Engineering interpenetrating network hydrogels as biomimetic cell niche with independently tunable biochemical and mechanical properties

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ABSTRACT

Hydrogels have been widely used as artificial cell niche to mimic extracellular matrix with tunable properties. However, changing biochemical cues in hydrogels developed-to-date would often induce simultaneous changes in mechanical properties, which do not support mechanistic studies on stem cell-niche interactions. Here we report the development of a PEG-based interpenetrating network (IPN), which is composed of two polymer networks that can independently and simultaneously crosslink to form hydrogels in a cell-friendly manner. The resulting IPN hydrogel allows independently tunable biochemical and mechanical properties, as well as stable and more homogeneous presentation of biochemical ligands in 3D than currently available methods. We demonstrate the potential of our IPN platform for elucidating stem cell-niche interactions by modulating osteogenic differentiation of human adipose-derived stem cells. The versatility of such IPN hydrogels is further demonstrated using three distinct and widely used polymers to form the mechanical network while keeping the biochemical network constant.

1. Introduction

Extensive efforts have been dedicated towards elucidating how cell behaviors are regulated by microenvironmental cues, including soluble factors, biochemical ligands [1,2] and mechanical signals [3–5]. To facilitate understanding cell-niche interactions, hydrogels have been widely used as artificial cell niche given their tissue-like water content as well as tunable chemical and physical properties [6,7]. However, few hydrogels developed to-date allow independent tuning of niche properties such as biochemical signals and mechanical stiffness. For example, extracellular matrix (ECM)-derived hydrogels such as collagen provide high mimeticity with abundant biochemical cues [8]. However, changing the concentration of collagen gel to vary biochemical ligand density will invariably induces simultaneous changes in the other hydrogel properties such as stiffness and degradation [9,10]. To overcome the limitations of ECM-based hydrogels, one strategy is to employ bioinert polymers to first construct a hydrogel network as “blank slate” to define mechanical properties, followed by incorporation of biochemical ligands to selected functional end groups. Both naturally-derived and synthetic polymers have been used to form such “blank slate” scaffolds including alginate, polyethylene glycol (PEG) and polyacrylamide [11–15]. Biochemical ligands can be subsequently incorporated by conjugating ECM-derived peptides to the un-crosslinked end groups within the bio-inert polymer network [16,17]. Such strategy allows tuning biochemical ligand density without altering the mechanical property of hydrogels to some extent. However, since the mechanical property and biochemical property is supplied by one polymer network, changes in the bio-inert polymer network will result in coupled changes in multiple niche properties. For example, polymer network degradation will lead to loss of biochemical ligands as well as a decrease in hydrogel stiffness. This makes it difficult, if not impossible, to interpret the contribution of various niche cues to the observed cellular responses.

To help elucidate the mechanisms of cell-niche interactions, it is highly desirable to develop biomimetic matrices that allows independent tunable niche properties (i.e. biochemical, mechanical, degradation etc.). To facilitate independent tuning of scaffold properties in 3D, IPN hydrogels have emerged as a promising platform to serve as an artificial stem cell niche with tailorable properties [18]. IPN is comprised of two or more polymer networks, and various attempts have been made to construct IPN-based...
scaffold for cell culture by mixing two or more polymers together. However, previous attempts often resulted in the formation of semi-IPNs, which were composed of physical entanglement of biochemical dynamic chains within a covalently-crosslinked mechanical network [19–22]. However, the distribution and stability of the biochemical ligands within such semi-IPN is hard to control, and batch-to-batch variance could be high. To improve the homogeneity and stability of the biochemical cues, chemical reagents or enzymes have been used to covalently crosslink the biochemical network ECM molecules [23,24], but these methods may introduce partial loss of the biological activity of the ECM molecules [25,26]. An alternative strategy on constructing IPN utilized sequential polymerization, which involves diffusing a second monomer in a pre-crosslinked network, followed by sequential crosslinking [27–29]. However, sequential polymerization often leads to heterogeneous IPN structure, and has been generally used in acellular applications for strengthening the mechanical property of hydrogels. Its application for cell culture remains a challenge due to the potentially high cytotoxicity during the complicated fabrication process and use of non-cell friendly crosslinking reactions. Therefore, methods need to be developed for constructing IPN as 3D cell niche with independently tunable niche properties using cell-friendly processes.

Here we report the development of a PEG-based IPN hydrogel platform to address the unmet need above by utilizing two distinct crosslinking mechanisms for forming interpenetrated networks. We have chosen PEG as the starting backbone materials given its bio-inert nature and ease for chemical modification. Biochemical precursors were crosslinked via amine-N-hydroxysuccinimide (NHS) coupling [30], while mechanical precursors were crosslinked by thiol-norbornene radical addition [31,32] (Scheme 1a). Both reactions can be carried out at physiological conditions, and will not significantly interfere with each other. To improve the homogeneous distribution of biochemical ligand density, we synthesized biochemical precursors via condensation reaction, which allows defined distance between adjacent pendant bioactive peptides (Scheme 1b). Biochemical network is formed by mixing PEG-derivative containing pedant bioactive peptide and NHS-terminated multi-arm PEG (Scheme 1c). Mechanical network is formed by mixing norbornene-terminated multi-arm PEG with thiol-terminated linear PEG (Scheme 1d). To allow cell-mediated degradation, we could also incorporate MMP-cleavable peptide into the mechanical network. In our IPN design, we vary the biochemical ligand density by changing the ratio of cysteine-containing peptides and cysteine incorporated to the pendant side of biochemical precursor polymers. This allows varying biochemical cues while maintaining the concentration of biochemical precursors, thereby avoiding the changes in crosslinking density or hydrogel stiffness. Thus the mechanical property of IPN can be tuned by changing the concentration and molecular weight of the mechanical precursors. Upon mixing together, the biochemical and mechanical precursors can crosslink simultaneously to form two independent networks (Scheme 1e). The properties of the as-formed IPN hydrogels were characterized by monitoring the stability of incorporated bioactive ligands over time, cell viability, enzymatic-mediated degradation, and ability to support cell spreading in 3D. Compressive mechanical testing was performed to determine the effects of varying the concentration of biochemical or mechanical blocks on the hydrogel stiffness. To assess the potential of IPN hydrogels as stem cell niche, human adipose-derived stem cells (hADSCs) were cultured on IPN hydrogel substrates with independently tunable biochemical cues and matrix stiffness, and outcomes were analyzed by cell morphology and osteogenic differentiation. Finally, the versatility of such IPN strategy was demonstrated using three distinct and widely used polymer network to supply the mechanical network including polyacrylamide [15,33], linear PEG-diacylate (PEGDA) [14,34], and multi-arm PEG [31,32] crosslinked via radical polymerization.

2. Materials and methods

2.1. Synthesis of biochemical precursors

To allow peptide incorporation in the biochemical precursors, we first synthesized linear PEG derivative with norbornene vinyl side groups (ExPEG–1KBN). Briefly, linear PEG–diol (Mw 1 KDa, Sigma) was dissolved in dichloromethane (Fisher), followed by adding 0.2 eq 4-dimethylaminoopyridine (Sigma), 1.5 eq triethylamine (Sigma) and 0.5 eq trans-5-norbornene 2,3-diacrylonyl chloride (Sigma). After 6 h, 0.5 eq N,N-diisopropylcarbodiimide (Sigma) was added, stirred overnight, then precipitated in ice cold ethyl alcohol. To incorporate RGD into the biochemical precursor (PEG–RGD), ExPEG–1KBN was dissolved in deionized water containing 12595 (0.05%, w/v) (Ciba) with predetermined concentration of CRGDS (Bio Basic). Under vigorous stirring, the solution was exposed to 365 nm UV light (4 mW/cm², XX-155 lamp, UV) for 2 min. Excess cysteine was added to the solution and exposed to UV for 8 min to block the remaining unreacted norbornene side chains. The resulting biochemical precursor solution was subsequently dialyzed against deionized water and lyophilized. Fluorescein probe incorporated PEG (PEGFC) was obtained by reacting PEG–RGD with 5 eq NHS-Fluorescein (Thermal Scientific) in dimethylformamide and dialyzing against deionized water for 3 days before lyophilized. The structure was confirmed by 1H NMR (Fig. S2), and the molecular weight was determined by H NMR (Mw ~ 12 KDa) and GPC (Mw ~ 21 KDa, Mw/Mn = ~ 2.4). The crosslinking precursor 8-arm-PEG succinimidylic glutarate (10 KDa, PEGS8) was purchased from Jenkem Inc.

2.2. IPN hydrogel formation

The monomers and polymer precursors include acrylamide (acrylamide: bis-acrylamide = 37:5; Fisher Scientific), PEGDA (5 KDa, Laysan Bio) and Barm-PEG-norbornene (10 KDa, PEG8BN) with PEG–diol (1.5 KDa, PEGD7). PEG8BN and PEGD7 were synthesized according to the literature [31,32]. The mechanical precursor solutions of acrylamide (12% (w/v)), PEGDA (10% (w/v)) and PEG8NB (6.25% (w/v)) with predetermined concentration of CRGDS (4 mW/cm²) were loaded into a chamber sandwiched between a glass slide and a coverslip, with a cover slide as the spacer (0.2 mm in thickness). Gelation was achieved by UV crosslinking (365 nm, 4 mW/cm²) for 2 min, and the IPN hydrogel sheets (∼2 × 5 × 0.2 cm, attached to the glass slide) could be obtained by detaching them from the cover slips. To make IPN hydrogels as 3D niche for cell encapsulation, IPN precursor solutions were loaded in a mold (∅ 6 x 26 mm) and crosslinked under UV (365 nm, 4 mW/cm²) for 2 min, and the IPN hydrogel sheets (∼2 × 5 × 0.2 cm, attached to the glass slide) could be obtained by detaching them from the cover slips. To make IPN hydrogels as 3D niche for cell encapsulation, IPN precursor solutions were loaded in a mold (∅ 6 x 26 mm) and crosslinked under UV (365 nm, 4 mW/cm²) for 2 min.

2.3. Stability of the biochemical network

To evaluate the stability of biochemical network within the IPN, fluorescein (FC) probe was incorporated into biochemical precursor (PEG–FC), and hydrogels without FC probe was included as control. The fluorescence intensity of the hydrogel was measured over multiple time points up to 4 days using a SpectraMax M2e multimode microplate reader (Molecular Devices).

2.4. Mechanical testing

Unconfined compression test was used to measure the stiffness of IPN hydrogels using Instron 5944 testing system (Instron) fitted with a 10 N load cell (Interface Inc.). All tests were conducted in PBS solution at room temperature. The compressive modulus was calculated using the linear curve fits of the stress vs strain curve for strain ranges of 10–20%. Four concentrations of mechanical precursors was examined (2.5, 5.0, 7.5 and 10% w/v) while keeping the PEG–RGD constant. The RGD concentration was varied by changing the substitution ratio of RGD (0.025, 1.0, 2.5 mm) in the biochemical precursor. All the hydrogels were incubated in PBS overnight to reach equilibrium swell before mechanical testing.

2.5. Cell culture

hADSCs were expanded in growth medium comprised of high-glucose Dulbecco’s minimal essential medium (DMEM, Invitrogen) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco), 100 U/mL penicillin/streptomycin (Pen Strep, Gibco) 10 mM β-Glucuronidase (β-Gal, ProProTech). Passage 5 hADSCs were used in all studies. To induce osteogenic differentiation, hADSCs were cultured in osteogenic differentiation medium as we previously reported [35].

2.6. Cell viability

To confirm that IPN precursors are suitable for cell culture, biochemical or mechanical precursors were added to hADSCs cultured in PBS solution at the highest concentration used for later studies. Cells were cultured for 24 h and cell...
viability was quantified using CellTiter 96® cell proliferation assay (MTS; Promega) following the manufacturer’s instruction. To determine the biocompatibility of IPN for 3D cell encapsulation, hADSCs were suspended in PEG-TE IPN precursor solution and exposed to 365 nm UV (4 mW/cm²) for 2 min for crosslinking. The cell-laden hydrogels were cultured in growth media at 37°C and 5% CO₂ for 24 h, and cut into thin slices to be stained with Live/Dead viability/cytotoxicity kit (Invitrogen), with live cells stained green and dead cells stained red.

2.7. Hydrogel degradation

PEG-TE IPN hydrogels were made to be MMP degradable by replacing PEG-DT into cysteine contain peptide (CGPQG IWGQC) in the gel mold. The hydrogels with normal PEG-DT was used as non-degradable control. The hydrogels were put in PBS with different concentration of collagenase and the wet weight was measured every day to get the mass loss curve. The hADSCs were encapsulated in IPN hydrogels with 2.5 mM RGD incorporated, and the cell morphology was examined using phase-contrast microscope.

2.8. Osteogenic differentiation of hADSCs on IPN substrates

hADSCs were cultured on PEG-TE substrates with different RGD concentration and mechanical stiffness at a seeding density of 2 × 10³/cm². Osteogenic induction medium [35] was used and changed three times per week. Cell morphology was examined using phase-contrast microscope.

2.9. Quantitative gene expression

After being cultured in osteogenic medium for 2 weeks, the RNA of the cells were extracted and purified with RNeasy Mini Kit (Qiagen). Reverse transcription reaction was performed using SuperScript III reverse transcriptase (Invitrogen). qRT-PCR was performed using a 7500 Fast Real-Time PCR System and SYBR Green master mix (Applied Biosystems). Relative expression level of target markers was determined using the comparative CT method, in which target gene expression was first normalized to the house-keeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), followed by secondary normalization by the control group (0.25 mM-54 kPa group). The qRT-PCR primer sequences used in this study are listed in Table S1.
2.10. Statistics

All experiments were performed in triplicate \((n = 3)\). The results were reported as mean ± standard deviation. Statistical analysis was performed using the one-way ANOVA with Tukey multiple comparisons \((\alpha = 0.05)\) as the posttest to compare all the groups.

3. Results

3.1. Bioactivity of incorporated biochemical cues

To confirm the stability of biochemical precursors within the IPN, we incorporated fluorescein (FC) into the biochemical precursor as a probe to allow real-time monitoring of the biochemical network within the IPN over time by tracking fluorescence intensity. Our results showed stable fluorescence signal in FC-containing IPN hydrogels over at least 4 days (Fig. 1a), indicating biochemical network remained well interlocked within the mechanical network. As a control, the PEG gel without FC incorporation showed minimal background fluorescence signal. To determine the bio-functionality of the peptide after IPN formation, IPN hydrogels containing CRGDS, a cell adhesive peptide, as biochemical cues were used as substrates for culturing hADSCs. CRGDS incorporation led to stable adhesion and extensive spreading of hADSCs after 72 h of culture on IPN substrates (Fig. 1b), confirming the retention of bioactivity of peptide after IPN formation. We then examined the potential of IPN for cell culture by culturing hADSCs in medium containing biochemical and mechanical precursors, as well as direct encapsulation of hADSCs in IPN in 3D. Both cell proliferation assay (MTS) and live-dead staining showed high cell viability at 24 h (Fig. 1c,d).

3.2. Enzymatic degradation and cell spreading

We then assess the degradability of IPN by culturing IPNs containing MMP-cleavable peptides in PBS containing increasing doses of collagenase, an enzyme that provides a trigger for MMP-cleavable peptides. IPN demonstrated a dose-dependent degradation, with

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**Fig. 1.** Characterization of IPN hydrogels to be used as cell niche. (a) Stability of the IPN network. FC probes were incorporated into the biochemical precursors, and showed stable signals over time. (b) CRGDS was incorporated into the IPN as a model peptide and led to rapid cell adhesion and spreading of human adipose-derived stem cells (hADSC) within 24 h. (c) Cell viability was checked after incubating in precursor solutions for 24 h using highest concentrations of biochemical and mechanical precursor used for 3D encapsulation study. (d) Live/dead staining of hADSCs 2 days after encapsulation in IPN hydrogel. (e) Degradation of IPN with MMP-cleavable peptide triggered by different concentration of collagenase. (f) hADSCs encapsulated in IPN with MMP-cleavable peptide were able to spread after 3 days of culturing.
increasing collagenase concentration leading to faster weight loss of IPN hydrogels (Fig. 1e). Without the collagenase (PBS) or using non-degradable IPNs containing no MMP-cleavable peptides, control IPN hydrogels remained stable with constant weight over time, indicating the degradation is specifically triggered by enzymatic stimuli. When encapsulated inside 3D IPN hydrogels containing CRGDS and MMP-cleavable peptides, hADSCs demonstrated extensive spreading within 2 weeks (Fig. 1f).

### 3.3. Decoupled biochemical and mechanical properties

To determine whether our IPN design allows decoupling of mechanical and biochemical properties of the resulting hydrogel network, we fabricated IPN hydrogels with varying concentration of mechanical precursors or biochemical ligand. Increasing the concentration of mechanical precursors from 2.5 to 10% (w/v) resulted significant increase in the stiffness of the IPN hydrogels, covering a broad range from brain tissue-like stiffness (0.9 kPa), muscle tissue-like stiffness (18.6 kPa) to stiffness range known to promote musculoskeletal tissue differentiation (54 or 91 kPa) (Fig. 2a, p < 0.0001). In contrast, increasing biochemical ligand (RGD) concentration from 0 to 2.5 mM resulted in negligible changes in the compressive modulus of IPNs. Together, these results show that our IPN strategy allows formation of biomimetic hydrogels for cell culture with independently tunable biochemical and mechanical properties.

### 3.4. Modulation of stem cell fate

We next demonstrated the potential of IPN hydrogels as biomimetic cell niche by culturing hADSCs on IPN substrates with independently tuned biochemical ligand density (RGD) and mechanical stiffness. We first maintained RGD density constant (2.5 mM), while increasing the IPN hydrogel stiffness from 0.9 to 91.3 kPa. Increasing hydrogel stiffness generally resulted in faster cell spreading and more cell proliferation over 3 days of culture (Fig. 3a). Specifically, hydrogels with stiffness of 54.6 kPa or above supported extensive cell spreading as early as day 1, and showed comparable level of cell proliferation by day 3. In contrast, cells remained round morphology on soft substrates (0.9–18.6 kPa) at day 1, and achieved cell spreading by day 3 with slow cell proliferation. Next, we kept the stiffness of IPN to be constant (54 kPa) while varying RGD concentration from 0.25 mM to 2.5 mM. While higher RGD density (2.5 mM) appeared to supported slightly faster cell spreading and proliferation at day 1, IPN hydrogels with RGD density all resulted in comparable cell spreading and proliferation by day 3, with cell morphology resembling those cultured on tissue plastic control (Fig. 3b).

Next, we assessed the effects of independent tuning of IPN biochemical and mechanical property on stem cell differentiation. hADSCs were cultured on IPN hydrogels for 14 days with supplementation of osteogenic medium. Osteogenic differentiation was measured by quantifying gene expression levels of two earlier bone markers including alkaline phosphatase (ALP) and core binding factor alpha-1 (CBFA1), as well as a mature bone marker osteocalcin (OCN). While keeping the RGD density constant at 2.5 mM, increasing IPN stiffness from 0.9 kPa to 54.6 kPa led to 6-fold increase in ALP gene expression (Fig. 4a). Further increase of IPN stiffness from 54.6 kPa to 91.3 kPa did not lead to significant changes in ALP expression (Fig. 4a). While keeping the hydrogel stiffness constant (54 kPa), increasing RGD density from 0.25 mM to 2.5 mM also resulted in a dose-dependent increase in ALP gene expression, with ~ 1 fold increase in ALP expression in IPN containing 2.5 mM RGD compared to IPN containing 0.25 mM RGD (Fig. 4a). Similar to the findings from the cell morphology, changes in hydrogel stiffness appeared to have a more dramatic effect on ADSC differentiation than changes in RGD density within the ranges tested in this study. Gene expressions of the other two osteogenic markers CBFA1 and osteocalcin in response to IPN stiffness change generally followed the same trend as ALP (Fig. 4b,c).

### 3.5. IPNs with different mechanical networks

To demonstrate the adaptability of this IPN strategy for forming hydrogels using tunable properties, we then demonstrated formation of IPNs using three different polymers to supply the mechanical network of IPN including polyacrylamide (PA), linear PEGDA, and multi-arm PEG (Fig. 5a–e). We have chosen these three polymers as they are the mostly commonly used for tuning hydrogel stiffness for cell studies, and all can be crosslinked using radical polymerization to form mechanical network within IPN. By tuning the concentration of three different mechanical blocks, we fabricated IPN hydrogels with comparable compressive modulus around the range of ~50 kPa (Fig. 5f). When mixed with biochemical precursors containing cell adhesive ligands (CRGDS), all three types of IPNs supported comparable level of cell adhesion and spreading (Fig. 5g). Increasing RGD density from 0.25 mM to 2.5 mM did not result in significant changes in cell morphology.

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**Fig. 2.** Mechanical stiffness of IPN hydrogels obtained from compress modulus testing. (a) Varying concentration of mechanical precursor led to broadly tunable stiffness of resulting IPN. (b) Varying concentration of biochemical ligands did not lead to any significant changes in the stiffness of IPN network. Data were presented as average ± standard deviations with 3 samples per group. All groups in (a) were statistically significant from each other (p < 0.0001).
4. Discussion

To facilitate elucidating how niche cues regulate cell fate, here we report the development and characterization of IPN hydrogels as biomimetic cell niche with independently tunable biochemical and mechanical properties. We have chosen two crosslinking mechanisms that can be carried out under physiological conditions without interfering each other, which allow simultaneous formation of two covalently-crosslinked networks. To introduce biochemical functionality into 3D hydrogel network, extracellular matrix (ECM)-derived peptides can be incorporated into the biochemical block polymer via chemical conjugation. Unlike ECM proteins, the relatively small size of peptides allows mimicking matrix functionality without significantly altering the mechanical stiffness and/or the diffusion properties of the resulting hydrogel network. One common strategy for incorporating peptides into PEG hydrogels involves reacting the peptide with mono-acrylated PEG-NHS, followed by copolymerizing into PEG diacrylate-based polymer network. However, this strategy simultaneously decreases the crosslinking density of resulting hydrogel, and may lead to changes in mechanical property as well as nutrients diffusion [16,36]. Alternatively, biochemical peptide can be incorporated by

![Representative micrographs of hADSCs cultured on IPN hydrogels on day 1 and day 3.](image)

(a) Mechanical stiffness was varied from 0.9 kPa to 91.3 kPa while keeping RGD concentration constant at 2.5 mM. (b) RGD concentration was varied from 0.25 mM to 2.5 mM while keeping mechanical stiffness constant at around 54 kPa. (c) Cells cultured on tissue culture plate (TCP) as control.

Fig. 3.

![Quantitative gene expression of osteogenic markers](image)

(a) ALP, (b) CBFA1 and (c) OCN of hADSCs cultured for 14 days in osteogenic medium. hADSCs were cultured on IPN hydrogels with varied mechanical stiffness from 0.9 kPa to 91.3 kPa but constant RGD concentration at 2.5 mM or with varied RGD concentration from 0.25 mM to 2.5 mM but constant mechanical stiffness at around 54 kPa. Data were presented as average ± standard deviations with 3 samples per group. Significant differences were marked as *p < 0.1, **p < 0.01, ***p < 0.001, and ****p < 0.0001 compared with 2.5 mM-0.9 kPa group; ##p < 0.01 compared with 0.25 mM-54 kPa group.

Fig. 4.
modifying end groups of multi-arm PEG, which allows tuning density of biochemical ligand by varying substitution rate but with very limited variability and poor control on homogeneous presentation of biochemical ligand density \[16,32,37\]. To overcome such limitations, we have devised a condensation reaction for synthesizing PEG derivatives as the biochemical precursor with uniform and tunable presentation of biochemical ligands (Scheme 1). Specifically, PEG-diol polymers were used as building blocks and coupled by trans-5-norbornene-2,3-diacrylbonyl chloride to generate a PEG derivative polymer with uniformly distributed active norbornene vinyl groups. Any cysteine-containing peptides can be incorporated to the PEG side chain via thiol-ene radical addition, a robust and facile reaction as reported previously \[31\].

The distance between two adjacent biochemical ligands is determined by the molecular weight of initial PEG-diol building block, which allows uniform ligands distribution with tunable density. The density of biochemical ligands may be further tuned by varying the substitution rate of the active norbornene vinyl groups. To reduce the density of biochemical ligands, some norbornene vinyl groups may be substituted using cysteine rather than cysteine-containing biochemical peptides. Furthermore, our platform allows facile incorporation of multiple types of biochemical peptides in 3D hydrogels by simply mixing different ratio of cysteine-containing peptides with the PEG precursors, which may provide a powerful tool for studying fundamental biological problems, such as elucidating the effects of varying peptide concentration and combination on regulating cell fate in 3D. One additional advantage of our IPN design over existing semi-IPN strategies is the enhanced stability of the biochemical network, as shown by the diffusion assay of fluorescently labeled biochemical ligands. Using cell adhesive peptide as a model ligand, we further demonstrated that IPN supported cell adhesion, spreading and osteogenic differentiation over time, confirming the retention of bioactivity of peptides after IPN formation.

The ability of extracellular matrix to be degraded plays an important role in mediating important cellular processes such as proliferation, spreading and migration. Both hydrolytic degradation and cell triggered enzymatic degradation can be employed to introduce homogeneous or cell-mediated matrix degradation \[7,38\]. To mimic the cell-responsive degradable nature of natural ECM, we incorporated enzymatically cleavable peptides into the linear PEG backbone of mechanical precursor. Specifically, we have chosen to incorporate a matrix metalloproteinase (MMP)-sensitive peptide (CGPQG YIWGQC) \[39,40\], which provides a unique advantage of our IPN strategy, which would be hard to achieve using conventional, single network-based hydrogels. In addition to the cell-instructive enzymatic degradation, ester linkage may also be incorporated into mechanical precursors to allow homogeneous hydrolytic degradation \[38\].

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**Fig. 5.** Three widely used hydrogels, including (a) polyacrylamide hydrogel crosslinked by radical polymerization of acrylamide and bis-acrylamide and PEG hydrogel crosslinked by (b) radical polymerization of PEG di-acrylate or (c) radical addition coupling of multi-arm PEG with thiol and norbornene groups. The IPN scaffolds (e) can be obtained by combining a, b, c with (d) uniform biochemical network. (f) Mechanical stiffness of IPN hydrogels made of PA, PEG-RP and PEG-TE mechanical precursors. The concentration was 13% (w/v), 10% (w/v) and 7.5% (w/v) correspondingly. (g) Representative micrographs of hADSCs cultured on IPN hydrogels based on different mechanical precursors. The RGD concentration were varied from 0.25 mM to 2.5 mM. The mechanical stiffness of the hydrogels were comparable and all around 50 kPa.
We demonstrated the potential of using IPN as biomimetic niche for studying stem cell-niche interactions by modulating osteogenic differentiation of hADSCs on IPN hydrogels with independently tunable biochemical and mechanical properties. Three RGD doses (0.25, 1.0 and 2.5 mg/ml) and four IPN stiffness (0.9–913 kPa) were varied independently, and MMP-degradable peptide was incorporated into the mechanical precursors to allow cell-responsive matrix degradation. Increasing hydrogel stiffness promoted osteogenic marker gene differentiations and cell spreading, and optimal osteogenic differentiation occurred at intermediate stiffness (~54 kPa), which is consistent with previous reports [41,42].

To demonstrate the versatility of this IPN platform, we used different polymers that can undergo radical polymerization to form the mechanical network of IPN while keeping the biochemical network constant. Besides the three polymers shown in this study, many other natural polymers such as dextran, hyaluronic acid and alginate can also be used for constructing the mechanical network. This is because most of these polymers can be easily modified with methacrylate end groups, which allow crosslinking via radical polymerization. Furthermore, the crosslinking mechanism of mechanical network can be changed from radical addition (photo-initiated or redox initiated) to Michael addition [43] to facilitate in situ polymerization without the need for radicals.

5. Conclusion

Here we report an IPN hydrogel platform with independently tunable biochemical and mechanical properties, which can be used as 3D biomimetic cell niche. Our IPN hydrogel allows homogeneous and stable presentation of biochemical ligands, and varying biochemical ligand concentration will not significantly change the matrix stiffness of the resulting IPN hydrogels. Unlike previous IPN strategies, our method is cell-friendly and allows simultaneous and independent formation of the biochemical and mechanical network. While we demonstrate the application of IPN hydrogels for directing stem cell differentiation as an example, the IPN hydrogels may also be utilized for studying other cell behavior such as cancer cell-niche interactions. Given the ability to decouple niche properties in such IPN hydrogel platforms, it also provides a robust material platform for developing combinatorial studies to help elucidate how complex niche signaling interact together to influence cell fate in 3D, and may also hold great promise for rapidly identifying optimal scaffold compositions for promoting desirable cellular processes and tissue formation.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2013.11.064.

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References


