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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 biomimicry 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 bio-inert 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

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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 pendent 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 pendent 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-diacrylate (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 (*ExtPEG-1KNB*). Briefly, linear PEG-diol (Mw 1 KDa, Sigma) was dissolved in dichloromethane (Fisher), followed by adding 0.2 eq 4-dimethylaminopyridine (Sigma), 1.5 eq triethyleneamine (Sigma) and 0.5 eq trans-5-norbornene 2,3-diacrybonyl chloride (Sigma). After 6 h, 0.5 eq N,N′-diisopropylcarbodiimide (Sigma) was added, stirred overnight, then precipitated in ice cold ethyl ether. To incorporate RGD into the biochemical precursor (*PEG-RGD*), ExtPEG-1KNB was dissolved in deionized water containing I2959 (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/cm^2, XX-15S lamp, UVP) 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 lyopholized. The structure was confirmed by ^{1}H NMR (Fig. S2), and the molecular weight was determined by ^{1}H NMR (Mw ~ 12 KDa) and GPC (Mw ~ 21 KDa, Mw/Mn = 2.4). The crosslinking precursor 8-armPEG succinimidyl glutarate (10 kDa, PEG8SG) was purchased from Jenkem Inc.

2.2. IPN hydrogel formation

The monomers and polymer precursors include acrylamide (acrylamide:bisacrylamide = 37.5:1, Fisher Scientific), PEGDA (5 kDa, Laysan Bio) and 8arm-PEG-norbornene (10 kDa, PEG8NB) with PEG-dithiol (1.5 kDa, PEGDT). PEG8NB and PEGDT 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 PEGDT (3.25% (w/v)) were mixed with biochemical precursors PEG-RGD (1% (w/v)) and PEG8SG (1% (w/v)) correspondingly for making IPNs of PA, PEG-RP and PEG-TE. All solutions were added with 0.05% (w/v) I2959 as photoinitiator. To form IPN hydrogels as substrates for cell culture, precursor solution was 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/cm^2) for 2 min, and the IPN hydrogel sheets (2 × 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 (ϕ 6 mm × 2.6 mm) and crosslinked under UV (365 nm, 4 mW/cm^2, 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 multimold 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, 0.25, 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 ng/mL basic fibroblast growth factor (bFGF, PeproTech). 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 96-well plates at the highest concentration used for later studies. Cells were cultured for 24 h and cell