washed in wash buffer five times, rinsed with running UHQ water, N_2 dried and then imaged with light microscope or confocal microscope. Fibronectin films (20 μg/ml, 30 min immersion, N_2 dried) were used as positive control. All the samples were prepared in triplicate.

2.8. Cell culture

3T3 fibroblast cells were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (v/v) (PAA) and 1% (v/v) penicillin/streptomycin and were incubated in a humidified atmosphere with 5% CO_2 at 37 ˚C. Cells were passaged every 3 days when approximately 80% confluence was reached.

2.9. Cell morphology

For morphological observation, 3T3 fibroblasts were seeded on coated glass wafers placed in 24-well culture plates at a density of 10000 cells/well. The morphology of the cells was directly examined by light microscope over 4 days. For F-actin stain, Alexa Fluor® 488 phalloidin (Invitrogen) was used on the cells cultured for 3 days. Specifically, cells were washed twice with pre-warmed PBS culture buffer and fixed in 3.7% (w/v) formaldehyde solution in PBS for 10 minutes at room temperature. Followed with PBS wash, cells were further treated with 0.1% (v/v) Triton X-100 in PBS for 5 minutes. Then the cells were incubated in phalloidin solution for 20 minutes.

2.10. MTT assay

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide, a yellow tetrazole) assay was performed on 3T3 cells. It is an indirect method to assess cell viability and proliferation since the activity of enzymes in mitochondria reduces the MTT to formazan crystals. Upon dissolving in DMSO, the solution shows a purple colour which can be quantified to equivalent cell number/viability by colorimetric assay at the wavelength of 570 nm.

2.11. Statistical analysis

Single factor analysis of variance (ANOVA) was used to assess the statistical significance of MTT assay results between groups. The data was shown as the mean ± standard deviation (n=3). The statistical analysis was performed with the software SPSS 16.0 at the confidence levels of 95% and 99%.

3. Results and Discussion

3.1. Secondary structures and stability of SF/G films

Successful cell attachment and growth demand a stable underlying substratum to support the sustainable bioactivity of extracellular matrix (ECM) proteins and functional growth factors [23, 24]. Failure of this property can cause problems in the initial cell attachment and further cell proliferation. The physical strength and stability of the native silk fibers are marked by the presence of crystallised β-sheets. SF in solution contains amorphous metastable conformations such as random coils and α-helices [25] which under certain conditions can be transformed into stable crystallised β-sheets. Such conditions include pH change, sheer force, methanol treatment (dehydrating solvent), metallic ions or heat treatment [26, 27]. In this work, methanol was used because of its efficiency and popularity in inducing stable β-sheets [28]. According to the previous research, immersion of such ultrathin films in the absolute methanol gave the optimal outcome in terms of both stability and time efficiency.

ATR-FTIR spectra (Fig. 1) show that after methanol treatment, amide I peak (C=O stretching) of the SF film shifted from 1645 cm^{-1} to 1622 cm^{-1}, which is a strong indication of the conformational transition from random coils to β-sheets [39]. The pure G films before (data not shown) and after methanol treatment diplay the amide I peaks at the same wavenumber of 1653 cm^{-1}, which indicates methanol has little or no effect on gelatin. The blended SF/G films upon methanol treatment show signature peaks of both the methanol treated SF film (1622 cm^{-1}) and G film (1653 cm^{-1}), however, these peak intensities respond to the proportion of the two proteins within the blended films. There is also a pronounced peak around 1460 cm^{-1} which is most likely to be the amide II peak (N-H bending). Beacause the protein films were very thin, the material in it is strongly confined by the substrate and this explains why the peak position shifted to the lower wavenumber than what have been reported in the literatures [29, 30].

Gelatin is a water-soluble heterogenous protein mixture derived from collagen. By blending G with SF followed with methanol treatment, the crystallised β-sheet structures were clearly able to retain G and prevent it from dissolving into the buffer. Fig. 2 shows the stability of the SF/G films in physiological conditions. Methanol treatment was performed on the SF film and the blended films but not on the G film. The thicknesses of the spin-coated films were roughly 10-12 nm for the SF based films and 28 nm for the G film. All the films were then immersed in Dulbecco's PBS buffer at 37 ˚C for 8 days which mimicked cell culture conditions. The SF films and blended films showed good stability over time. Although there was some decrease in the surface excess for the film with high ratios of G content (e.g. 20%, w/w) during the first day, the remaining parts were stable. In contrast, the G film alone was largely solubilised in buffer within hours.

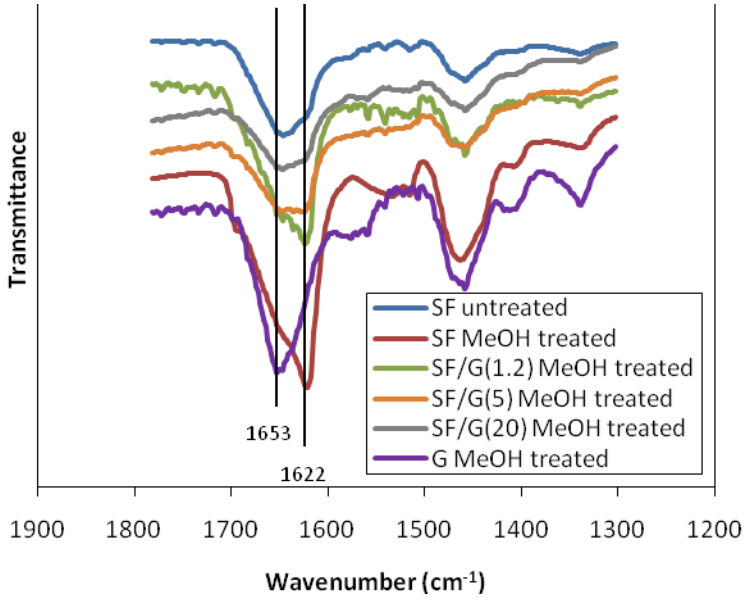

Fig. 1. ATR-FTIR spectra of the SF/G films with or without methanol treatment. SF/G(1.2), SF/G(5), SF/G(20): blended films containing 1.2%, 5% and 20% (w/w) gelatin.

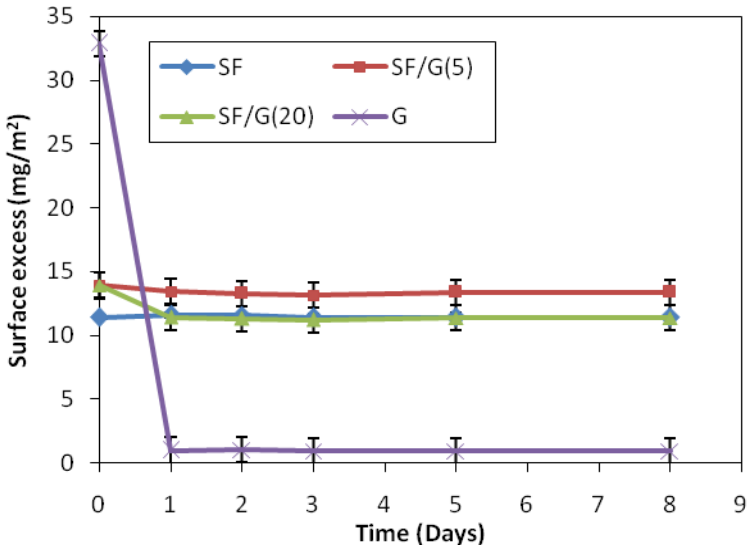

Fig. 2. Stability of the spin-coated SF/G films. All films were immersed in Dulbecco's PBS buffer at 37°C for 8 days. SF/G(5), SF/G(20): blended films containing 5% and 20% (w/w) gelatin. Error bars indicate standard deviation from 3 different locations on the SiO_2 wafer. All films were spin-coated at 4 mg/ml. SF and SF/G blended films were measured after methanol treatment initially.

Amorphous SF/G blends appear transparent and miscible at any ratio. Upon exposure of as-cast films to methanol, the amorphous part of SF transformed into stable crystallised β-sheets. The presence of G did not affect the dehydrating process induced by methanol. In fact, as proposed by Gil *et al*. [30], the SF/G blends upon SF crystallisation could yield physically cross-linked interpenetration networks in which G exhibits a triple helix structure at ambient temperature. The abundant hydrophobic crystallised SF serves to protect G from the full exposure to the aqueous environment and thus lock it within the network. A previous thermal calorimetry study [29] demonstrated that increasing crystallised SF content from 0 to 75% (w/w) in the SF/G blends increased the transition temperature of G going through the helix to the coil state (i.e. gel to liquid) from 35 °C to 50 °C (peak value). This shift can also explain the mechanism of how SF stabilises G. Namely, apart from shielding G from directly contacting water molecules, crystallised SF can maintain G in the more stable conformation of triple helical structures at elevated temperatures (e.g. 37 ˚C).

3.2. Surface morphology of SF/G films

The surface morphology of spin-coated SF/G films was characterised by AFM in the tapping mode (Fig. 3). All the SF based films were methanol treated, water rinsed and N_2 dried. This procedure turns a spin-coated SF film into a rougher surface. The clustering on the surface indicates aggregation of β-sheet structures of SF. When G was involved, the shape of each clustering became smaller and more rounded granules were formed. For the film with 20% (w/w) G, the surface was almost completely packed with small rounded granules. The average surface roughness was reduced from 7.1 nm for the pure SF film to 5.8 nm for the 5% (w/w) SF/G film. Further increasing G content to 20% (w/w) only changed the surface morphology but did not reduce the roughness. This smoothing effect and morphological changes must be related to the involvement of G in the dehydrating process upon exposure to methanol. As previously shown, SF/G films were fairly stable against hydration and hydrogen bonding between SF molecules and also between SF and G molecules must play a role.

In contrast, the surfaces for the spin-coated pure G films were smooth with an average roughness of 0.44 nm, close to the SiO_2 surface (0.24 nm).

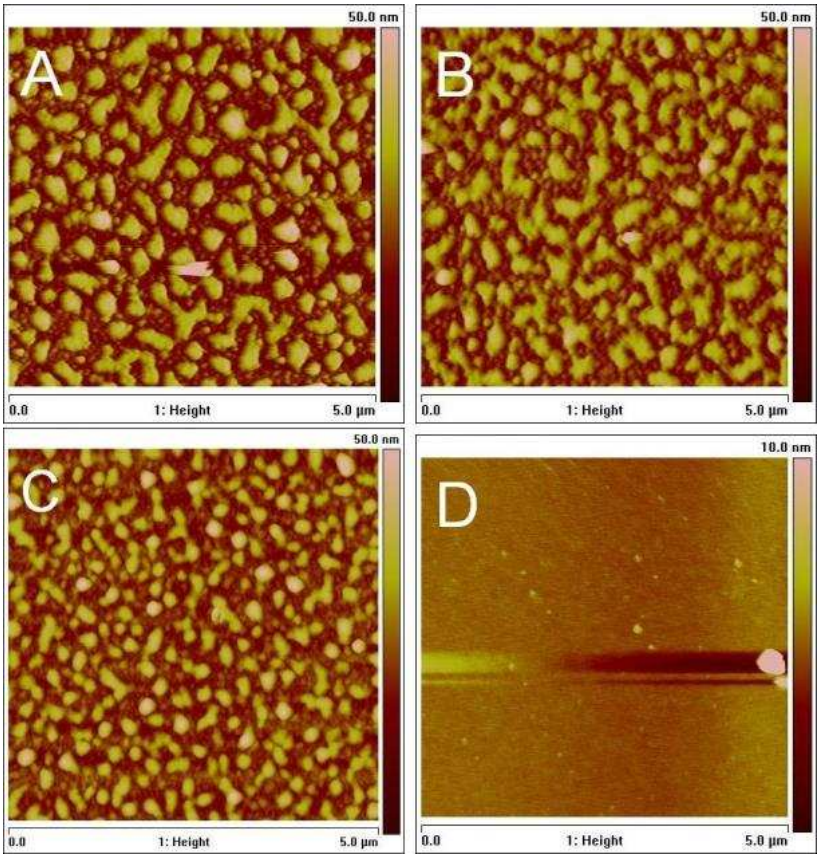

Fig. 3. AFM height images of the SF/G film surfaces. (A): SF film; (B): SF/G blended film with 5% (w/w) gelatin; (C): SF/G blended film with 20% (w/w) gelatin; (D): gelatin film. All films were spin-coated at 4 mg/ml. SF based films were methanol treated.

3.3. RGD identification

Arginine-glycine-aspartic acid (RGD) is the short peptide sequence found in many ECM proteins that is widely recognised as a cell-binding ligand by at least one type of integrins (receptors) on cell surfaces [4, 31]. RGD-containing proteins (e.g. fibronectin, vitronectin, fibrinogen, laminin, collagen/gelatin) together with their integrins constitute a versatile recognition system for cell attachment, migration, differentiation and signalling [4, 21, 31, 32]. Once immobilised on the surface, such tripeptide motifs promote cell attachment, but when solubilised in solution they can inhibit cells from anchoring [32-34]. In this work, the SF films were modified by blending with a given amount of G. RGD motifs can be hidden by chain entanglements and β-sheet structures, making it unavailable for cellular interactions [22]. Therefore, it is important to confirm the existence of RGD sequences on a given film surface. Surface RGD motifs can be visualized by specific binding of an integrin mimicking peptide (CWDDGWLC-biotin) [21, 22]. The biotin labelled peptide can then be probed by streptavidin-colloidal gold conjugate (Fig. 4). With the help of silver enhancer or fluorescein label, the RGD binding sites can be observed under the microscope (Fig. 5 and Fig. 6). Signs of gold binding were not observed for the pure SF film because SF from domesticated *Bombyx mori* silkworm does not contain any RGD sequence. However, clear dark spots and fluorescent spots appeared on the SF/G blended films demonstrating the existence of RGD motifs on the film surface. Moreover, the higher ratios of G (10%, w/w) resulted in more RGD surface presentation than the lower ratios (5%, w/w). These RGD motifs can initiate increased affinity between cells and film surface.

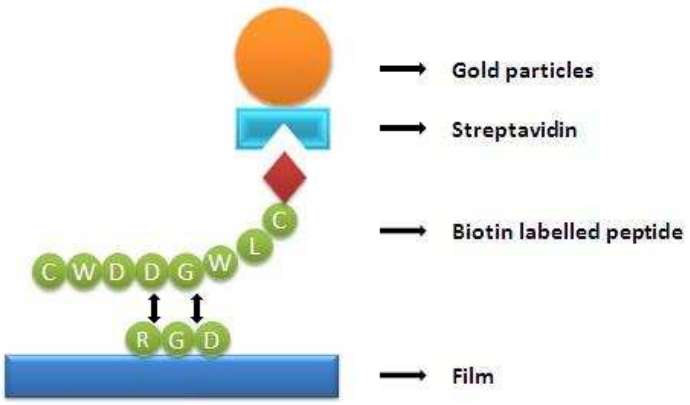

Fig. 4. Schematic diagram of RGD identification method using an integrin mimicking peptide.

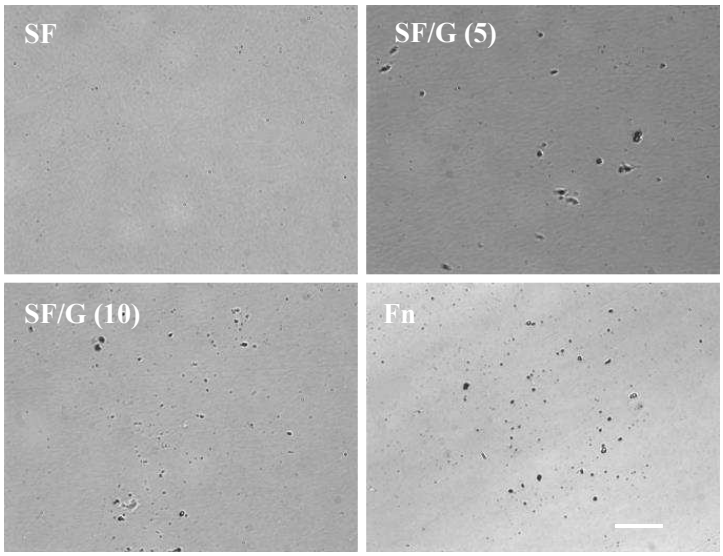

Fig. 5. Light microscopic images identifying the existence of RGD motifs visualised as black spots by binding with CWDDGWLC-biotin-strepavidin-gold nanoparticles enhanced with silver on the surface of gelatin modified SF films. SF/G(5), SF/G(20): blended films containing 5% and 20% (w/w) gelatin; Fn: fibronectin film as control. Scale bar represents 50 μm.

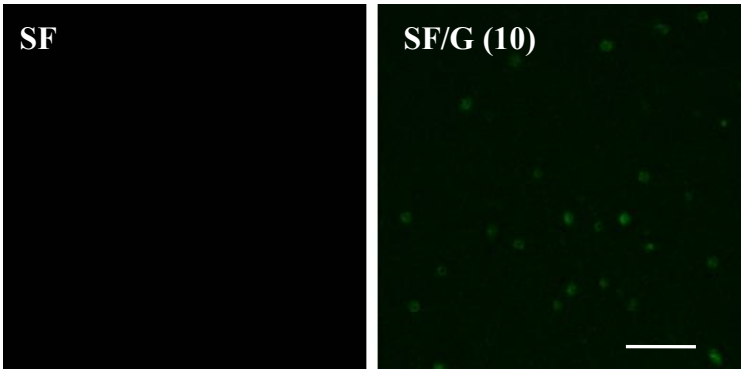

Fig. 6. Confocal microscopic images identifying the existence of RGD motifs visualised as fluorescent spots by binding with CWDDGWLC-biotin-strepavidin-gold nanoparticles labelled with Alexa Fluor® 488 on the surface of gelatin modified SF films. SF/G(10): blended films containing 10% (w/w) gelatin. Scale bar represents 50 μm.

3.4. Morphology of 3T3 fibroblast cells

3T3 fibroblast cells were cultured on the SF/G blended films in order to examine cellular responses. The morphology of the cells was monitored under the light microscope over 4 days as shown in Fig. 7. At 16 hours, cells remained small and rounded on the pure SF films showing weak or no attachment. However, clear cell attachment was presented on the blended films as well as on the G films. Even a very small amount of G content (0.6%, w/w) improved cell attachment. When the G content was increased to 1.2% (w/w) or above, most cells had already attached and spread on the surface. It is interesting to note that although many cells seemed to attach on the pure G film, the majority of cells had not yet spread at this stage. This could be due to the instability of the gelatin film when initially immersed in the culture medium. As G was spin-coated and not physically adsorbed or chemically bound to the substrate, the loosely attached molecules were readily dissolved into the medium within 24 hours as characterised by ellipsometry. Thus, it failed to provide a stable support for cells to attach at the early stage. Over time, attached cells grew and proliferated. Mature cells were spindle-shaped and exhibited a flatter configuration on the blended films than on the pure SF films. On day 4, cell clusters were found on the pure SF films and blended films containing the least amount of G (0.6%, w/w). However, on blended films with higher G contents (1.2%-20%, w/w), cells were more evenly distributed and reached above 80% confluence, demonstrating better cytocompatibility of these blended film surfaces.

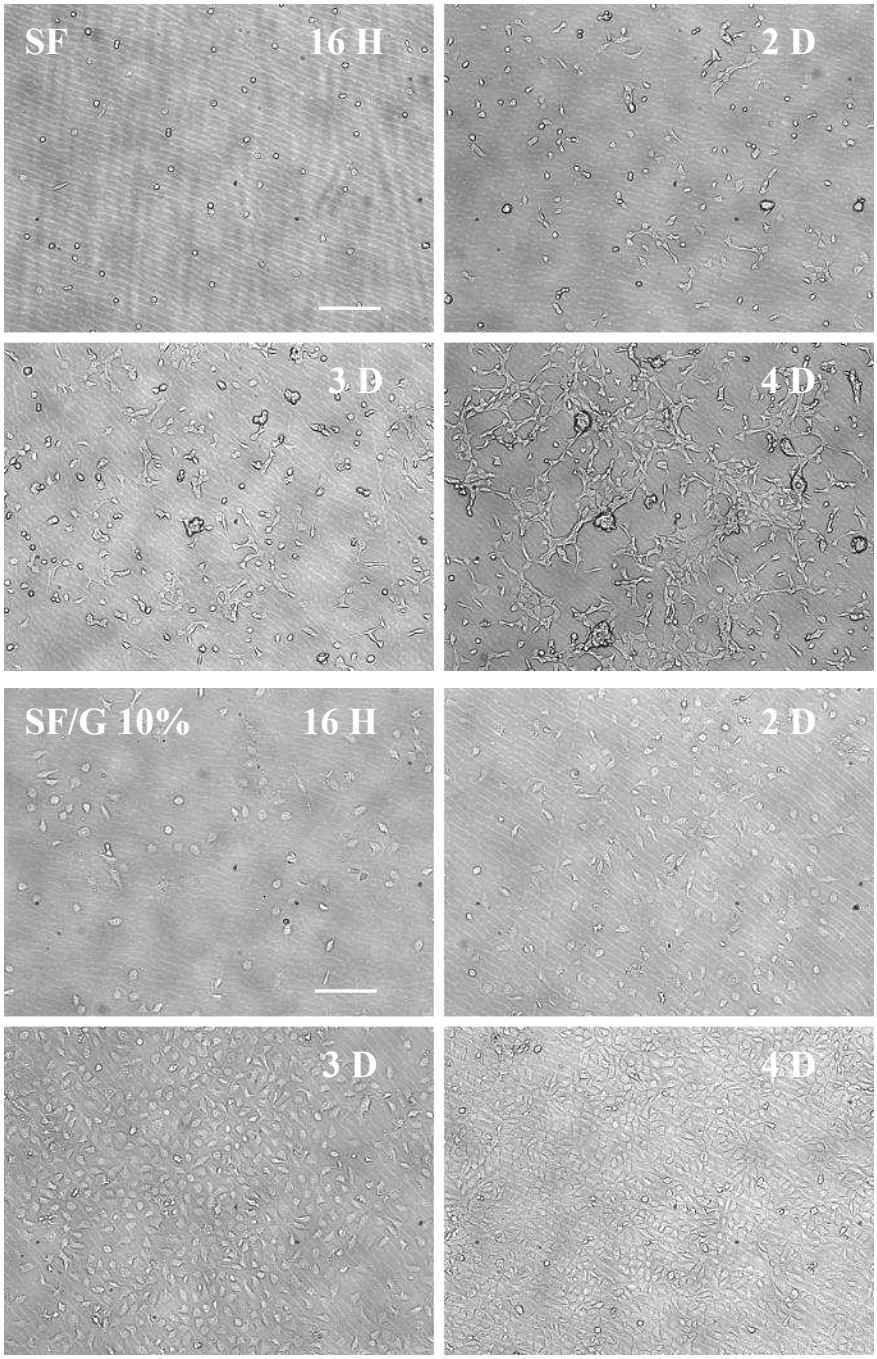

Fig. 7. Light microscopic images of live 3T3 fibroblasts on the pure SF films and G modified SF films at a weight ratio of 10% from 16 hours to 4 days. Scale bar represents 100 μm.

Dramatic differences in the actin microfilament arrangement of cells were found between the blended films and the other substrates. Fig. 8 shows the F-actin cytoskeleton of 3T3 fibroblasts cultured on the SF film, blended films, G film and commercial polystyrene culture substratum. One clear difference is the greater distribution of filaments for each individual cell with increasing G content in the

blended film, reflecting larger cell spreading area. The other difference is that cells growing on the blended films had clear filopodia which were missing or not obvious on the other substrata. Filopodia play important roles in cell migration, cell-ECM adhesion and cell-cell adhesion [35]. Integrins and cadherins, which are cell adhesion molecules, are often found in the tips of filopodia or along the shafts [36, 37]. Extensive studies proved that initiation and elongation of filopodia are controlled by a key set of proteins such as fascin and myosin-X [35]. Filopodia was only found to exit for the cells on the blended films, which shows that there was better cell interaction for the G modified films. Although the mechanism of filopodia formation in relation to SF/G films and the proteins involved are unknown, the presence of RGD sequences may promote the functioning of the RGD-integrin recognition system.

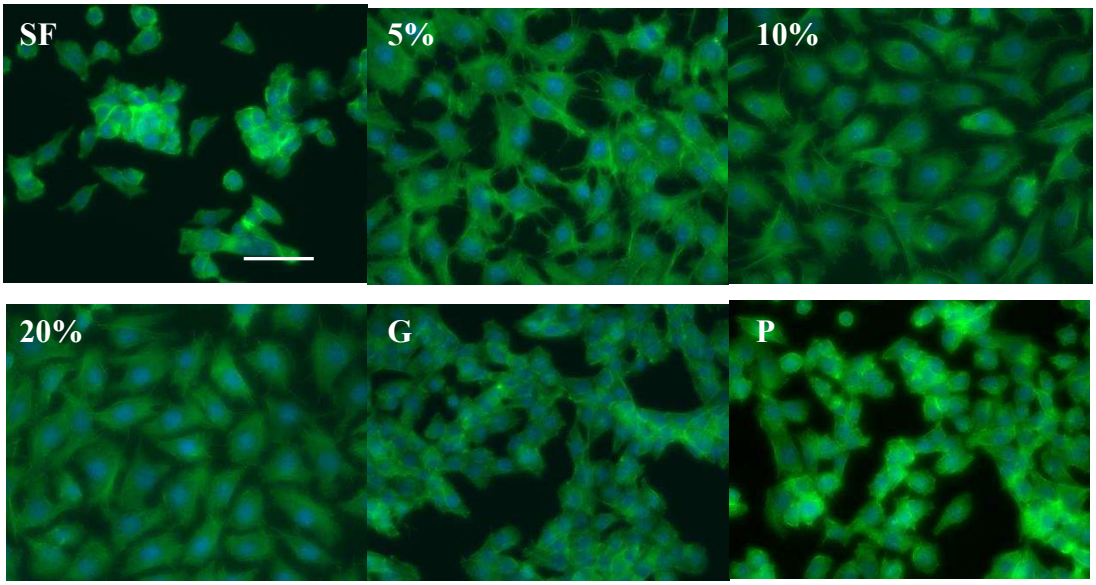

Fig. 8. Fluorescence micrographs of 3T3 fibroblasts growing on SF film, modified SF films containing 5%, 10% and 20% (w/w) gelatin, gelatin film(G) and polystyrene surface (P) on day 3. F-actin cytoskeleton was stained by Alexa Fluor® 488 phalloidin (green), and the cell nuclei were stained by DAPI (blue). Scale bar represents 50 μm.

3.5. Viability of 3T3 fibroblast cells

The viability of 3T3 fibroblasts cultured on different films was evaluated by MTT assay (Fig. 9). The MTT is a yellow tetrazolium salt which can be reduced to blue formazan by mitochondria enzyme from living cells. The amount of reduced formazan is directly proportional to the cell number, thus, it can also reflect cell proliferation [38]. Statistical analysis revealed that cell attachment (16 h) on the modified SF films containing 1.2%-

20% (w/w) G and pure G film was significantly better ($p<0.05$) than that on the pure SF film. On the 4th day, cells growing on the SF films containing 5%-20% (w/w) G displayed significantly higher viability ($p<0.01$) than that on the pure SF film. Moreover, cells growing on the SF films containing 10% and 20% (w/w) G had significantly higher viability ($p<0.05$) than that on the pure G film.

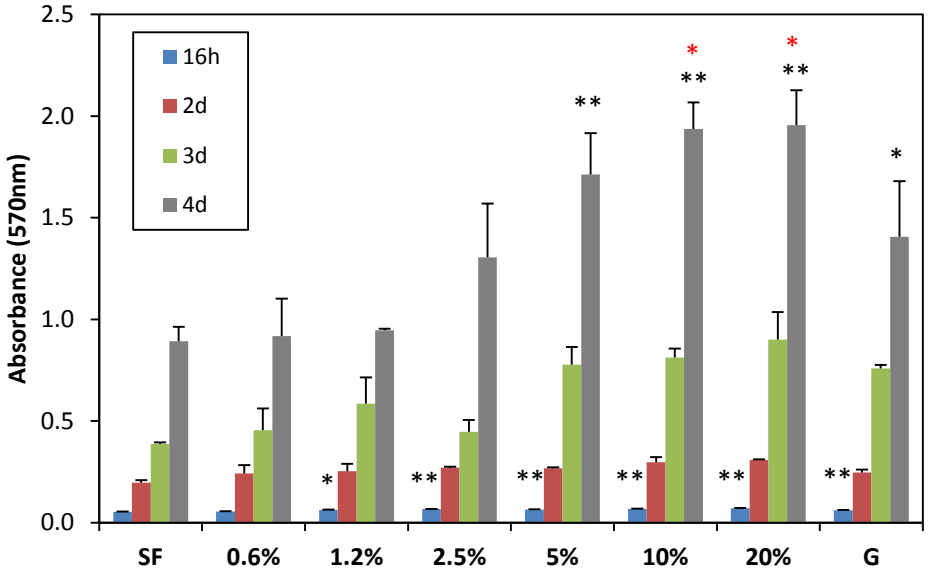

Fig. 9. MTT results showing the viability of 3T3 fibroblast cells on SF film, blended films with 0.6%, 1.2%, 2.5%, 5%, 10% and 20% (w/w) gelatin and gelatin film (G). Error bars represent means+SD for n=3. * ($p<0.05$) and ** ($p<0.01$) indicating significant diffenrece were determined by ANOVA using SF coated films as control; similarly, * ($p<0.05$) indicates significant difference using gelatin coated films as control.

4. Conclusions

This study reports the successful fabrication of nanometre-scale SF/G blended films using the spin coating method, providing reliable control of film surface structure and composition. The as-cast films show good stability in physiological conditions. Preliminary cell culture experiments with 3T3 fibroblast cells demonstrated that SF/G blended films promoted cell attachment and proliferation compared with pure SF films and in some cases, even better than pure gelatin films. The main reason for this enhanced cell performance must be improved film stability arising from SF and improved surface biocompatibility arising from gelatin. All the results obtained suggest these ultrathin SF/G films are good surface coating materials and have great potential in

tissue engineering and biomedical engineering where such blended films are either used as bulk material or for coating purposes.

References

1. Han, L.; Mao, Z.; Wu, J.; Zhang, Y.; Gao, C., Influences of Surface Chemistry and Swelling of Salt-Treated Polyelectrolyte Multilayers on Migration of Smooth Muscle Cells. *Journal of the Royal Society Interface* **2012**, 9 (77), 3455-3468.

2. Arima, Y.; Iwata, H., Effect of Wettability and Surface Functional Groups on Protein Adsorption and Cell Adhesion Using Well-Defined Mixed Self-Assembled Monolayers. *Biomaterials* **2007**, 28, 3074-3082.

3. Richert, L.; Boulmedais, F.; Lavalle, P.; Mutterer, J.; Ferreux, E.; Decher, G.; Schaaf, P.; Voegel, J-C.; Picart, C., Improvement of Stability and Cell Adhesion Properties of Polyelectrolyte Multilayer Films by Chemical Cross-Linking. *Biomacromolecules* **2004**, 5, 284-294.

4. Ruoslahti, E.; Pierschbacher, M. D., New Perspectives in Cell Adhesion: RGD and Integrins. *Science* **1987**, 238, 491-497.

5. Minoura, N.; Tsukada, M.; Nagura, M., Physiochemical Properties of Silk Fibroin Membrane as a Biomaterial. *Biomaterial* **1990**, 11, 430-434.

6. Minoura, N.; Tsukada, M.; Nagura, M., Fine Structure and Oxygen Permeability of Silk Fibroin Membrane Treated with Methanol. *Polymer* **1990**, 31, 265-269.

7. Chen, J.; Minoura, N.; Tanioka, A., Transport of Pharmaceuticals through Silk Fibroin Membrane. *Polymer* **1994**, 35, 2853-2856.

8. Meinel, L.; Hofmann, S.; Karageorgiou, V.; Kirker-Head, C.; Mccool, J.; Gronowicz, G.; Zichner, L.; Langer, R.; Vunjak-Novakovic, G.; Kaplan, D. L., The Inflammatory Responses to Silk Films *in vitro* and *in vivo*. *Biomaterials* **2005**, 26, 147-155.

9. Minoura, N.; Aiba, S-I.; Gotoh, Y.; Tsukada, M.; Imai, Y., Attachment and Growth of Cultured Fibroblast Cells on Silk Protein Matrices. *Journal of Biomedical Materials Research* **1995**, 29, 1215-1221.

10. Minoura, N.; Aiba, S-I.; Higuchi, M.; Gotoh, Y.; Tsukada, M.; Imai, Y., Attachment and Growth of Cells on Silk Fibroin. *Biochemical and Biophysical Research Communications* **1995**, 208 (2), 511-516.

11. Inouye, K.; Kurokawa, M.; Nishikawa, S.; Tsukada, M., Use of *Bombyx Mori* Silk Fibroin as a Substratum for Cultivation of Animal Cells. *Journal of Biochemical and Biophysical Methods* **1998**, 37, 159-164.

12. Seo, K. H.; You, S. J,; Kim, C-H.; Lee, W. K.; Lim, Y. M.; Nho, Y. C.; Jang, J. W., *In Vitro* and *In Vivo* Biocompatibility of γ-Ray Crosslinked Gelatin-Poly (vinyl Alcohol) Hydrogels. *Tissue Engineering and Regenerative Medicine* **2009**, 6, 414-418.

13. Tritanipakul, S.; Kanokpanont, S.; Kaplan, D. L.; Damrongsakkul, S., Morphology and *In Vitro* Biocompatibility of Hydroxyapatite-Conjugated Gelatin/Thai Silk Fibroin Scaffolds. *13th International Conference on Biomedical Engineering IFMBE Proceedings* **2009**, 23 (4), 1377-1380.

14. Mandal, B. B.; Mann, J. K.; Kundu, C. C., Silk Fibroin/Gelatin Multilayered Films as a Model System for Controlled Drug Release. *European Journal of Pharmaceutical Sciences* **2009**, 37, 160-171.

15. Fan, H.; Liu, H.; Toh, S. L.; Goh, J. C. H., Enhanced Differentiation of Mesenchymal Stem Cells Co-Cultured with Ligament Fibroblasts on Gelatin/Silk Fibroin Hybrid Scaffold. *Biomaterials* **2008**, 29, 1017-1027.

16. Wang, S.; Zhang, Y.; Yin, G.; Wang, H.; Dong, Z., Electrospun Polylactide/Silk Fibroin-Gelatin Composite Tubular Scaffolds for Small-Diameter Tissue Engineering Blood Vessels. *Journal of Applied Polymer Science* **2009**, 113, 2675-2682.

17. Xiao, W.; He, J.; Nichol, J. W.; Wang, L.; Hutson, C, B.; Wang, B.; Du, Y.; Fan, H.; Khademhosseini, A., Synthesis and Characterisation of Photocrosslinkable Gelatin and Silk Fibroin Interpenetrating Polymer Network Hyfrogels. *Acta Biomaterialia* **2011**, 7, 2384-2393.

18. Wilaiwan, S.; Yaowalak, S.; Yodthong, B.; Prasong, S., Silk Fibroin/Gelatin Hybrid films for Medical Applications: Study on Chlorhexidine Diacetate. *Journal of Biological Sciences* **2010**, 10 (5), 455-459.

19. Prasong, S., Characterisation of Tetracycline-loaded Thai Silk Fibroin/Gelatin Blend Films. *Journal of Applied Sciences* **2010**, 10 (22), 2893-2898.

20. Watcharin, O-C.; Yaowalak, S.; Wilaiwan, S.; Prasong, S., Morphology, Secondary Structure and Thermal Properties of Silk Fibroin/Gelatin Blend Film. Pakistan Journal of Biological Sciences 2009, 12 (23), 1526-1530.

21. Pasqualini, R.; Koivunen, E.; Ruoslahti, E., A Peptide Isolated from Phage Display Libraries Is a Structural and Functional Mimic of an RGD-Binding Site on Integrins. *The Journal of Cell Biology* **1995**, 130 (5), 1189-1196.

22. Morgan, A. W.; Roskov, K. E.; Lin-Gibson, S.; Kaplan, D. L.; Becker, M. L.; Jr., C. G. S., Characterisation and Optimisation of RGD-Containing Silk Blends to Support Osteoblastic Differentiation. *Biomaterials* **2008**, 29, 2556-2563.

23. Branch, D.; Wheeler, B.; Brewer, G. J.; Leckband, D. E., Long-Term Stability of Grafted Polyethylene Glycol Surfaces for Use with Microstamped Substrates in Neuronal Cell Culture. *Biomaterials* **2001**, 22, 1035-1047.

24. Richert, L.; Boulmedais, F.; Lavalle, P.; Mutterer, J.; Ferreux, E.; Decher, G.; Schaaf, P.; Voegel, J-C.; Picart, C., Improvement of Stability and Cell Adhesion Properties of Polyelectrolyte Multilayer Films by Chemical Cross-Linking. *Biomacromolecules* **2004**, 5, 284-294.

25. Canetti, M.; Seves, A., CD and Small-Angle X-Ray Scattering of Silk Fibroin in Solution. *Biopolymers* **1989**, 28, 1613-1624.

26. Matsumoto, A.; Lindsay, A.; Abedian, B.; Kaplan, D. L., Silk Fibroin Solution Properties Related to Assembly and Structure. *Macromolecular Bioscience* **2008**, 8, 1006-1018.

27. Putthanarat, S.; Zarkoob, S.; Magoshi, J.; Chen, J. A.; Eby, R. K.; Stone, M.; Adams, W. W., Effect of Processing Temperature on the Morphology of Silk Membranes. *Polymer*, **2002**, 43, 3405-3413.

28. Vepari, C.; Kaplan, D. L., Silk as a Biomaterial. *Progress in Polymer Science*, **2007**, 32, 991-1007.

29. Gil, E. S.; Spintak, R.; Hudson, S. M., Effect of β-Sheet Crystal on the Thermal and Rheological Behaviour of Protein-Based Hydrogels Derived from Gelatin and Silk Fibroin. *Macromolecular Bioscience* **2005**, 5, 702-709.

30. Gil, E. S.; Frankowski, D. J.; Bowman, M. K.; Gozen, A. O.; Hudson, S. M.; Richard, J. Spontak., Mixed Protein Blends Composed of Gelatin and *Bombyx mori* Silk fibroin: Effects of Solvent-Induced Crystallisation and Composition. *Biomacromolecules* **2006**, 7, 728-735.

31. Kardestuncer, T.; McCarthy, M. B.; Karageorgiou, V.; Kaplan, D.; Gronowicz, G., RGD-Tethered Silk Substrate Stimulates the Differentiation of Human Tendon Cells. *Clinical Orthopaedics and Related Research* **2006**, 448, 234-239.

32. Ruoslahti, E., RGD and Other Recognition Sequences for Integrins. *Annual Review of Cell and Developmental Biology* **1996**, 12,697-715.

33. Gehlsen, K. R.; Argraves, W. S.; Pierschbacher, M. D.; Ruoslahti, E., Inhibition of *In Vitro* Tumor Cell Invasion by Arg-Gly-Asp-Containing Synthetic Peptides. *The Journal of Cell Biology* **1988**, 106, 925-930.

34. Iwamoto, H.; Sakai, H.; Tada, S.; Nakamuta, M.; Nawata, H., Induction of Apoptosis in Rat Hepatic Stellate Cells by Disruption of Integrin-Mediated Cell Adhesion. *Journal of Laboratory and Clinical Medicine* **1999**, 134 (1), 83-89.

35. Mattila, P.; Lappalainen, P., Filopodia: Molecular Architecture and Cellular Functions. *Nature* **2008**, 9, 446-454.

36. Galbraith, C. G.; Ymada, K. M.; Galbraith, J. A., Polymerising Acting Fibres Position Integrin Primed to Probe for Adhesion Sites. *Science* **2007**, 315, 992-995.

37. Steketee, M. B.; Tosney, K. W., Three Functionally Distinct Adhesions on Filopodia: Shaft adhesions control lamellar extension. *The Journal of Neuroscience* **2002**, 8071-8083.

38. Mosmann, T., Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays. *Journal of Immunological Methods* **1983**, 65, 55-63.

39. Hu, X.; Kaplan, D.; Cebe, P., Determining Beta-Sheet Crystallinity in Fibrous Proteins by Thermal Analysis and Infrared Spectroscopy. *Macromolecules* **2006**, 39, 6161-6170.