Supramolecular hydrogels inspired by collagen for tissue engineering†

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Supramolecular hydrogels are promising biomaterials for cell culture in 2-D and 3-D environments. Inspired by the chemical structure of collagen, which bears the repeating tripeptide of glycine-Xaa-4*R*-hydroxyproline (GXO; Xaa is any one of the natural amino acids), we designed and synthesized a small library of supramolecular hydrogelators (a total of 6). We found that four of the hydrogels were suitable for NIH 3T3 cell culture in the 2-D environments. Gel 2, the best hydrogel, has properties that are similar to those of collagen for 3T3 cell culture. These findings not only provide more supramolecular hydrogel candidates for tissue engineering, but also offer a new strategy for designing biomaterials that mimic nature.

1. Introduction

Here, we report on a small library of self-assembled small molecules that mimic collagen for the purpose of tissue engineering. Supramolecular hydrogels, formed by the self-assembly of small molecules, possess three-dimensional fibril networks that share similarities with extra-cellular matrices (ECM).[1] They are, therefore, thought to be a class of promising materials for tissue engineering and regeneration biomedicine. The repeating RAD (arginine-alanine-aspartic acid) peptide-based supramolecular hydrogels can provide bioactive scaffolds for cell attachment, patterning, proliferation, and differentiation;[2] self-assembled peptide amphiphiles (PAs) have been demonstrated as efficient three-dimensional matrices for the regeneration of tissues and organs;[3] and aromatic short peptide-based hydrogels have shown to be good for cell cultures in 2-D or 3-D environments.[4] We believed that the bioactivity of these self-assembled molecules relied on the fact that they mimicked naturally occurring biomolecules—the molecular design of the RAD peptide is based on the bioactive RGD (arginine-glycine-aspartic acid) peptide; the self-assembled PAs mimic lipids that are the major components in the cell membrane; and the central self-assembled motif of Phe-Phe on the aromatic short peptides comes from an amyloid that exhibits fibril structures. Therefore, one of the strategies to design novel self-assembled molecules that are used for tissue engineering and regeneration medicine relies on the chemical structures of other naturally occurring biomolecules.

Collagen is a group of naturally occurring proteins, and it is the most abundant protein in mammals, making up about 30% of the overall body protein content.[5] As a fibrous protein, collagen has a triple-helical structure, which is comprised of strands with a repeating Gly-Xaa-Yaa sequence. The 4(*R*)-hydroxy-L-proline (4-Hyp or O) residue occurs most often in the Yaa positions, and the Xaa position usually can be any one of the other natural amino acids. Extensive efforts have been applied to synthesize oligopeptides with a repeating Gly-Xaa-Yaa sequence to mimic collagen, and strategies to improve the stability of the triple-helical structure of these collagen-mimicking molecules have also been developed.[6] However a molecule that mimics collagen and possesses the ability to form supramolecular hydrogels has not yet been reported. In this paper, a small library of self-assembled short peptides (naphthyl-modified) was designed and synthesized. These self-assembled small molecules shared similarity in their chemical structures to that of collagen, bearing the collagen tripeptide of Gly-Xaa-4-Hyp (GXO). In addition their potential for tissue engineering was also tested using an *in vitro* cell culture assay.

2. Experimental

2.1 Formation of hydrogels

1.0 equiv. of **1**, **2**, **3**, **4**, **5**, or **6** and 1.0 equiv. of Na_2CO_3 were firstly suspended in phosphate buffer saline (PBS, pH = 7.4), and then the suspensions were heated by a hot water bath until clear solutions were obtained. The gels formed after cooling back to room temperature.

2.2 Cell culture

Mouse embryonic fibroblast cells (NIH 3T3) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS (fetal bovine serum, Gibco, USA) and 1% Penicillin/Streptomycin solution. Prior to 2D-culture, cells within a sub-confluent monolayer were trypsinized using trypsinase (0.25%)–EDTA (0.02%) solution and resuspended in complete

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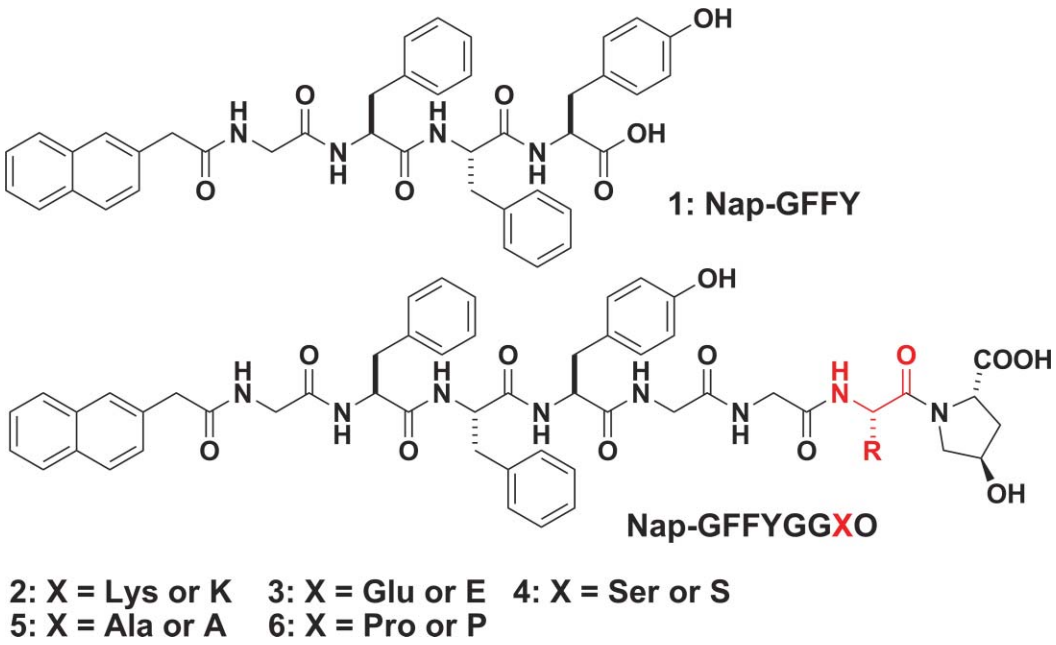

Scheme 1 Chemical structures of all compounds used for hydrogelation.

medium (DMEM plus 10% FBS and 1% Penicillin/Streptomycin solution). Prior to preparation of the hydrogel of **1**, **2**, **3**, **4**, **5**, or **6**, the powders were weighed into separate vials and sterilized by UV light for 60 min. The six compounds were suspended in PBS buffer (pH = 7.4) containing 1.0 equiv. of Na_2CO_3 at a final concentration of 0.3 wt% of the hydrogelators. 50 μL of the hydrogels were formed by a heating–cooling cycle (heating to about 90 °C by a hot water bath) in each well in a non-coating 96-well plate. For the purpose of buffer exchange, one hour after the formation of the hydrogels, 100 μL of complete medium was added on the top of the hydrogels, pipetted off the medium after half an hour. This procedure was repeated for three times. Then 200 μL of 3T3 cell suspension in complete medium (DMEM plus 10% FBS and 1% Penicillin/Streptomycin solution) containing 1.0×10^4 cells was pipetted into each insert of the 96-well plate. As controls, 200 μL complete medium was added in each well without cells. The 96-well plate was maintained in a 37 °C/5% CO_2 incubator.

2.3 Live–dead assay

Viability of encapsulated cells was tested by a Live–Dead assay (Sigma-Aldrich) performed 24 h post culture. The cell–gel constructs were washed three times with DMEM medium without FBS, and then a 60 μL aliquot of the assay solution containing 4 μM EthD-1 (ethidium homodimer-1) and 2 μM calcein AM was pipetted onto each cell–gel construct. After 30 min incubation at room temperature, the constructs were observed using a Nikon Eclipse TE2000-U fluorescence microscope with excitation filters of 450–490 nm (green, Calcein AM) and 510–560 nm (red, EthE-1).

2.4 MTT assay

To quantify cell proliferation inside the cell–gel constructs, an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed at a series of time points. A 2D-culture standard was made by encapsulating cells into the hydrogels following the above 2D-culture procedure (seeding concentrations: 1.0×10^4/well). 50 μL of the hydrogels without the cells were formed as blanks. To perform the MTT assay, each cell–gel construct was incubated with 20% (v/v) MTT reagent in complete medium. The plates were then kept for 4 h, and the medium was pipetted off. Formazan (MTT metabolic product) was resuspended in 200 μL of DMSO, and placed on a shaking table for 15 min to thoroughly mix the formazan into the solvent. Absorbance of the solution was measured by a LabSystems Ascent colorimetric plate reader at a wavelength of 492 nm and the absorbance values obtained in the blanks were subtracted from those obtained in the samples to determine the cell proliferation rate. The experiment was repeated six times.

3. Results and discussion

3.1 Molecular design

We recently found that Nap-Gly-Phe-Phe-Tyr-COOMe (Nap-GFFY-COOMe) can gel water at a minimum gelation concentration of 0.01 wt% by the enzyme of phosphatase (from Nap-GFFYp-COOMe).[7] Based on this information and the repeating tripeptide of Gly-Xaa-4-Hyp on collagen, we designed a small library of small molecules that had a chemical structure similar to that of Nap-Gly-Phe-Phe-Tyr-Gly-Gly-Xaa-4-Hyp (Nap-GFFYGGXO in Scheme 1). The Xaa is lysine, glutamic acid, serine, alanine, and proline for compounds **2**, **3**, **4**, **5**, and **6**, respectively. Compound **1** is Nap-GFFYG without the tripeptide of GXO as the control compound. All compounds **1–6** can be easily synthesized *via* solid-phase peptide synthesis and purified by the reverse-phase HPLC in high yields.

3.2 Gelation test

The gelation ability of all of the compounds that we synthesized was tested—all of them could form hydrogels in the phosphate

buffer saline (PBS, pH = 7.4) upon a heating–cooling cycle. The gels formed by **1–6** were named as gel 1 (Fig. 1A-I, clear), gel 2 (Fig. 1A-II, clear), gel 3 (Fig. 1A-III, clear), gel 4 (Fig. 1A-IV, clear), gel 5 (Fig. 1A-V, clear), and gel 6 (Fig. 1A-VI, opaque). Their minimum gelation concentrations were 0.10, 0.06, 0.08, 0.06, 0.04, and 0.04 wt%, respectively, which means that 1 molecule of **1**, **2**, **3**, **4**, **5**, or **6** can gel 42 100, 97 500, 73 200, 94 200, 139 000, or 143 000 molecules of H_2O, respectively. We then evaluated the property of all the hydrogels. As usually,[8] gels with a higher concentration of gelators were more stable than those with fewer amounts of gelators. That is, gels with a higher concentration of gelators possess higher gel–sol phase transition pH values (pH_{gs}) and temperature (T_{gs}). For example, the T_{gs} of gel 2 is 53, 67, 80, 84 and 88 °C when the concentration of **2** in the hydrogels is 0.1, 0.2, 0.3, 0.4 and 0.5 wt%, respectively (Fig. 1B). Since all of the compounds could form stable hydrogels in PBS buffer at the concentration of 0.3 wt%, we choose the hydrogels at this concentration for further analysis. Comparing the T_{gs} value of all the hydrogels at 0.3 wt% of the gelators, it is in the order gel 2 > gel 1 > gel 5 > gel 4 > gel 6 > gel 3. Furthermore, the pH_{gs} value of the gels at 0.3 wt% of the gelators showed a similar trend of gel 2 > gel 1 ~ gel 5 > gel 4 > gel 6 > gel 3. The lowest pH_{gs} and T_{gs} values of gel 3 might be due to the additional carboxylic acid at the side chain of glutamic acid that will improve the solubility of **3** in the weak basic PBS buffer. Actually, it takes a longer time for **3** to form the hydrogels after the heating–cooling cycle has been applied (about 20 min).

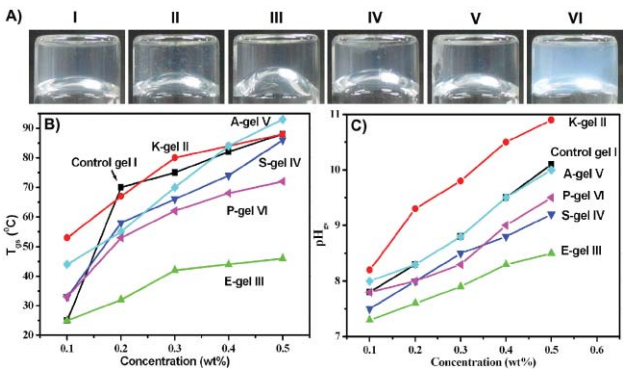

Fig. 1 A) Optical images of the hydrogels with 0.3 wt% of the gelators, I) gel 1, II) gel 2, III) gel 3, IV) gel 4, V) gel 5, and VI) gel 6, and the effect of concentration of gelators on B) gel-sol phase transition temperature of the hydrogels (T_{gs}) and C) gel–sol phase transition pH value of the hydrogels (pH_{gs}).

3.3 Rheology

A dynamic frequency sweep was applied for all the hydrogels to evaluate their mechanical properties. As shown in Fig. 2A and 2B, gel 1 and gel 2 exhibited weak frequency dependences from 0.1–100 rad s^{-1}, with G′ dominating G″, indicating that they were hydrogels. The values of G′ of gel 3–5 were lower than 1 Pa and the values of G′ changed to smaller than those of G″ at high frequency regions (from 30 to 100 rad s^{-1}), suggesting that they were weak gels. Gel 6 showed a frequency dependence from 0.1 to 100 rad s^{-1}, with the value of G′ keeping an increase, and the value of G′ was lower than 10, which also indicated that it was a relatively weak

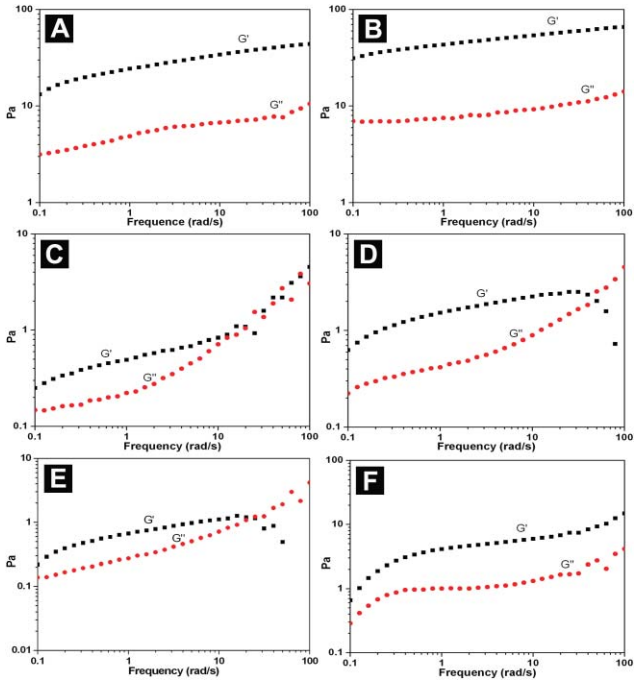

Fig. 2 Dynamic frequency sweep at the strain of 2%, A) gel 1, B) gel 2, C) gel 3, D) gel 4, E) gel 5, and F) gel 6.

gel. Comparing the six kinds of hydrogels, gel 1 and gel 2 were relatively stronger gels than the other four kinds of hydrogels.

3.4 Morphology

Transmission electron microscopy (TEM) was used to characterize the morphology of microstructures in the hydrogels. As shown in Fig. 3, all hydrogels except gel 3 exhibited fibril structures, but

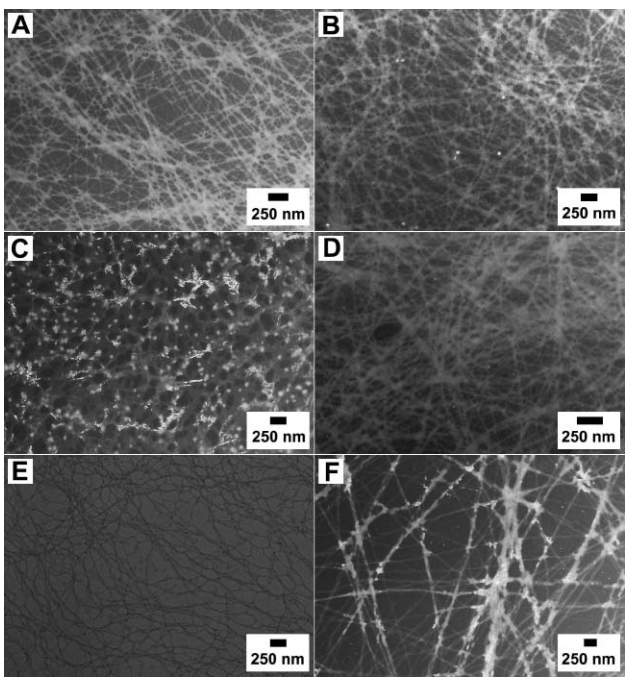

Fig. 3 TEM images of A) gel 1, B) gel 2, C) gel 3, D) gel 4, E) gel 5, and F) gel 6.

the size of the fibers differed: 15–20 nm, 20–30 nm, 18–35 nm, 20–25 nm, and 40–200 nm for gel 1, gel 2, gel 4, gel 5, and gel 6, respectively. The small fibrils entangle with each other and form large bundles that serve as the matrix for the hydrogels. Comparing the morphology of the microstructures in the hydrogels, we found that gel 3 exhibited a different one—the fibers were less uniform (50–150 nm), and they strongly aggregated with each other to form a dense network within the hydrogel. This finding about gel 3 might be due to the fact that gel 3 is a weak and viscous hydrogel, as indicated in Fig. 1A-III.

3.5 Molecular arrangements

As a powerful tool to study the secondary structure of proteins and peptides, circular dichroism (CD) is helpful in providing detailed information about the superstructures of self-assembled peptide derivatives in the gel phase.[9,10] As shown in Fig. 4A, compounds **1–4** exhibited a pronounced trough at about 203 nm (ππ* transition), suggesting that they adopt disordered structures (random coil) in their gel stages.[10] Compound **5** shows a distinct peak at about 200 nm (ππ* transition) and a small trough at 223 nm (nπ* transition), indicating that compound **5** may adopt antiparallel β-pleated sheets (β-helices).[10] Compound **6** possesses the α-helical conformation in gel 6, characterized by a positive peak near 196 nm (ππ* transition) and negative bands at 206 and 218 nm (nπ* transition) in its CD spectrum.[10] There are small troughs at about 240 nm (nπ* transition) for gel 2, gel 4, and gel 6, suggesting that small amounts of compound **2**, **4**, and **6** also possess the β-turn like arrangement in the gel stage.[11] None of the compounds exhibits both the distinct negative peak near 202 nm and the positive peak at about 224 nm, indicating that none of them forms a triple helix structure in their corresponding gel phase.

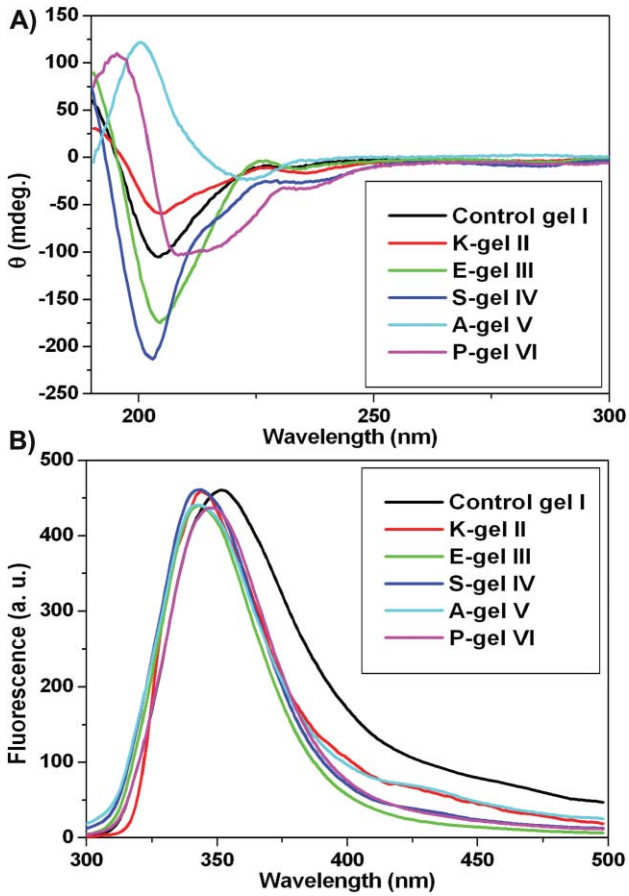

Fig. 4 A) Circular dichroism (CD) spectra and B) fluorescence spectra of the hydrogels (λ_{exc} = 272 nm).

To understand the aromatic interaction between naphthalene groups of all compounds in the gel phase, we obtained the emission spectra of all compounds in the solution and gel phase (Fig. 4B). Compounds **1–6** showed similar emission peaks in the solution phase—all possess distinct peaks centered at about 338 nm, which implies that they form small aggregates even in the dilute solution phase. Compounds **2–6** exhibited distinct peaks at about 343 nm in their corresponding gel phases, which correspond to the ^1L_b transition of naphthalene.[12] This peak red-shifted to 352 nm for **1** in gel 1. This result indicates that the additional tripeptide of GXO helps to form more extensive hydrogen bonds between peptides, thus weakening π–π interactions between aromatic moieties. For all compounds in the gel phase, pronounced shoulder peaks that are at an approximate distance of 368 nm from the partial excimer of naphthalene and the peaks that exist at about 400 nm from the excimer peak of naphthalene are also observed.[12] Both peaks suggest that naphthalene groups are restricted and stack efficiently in the gel phase.

3.6 Cell culture

In order to evaluate the biocompatibility of the hydrogels and study the possibility of using hydrogels for tissue engineering,[13] an *in vitro* cell culture experiment on the top of the hydrogels was applied in the 2-D environments. After the formation of the hydrogels and buffer exchange, mouse embryonic fibroblast cells (3T3) in the culture medium were put on the top of the hydrogels. The live–dead assay was performed 72 h post culture. As shown in Fig. 5A–5D, 3T3 cells attached well to the surface of gel 1, gel 2, gel 3, and gel 4. The 3T3 cells adopted spindle or polyhedron shapes at the surface of the hydrogels, and most of them were alive, as indicated by the cells shown in green. Meanwhile, less 3T3 cells were observed at the surface of both gel 5 and gel 6. On top of these two hydrogels, most of the cells in green color (live cells) maintained a round morphology, and many red cells (dead ones) were observed, which meant that both gel 5 and gel 6 were unsuitable for 3T3 cell culture. An MTT assay was also used to determine the proliferation rate of 3T3 cells on top of six kinds of hydrogels and in control experiments (as a culture, 96-well plates and cell culture dishes were coated with cells cultured in collagen). Fig. 5I shows the results that demonstrate that 3T3 cells in all groups kept increasing during the 3-day culture period. The cell density at day 3 was 145%, 96%, 94%, 93%, 78%, and 79% higher than that at day 1 on the top of gel 1, gel 2, gel 3, gel 4, gel 5, and gel 6, respectively. Comparing the cell number in collagen-coated 96-well plates at day 3, 67%, 83%, 59%, 54%, 43%, and 39% of the cell numbers were recorded in gel 1, gel 2, gel 3, gel 4, gel 5, and gel 6, respectively. These results indicate that gel 1, gel 2, gel 3, and gel 4 had the potential to be used for 3T3 cell culture, probably due to the fact that compounds **1–4** shared similar random coil conformations in the hydrogels. The results also indicated that gel 2 was the most suitable gel for 3T3 cell culture among the six kinds of hydrogels described here, which is most likely due to the

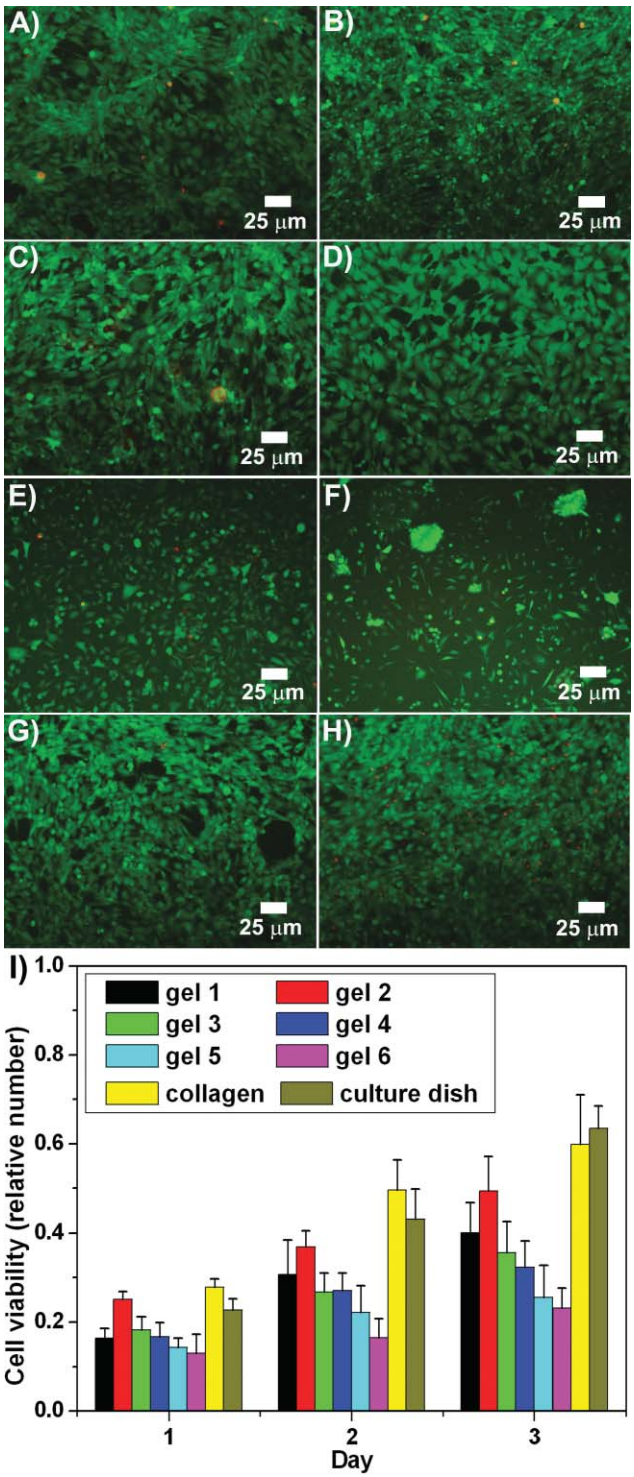

Fig. 5 Cell adhesion and morphology in the hydrogels 72 h post culture (determined by the live–dead assay; live cells are shown in green and dead ones in red): A) gel 1, B) gel 2, C) gel 3, D) gel 4, E) gel 5, F) gel 6, G) collagen, and H) culture dish and the MTT assay result of cell proliferation rate for 3 days (cells cultured on a collagen-coated 96-well plate and in a cell culture dish as controls).

presence of the cationic lysine side chain on compound **2** that can increase the binding to the negative cell surface.[14] The cell numbers on both gel 1 and gel 2 were relatively higher than those on the other gels, which suggested that not only the peptide sequence but also the mechanical property of the hydrogels might have influence for their potentials as cell culture materials. The unsuitability of both gel 5 and gel 6 for 3T3 cell culture might arise from the hydrophobic side chains on alanine and proline.

4. Conclusions

In summary, a small library of small molecules inspired by collagen was designed and synthesized. The results indicated that mechanical property, peptide conformation, and peptide sequence influence the 3T3 cell viability being cultured on top of the hydrogels formed by them—peptides with random coil conformation, proper mechanical strength, and suitable amino acids (aromatic phenylalanine on 1 and positive lysine one 2) might provide better environments for 3T3 cell growth. Though only 3T3 cells were tested in our hydrogel systems, it provided a simple platform to study the influence of mechanical property and peptide conformation/sequence on other more useful cells' viability in the hydrogel scaffold.

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