The effects of varying poly(ethylene glycol) hydrogel crosslinking density and the crosslinking mechanism on protein accumulation in three-dimensional hydrogels

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ABSTRACT

Matrix stiffness has been shown to play an important role in modulating various cell fate processes such as differentiation and cell cycle. Given that the stiffness can be easily tuned by varying the crosslinking density, poly(ethylene glycol) (PEG) hydrogels have been widely used as an artificial cell niche. However, little is known about how changes in the hydrogel crosslinking density may affect the accumulation of exogenous growth factors within 3-D hydrogel scaffolds formed by different crosslinking mechanisms. To address such shortcomings, we measured protein diffusivity and accumulation within PEG hydrogels with varying PEG molecular weight, concentration and crosslinking mechanism. We found that protein accumulation increased substantially above a critical mesh size, which was distinct from the protein diffusivity trend, highlighting the importance of using protein accumulation as a parameter to better predict the cell fates in addition to protein diffusivity, a parameter commonly reported by researchers studying protein diffusion in hydrogels. Furthermore, we found that chain-growth-polymerized gels allowed more protein accumulation than step-growth-polymerized gels, which may be the result of network heterogeneity. The strategy used here can help quantify the effects of varying the hydrogel crosslinking density and crosslinking mechanism on protein diffusion in different types of hydrogel. Such tools could be broadly useful for interpreting cellular responses in hydrogels of varying stiffness for various tissue engineering applications.

1. Introduction

Matrix stiffness has recently been recognized as playing an important role in regulating cell fate and tissue development. It has been shown that stem cells specify their lineage and commit to their fate by matrix stiffness, which mimics specific tissue-level elasticity [1–3]. Matrix stiffness has also been shown to directly influence other cellular fate processes, such as cell cycle, in a variety of cell types, including myofibroblasts [4], epithelial cells, vascular smooth muscle cells and osteoblasts [5]. Cancer cells are also known to be mechanosensitive, and increasing matrix stiffness resulted in an increase in cell growth, spreading and migration [6].

To study the effects of varying matrix stiffness on cell behavior, poly(ethylene glycol) (PEG) hydrogels have been widely used to create a biomimetic artificial niche with tunable biochemical and biomechanical cues [7]. The biomechanical cue, specifically stiffness of the PEG hydrogel, can be easily tuned by varying the molecular weight or concentration of the PEG [8]. Increasing the molecular weight or decreasing the concentration of PEG reduces the crosslinking density of the hydrogel, which results in a softer gel. However, varying the crosslinking density simultaneously changes the mesh size of the hydrogel network, which, in turn, can influence protein diffusion in 3-D hydrogels [9,10]. Previous studies have shown that increasing the gel crosslinking density by increasing the molecular weight or decreasing the concentration of PEG can lead to decreasing diffusivity of different solutes (vitamin B_{12}, insulin, myoglobin, trypsin inhibitor, carbonic anhydrase, ovalbumin, bovine serum albumin (BSA), IgG) [9–11].

However, varying the gel crosslinking density influences not only the protein diffusivity but also the protein accumulation within hydrogels. To date, most studies have only looked at protein diffusivity (how fast proteins can diffuse within hydrogels), ignoring protein accumulation (how much proteins can actually go into 3-D hydrogels), which can directly influence the encapsulated cells. A

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recent study has shown that varying the hydrogel crosslinking density can alter the stem cell differentiation, which is unlikely to be related to mechanotransduction [12]. Since cells are known to be sensitive to available soluble factors, it is important to characterize the effects of varying the hydrogel crosslinking density on exogenous protein accumulation within 3-D hydrogels, thereby allowing the correct interpretation of the mechanisms that regulate cellular responses in hydrogels with varying stiffness.

Protein accumulation can also be affected by the crosslinking mechanism of PEG hydrogels. Although it is known that the crosslinking mechanism can affect network homogeneity [13], it is poorly understood how different crosslinking mechanisms can affect protein accumulation in 3-D hydrogels. Typically, PEG hydrogels can be crosslinked via two mechanisms: chain-growth (CG) or step-growth (SG) polymerization (Fig. 1). CG polymerization is generally less controllable, and is known to form a more heterogeneous gel network. Because monomers are crosslinked via polymer kinetic chains, the crosslink functionality is hardly controlled and network defects (e.g. loop) are more likely to form. On the other hand, SG polymerization generally results in a more homogeneous hydrogel network that is formed by at least two different monomers with defined functionality and mutually reactive end groups [13]. Given that different crosslinking mechanisms can affect the network homogeneity, it is important to understand how the crosslinking mechanism affects protein accumulation in 3-D PEG hydrogels.

Therefore, the goal of this study was to investigate the effects of varying crosslinking density and crosslinking mechanism of PEG hydrogel on protein accumulation within the hydrogel. Specifically, we varied the hydrogel crosslinking density and the crosslinking mechanism, and investigated their effects on protein accumulation within hydrogels. To control the crosslinking density, the PEG molecular weight or concentration was varied. To study how the crosslinking mechanism affects protein diffusion, CG and SG polymerization were used to form PEG hydrogels. To mimic accumulation of exogenously supplemented soluble growth factor in 3-D tissue engineering scaffolds, premade PEG hydrogels were immersed in protein solution for 24 h to load the protein. After 24 h of loading, gels were placed in fresh PBS for 24 h, followed by gel homogenization to take into account any protein that remain entrapped in the hydrogel. Protein accumulation within hydrogels was calculated from the protein release accumulated over 24 h and the protein that remained entrapped in the hydrogels. Protein diffusivity was calculated by fitting the protein fractional release curve to a 3-D Fickian model.

2. Materials and methods

2.1. Materials

Poly(ethylene glycol) (PEG, mol. wt. 2, 3, 4, 10 kDa), K_2CO_3, dichloromethane (DCM), acryloyl chloride, potassium iodide (KI), Celite® 521, 4-(dimethylaminio)pyridine (DMAP), N,N′-diisopropylcarbodiimide (DIC), 5-norbornene-2-carboxylic acid, sodium hydride (NaH), allyl bromide, 2,2-dimethoxy-1,2-diphenylethan-1-one (DMPA), dithiothreitol, tetrahydrofuran (THF) and thioacetic
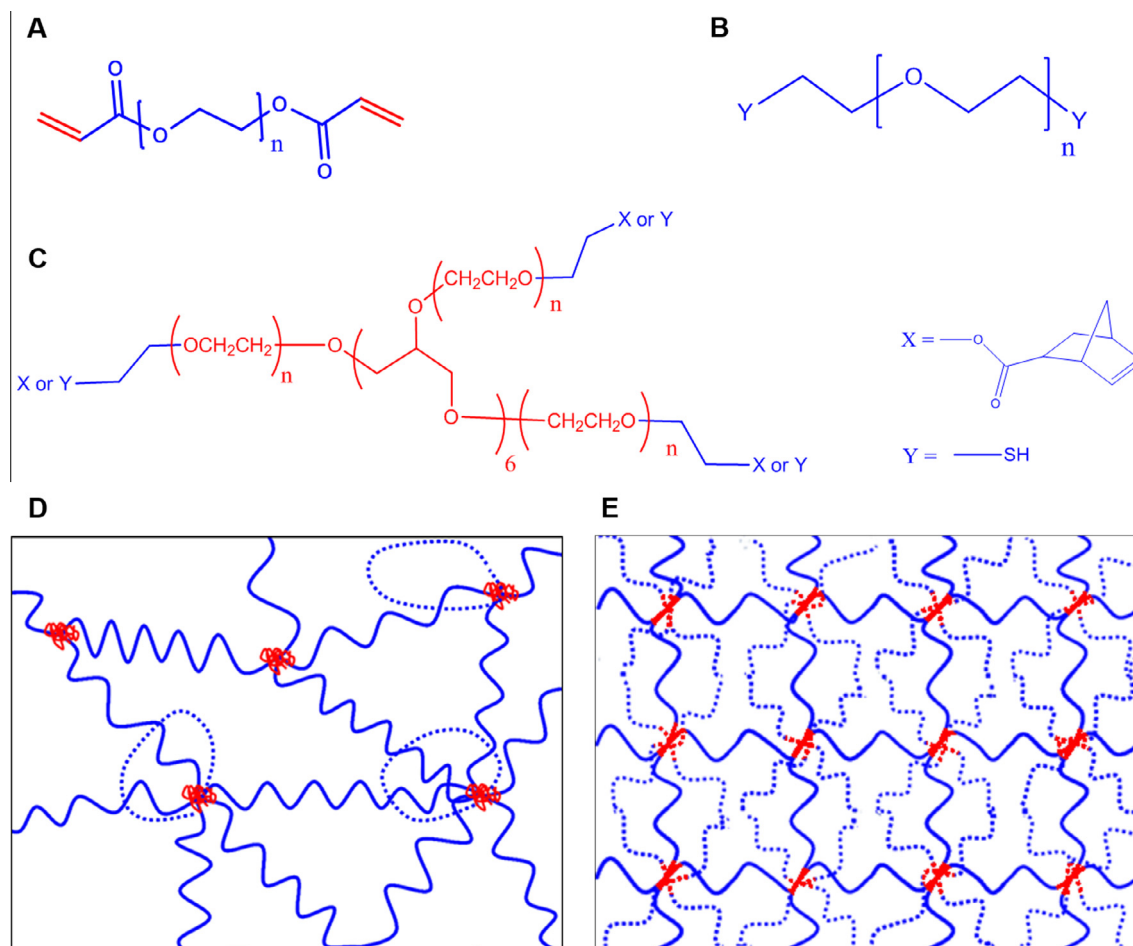

Fig. 1. Chemical structure of polymers and schematic of PEG hydrogel network formed by different crosslinking mechanisms. Chemical structure of (A) PEGDA, (B) PEG-dithiol, (C) 8-arm PEG–norbornene (X) (8-arm PEG-NB) or 8-arm PEG–thiol (Y) (8-arm PEG-SH). (D) CG gels using PEGDA. (E) SG gels using norbornene- and thiol-terminated PEG. Blue line: polymer chains between crosslinking points; red line: crosslinking points; solid line: on the same plane; dotted line: on different planes.

acid were purchased from Sigma–Aldrich (St Louis, MO). 4-(2-Hydroxyethoxy)phenyl-(2-hydroxy-2-propyl)ketone (Irgacure 2959) was purchased from BASF (Florham Park, NJ). PEG diacrylate (PEGDA, mol. wt. 5 kDa) was purchased from Laysan Bio (Arab, AL). 8-arm PEG (mol. wt. 10 kDa) and 8-arm PEG-SH (mol. wt. 10 kDa), were purchased from JenKem Technology (Allen, TX). PEGDA (mol. wt. 2, 3, 4, 10 kDa), 8-arm PEG-norbornene (8-arm PEG-NB, mol. wt. 10 kDa) and PEG-diSH (mol. wt. 1.5, 10 kDa) were synthesized in house. Regular BioRad Protein Assay was purchased from BioRad (Hercules, CA).

2.2. Synthesis

To synthesize PEGDA (mol. wt. 2, 3, 4, 10 kDa), linear PEG (2, 3, 4, 10 kDa) was dissolved in DCM with the addition of 3 eq (mole equivalent with respect to hydroxyls) K_2CO_3. Next, 3 eq acryloyl chloride and 0.1 eq KI were added to the solution. The solution was then stirred at 4 °C overnight and filtered using Celite® 521 to remove any undissolved particles. The product was collected by evaporation of the solvent and precipitation in cold ether. It was purified by dialysis against deionized water (MCO 1 kDa) for 2 days, followed by lyophilization to collect the purified product. The structure of the products was confirmed by ^{1}H nuclear magnetic resonance (NMR) and the conversion ratio was over 95%.

8-arm PEG-norbornene (10 kDa) and PEG-dithiol (1.5, 10 kDa) were synthesized as previously reported [14,15]. To synthesize norbornene-terminated 8-arm PEG, PEG (10 kDa) was dissolved in DCM and 0.2 eq DMAP and 3 eq 5-norbornene-2-carboxylic acid were added. After cooling the solution in an ice bath, 3 eq DIC was added. After stirring overnight, the reaction mixture was filtered and concentrated by evaporating away most of the solvent. The concentrated solution was then poured into ice-cold diethyl ether to precipitate the product. For the synthesis of PEG-dithiol (1.5, 10 kDa), linear PEG-diol (1.5, 10 kDa) was dissolved in THF, and 5 eq NaH and 2 eq allyl bromide were added. After stirring overnight, the solution was filtered and concentrated, then precipitated in ice-cold diethyl ether. The resulting PEG allyl was then dissolved in DCM containing 0.5% (w/v) DMPA and 2 eq thioacetic acid. The solution was then exposed to 365 nm UV (4 mW cm^{-2}; UVP XX-15S lamp) for 1 h and the PEG thioester was precipitated in ice-cold ether. The product was then dissolved in ammonium methanol and 0.5 eq dithiothreitol was added to avoid disulfide formation. After stirring for 3 h, the product was precipitated in ice-cold ether. The conversion ratio for both products was over 95%, as confirmed by ^{1}H NMR.

2.3. Hydrogel formation

Two different hydrogel structures were formed via CG polymerization and SG polymerization (Fig. 1). A total of 24 groups were examined with different crosslinking densities and crosslinking mechanisms (Table S1). To form CG hydrogels, different concentrations (10, 15, 20% (w/v)) of PEGDA (mol. wt. 2 k, 3 k, 4 k, 5 k, 10 kDa) were dissolved in phosphate-buffered saline (PBS) containing Irgacure 2959 (0.05% (w/v)). For SG hydrogels, norbornene-terminated PEG (8-arm PEG10 kDa-NB) and thiol-terminated PEG (8-arm PEG10 kDa-SH, linear PEG1.5 k-dithiol, linear PEG10 k-dithiol) were mixed in a stoichiometrically balanced ratio in PBS solution containing Irgacure 2959 (0.05% (w/v)). The molecular weight of the SG gel (2.5 k, 4 k, 12.5 kDa) was defined as the molecular weight between two adjacent crosslinks. To form hydrogels, 50 μl of the precursor solution was loaded into a cylindrical gel mold (5 mm diameter × 3 mm thickness) and exposed to UV light (365 nm, 4 mW cm^{-2}, 5 min). During UV exposure, CG-polymerized gels using PEGDA were formed by polyacrylate kinetic

chains, while SG-polymerized gels were formed by mutually reactive groups, norbornene groups and thiol groups.

2.4. Mechanical test

The PEG hydrogel stiffness was measured by unconfined compression tests using an Instron 5944 materials testing system (Instron Corporation, Norwood, MA), as previously reported [16]. Briefly, the specimen diameter and thickness were measured using digital calipers and the material testing system's position read-out, respectively. Before each test, a preload of approximately 2 mN was applied to the hydrogels. All tests were conducted in PBS solution at room temperature. The upper platen was then lowered at a rate of 1% strain s^{-1} to a maximum strain of 30%. Load and displacement data were recorded at 100 Hz. The compressive modulus was determined for strain ranges of 20–30% from the linear curve fit of the stress vs. strain curve in each strain range.

2.5. Protein release test

To quantify the protein diffusivity and protein accumulation within 3-D hydrogels, BSA was chosen as the model solute for diffusion. To mimic exogenously supplemented growth factor accumulation in 3-D tissue engineering scaffolds, premade hydrogels were immersed in BSA solution (4 mg ml^{-1}) at room temperature for 24 h to achieve uniform protein loading. For protein diffusivity on the order of 10^{-7} cm^2 s^{-1}, the diffusion time is 15 h for a diffusion length of 1.5 mm ($t \sim L^2/4D$). Therefore, a 24 h loading time should be sufficient to achieve equilibrium protein concentrations within the gels. The loading time observed in similar studies of various model proteins through even denser polymer networks further support the rationale for a 24 h loading time [9].

For protein release, BSA-loaded hydrogels were immersed in fresh PBS. To maximize the driving force for diffusion, all of the supernatant was collected and replaced by fresh PBS at different time points (t = 5, 10, 15, 20, 25, 30, 45, 60, 90 and 120 min and 24 h). To release any proteins that remained entrapped in the hydrogels after release for 24 h, the hydrogels were mechanically broken down in fresh PBS using a homogenizer. To quantify the protein in the supernatant, a Micro-BCA™ protein assay was performed following the manufacturer's protocol.

Protein accumulation was calculated from the protein released into supernatant at different time points and the protein that remained trapped in the gel after the 24 h release. The protein concentration within hydrogels was calculated by dividing the protein accumulation (mass) by the free water content (vol.) within the gels. The free water content (vol.) was calculated by using the free water content (mass) from a swelling ratio test, assuming the PBS density to be 1 mg ml^{-1}.

To determine the protein diffusivity (D) within different hydrogels, fractional release profiles were fitted to the following equation, which represents a short-time approximation of a 3-D Fickian diffusion model: a disk-shaped gel with uniform initial concentration and equal surface concentration [17] (the short-time solution of the 3-D Fickian diffusion model is valid for the first 65–70% of total release):

$$\frac{M_t}{M_\infty} = 4\left(\frac{Dt}{\pi a^2}\right)^{\frac{1}{2}} - \pi\left(\frac{Dt}{\pi a^2}\right) - \frac{\pi}{3}\left(\frac{Dt}{\pi a^2}\right)^{\frac{3}{2}} + 4\left(\frac{Dt}{\pi a^2}\right)^{\frac{1}{2}}$$
$$- \frac{2a}{l}\left(8\left(\frac{Dt}{\pi a^2}\right) - 2\pi \cdot \left(\frac{Dt}{\pi a^2}\right)^{\frac{3}{2}} - \frac{2\pi}{3} \cdot \left(\frac{Dt}{\pi a^2}\right)^2\right)$$

Here M_t is the accumulated protein release up to the time point t; M_∞ is the accumulated protein release at infinite time, which was determined by the protein accumulation; and D is the diffusivity

of the BSA within the gel. Given that gels have different swelling ratios, the final swollen gel geometry was taken into account when calculating the protein diffusivity (a is the gel diameter and l is the gel thickness).

2.6. Swelling test for network mesh size calculation

Hydrogel samples were swollen in PBS at room temperature for 24 h and weighed to obtain the equilibrium swollen mass (M_s). To obtain the dry polymer mass (M_d), the samples were placed in deionized water to remove the PBS salts, frozen and lyophilized overnight. The volumetric swelling ratio (Q_v) was calculated from the mass swelling ratio (M_s/M_d) using the densities of PBS (1.01 g ml^{-1}) and PEG (1.18 g ml^{-1}) [18]:

$$Q_v = \frac{M_s/\rho_{gel}}{M_d/\rho_{PEG}}, \rho_{gel} = \rho_{PBS} \times \left(1 - \frac{M_d}{M_s}\right) + \rho_{PEG} \times \frac{M_d}{M_s} \qquad (1)$$

Q_v was used to calculate the hydrogel network mesh size (ξ) according to the Flory–Rehner theory [19].

2.7. Statistical analysis

Data are presented as mean ± standard deviation. The standard deviation was calculated based on three replicates ($n = 3$). A Student's t-test was used to compare data sets and a p value less than 0.05 was considered statistically significant.

3. Results

3.1. Stiffness of PEG hydrogels

To evaluate how changing gel stiffness by varying gel crosslinking density can simultaneously alter protein accumulation within 3-D hydrogels, we first evaluated the effect of varying the PEG crosslinking density on gel stiffness. Regardless of the PEG crosslinking mechanism, increasing the PEG concentration or decreasing the PEG molecular weight increased the hydrogel stiffness (Fig. 2). Decreasing the PEG molecular weight (CG 20% (w/v) gel: from 10 k to 2 k) led to a 15-fold increase in gel stiffness (Fig. 2A). Similarly, increasing the PEG concentration (CG 2 k gel: from 10 to 20% (w/v)) resulted in a 10-fold increase in stiffness.

To study the effect of different crosslinking mechanisms on gel stiffness, we compared the stiffness of CG and SG gels with the same molecular weight ($M_c = 4$ kDa) and concentration (10, 15 and 20% (w/v)). The CG gels generally demonstrated higher stiffness than the corresponding SG gels (Fig. 2). For the CG gels, increasing the PEG concentration (from 10 to 20% (w/v)) led to a

5-fold increase in stiffness, while it led to 3-fold increase for the SG gels.

3.2. Protein diffusion profile and diffusivity

To investigate the effects of varying the PEG crosslinking density and crosslinking mechanism on protein diffusion, we next conducted protein release experiments using BSA as the model solute. In general, increasing the PEG molecular weight or decreasing the PEG concentration increased the amount of protein released during the 2 h release period (Fig. 3). For the CG gels, a substantial increase in protein release was observed when increasing the PEG molecular weight from 5 k to 10 k, while increasing the PEG molecular weight from 2 k to 5 k did not change the diffusion profiles significantly (Fig. 3A–C). For the SG gels, a 10-fold increase in protein release was observed when the PEG molecular weight was increased from 4 k to 12.5 k, whereas 2 the.5 k and 4 k groups showed similar diffusion patterns (Fig. 3D–F). Also, increasing the PEG concentration from 10% to 15% (w/v) resulted in the greatest decrease in protein release for both CG and SG gels. For example, protein release (SG 12.5 k gels) dropped by 57% when the PEG concentration was increased from 10% to 15% (w/v). However, a further increase in PEG concentration from 15% to 20% (w/v) resulted in only an 11% decrease in protein release.

To examine the effect of the crosslinking mechanism on protein diffusion, CG 4 k and SG 4 k gels were specifically chosen and compared with regard to protein release (Figs. 3 and S1). We found that CG gels (4 k, 10% (w/v)) released twice as much protein as the corresponding SG gels during 2 h.

Protein diffusivity is a commonly used parameter to describe protein diffusion within a 3-D hydrogel. We calculated the diffusivity by fitting the fractional release profile to a 3-D Fickian diffusion model. The 2 h release was sufficient to calculate the BSA diffusivity since the short-time approximated solution of the 3-D Fickian model was valid for fitting the initial 65–70% of total release. While increasing the molecular weight from 2 k to 10 k resulted in a 2-fold increase in the protein diffusivity of the CG gels, an unexpected increase in protein diffusivity was observed in the CG gels when increasing the PEG concentration from 10% to 20% (w/v) across all of the molecular weight groups examined (Fig. 4). On the other hand, in the SG gels, varying the molecular weight or concentration had only a mild effect on the protein diffusivity, and the diffusivity in the SG gels across all of the different concentrations (10–20% (w/v)) and molecular weights (2.5–12.5 k) showed comparable values (1.5–2.5 × 10^{-7} cm^2 s^{-1}). Unlike the protein diffusion profile (Fig. 3), no substantial jump in protein diffusivity was observed (Fig. 4).
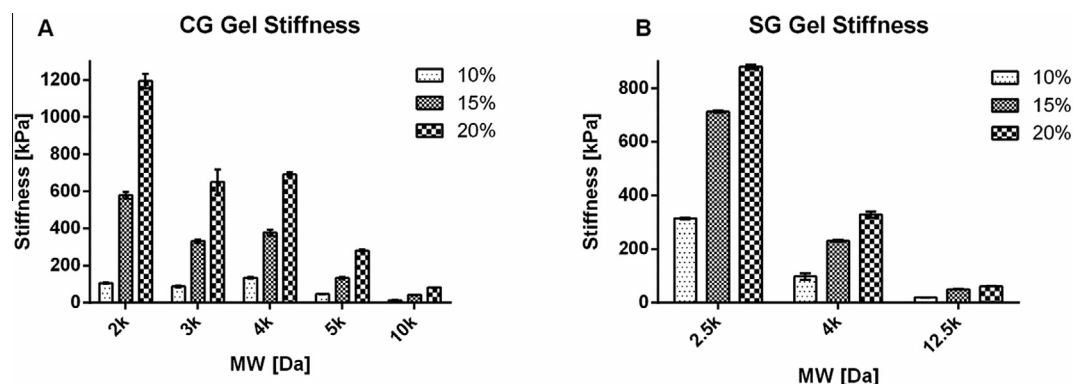

Fig. 2. The effects of varying PEG molecular weight (2, 2.5, 3, 4, 5, 10 and 12.5 kDa) or concentration (10, 15 and 20% (w/v)) on stiffness of (A) CG- or (B) SG-polymerized hydrogels.
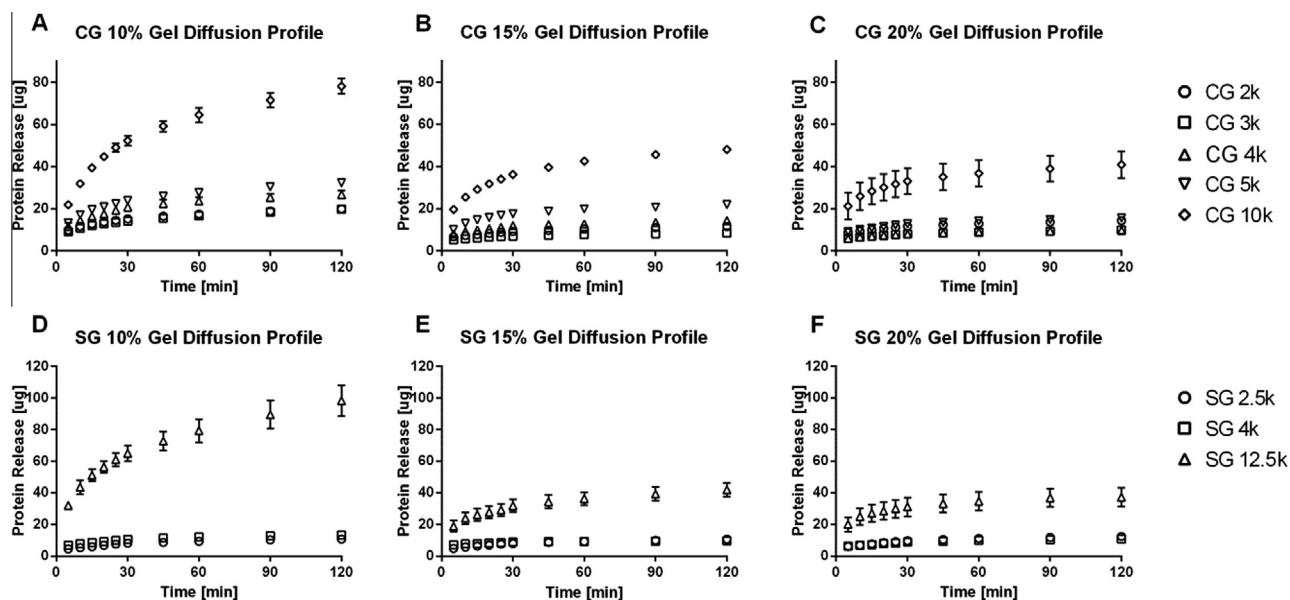
Fig. 3. The effects of varying PEG molecular weight (MW) or concentration (conc.) on protein release from (A–C) CG- or (D–F) SG-polymerized gels. Increasing the MW or decreasing the conc. led to increasing protein release. A substantial increase in protein release was observed when the MW was increased from 5 k to 10 k (CG) or from 4 k to 12.5 k (SG). Similarly, a substantial increase in protein release was observed when the conc. Was decreased from 15% to 10% (both CG and SG).
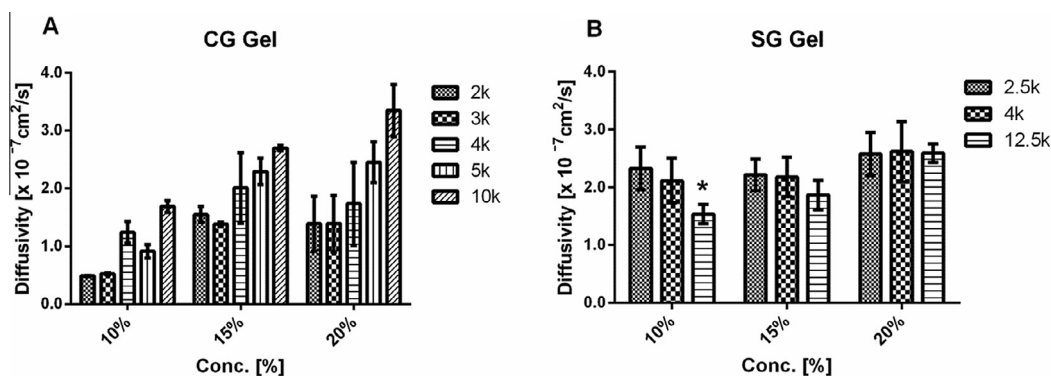

Fig. 4. The effects of varying PEG MW or conc. on protein diffusivity in (A) CG- or (B) SG-polymerized gels. As the PEG MW or conc. increases, the diffusivity also increases within the CG gels, whereas the diffusivity change was not significantly different among the SG gels. The asterisk indicates significant difference with respect to 2.5 k 10% ($p < 0.05$).

3.3. Protein accumulation within hydrogels

In addition to protein diffusivity, we hypothesized that varying the gel crosslinking density and crosslinking mechanism would affect protein accumulation within the hydrogels. The protein accumulation was calculated by adding up the accumulated protein release during 24 h and any protein entrapped within the hydrogels. In general, increasing the PEG molecular weight or decreasing the PEG concentration resulted in increased protein accumulation in both the CG and SG gels (Fig. 5A and B). For the CG gels, a substantial increase in protein accumulation was observed when the PEG molecular weight was increased from 5 k to 10 k. A similar trend was observed when the PEG molecular weight was increased from 4 k to 12.5 k in the SG gels. For example, increasing the molecular weight from 4 k to 12.5 k resulted in a 10-fold increase in protein accumulation in the SG gels, while the increase was only 1.3-fold when increasing molecular weight from 2.5 k to 4 k (Fig. 5B). Similarly, decreasing the concentration (from 15% to 10% (w/v)) resulted in a 2.6-fold increase in protein accumulation from the SG gels (12.5 k), while decreasing the concentration (from 20% to 15% (w/v)) resulted in only a 1.2-fold increase (Fig. 5B). We then calculated the network mesh size to

compare it with the hydrodynamic diameter of BSA (7.2 nm). Interestingly, only the 10 k CG gels and 12.5 k SG gels had mesh sizes larger than the BSA hydrodynamic diameter (7.2 nm) (Fig. 5C and D).

Since it is the protein concentration that cells directly sense within hydrogels, the protein concentration was calculated by dividing the protein accumulation by the water content in the gels. The protein concentration increased with increasing PEG molecular weight and decreasing PEG concentration similar to the protein accumulation trend (Fig. 5 and 6). For 4 k PEG, the CG gels showed higher protein concentrations than the SG gels regardless of the PEG concentration (10–20% (w/v)). For example, the protein concentration in the CG 4 k 10% (w/v) gels was 2.2-fold higher than that in the corresponding SG gels.

4. Discussion

PEG hydrogels have been widely used as 3-D cell niches to examine the effects of matrix stiffness on cell fate in vitro [20–22]. To obtain hydrogels with tunable stiffness, the PEG hydrogel crosslinking density is often varied by varying the PEG molecular
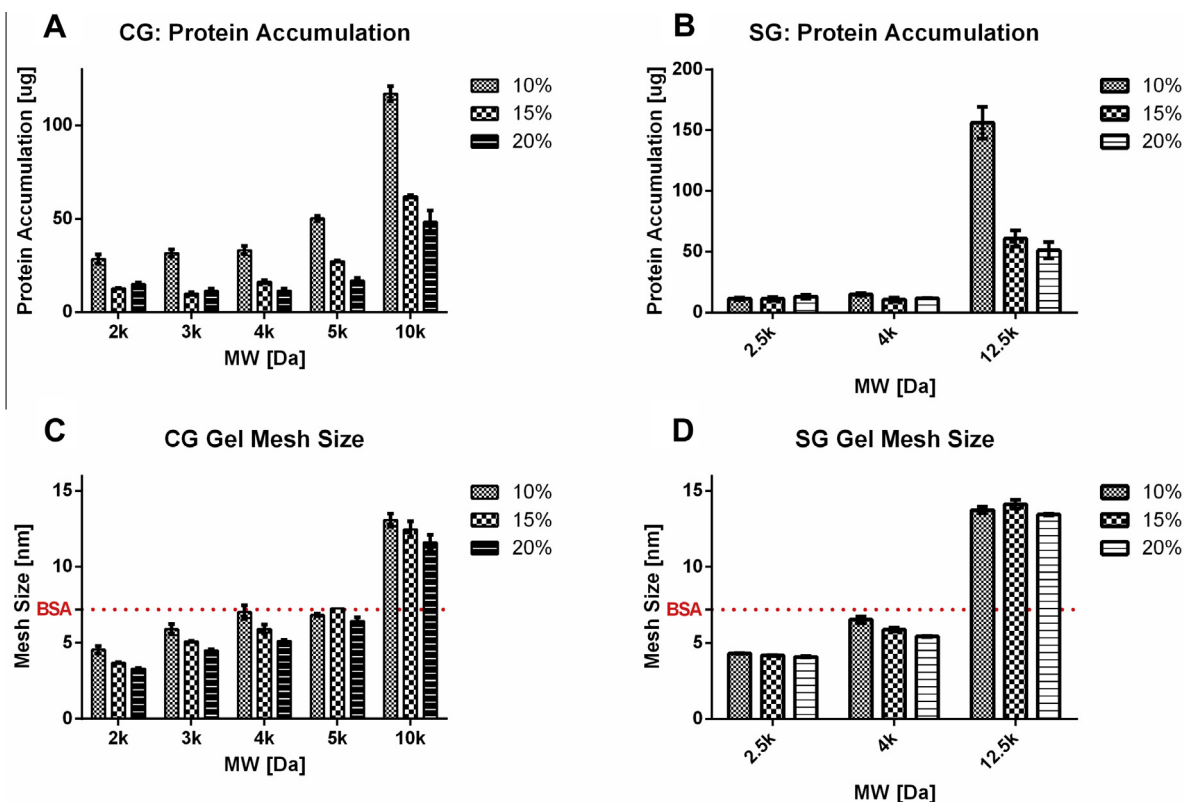
Fig. 5. (A, B) The effects of varying the PEG MW or conc. on protein accumulation in CG-polymerized gels or SG-polymerized gels. (C, D) CG or SG gel mesh size calculated from the swelling ratio. A substantial increase in protein accumulation was observed when the calculated mesh size exceeded the BSA hydrodynamic size (CG gel: MW from 5 k to 10 k; SG gel: MW from 4 k to 12.5 k).
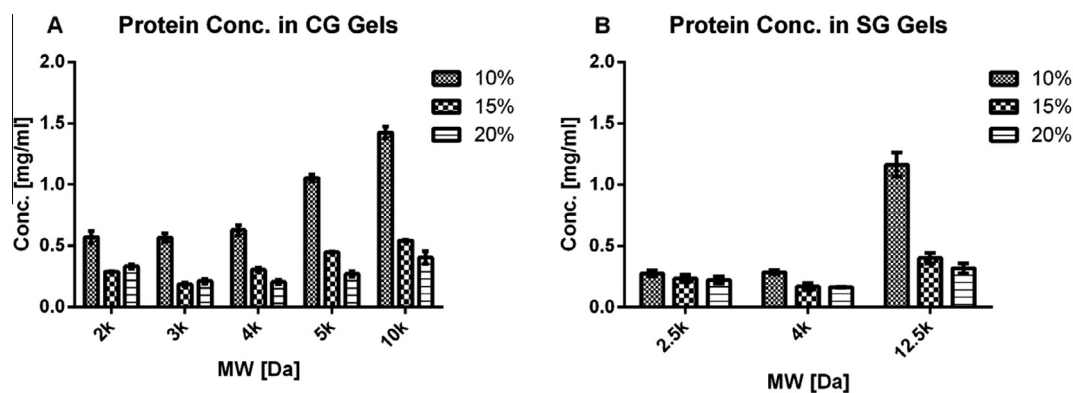

Fig. 6. The effects of varying PEG MW or conc. on protein concentration in (A) CG- or (B) SG-polymerized gels.

weight or concentration [9,20,23]. While various studies have highlighted that varying the PEG hydrogel stiffness can result in changes in cellular fate, most studies did not evaluate the potential differences in protein accumulation induced by such changes in 3-D hydrogel scaffold where cells are known to be sensitive to available growth factors [3,12]. As such, this study aimed to investigate how protein accumulation is affected by varying the PEG hydrogel crosslinking density when PEG hydrogels are formed via two different crosslinking mechanisms. To obtain PEG hydrogels with tunable stiffness (10–1000 kPa), we fabricated PEG hydrogels with varying crosslinking density and mechanisms. We then loaded proteins within 3-D PEG hydrogels and characterized both the protein accumulation and the protein diffusivity. As the model protein, BSA was chosen over fluorescein isothiocyanate–dextran, which is another solute that is commonly used for diffusion studies

[9–11]. This is because dextran is a linear and flexible polymer, which may demonstrate a diffusive behavior that is distinct from that of globular proteins such as growth factors.

While decreasing the gel crosslinking density generally led to increased protein accumulation (Fig. 5A and B), our results suggest that a critical mesh size may exist for protein accumulation. We calculated the gel mesh size based on the swelling ratio and found that only the CG 10 k and SG 12.5 k gels had bigger calculated mesh sizes than the hydrodynamic diameter of BSA (Fig. 5C and D). Together, these results suggest that, when the gel mesh size exceeds the hydrodynamic size of the solute protein, protein diffusion is less sterically hindered, leading to a substantial increase in protein accumulation within hydrogels.

We observed that protein accumulation drops substantially when the PEG concentration increases from 10% to 15%, despite

the calculated mesh size of the two hydrogel networks being comparable and larger than the hydrodynamic radius of BSA (Fig. 5). Given the protein-repulsive nature of PEG, it is possible that increasing the PEG concentration from 10% to 15% results in a substantial increase in protein repulsion despite the comparable hydrogel mesh size. Furthermore, the mesh size is calculated from the hydrogel swelling ratio, and represents only the average value of the hydrogel network mesh size without accounting for the network heterogeneity. Together, these factors may explain the different trends observed in protein accumulation and hydrogel mesh size.

We also found that protein accumulation can be substantially affected by the PEG hydrogel crosslinking mechanism chosen (CG vs. SG). For example, the CG gels (4 k, 10% (w/v)) resulted in 2.2-fold more protein accumulation than the corresponding SG gels, although the calculated mesh sizes for the two types of gel were comparable (Figs. 3 and 5 and S1). Given that the swelling ratio reflects only the hydrogel bulk property and does not take into account the heterogeneity of the mesh size, the greater protein accumulation within the CG gels suggests that there is higher heterogeneity in the CG network, which may have facilitated protein accumulation via local network defects [13]. Many previous studies have shown that the hydrogel network structure is more homogeneous when the hydrogel is crosslinked between mutually reactive end groups of multi-arm PEG [24–27]. It is possible that some end groups of PEG polymers might not fully react during the polymerization process, which may leave unreacted acrylate or thiol/-ene end groups. However, we expect such unreacted end groups will be minimal and their contribution to the observed differential protein accumulation in different hydrogels to be negligible. Therefore, the hydrogel crosslinking mechanism may directly lead to differential protein accumulation due to changes in network heterogeneity.

Similar to previous reports, our results showed an increasing trend in protein diffusivity when the PEG molecular weight was increased in the CG gels (Fig. 4) [9,10]. However, our data unexpectedly showed an increase in protein diffusivity with increasing PEG concentration in these gels (Fig. 4). This counter-intuitive observation may be explained by a phenomenon called macromolecular crowding. Previous studies have shown that proteins favor compact conformation in a crowded environment as this increases stabilization [28–31]. In particular, Tokuriki et al. [28] showed that globular proteins adopted more compact conformations and exhibited higher diffusivity in the presence of a higher concentration of PEG. In addition to this, the unexpected trend of protein diffusivity with regards to PEG concentration might in part be due to underestimating the protein accumulation (M_∞) in the hydrogels of higher PEG concentration, which would lead to an overestimation of the fractional release. It is possible that some proteins remain entrapped in hydrogel debris even after the gel homogenization procedure and more proteins are likely to be entrapped in PEG hydrogels formed with a higher monomer concentration.

This study highlights that the trend of protein accumulation does not follow the trend of protein diffusivity (Figs. 4–6). A previous study concluded that differences in protein diffusivity was not significant to alter stem cell response to varying hydrogel mechanical properties. However, this study suggests that protein accumulation within hydrogels of varying stiffness can differ substantially despite changes in protein diffusivity being insignificant. Therefore, we think protein accumulation must be considered as an important parameter in addition to protein diffusivity when PEG hydrogel crosslinking density is varied.

5. Conclusion

Here we report that both hydrogel crosslinking density and the crosslinking mechanism have a crucial effect on protein diffusivity and accumulation within the hydrogel. In this study, we highlight protein accumulation within hydrogels as an important parameter, in addition to protein diffusivity, when varying the hydrogel crosslinking density. This study shows that protein accumulation increases substantially above a critical mesh size, which cannot be predicted from the protein diffusivity trend. Furthermore, our findings show that the crosslinking mechanism can affect protein accumulation within hydrogels, which may be induced by network heterogeneity. We believe this work helps in understanding the effects of varying the crosslinking density and the crosslinking mechanism on protein accumulation and emphasizes the need to consider protein accumulation to appropriately interpret cellular responses to different mechanical cues.

Appendix A. Figures with essential color discrimination

Certain figures in this article, particularly Figs. 1 and 5 are difficult to interpret in black and white. The full color images can be found in the on-line version, at http://dx.doi.org/10.1016/j.actbio.2014.05.023.

Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actbio.2014.05.023.

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