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Cytocompatibility of self-assembled β-hairpin peptide hydrogel surfaces

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Abstract

MAX1 is a 20 amino acid peptide that undergoes triggered self-assembly to form a rigid hydrogel. When dissolved in aqueous solutions, this peptide exists in an ensemble of random coil conformations rendering it fully soluble. The addition of an exogenous stimulus results in peptide folding into β-hairpin conformation. This folded structure undergoes rapid assembly into a highly crosslinked hydrogel network. DMEM cell culture media is one stimulus able to initiate folding and consequent self-assembly of MAX1. The cytocompatibility of this gel towards NIH 3T3 murine fibroblasts is demonstrated. Gels were shown to be non-toxic to the fibroblast cells. MAX1 hydrogels also foster the ability of the cells to attach to the hydrogel scaffold in the absence or presence of serum proteins. Additionally MAX1 hydrogels were able to support fibroblast proliferation to confluency with little effect on the rheological properties of the scaffold. MAX1 hydrogels meet the preliminary mechanical and cytocompatibiltiy requirements of a tissue engineering scaffold.
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1. Introduction

Several approaches resulting in successful tissue regeneration exist. One approach involves using prefabricated materials designed to illicit cellular response. Those materials can be implanted into a host, and indigenous cellular machinery acts to construct nascent tissue. For example, OP-1® (Stryker Biotech in Hopkinton, MA) is a collagen matrix composite infused with a bone morphogenetic protein (BMP-7), which signals bone marrow cells to turn on bone regeneration after the matrix is implanted at non-union sites of broken bones [1]. Another approach, autologous tissue regeneration, involves introducing a patient's own cells ex vivo to a polymer scaffold; cell adhesion followed by proliferation leads to new tissue formation [2]. An example of a commercially available autologous tissue regeneration system is Bioseed®-S (BioTissue Technologie in Frieburg, Germany), which combines a fibrin adhesive and skin cells (grown in culture after isolation from the patient) to create a skin graft for treating poorly healing wounds [3]. The choice of material scaffold is critical to the success of either approach with many tissue types still lacking optimal scaffolds. As a result a variety of materials are being considered as potential tissue engineering scaffolds, including both natural [4–14] and synthetic [15–23] polymers. Biologically derived materials may offer advantages, including possible biodegradability and resemblance to the natural extracellular matrix in which cells are supported in vivo [24].

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A large portion of these potential tissue engineering scaffolds are hydrogels, polymeric scaffolds composed primarily of water. The specific requirements for hydrogel tissue engineering scaffolds vary with the desired application, yet there are several general material and biological requirements that should be considered. Structurally, the scaffold should be mechanically rigid and self-supporting in the presence of the desired cells [25]. Porosity is also required for tissue engineering applications, as both cellular nutrients and waste must be able to diffuse through the gel. For in vivo applications, materials must be both cytocompatible and biocompatible. Cytocompatible scaffolds will not only be non-toxic to cells, but will also support cell adhesion and proliferation. Biocompatiblity requires that the scaffold does not elicit an immune response from the host. Finally, in vivo biodegradation of the scaffold, although not a requirement, is advantageous for some applications [24].

A family of peptides has been developed whose ability to self-assemble into hydrogel material is directly linked to their intramolecularly folded state. These peptides adopt random coil conformations in aqueous solution and are freely soluble until intramolecular folding is triggered by the addition of a stimulus. Upon folding, the peptides adopt a conformation conducive to self-assembly. Assembly ultimately leads to the formation of a structurally rigid hydrogel without the need for incorporation of covalent crosslinks (Fig. 1A). This novel, chemically benign mechanism allows material formation in a temporally resolved manner that should facilitate both the prefabrication of materials for eventual host incorporation and autologous approaches to tissue engineering. We have recently reported peptides that undergo triggered folding and either reversible or irreversible assembly in response to changes in the pH [26], temperature [27], or ionic strength [28]. Herein, we investigated the cytocompatibility of a hydrogel formed from one of these peptides whose folding and self-assembly can be triggered by the addition of cell growth media.

MAX1 is a 20 amino acid peptide that folds to form an amphiphilic β-hairpin. The sequence of MAX1 (H_2N-VKVKVKVKV^{D}PPTKVKVKVKV-$CONH_2$) includes alternating lysine and valine residues on two β-strands flanking a type II′ β-turn (Fig. 1B). Folding of the peptide is directly linked to self-assembly; therefore, the conditions that favor hairpin formation also lead to material formation. Initial studies indicate that MAX1 folding and self-assembly are contingent upon elimination of charge repulsion between neighboring lysine residues via deprotonation at high pH (9.0) [26] or charge screening with exogenous salt at physiological pH (7.4). For example, when MAX1 is dissolved in water at a concentration of 4 wt%, it is unfolded and the resulting solution is freely flowing, having the viscosity of water. When an equal volume of buffered (pH 7.4), concentrated saline solution is added (resulting in 150 mM NaCl, 2 wt% peptide), the peptide folds and assembles into rigid hydrogel material. The nanoscale structure of the resultant hydrogel is consistent with the formation of short (20–200 nm) β-sheet-rich fibrils that are non-covalently crosslinked by interfibril junctions. Junctions presumably result from the non-specific

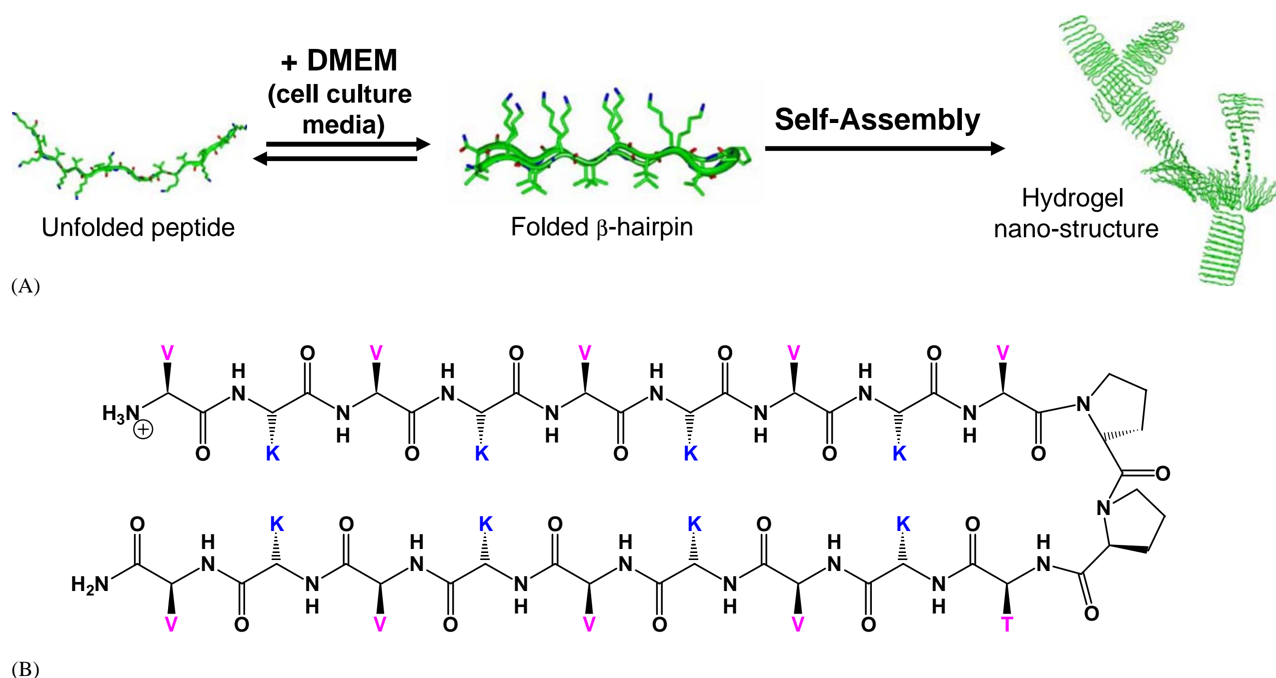

Fig. 1. (A) Model for the folding and self-assembly of MAX1. (B) Folded sequence of MAX1.

collapse of the valine-rich faces of the amphilic hairpins during assembly, Fig. 1A. This model is supported by TEM as well as neutron and X-ray scattering experiments [28]. Resultant hydrogels are structurally rigid as assessed by rheology yet porous on both the nano- and micro-length scales as evident by cryo-TEM and confocal microscopies [26]. Therefore, these gels meet the general mechanical requirements for use as a tissue engineering scaffold.

We were interested in nucleating the folding event by the addition of cell culture growth media instead of saline to facilitate formation of a prefabricated hydrogel impregnated with cellular nutrients for use in tissue engineering. The high salt content of cell culture media (~165 mM) and physiologically compatible pH of 7.4 make it a candidate for initiation of MAX1 folding and hydrogel formation [28]. Fig. 1A contains a model for the folding and self-assembly of MAX1. Here the addition of Dulbecco's Modified Eagle's Medium (DMEM) to an aqueous peptide solution results in the screening of the lysine residue charge density and leads to hairpin folding, self-assembly, and ultimate hydrogelation. In this paper, the rheological material properties of MAX1 hydrogels are investigated in the presence of cell culture media, as well as in the presence of proliferating NIH 3T3 murine fibroblast cells, to access the time-dependent effect of gel exposure to cells. Importantly, the cytocompatibility of MAX1 hydrogel is quantitatively demonstrated with this model cell line. Fibroblast cells were chosen for this study due to their predominance in connective tissue, their robust cell growth, and their distinct morphology change upon adhesion to surfaces. The gel non-cytoxicity, robust cell adhesion, and vigorous cell proliferation of adherent cells on the surface of these hydrogels will be discussed.

2. Experimental

2.1. Materials

Trifluoroacetic acid (TFA), piperidine, thioanisole, ethanedithiol, anisole, and trichloroacetic acid (TCA) were purchased from Acros. Appropriately side-chain protected Fmoc-amino acids, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and 1-hydroxy-benzotriazole (HOBT) were purchased from Senn Chemicals. NIH 3T3 cells were obtained from American Type Culture Collection (ATCC). DMEM, bovine serum albumin (BSA), and Gentamicin were purchased from Sigma. Trypsin (0.25%) ethylenediaminetetraacetic acid (EDTA), L-Glutamine, and phosphate buffered saline (PBS) were obtained from Cellgro. Calf serum was purchased from Colorado Serum Company. ^{3}H-Thymidine (69 μCi/mmol) was purchased from ICN Biomedicals. Scinti-Safe* Econo 2 Scintillation Cocktail was supplied by Fisher Scientific. Cell growth conditions are 90% DMEM with 50 μg/mL Gentamicin, 10% calf serum and incubation at 37 °C and 5% CO_2 unless otherwise noted.

2.2. Peptide synthesis

MAX1 was prepared on Rink amide resin via automated 9-Fluorenylmethoxycarbonyl (Fmoc)-based peptide synthesis employing an ABI 433A peptide synthesizer and HBTU/HOBT activation and purified by HPLC, according to previously published protocol [26].

2.3. Hydrogel preparation

MAX1 was dissolved in sterile H_2O to a concentration of 4 wt%. Aliquots (75 μL) of this peptide stock were added to desired wells in a 48 well tissue culture plate (Costar 3548). DMEM (75 μL, serum-free) was added to the same wells and the solution (2 wt% in peptide) was incubated for several hours to allow the gel to set. The resultant gels were equilibrated to cell culture conditions by addition of DMEM (200 μL) and incubation at 37 °C and 5% CO_2 overnight. This media (200 μL) is removed prior to addition of cells.

2.4. Rheology

Dynamic oscillatory experiments were performed on a Rheometrics ARES rheometer with a 25 mm parallel plate geometry at 37 °C. A dynamic time sweep gel formation experiment (Fig. 3A) monitored the storage (G') and loss (G'') moduli at 6 rad/s constant frequency and 1% constant strain for 2 h. The 2 wt% MAX1 hydrogel was prepared as described above (350 μL) and loaded onto the instrument immediately after addition of the serum-free media. Frequency sweep experiments (Fig. 3B and Fig. 8) were carried out at 1% strain between 0.1 and 100 rad/s on preformed MAX1 hydrogels. These hydrogels had been incubated under cell culture conditions (37 °C and 5% CO_2), to determine the effect of serum proteins and fibroblast cells on gel rigidity. Gels preformed by the addition of serum-free DMEM were incubated for 3 h. After which time, either: DMEM without serum; DMEM with 10% serum (Fig. 3); or DMEM with 50,000 NIH 3T3 cells/cm^2 and 10% serum was added (Fig. 8). Gel rigidity was measured on separate identically prepared samples for each condition 24 and 96 h after the appropriate additions were made. Since hydrogels do not undergo further swelling after their initial preparation, reagents can be added and removed without affecting the gel

surface morphology. Thus the media (with or without serum) was removed and replaced each day on the 96 h hydrogel samples without disturbing the surface. Gels were transferred to the rheometer and allowed to equilibrate for 30 min before frequency sweep data was measured.

2.5. Cell viability assays

Stock NIH 3T3 cells used in this study were maintained on tissue culture treated polystyrene (TCTP) plates then trypsinized, and counted using a hemacytometer. The resulting cell suspension was diluted with DMEM (containing 10% calf serum) for addition to the 8 well confocal microscope cell culture plate. Cells were plated in triplicate (40,000 cell/cm^2 in 400 μL) on the control TCTP culture plate and on the MAX1 hydrogel surface. The plate was incubated at 37 °C and 5% CO_2 for 5 h to allow cell attachment and spreading to occur. Media was removed and each well was washed with 200 μL DMEM (serum free) to remove serum proteins by diffusion from the gel. The presence of serum proteins can lead to high background fluorescence. A stock solution of 1 μM calcein AM and 2 μM ethidium homodimer in PBS was prepared according to the live/dead assay (Molecular Probes ♯L3224) package instructions, and 200 μL of this stock was added to each well. Photographs of cells were obtained using 10 × magnification on a Zeiss 510 LCM confocal microscope. Green cells indicate viability as the cell permeable calcein AM is hydrolyzed to fluorescent calcein which is cell impermeable. Red coloring indicates cell death due to an increase in ethidium homodimer fluorescence upon DNA binding through incorporation into cells with compromised membranes.

2.6. Cell attachment assays

NIH 3T3 cells were radioactively labeled by addition of ^{3}H-thymidine (5 μCi/mL) to cells growing in a T-150 maintenance flask overnight prior to the attachment assay. After removal of radioactive media, unincorporated ^{3}H-thymidine was removed by incubating the cells with unlabeled media for 1 h. Cells were then trypsinized, transferred to a tube where the trypsin was quenched with media containing serum, and centrifuged at 1500 rpm for 2 min. The resulting cell pellet was resuspended in media, the cells were counted using a hemocytometer, and diluted for addition of 10,000 cells suspended in 200 μL DMEM (containing no calf serum) to a 48-well cell culture plate. Cells were plated in triplicate on the TCTP culture plate, BSA-coated control plate, and on the hydrogel surface. The plate was incubated at 37 °C and 5% CO_2 for 5, 15, 30, or 70 min. At each time point, media was removed from each well and transferred to a scintillation vial (containing 500 μL 1 N NaOH). Each well was washed gently 2 × with PBS (200 μL) and added to the above vial (♯1). Vial ♯1 was counted to determine the amount of non-attached and weakly attached cells. Then DNA from strongly attached cells was solubilized with 1 N NaOH (500 μL) and transferred to a separate scintillation vial (♯2) for counting. Scintillation counting was performed on a Packard 1500 Tri-Carb liquid scintillation analyzer. The percent of strongly attached cells was calculated for each timepoint according to the following equation:

$$\% \text{ strongly attached} = \mathrm{DPM}_{\text{vial }\sharp 2}/(\mathrm{DPM}_{\text{vial }\sharp 1} + \mathrm{DPM}_{\text{vial }\sharp 2}) \times 100\%$$

2.7. Cell loading assay

In order to establish rate profiles for cell proliferation on hydrogel surfaces, the optimal initial cell seeding density first needs to be established. This ensures that cell confluency is not reached within the time frame of the proliferation rate experiment thus not complicating proliferation with cellular behavior at confluency. Quiescent NIH 3T3 cells were trypsinized, counted using a hemocytometer, and diluted in 400 μL DMEM (containing 10% calf serum) for addition to a 48-well cell culture plate. Each cell loading density was plated in triplicate on both the TCTP culture plate and the hydrogel surface. The plate was incubated at 37 °C and 5% CO_2 and media was replaced after 24 h. Pictures of the cells were taken using 10 × magnification on a Nikon Eclipse TE2000U microscope. After 48 h, media was removed from each well and replaced with 200 μL DMEM (serum free) containing ^{3}H-thymidine (1 μCi/mL). Following incubation for 2 h, the radioactive media was removed and the cells were washed 6 × with DMEM (500 μL, gel samples only) and 2 × PBS (200 μL) to remove unincorporated ^{3}H-thymidine. Cellular DNA was then solubilized with 1 N NaOH (500 μL) and transferred to a scintillation vial for counting with 5 mL ScintiSafe cocktail.

2.8. Cell proliferation rate assay

The rate of cell proliferation was monitored in a manner that is essentially identical to that described for the cell loading assay with the following modification to protocol. Five plates were prepared simultaneously as described above and incubated under cell growth conditions. One plate was removed at each time point (6, 12, 24, 48, and 72 h) and assayed by addition of ^{3}H-thymidine following the procedure above. The growth media was refreshed every 24 h to maintain an adequate supply of cell nutrients in the 48 and 72 h plates.

3. Results and discussion

3.1. Hydrogel formation and mechanical characterization

The folding and self-assembly of MAX1 into hydrogel can be triggered by the addition of buffered saline to an aqueous solution of the peptide. Circular dichroism (CD) spectroscopy shows that when MAX1 is dissolved in aqueous buffer (pH 7.4) without additional salt, it adopts an ensemble of random coil conformations. However, when the saline concentration is adjusted to 150 mM, MAX1 folds into a β-hairpin and self-assembles affording a 2 wt% β-sheet rich hydrogel (see supporting information) [28]. Treating an aqueous solution of MAX1 with DMEM cell culture media should have a similar effect due to the total salt content of the media (165 mM). Although it is impossible to directly observe the folding and self-assembly of the peptide by CD in the presence of opaque cell culture media, hydrogelation can be monitored visually. Fig. 2 shows the hydrogelation that occurs when vials containing an aqueous solution of MAX1 and DMEM are mixed; a 2 wt% hydrogel results in which media has been homogeneously incorporated throughout the gel. The kinetics of hydrogel formation can be followed by time sweep rheology. In Fig. 3A, both the storage modulus (G', a measure of the elastic response of the material) and the loss modulus (G'', a measure of the viscous response) are measured at 37 °C as a function of time immediately after the addition of serum-free DMEM to aqueous MAX1. Within 30 min, self-assembly affords a significantly rigid ($G' > 1500$ Pa) hydrogel that is

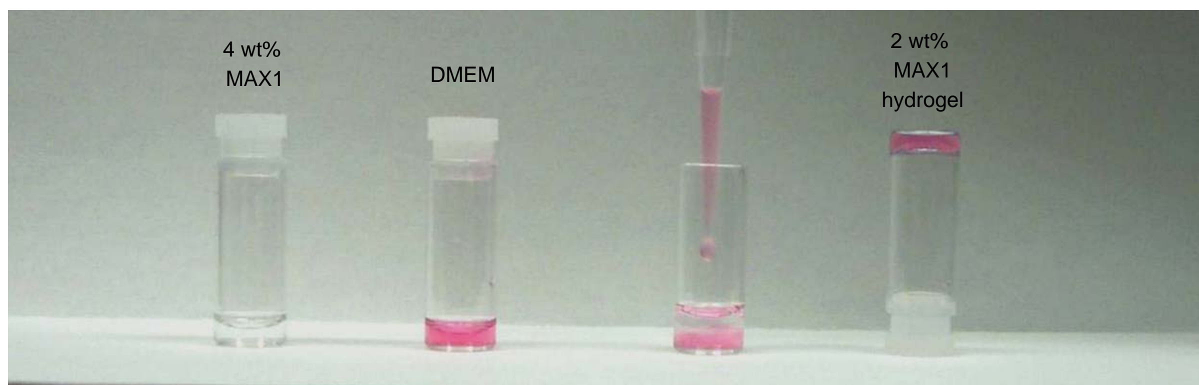

Fig. 2. Formation of 2 wt% MAX1 hydrogel with DMEM cell culture media. Vial 1 contains a clear aqueous solution of 4 wt% MAX1. Vial 2 contains DMEM cell culture media. Addition of vials 1 and 2 together results in the formation of a mechanically rigid self-supporting hydrogel.

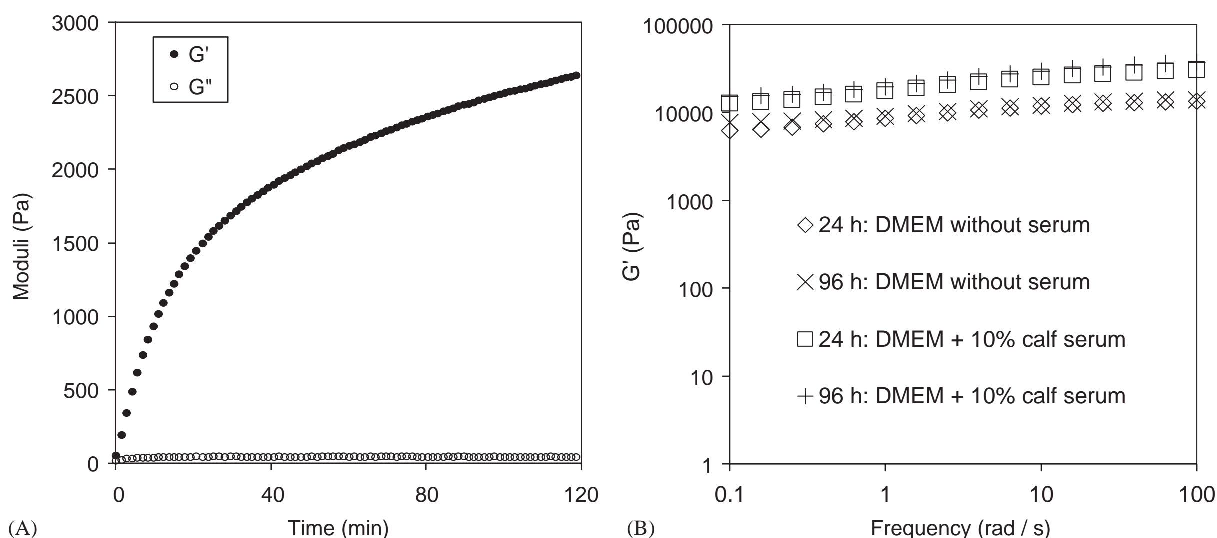

Fig. 3. (A) Time-dependent rheology (freq = 6 rad/s) of 2 wt% MAX1 hydrogel formed upon addition of DMEM to an aqueous solution of MAX1. Closed symbols (●) represent the storage modulus (G′) and open symbols (○) represent the loss modulus (G″). (B) Frequency sweep rheology monitoring the storage modulus of hydrogels under cell culture conditions in the presence and absence of 10% calf serum. Loss modulus not shown for clarity.

self-supporting, with the storage modulus continuing to increase with time ($G' > 2500$ Pa at 2 h). The loss modulus is nearly linear and much smaller than the storage modulus over the entire time range, indicating that the resulting material is rigid and heavily, non-covalently cross-linked in accordance with the nanostructure shown in Fig. 1.

Following the demonstration that DMEM is capable of triggering MAX1 hydrogelation, the material properties of the resultant hydrogels were investigated under cell culture conditions. After hydrogelation is initiated with the addition of serum-free DMEM, gels were treated with additional media and incubated in a high humidity, 5% CO_2 atmosphere at 37 °C. Fig. 3B shows frequency sweep data that measures the storage modulus of preformed gels after incubation in the presence and absence of calf serum under these conditions. Within the studied frequency range, G' was always significantly greater than G'', thus only G' is shown for clarity. Comparing the storage modulus in Fig. 3A ($t = 120$ min) to the moduli in Fig. 3B, it is clear that the rigidity of the gels dramatically increases after they are exposed to cell culture conditions. When gels are incubated in the absence of serum, storage moduli of about 10,000 Pa are realized at 24 h and no significant change occurs thereafter. The addition of 10% calf serum to a separate, identically prepared, hydrogel increases the rigidity slightly. Collectively, these data indicate that soon after peptide folding is triggered, hydrogels are formed of sufficient rigidity for cell seeding. However, incubation of the hydrogel for an additional 24 h in the absence or presence of calf serum results in a more rigid gel. Typically for cell studies, we initiate folding with serum-free DMEM and allow the gel to cure for several hours. After this time, media with or without 10% calf serum (depending on the study) is added and the gel is incubated under cell culture conditions overnight.

3.2. Fibroblast compatibility on hydrogels

Cytocompatibility of 2 wt% MAX1 hydrogels was assessed by investigating the cytotoxicity of the gel towards fibroblasts, the ability of cells to attach to the hydrogel surface and finally the ability of cells to proliferate on the hydrogel surface. Cytotoxicity was qualitatively investigated using a live/dead cell viability assay. Cells were seeded onto preformed hydrogels under growth conditions and incubated for 5 h. In this assay, calcein AM hydrolysis in live cells produces a green fluorescent signal while ethidium homodimer is excluded from live cells and produces a red fluorescent signal only in compromised cells. Fig. 4 shows that cell viability on the hydrogel surface is equivalent to that of the control TCTP surface, indicating that the hydrogels are non-cytotoxic. The figure also qualitatively shows that the cells are able to attach and have begun to adopt a spread morphology on the gel. Interestingly NIH 3T3 cells can be maintained on 2 wt% MAX1 hydrogels and will proliferate beyond confluency over extended periods of time (at least 3 weeks) as long as fresh growth media is provided periodically. It is apparent from this qualitative assay that NIH 3T3 cells are able to grow freely on MAX1 hydrogels as long as required nutrients are supplied.

Although the live/dead assay indicates attachment of the fibroblast cells to the MAX1 hydrogel, it is likely that the serum proteins in the growth media facilitate this attachment under growth conditions. Therefore an attachment assay was performed in the absence of serum to allow assessment of the interaction of the cells directly with the hydrogel surface. Fig. 5 shows the results of an assay in which cell attachment was measured as a function of time and surface identity. Fibroblasts were pre-labeled with ^{3}H-thymidine and seeded onto MAX1 hydrogel, a TCTP control surface and a BSA negative control surface. The data shows the percent of initially

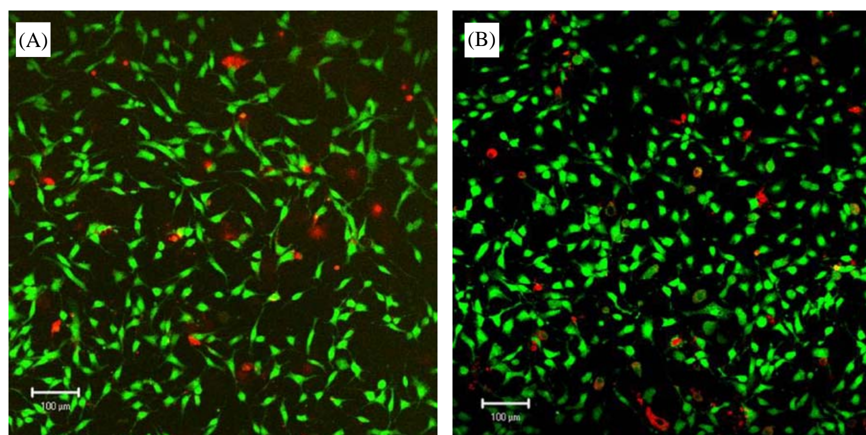

Fig. 4. Live/dead cytotoxicity assay on 40,000 cells/cm^2 murine NIH 3T3 cells 5 h after introduction onto (A) 2 wt% MAX1 hydrogels or (B) TCTP control plates. Viable cells fluoresce green and compromised cells fluoresce red. Scale bar represents 100 μM.

seeded cells that become strongly attached. Attachment of NIH 3T3 cells to both the MAX1 hydrogel and the TCTP control was rapid with nearly all of the cells strongly attached within 15 min. Attachment of the cells to MAX1 hydrogels is marginally faster than to the control plate. Heat denatured BSA coated surfaces were included as a negative control for cell attachment [29], and as expected cells minimally attached to this surface. This assay demonstrates cell compatibility with the MAX1 hydrogel surface suggesting that the hydrogel by itself is conducive to cell attachment and serum proteins need not be present. This is certainly not a requirement for the material, because in many tissue engineering processes nutrients such as serum will typically be supplied to the cells as they are seeded onto a support material. However, some applications may necessitate the absence of serum proteins, and MAX1 hydrogels foster strong attachment both in the absence or presence of serum proteins. Following cell attachment, viable fibroblasts will adopt a "spread" morphology. Photographs taken during a proliferation assay (vide infra) show that cells introduced onto MAX1 hydrogel attach and adopt the elongated morphology expected for healthy fibroblasts, Fig. 6B and C.

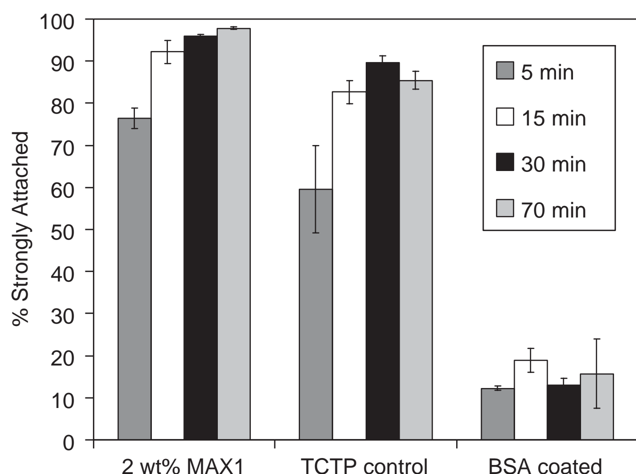

Fig. 5. Percentage of NIH 3T3 cells that are strongly attached to the indicated surfaces after incubating for 5 min (dark gray bars), 15 min (white bars), 30 min (black bars) or 70 min (light gray bars) in serum free DMEM. Polystyrene surfaces coated with heat denatured BSA are used as a negative control for attachment.

3.3. Fibroblast proliferation on hydrogels

Successful tissue engineering scaffolds are not only non-cytotoxic and supportive of cell adhesion, they must also foster cell proliferation. The photographs in Fig. 6B and C were taken of cells proliferating on hydrogel (at identical spatial plate coordinates) on consecutive days. Qualitatively, proliferation is indicated by the decreasing surface space between cells over time. The rate of cell proliferation can be quantitated using a ^{3}H-thymidine incorporation assay. This assay necessitates that an optimal cell-loading density first be determined so that proliferation can be followed over a desired time period without complications from crowding and nutrient depletion. A varying number of cells were introduced onto hydrogel and allowed to proliferate for 48 h after which time ^{3}H-thymidine was added. Only cells that are undergoing DNA replication will incorporate the radioactive label. Therefore, only viable proliferating cells are counted. Again, TCTP serves as the control surface. Fig. 6A indicates that cell proliferation increases with increasing cell loading density. This result is expected as larger numbers of cells will incorporate more thymidine. The decrease in signal at the highest cell numbers is also expected due to two factors: contact inhibition of cell growth as the cells become crowded,

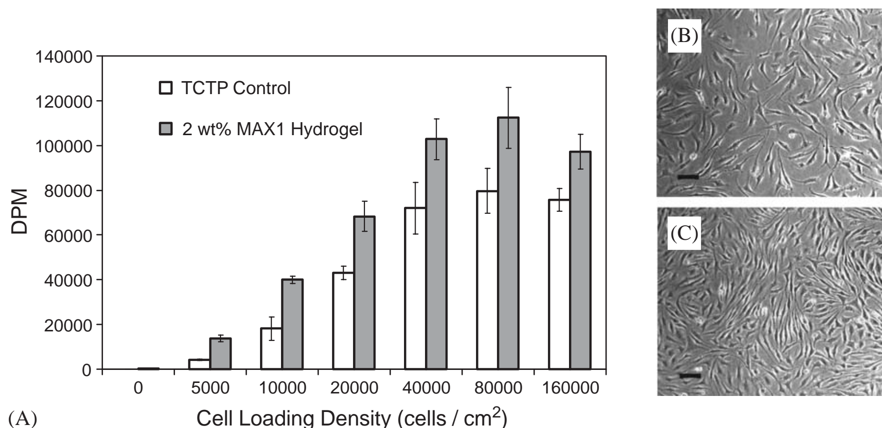

Fig. 6. (A) Cell loading density assay measuring cell proliferation level after 48 h incubation. Degradations per minute (DPM) for 3H-thymidine incorporated into NIH 3T3 cells during DNA synthesis after the cells have been incubated on the surface for 48 h. Photographs of NIH 3T3 cells introduced onto 2 wt% MAX1 hydrogels at 20,000 cells/cm^2 were taken at the same well coordinates after (B) 24 h, and (C) 48 h incubation. Scale bar represents 50 μM.

and slow growth when too many cells consume all available nutrients supplied in the media. This does not indicate a decrease in viability when the cells are past confluency, but simply a decrease in the proliferation rate. Ultimately, 20,000 cells per cm^2 was chosen as the optimal cell seeding density for the rate assay (Fig. 7A) due to the high level of cell proliferation, and the observation that this number leads to confluency after about 48 h of incubation (Fig. 6C). A lower density of 5000 cells per cm^2 was also assayed to determine if sparsely populated gel surfaces would perform well.

Fig. 7 displays the proliferation rate profile of NIH 3T3 cells over three days; although optimal seeding densities were determined for a 48 h proliferation assay, data at prolonged times were collected to investigate the effects of cell crowding on proliferation. Data in Fig. 7A indicates that at 48 h, fibroblasts are proliferating on the MAX1 surface at least as well as on the TCTP control surface. At 72 h, when cells have grown well past confluency, proliferation continues, indicating that cell crowding has little effect as long as fresh nutrients are supplied. The second condition of lower cell loading density (5000 cells/cm^2) more intimately investigates the interaction of the NIH 3T3 cells with the surface by decreasing cell-to-cell interactions [30–32]. When the initial cell seeding density is lower, the cells appear to proliferate at a similar or slightly faster rate on the hydrogel surface compared to the control. Collectively, the data in Fig. 7 suggests that the hydrogel surface performs slightly better than the TCTP control surface in supporting cell proliferation. TCTP plates offer an optimal surface for maintenance of cells in culture. In fact, because the cells used in these studies have been maintained on TCTP plates prior to their uses in the assays, they might be expected to behave more favorably on the control surfaces. The ability of MAX1 hydrogels to even match the cell adhesive and proliferation capabilities of the cell culture plates is significant.

Finally, the mechanical properties of the peptide hydrogel were investigated after prolonged exposure to proliferating cells. Storage moduli of gels that have been seeded with fibroblasts were measured after 96 h. The photograph in Fig. 8 indicates that cells have reached

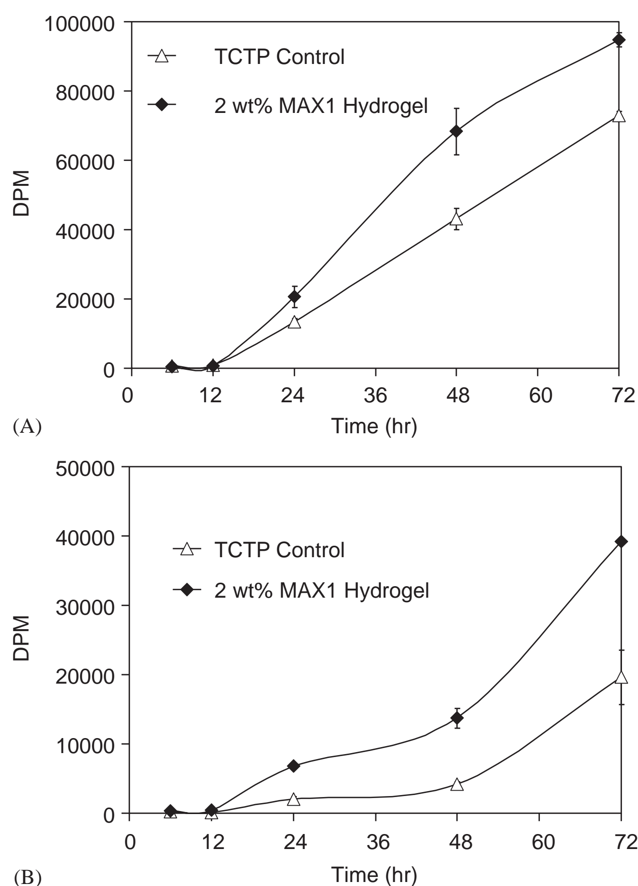

Fig. 7. Cell proliferation rate assay comparing the ability of cells to proliferate on 2 wt% MAX1 hydrogel to a TCTP control surface. DPM represent the level of cell proliferation occurring after the cells have been incubated on the surface for the designated time. NIH 3T3 cells were initially plated under growth conditions at (A) 20,000 cells/cm^2 and (B) 5000 cells/cm^2. Lines are included to aid visualization.

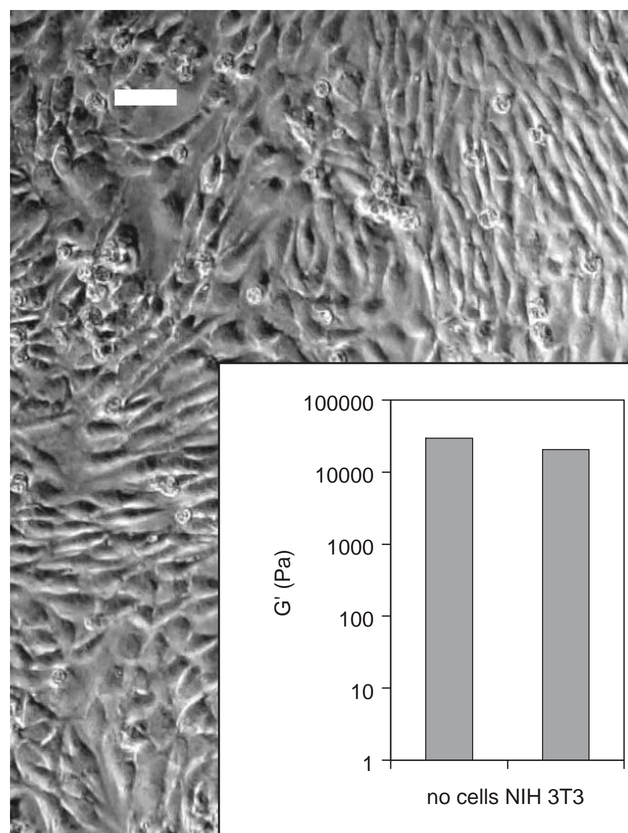

Fig. 8. Photograph (scale bar is 50 μM) of NIH 3T3 cells on the 2 wt% MAX1 hydrogel after 72 h shows that cells have proliferated to confluency. (Inset) Storage modulus, obtained from frequency sweep measurements, of hydrogel at 96 h in the absence and presence of fibroblast cells.

confluency by 72 h and visual assessment concluded that at 96 h confluency was well exceeded. The inset compares the storage modulus of gels incubated under growth conditions in the absence and presence of over-confluent cells. This data suggest that growth of NIH 3T3 cells on the surface of the hydrogel does not have a significant effect on the gel's rigidity even over 4 days. The scaffolding provided by self-assembled MAX1 is robust in the presence of proliferating fibroblast cells. Interestingly, molecular level control of the primary sequence of MAX1 may enable incorporation of protease sites in future designs to control biodegradation for specific applications [19,33,34].

4. Conclusion

MAX1, a de novo designed peptide, undergoes hydrogelation on cue in response to the introduction of cell culture media. This peptide remains unfolded and freely soluble in aqueous solution of low ionic strength. Adjusting the ionic strength to physiological levels (150–165 mM salt) results in peptide folding affording β-hairpin conformers that self-assemble into hydrogel. This process allows temporal control over material formation using biologically compatible media to initiate material formation. Rheological studies indicate that mechanically rigid gels are obtained within 30 min after the initiation of hydrogelation ($G' = 1500$ Pa). Upon exposing the gels to cell culture conditions material rigidity increases ($G' = 10,000$). Cytocompatibility studies indicate that MAX1 hydrogel surfaces are non-toxic towards model NIH 3T3 fibroblasts; they are also conducive towards cell attachment both in the absence or presence of serum and foster cell proliferation. The abililty of MAX1 hydrogels to allow cell attachment in the absence of serum is advantageous; many gel scaffolds under investigation must be functionalized with additional cell binding epitopes to foster cell attachment [8,9,12,15,16,22,23]. Additional rheological studies show that the ridigity of hydrogels is little affected by fibroblasts that have proliferated well beyond confluency on their surface. These experiments collectively indicate that MAX1 hydrogels are cytocompatible towards model fibroblasts and maintain their mechanical rigidity during cell proliferation, making them attractive candidates for use in tissue engineering.

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Appendix A. Supplementary Materials

The online version of this article contains additional supplementary data. Please visit doi:10.1016/j.biomaterials.2005.01.029.

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