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Influence of Cell-Adhesive Peptide Ligands on Poly(ethylene glycol) Hydrogel Physical, Mechanical and Transport Properties

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Abstract

Synthetic three-dimensional (3D) scaffolds for cell and tissue engineering routinely utilize peptide ligands to provide sites for cell adhesion and to promote cellular activity. Given the fact that recent studies have dedicated great attention to the mechanisms by which cell behavior is influenced by various ligands and scaffold material properties, it is surprising that little work to date has been carried out to investigate the influence of covalently-bound ligands on hydrogel material properties. Herein we report the influence of 3 common ligands utilized in tissue engineering, namely RGD, YIGSR, and IKVAV on the mechanical properties of cross-linked poly(ethylene glycol) (PEG) hydrogels. The effect of the ligands on hydrogel storage modulus, swelling ratio, mesh size, and also on the diffusivity of bovine serum albumin (BSA) through the hydrogel were investigated in detail. We identified conditions at which these ligands strikingly influence the properties of the material: the extent of influence and whether the ligand increases or decreases a specific property is linked to ligand type and concentration. Further, we pinpoint mechanisms by which the ligands interact with the PEG network. This work thus provides specific evidence for interactions between peptide ligands and cross-linked PEG hydrogels that significantly impact hydrogel material and transport properties. As a result, this work may have important implications for interpreting cell experiments carried out with ligand-modified hydrogels because the addition of ligand may affect not only the scaffold's biological properties, but also key physical properties of the system.

Keywords

ligand; PEG hydrogel; swelling ratio; mesh size; storage modulus

1. Introduction

The adhesive ligand sequence RGD (arg-gly-asp) from fibronectin was first discovered in 1984 by Pierschbacher and Ruoslahti et al. [1]. It was recognized as the minimal active amino acid sequence necessary to promote cell adhesion. Soon after this report, it was confirmed that the RGD sequence is the cell attachment site of many other adhesive proteins such as collagen type I and laminin [2–6]. Since then, RGD, as well as other adhesive ligands such as the laminin derived YIGSR (tyr-ile-gly-ser-arg) and the fibrinogen derived IKVAV (ile-lys-val-ala-val), have been used in hundreds of applications to induce specific

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cell behaviors [7–10]. These applications include bone biology [11], cardioprogenitor differentiation [12], fibroblast cell migration [13,14], vascular healing [15], axon guidance [16], support of differentiating marrow stromal osteoblasts [17], and stem cell applications [18].

Despite the fact that ligands retain only 10% to 30% of their biological activity as compared to the whole protein [19,20], chemical conjugation of polymers with short peptides has several advantages: 1) higher stability against conformational change; 2) control of ligand density; 3) control of ligand orientation to possibly provide more favorable ligand-receptor interactions and cell adhesion; 4) benefits in terms of minimizing immune response and infection; 5) higher stability during sterilization; 6) easier storage and characterization; and 7) cost effectiveness [21].

While adhesive ligands have also been incorporated into natural materials (e.g., collagen [22,23], fibrin [24], and laminin [25]) to enhance cell adhesion and proliferation, they have become a crucial component of synthetic materials for tissue engineering. Even though natural materials are inherently biocompatible and bioactive, synthetic materials offer a wider range of properties and lend themselves to easier processing and modification [26]. Furthermore, synthetic materials are an invaluable resource when deciphering the intricate relationship between the matrix components and their possible individual influence on cell behavior. For example, synthetic materials have well defined and controllable mechanical properties so they can serve as the “blank slate” or inert structural backbone of a scaffolding matrix. However, since the scaffolds lack bioactivity, whole proteins or short ligands must be incorporated to afford biological properties to the system. This approach allows for delineation of the effects of mechanical properties from the biological properties of the materials, a recognized issue of growing importance for the field of tissue engineering. Scaffold mechanical properties, such as stiffness, which is well represented by the storage modulus of the material, have been shown to have a tremendous effect on cell behavior and to have important implications for development, differentiation, disease progression and regeneration [27]. Specifically, material stiffness has been linked to promoting malignant behavior in tumors [28], to directing stem cell lineage [29], to guiding fibroblast cells movement [30], and to the spreading and organization of aortic smooth muscle cells [31].

To date, it has been generally assumed that an addition of a small amount of ligand to a matrix does not interfere significantly with the mechanical properties of the material and that these two properties can be tuned independently. While a tremendous amount of studies have been carried out on the influence of ligands on cell behavior, few studies have been undertaken to investigate the possible influence of ligands on the mechanical properties of the material [31].

This project was aimed at investigating the influence of the 3 most commonly used ligands in tissue engineering, namely RGDS, YIGSR, and IKVAV on the mechanical properties of a cross-linked poly(ethylene glycol) (PEG) hydrogel. Ligands covalently bound to the PEG hydrogel were compared to a negative control of bound monofunctional PEG-thiol (PEG-SH) of similar molecular weight. We tested the effect of the ligands on hydrogel stiffness, swelling ratio, mesh size, and also on the diffusivity of a model protein, bovine serum albumin (BSA), through the hydrogel. We have identified conditions under which the ligands profoundly influence hydrogel properties and performed additional tests in order to pinpoint the possible causes for these effects.

2. Materials and methods

All reagents were acquired from Fisher Scientific or Sigma Aldrich unless otherwise noted.

2.1. Synthesis of 4-arm poly(ethylene glycol)-vinyl sulfone (4-arm PEG-VS)

The synthesis of 4-arm PEG-VS was adapted from a previous protocol [32,33] where 4-arm PEG-OH (10 kDa; Nektar, Huntsville, LA) was modified in the presence of excess divinyl sulfone. Briefly, PEG was dried by azeotropic distillation in toluene using a Dean-Stark trap and then dissolved in dry dichloromethane. Sodium hydride was added under Ar at a 5-fold molar excess over OH groups. After hydrogen evolution divinyl sulfone was added immediately at a 50-fold molar excess to OH groups. The solution was allowed to react for 3 d at room temperature under Ar and with constant stirring. The solution was then filtered and reduced in volume (~30 ml) by rotary evaporation. The polymer was recovered in ice-cold diethyl ether and dried under vacuum. The dry polymer was then dissolved in deionized water containing sodium chloride and extracted three times in dichloromethane. After drying with sodium carbonate, the polymer product was reduced in volume, precipitated, and dried as described above. The product was stored under Ar at -20°C until use. Derivatization was confirmed by ^1H NMR (CDCl_3): 3.6 ppm (982H, PEG backbone), 6.1 ppm (4H, 1H, $=\text{CH}_2$), 6.4 ppm (4H, 1H, $=\text{CH}_2$), 6.8 ppm (4H, 1H, $-\text{SO}_2\text{CH}=\text{}$). The typical yield from this procedure was 80–90% and the degree of end group conversion, as shown by NMR, was 93–98%.

2.2. Adhesive ligands

The adhesive ligands GRCD-*YIGSR*-PD, GRCD-*IKVAV*-PD, GRCD-*RGDS*-PD and GRCD-*YIGSR* were purchased from CPC Scientific Inc., San Jose, CA (>90% purity; Table 1). The italic font designates the bioactive peptide sequence. The ligands were terminated on one end with the sequence GRCD which contains a cysteine residue to allow for covalent attachment to 4-arm PEG-VS [34]. The PD flanking group was chosen based on work by Lutolf et. al. [35].

To minimize weighing error, the ligand was aliquoted in advance in 5% acetic acid solution. The acidic solution kept the thiol groups of the cysteine residue protonated and thus prevented disulfide bonding between the peptides prior to reaction with PEG-VS. Each aliquot was used for up to 2 wks before discarding. The ligands were chosen such as to represent a large span of biochemical properties (Table 1) and thus a range of possible effects on hydrogel structure and function. Additionally, the sequences RGDS, IKVAV, YIGSR are the most commonly used in tissue engineering, including a system of our particular interest, neural tissue engineering [36,37].

2.3. Formation of PEG-based hydrogels

PEG hydrogels were made with a PEG-dithiol cross-linker (Laysan Bio Inc., Arab AL) and functionalized with adhesive ligands (Figure 1) with negative controls consisting of hydrogels without ligand and hydrogels made with mono-functional PEG-thiol (PEG-SH, 1000 Da) which is the same molecular weight as the adhesive peptide ligands but has the properties of the hydrogel backbone polymer.

Briefly, adhesive ligand was added to 4-arm PEG-vinyl sulfone (PEG-VS) dissolved in 0.3 M triethanolamine (TEA) solution of pH 8.2 at a large stoichiometric deficit and left to react for 30 min. The cross-linker, PEG-dithiol was then added to give a final ratio of VS:SH of 1:1 for all hydrogel densities and types. TEA of pH 8.2 was used to dilute reagents to the desired final PEG concentration and hydrogel volume. The solution was vortexed for 30 sec and transferred to the center of a glass slide pre-treated with RainX (Sopu Products, Houston, TX) to provide a hydrophobic surface. Silicone spacers (1- or 2.5-mm thick cut from CoverWell perfusion chambers, Grace Bio-Labs, Bend, OR) were placed at the ends of the glass slide and a second hydrophobic slide was placed on top. The two slides were clamped together over the spacers with binder clips. The slides were then transferred to a

humidified incubator to allow the hydrogel to cross-link at 37°C. Gelation occurred in several minutes but the hydrogels were left in the incubator for 1–2 h to achieve maximum cross-linking.

2.4. Efficiency of ligand incorporation in the PEG hydrogel

The fluorescently labeled ligand 5FAM-GRCD-RGDS-PD was used to estimate the efficiency of ligand incorporation in the PEG hydrogel, where 5FAM stands for 5-carboxyfluorescein ($\lambda_{\text{ex}} = 490 \text{ nm}$, $\lambda_{\text{em}} = 520 \text{ nm}$). The ligand was added to the PEG-VS as described above and allowed to react for 30 min. The solution was then diluted with PBS and the unbound ligand was removed via a centrifugal filter device (Millipore, Bedford, MA; 5 kDa molecular weight cut-off). The amount of unbound ligand was quantified via measurement of the filtrate fluorescence with a Cary Eclipse spectrophotometer (Varian Inc., Walnut Creek, CA) and comparison to a calibration curve with lower ligand sensitivity limit of 0.1 μM .

In order to determine the extent to which ligand diffused out of the hydrogel prior to further characterization of the hydrogel properties (at 24 h), the hydrogels were prepared as described above. The hydrogels were then positioned in a cuvette filled with 10 mM PBS, pH 7.4 and were kept at room temperature and not moved until the end of the experiment. Over 24–72 h the fluorescence of the hydrogel and the supernatant PBS solution was measured. The PBS was transferred to a separate cuvette via a syringe and its fluorescence as well as that of the hydrogel itself were determined separately. After the measurement, the PBS was returned to the original cuvette containing the hydrogel. In cases where only the fluorescence of the hydrogel was measured, the supernatant PBS was replaced with fresh PBS prior to each measurement. To improve the accuracy of the measurements and avoid evaporation of the PBS, the solution was kept capped at all times. The experiment was performed at room temperature. The solution containing cuvette was covered with foil at all times (except during measurements) to prevent fluorophore photobleaching.

2.5. Hydrogel characterizations

Rheological measurements were performed with an AR 2000ex rheometer (TA Instruments) in parallel plate geometry with a 20-mm diameter acrylic upper plate, at 22°C, a frequency of 1–10 rad/s, and a constant 2% strain [38]. The hydrogel samples were prepared to yield discs of 20-mm diameter and 1-mm thick (thickness < diameter/4 per TA Instruments guide) following swelling in 10 mM phosphate buffer saline (PBS), pH 7.4. The excess water from the hydrogel surface was carefully blotted before measurement. Storage modulus (G') at 1 rad/s was reported for each sample.

To estimate swelling ratio, hydrogels (50 μL) were soaked in 10 mM PBS, pH 7.4. Hydrogel samples were collected at regular intervals and their mass after swelling (M_S) was measured. The hydrogels were then dried in an oven at 80°C for 24 h and their dry mass (M_D) was measured. The swelling ratio based on hydrogel mass (Q_M) was calculated using Equation 1 [39]:

$$Q_M = \frac{M_S}{M_D}. \quad (1)$$

Flory-Rehner calculations were used to determine hydrogel mesh size (ξ). First the molecular weight between cross-links (M_c) was calculated by Equation 2 [40]:

$$\frac{1}{\overline{M}_c} = \frac{2}{\overline{M}_n} - \frac{\overline{v}_1(\ln(1 - v_2) + v_2 + \chi_1 v_2^2)}{v_2^{1/3} - \frac{v_2}{2}} \quad (2)$$

where \overline{M}_n is the number-average molecular weight of the un-cross-linked hydrogel, V_1 is the molar volume of the solvent (18 cm³/mol for water), v_2 is the polymer volume fraction in the equilibrium swollen hydrogel, \overline{v} is the specific volume of the polymer (ρ_s/ρ_p), and χ_1 is the polymer-solvent interaction parameter (0.426 for PEG-water [39, 40]).

Mesh size was then determined as described by Canal and Peppas [41]. The root-mean-square end to end distance of the polymer chain in the unperturbed state ($(\overline{r_0^2})^{1/2}$) was calculated using Equation 3:

$$(\overline{r_0^2})^{1/2} = l C_n^{1/2} n^{1/2} \quad (3)$$

where l is the average bond length (0.146 nm [42,43]), C_n is the characteristic ratio of the polymer (typically 4.0 for PEG [43,44]) and n is the number of bonds in the crosslink [13]:

$$n = 2 \frac{\overline{M}_c}{M_r} \quad (4)$$

where M_r is the molecular weight of the repeat unit (44 for PEG). Mesh size was then calculated by

$$\xi = v_2^{-1/3} (\overline{r_0^2})^{1/2}. \quad (5)$$

The diffusion coefficient was estimated from bulk diffusion experiments. Briefly, the hydrogels were cross-linked in the presence of the solute BSA to achieve a final solute concentration in the hydrogel of 2% w/v. The hydrogels were then placed in 15-ml tubes filled with 10 mM PBS of pH 7.4 and mixed end-over-end at 22°C. At specified sample collection times, 1 ml of solution was transferred to a microfuge tube and was replaced in the 15-ml tube with fresh PBS. The solute content of each sample was analyzed with the Bio-Rad protein assay using the manufacturer's microassay procedure.

Diffusion coefficient was calculated via a modified form of the Fick's law for short release times [45]:

$$\frac{M_i}{M_{inf}} \cong 2 \left[\frac{D_e t}{\pi \delta^2} \right]^{1/2}. \quad (6)$$

From the Equation 6, it follows that M_i/M_{inf} is directly proportional to $t^{1/2}$ and therefore a plot of M_i/M_{inf} versus $t^{1/2}$ can be used to find D_e . A mass balance was performed to calculate M_i :

$$M_i = C_i V + \sum C_{i-1} V_s \quad (7)$$

where C_i is the concentration of solute in the release solution at time i , V is the total volume of the release solution (15 ml) and V_s is the sample volume (1 ml).

2.6. Statistical analysis

The results of all experiments are the mean values ($\pm SD$) of triplicate samples performed in minimum three independent experiments. Comparisons between multiple samples were performed with single factor analysis of variance (ANOVA). Comparisons between two samples were performed with two-tailed Student's t -test. Differences between data sets were considered significant when $p < 0.05$.

3. Results and Discussion

3.1. Incorporation of ligand into the PEG hydrogel

The fluorescent ligand 5FAM-GRCD-RGDS-PD was incorporated into the PEG hydrogel and its fluorescence was measured over time to estimate whether any unbound peptide was released from the hydrogel. Figure 2a shows that the fluorescence of the ligand in the hydrogel or in the supernatant did not change appreciably over the course of the experiment (4 and 24 h). The average fluorescence of the PBS solution was lower than the average fluorescence of the hydrogel indicating that some ligand was not bound covalently to the hydrogel and therefore was free to diffuse out of the hydrogel during the soaking in PBS. Figure 2b shows that the fluorescence of the hydrogel did not change over 72 h which also indicated that all of the ligand was released initially (in < 4 h) and the ligand remaining in the hydrogel was stably incorporated; therefore, characterization of hydrogel properties (e.g., G') was not affected by unbound ligand. Additionally, fluorescent ligand was reacted with PEG-VS and after the reaction was complete, the solution was filtered to recover the unbound ligand. The unbound ligand from this experiment was estimated to be $36.1 \pm 0.3\%$.

There are several possible explanations for the apparent incomplete incorporation of the ligand. First, it is possible that a small amount of free dye is present in solution. The excess dye was cleaned with a one-step high-performance liquid chromatography (HPLC) purification (CPC Scientific) and the purification efficiency was not tested subsequently. Second, it is possible that despite the preventative measures, a fraction of the ligands had reacted with each other forming dimers by disulfide bonding thus taking up thiol groups that otherwise would be covalently bound to the VS group of the PEG polymer. Disulfide formation could have occurred in the aliquot or after the ligand was added to the PEG-VS solution. The competitive disulfide formation after the peptide was added to the PEG-VS solution would be closely related to the initial concentration of ligand: more ligand would lead to the formation of more disulfides [34,46]. Lastly, the reaction efficiency of the cysteine thiol binding to the VS group of the PEG by Michael-type addition strongly depends on the surrounding amino acids and their charges: Lutolf et al. [34] demonstrated that positively charged amino acids positioned near the thiol resulted in a faster addition reaction rate. Specifically, the binding efficiency constant for the sequence GRCD (used in our project) to PEG-diacrylate (PEGDA) was 58.3 L/mol.min compared to 124.0 L/mol.min for GRCR. Therefore, it is possible that choosing a peptide with a sequence such as GRCR-RGDS-PD, would improve the binding efficiency.

3.2. Effect of ligand type on material properties: general findings

Q_M is defined as the mass of the swollen hydrogel divided by the mass of the dry hydrogel and is a measure of the hydrophilicity of the polymer. When the polymer is cross-linked and forms a 3D structure, Q_M is also a measure of how much water is incorporated into the structure (i.e., the water to polymer ratio). Given a mesh network (a good approximation of the PEG hydrogel) as shown in Figure 3a: more cross-link points will result in tighter mesh;

consequently, less water will be incorporated in the network. On the other hand, if some of the cross-links are disrupted, local “pockets” with larger mesh would be created that are able to incorporate more water. Hence, by adding ligand and consequently inhibiting the complete cross-linking of the hydrogel, we should see increase in Q_M (up to a certain maximum peptide concentration that would occupy sufficient cross-linking sites to prevent polymer-polymer bonds and result in a soluble macropolymer). If this hypothesis holds true, it would indicate that the properties of the ligand and the PEG polymer are comparable and there is no ligand-polymer interaction.

It is also likely that Q_M would not be affected by the incorporation of ligand. Note that at 100 μM ligand or PEG-SH, only up to 0.7% of the VS groups would be occupied. The rest of the VS groups would be free for gelation with the PEG-dithiol cross-linker. This decrease in available cross-linkable VS sites may have a negligible effect; thus the net homogeneity of the resultant hydrogel network would not be affected and Q_M would not be altered.

Figure 3b shows the influence of ligand type on Q_M of the PEG hydrogel. Compared to hydrogels made without ligand or PEG-SH, Q_M was ~14% higher when PEG-SH was incorporated into the hydrogel, which was expected because the PEG-SH occupied cross-link sites but was not expected to interact with the surrounding PEG network. For the peptide ligands, the addition of ligand either had no effect (as for RGDS and IKVAV), or decreased Q_M (as for YIGSR and YIGSRPD).

For all general purposes, especially cell attachment and protein adhesion, PEG is considered an inert polymer [47], an observation which would not explain the above findings. However, Kokufuta et. al. [48] and Xia et. al. [49] have shown that PEG was able to form a complex with pepsin under the appropriate conditions. The complex formation had been attributed to hydrogen bonding of the carboxyl and phenolic OH groups in pepsin. However, the hydrogen bonding would only be possible when these acidic side chains are protonated, i.e., this effect would depend on the pH of the surrounding solution. The pH of the PEG solution was 8.2 during gelation and 7.4 after gelation when the fully cross-linked 3D hydrogel was soaked in PBS. Considering the full amino acid sequence of all ligands, one may note that the only amino acid present that contains a carboxylic side chain is D (aspartic acid). It has a pKa of 3.9 [50] and thus would be protonated at pH ~3 or below. Therefore, under the selected experimental conditions, the carboxylic acid of D would be unprotonated and could not participate in hydrogen bonding with the ether oxygen of the PEG polymer. Furthermore, D is present in all ligands (in the flanking sequences GRCD and PD) and thus could not explain the differences in their influence over the material properties.

All ligands as well as the control PEG-SH were of similar molecular weight (~1000 Da), thus it is not likely that ligand size alone could explain the observed results. Therefore, the effect of ligand incorporation could not be generalized and specific ligand properties must be considered to explain their various effects. We first examined the individual properties of the ligand peptide sequences used in this work (Figure 4). Because we observed a completely different influence associated with addition of PEG-SH to the hydrogel as compared to addition of peptide ligands, we examined the major properties that clearly distinguish the two. First, we considered the net charge of the ligands (Figure 4a). PEG itself (including PEG-SH) is uncharged, while each of the ligands contained charged polar amino acids. However, considering net charge alone, we did not observe obvious trends in the data.

Next, we considered the possibility that negatively charged or polar uncharged amino acids were forming ionic or weaker Van der Waals bonds with the partially positively charged ether oxygen of the PEG. An interaction of this type could potentially cause the ligand to align parallel to the PEG polymer chain or even create a weak bond (temporary or

permanent) with an opposing PEG chain and thus influence the mesh structure. Such bond is more likely to be created at solution pH close to that of the ligand pI. Therefore, we examined whether any trends existed between Q_M and ligand pI (Figure 4b). Again, we did not observe a clear trend in the data but in general the ligands with the highest pI, YIGSR and YIGSRPD, were the ones that had the greatest effect on Q_M versus gels without ligand (see also Figure 3b). The YIGSR ligand with the largest influence (20% lower Q_M than hydrogels with no ligand) had the highest pI of 8.9, which is the closest to the pH of the hydrogel solution during cross-linking (pH 8.2). Therefore, it is possible that some ligand types (in our case YIGSR and YIGSRPD) bonded via weaker ionic or Van der Waals interactions with the 4-arm PEG-VS after they had been covalently incorporated into the PEG network (via the unpaired cysteine residue). It is also possible that these interactions resembled the manner of a cross-linker, connecting (permanently or temporarily) opposing PEG chains or creating folds within a single PEG chain. Such an interaction would not only result in a disrupted non-homogeneous mesh but would also lead to a smaller mesh since the molecular weight of the ligand (1000 Da) is 3-fold smaller than that of the cross-linker used (3400 Da).

Because Q_M is a measure of the hydrophilicity of the network environment, it is possible that the addition of ligand introduced local areas of hydrophobicity to the otherwise fully hydrophilic polymer. To explore this hypothesis, we specifically examined the hydrophobicity index of each ligand type (Figure 4c). We did not observe a clear trend in the data. In fact, it appeared that the ligand with highest hydrophobicity index, RGDS, did not exhibit a significant influence on Q_M versus hydrogels without ligand. Therefore, change in the hydrophobicity of the hydrogel environment alone could not explain the observed results.

To explore other possible effects of ligand type on hydrogel properties, we also determined hydrogel ξ and G' . The concept of Q_M is closely related to ξ and these two hydrogel properties are proportional. Figure 5a shows the effect of ligand type on ξ of the hydrogel, and as expected, the data followed the same trends observed in Figure 3b. As discussed earlier, addition of PEG-SH to the hydrogel occupied sites that otherwise would be available for cross-linking thus leading to an increase in ξ . On the other hand, ligands also occupied sites available for cross-linking but interactions with the PEG chains either counter-balanced that effect or lead to a decrease in the overall ξ of the hydrogel.

Another important scaffold property that could be influenced by the addition of ligand is G' . Figure 5b shows the influence of the ligand type on PEG hydrogel G' . Based on the data presented in Figure 3b and 5a we expected that the addition of the control PEG-SH would lead to an increase in G' but we did not see a significant difference in G' between the hydrogels made without ligand and hydrogels made with PEG-SH. This result could be explained with the fact that even though PEG-SH occupied cross-linking sites, the overall polymer density was not altered. As with Q_M and ξ , addition of RGDS and IKVAV also did not have a significant effect on G' but the influence of YIGSRPD and YIGSR ligands in this case was most pronounced and resulted in 10% and 19% increases in G' , respectively. This outcome confirmed our initial observation that some inherent property of the YIGSR and YIGSRPD ligands may be responsible for altering PEG hydrogel properties.

3.3. Effect of ligand type and pH on material properties: focus on YIGSR

To further explore the concept that the difference in material properties observed upon addition of ligand is inherently linked to ligand type, we examined the two ligands that had the most effect on Q_M , namely YIGSR and YIGSRPD. The unique amino acids that they have in common but do not share with the other ligands were Y (tyrosine; polar, uncharged and contains a benzene ring) and I (isoleucine; non-polar and hydrophobic). Y is also unique

in that it contains a phenolic OH group [50]. The pKa of the Y side chain is 10.1 [50]. Under the current experimental conditions, the side chain would be protonated and therefore able to form a hydrogen bond with the ether oxygen of the PEG polymer (Figure 6a).

The impact of a bond between Y and the PEG network could have several outcomes. For example, this hydrogen bond could reduce polymer hydrophilicity by altering the nature of the ether oxygen of the backbone chain or more importantly reducing the mesh size of the network by connecting opposing PEG chains or creating entanglements in a single PEG chain. A hydrogen bond between the YIGSR or YIGSRPD and the PEG polymer chain could therefore explain the decrease in swelling ratio and mesh size and increase in G' upon addition of these ligands to a PEG-based hydrogel.

A pH experiment was specifically designed to test whether the interaction between the YIGSR ligand and the PEG polymer was due to hydrogen bonding between the phenolic OH group of the Y amino acid and the ether oxygen of the PEG repeat unit (Figure 6a). Figure 6b shows the effect of pH on PEG hydrogels made with or without YIGSR ligand. In the pH range of 5.6–10.5 we noted three distinct effects of YIGSR incorporation. First, in the acidic pH range (5.6 and 6.6) there was no difference in the Q_M of the hydrogels made with and without YIGSR. This finding supported our hypothesis that an acidic environment provides an abundance of protons that would compete with the phenolic OH group of the Y amino acid, occupy hydrogen bonding sites and thus decrease or completely eliminate the influence of the ligand on the bulk PEG properties. As noted previously (Figure 3a), at pH 7.4 the presence of ligand lead to a decrease in hydrogel Q_M . However, in a basic environment (pH 8.6 and 10.5), we observed a shift in the hydrogel Q_M : hydrogels made with YIGSR had higher Q_M than hydrogels without ligand. The difference between the two types of hydrogels was significant at pH 10.5, which is above the pKa of the Y side chain. This shift could be explained by the fact that the phenolic OH groups became deprotonated at basic pH and were no longer available for hydrogen bonding with the PEG polymer. With deprotonated phenolic OH groups, the YIGSR ligand behaved more like the control PEG-SH (i.e., did not interact with the PEG polymer but simply occupied cross-linking sites).

3.4. Effect of ligand concentration on material properties

All of the above data was collected at a single ligand concentration. However, in building tissue engineering scaffolds, ligands are used at a range of concentrations. For example, comparing between RGDS, YIGSR and IKVAV ligands, Gunn et. al. [37] has shown that the concentration of ligand required for optimal neurite extension on 2D PEGDA hydrogels depended on which specific ligand was incorporated. In this work, the authors had also linked the extent of neurite outgrowth to the change in mechanical properties of the hydrogels without attempting to establish a connection between the ligand concentration and the resulting hydrogel mechanical properties. In general, adhesive ligand concentration which influences neurite outgrowth independent of scaffold mechanical properties, has not been consistently controlled, nor quantitatively measured [51]. Thus, we have chosen an array of RGDS, IKVAV and YIGSR concentrations widely used in building tissue engineering scaffolds (10, 100, 300 μ M) [35] and tested the influence of ligand concentration on hydrogel materials properties. (Between the two similar sequences, YIGSR and YIGSRPD, we chose YIGSR for further testing as it showed the greatest impact on material properties.)

Figure 7a shows that the only RGDS concentration to have a significant influence on Q_M was 300 μ M, which led to a 12% increase in Q_M compared to hydrogels synthesized without ligand. On the other hand, Figures 7b and 7c indicate that addition of IKVAV and YIGSR ligand either had no effect, or in fact resulted in reduced values of Q_M . The greatest influence of IKVAV was noted at 10 μ M ligand (20% decrease in Q_M versus hydrogels

without ligand), whereas the greatest impact of YIGSR was observed at 100 μM (21% decrease). Hence, in contrast to the results presented in Figure 3b (which showed that the effect of YIGSR on Q_M was larger than that of IKVAV), we saw that both IKVAV and YIGSR had the same overall influence on the hydrogel Q_M but at different concentrations. Figure 7d shows that addition of 10 μM of the control PEG-SH did not affect the hydrogel Q_M while addition of 100 and 300 μM both lead to a 20% increase in Q_M , which is consistent with Figure 3b.

Thus, we observed that both IKVAV and YIGSR exhibited an “optimal” concentration at which their influence on the material Q_M was at its maximum, but RGDS ligand did not exhibit the same behavior within the range of ligand concentrations explored in this study. In fact, at the highest concentration (300 μM) the RGDS influence on the material properties resembled that of the PEG-SH. Based on this observation, it is possible that RGDS did not react in any significant way with the PEG hydrogel and did not change the hydrogel network environment.

Note that RGDS has the highest hydrophobicity index. Since the addition of a high quantity of the ligand resulted in an increase in Q_M rather than a decrease, we can conclude that the decrease in the overall hydrophilicity of the PEG network upon addition of ligand under these conditions was negligible and not responsible for the overall change in hydrogel properties. Additionally, the pI of the RGDS ligand (4.2, the lowest for all studied ligand types), however, was far below the pH of the hydrogel solution upon cross-linking (8.2) and after cross-linking (7.4). Therefore, it is unlikely that the RGDS ligand would interact with the PEG polymer (apart from the covalent addition of the cysteine thiol to the PEG's VS reactive group).

Figure 8 shows the influence of ligand concentration on hydrogel ξ . These data follow the same trend as measurements of Q_M (Figure 7). The addition of RGDS did not significantly influence ξ at concentrations below 300 μM (Figure 8a). Both IKVAV (at 10 μM) and YIGSR (at 100 μM) lead to ~ 3 nm decrease in ξ (Figure 8b and 8c, respectively). Addition of the control PEG-SH did not significantly alter in the studied concentration range (Figure 8d).

Figure 9 shows the influence of ligand concentration on G' . The addition of RGDS did not significantly alter G' at concentrations below 300 μM (Figure 9a). Both IKVAV (at 300 μM) and YIGSR (at 100 μM) lead to $\sim 15\%$ increase in G' (Figure 9b and 9c, respectively). Addition of the control PEG-SH did not change G' significantly in the studied concentration range (Figure 9d).

Figures 7–9 indicate that IKVAV influenced the hydrogel properties in a similar fashion as YIGSR, albeit at different concentrations. Upon considering the amino acid sequence for IKVAV, we note that the amino acids that are not shared with the other ligands are lysine (K), valine (V) and aspartic acid (A). A and V are non-polar hydrophobic amino acids, but as mentioned above, we hypothesize that the hydrophobicity of the tested ligands does not significantly alter hydrogel material properties. K is a basic amino acid with a pKa of the side chain of 10.79. Therefore, it was possible that at the pH of the hydrogel microenvironment, these fully protonated basic groups form hydrogen bonds with PEG, a phenomenon that had been observed by Azegami et. al. [52] when mixing human serum albumin with PEG at pH 8.

The other basic amino acid found in all tested ligands was R (arginine) where it was present in the flanking sequence GRCD and was also part of the active sequence for YIGSR, YIGSRPD and RGDS. However, based on its proximity to cysteine (C), the site of covalent bonding to 4-arm PEG-VS, we would expect that only the R of YIGSR and YIGSRPD

would be available for hydrogen bonding. (In the case of GRCD sequence, R was an α substituent and in the case of RGDS (GRCDRGDSPD) it was a β substituent and potentially physically restricted, i.e., not available for hydrogen bonding).

In summary, the results of the ligand types and concentrations presented in Figures 7–9 lead to the conclusion that ligand concentration must be considered when discussing the influence of ligand type on the material properties of hydrogels because a single concentration may not result in similar effects or trends in all cases.

3.5. Effect of polymer density on material properties

Figure 10 depicts the influence of YIGSR ligand on material properties examined in hydrogels of 5–20% w/v polymer density. At the lowest polymer density, the addition of YIGSR did not have an effect on Q_M (Figure 10a), ξ (Figure 10b), or G' (Figure 10c). For higher polymer concentrations (10, 15, 20% w/v), addition of ligand resulted in decreased Q_M and ξ and increased G' (though the difference in G' at 20% was not significant). Since ligand concentration was kept constant for all polymer densities, and in the range of 10–20% w/v polymer, no other trends were noted, these results ruled out the possibility that the ratio of ligand to polymer concentration had a significant influence. Moreover, if the influence of the ligand on hydrogel properties was due to consumption of cross-linking sites, the extent of this effect would have been inversely proportional to polymer density; this result was generally not observed. The lack of influence of ligand on the properties of the 5% w/v hydrogels could be explained by the very large Q_M of the hydrogel at this polymer density. With high swelling, it is possible that weak bonds (such as hydrogen bonds) were insufficiently strong and temporary in effect; thus, the overall contribution of entanglements resulting from such bonds could be negligible.

3.6. Effect of ligand on bulk diffusion in the PEG hydrogels

Figure 11 shows the effect of ligand on BSA diffusivity in the PEG hydrogels. All ligand types resulted in decreased diffusivity by ~30%, whereas addition of the control PEG-SH decreased diffusivity of BSA by ~60%. The charge and hydrophobicity of the ligand amino acids likely interact with BSA and obstruct its diffusivity in the otherwise inert hydrogel. However, the discussion above may suggest another ligand-specific mechanism that could restrict the diffusion of soluble molecules in the PEG hydrogels. The ~2-fold decrease in BSA diffusivity in hydrogels made with PEG-SH as compared to hydrogels made with peptide ligands could be explained by the fact that covalently-bound PEG-SH did not interact with the PEG polymer chain. Instead, it may act as an additional free chain rotating in the mesh of the hydrogel, thus obstructing the diffusion of BSA inside the hydrogel. The peptide ligands on the other hand, may interact with PEG polymer chains as described above. In this way, the net effect of ligands in the hydrogel was a reduced obstacle to the diffusion of BSA inside the mesh.

4. Conclusions

The peptide ligands studied here, namely RGDS, IKVAV, YIGSR and YIGSRPD are recognized as adhesive sequences derived from larger proteins that promote integrin-mediated cell adhesion and therefore are routinely used for adding biological activity to inert synthetic materials. In general, their influence on the cell behavior is typically assessed without note as to their influence on the material properties when the ligands are added to hydrogel scaffolds.

This work determined the influence of ligands covalently bound to PEG hydrogels on the materials' physical, mechanical and transport properties. We demonstrated that the ligands

affect the Q_M , ξ , and G' of the PEG hydrogel, as well as BSA diffusivity through the hydrogel and the extent of this effect was specific to the ligand type and concentration. The YIGSR and the YIGSRPD ligands had the most pronounced effect on the material properties due to hydrogen bonding between the phenolic OH group of the Y and the ether oxygen of the PEG polymer as confirmed by additional testing of hydrogel properties at various pH. Generally, the addition of YIGSR, YIGSRPD and IKVAV ligands lead to a decrease in Q_M and ξ as well as an increase in G' , while addition of a control monofunctional PEG-SH and RGDS ligand had the opposite effect on the material properties. The addition of all ligands as well as the PEG-SH control led to a decrease in BSA diffusivity. The concentrations of RGDS, IKVAV, YIGSR and PEG-SH at which the greatest change in properties was observed were 300 μM , 10 μM , 100 μM and $>100 \mu\text{M}$, respectively; this finding emphasizes the specificity of the ligand/polymer interaction. Polymer densities wherein incorporation of ligands affected hydrogel material properties occurred at 10 and 20% w/v, but not at 5% w/v possibly due to large Q_M at this lowest polymer density.

This work provides specific evidence for interactions between peptide ligands and cross-linked PEG hydrogels that significantly impact hydrogel material and transport properties. Therefore, this work may have important implications for interpreting the results of cell experiments carried out with ligand-modified hydrogels because the addition of ligand may affect not only the scaffold's biological properties, but also key physical properties of the system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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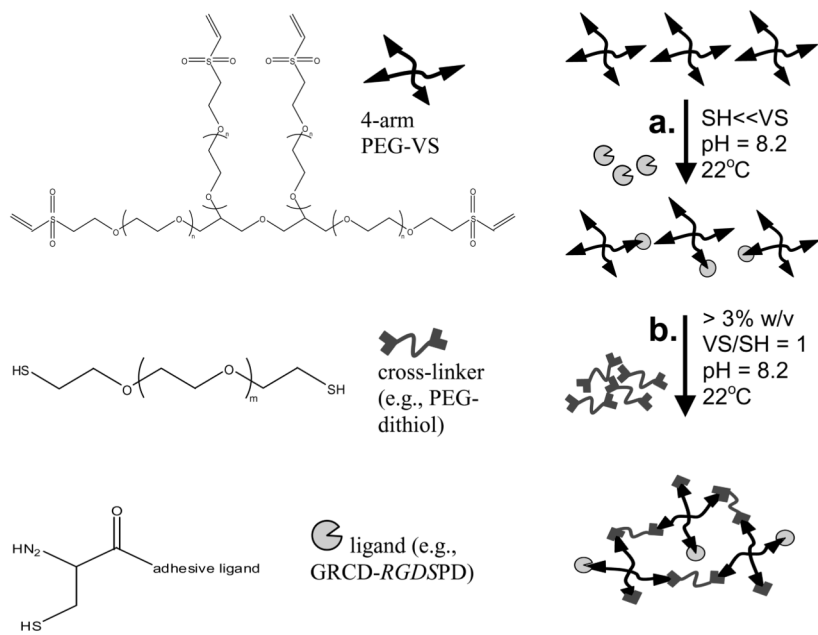


Figure 1.

Schematic of the Michael-type addition reaction to form PEG hydrogels with covalently incorporated ligands. In the first step (a), 4-arm PEG-VS is functionalized with adhesive ligands via an unpaired cysteine residue. The ligand is added at a large stoichiometric deficit to VS groups. In a second cross-linking step (b), a PEG-dithiol cross-linker is added such that total VS/SH = 1:1 to form a 3D hydrogel network.

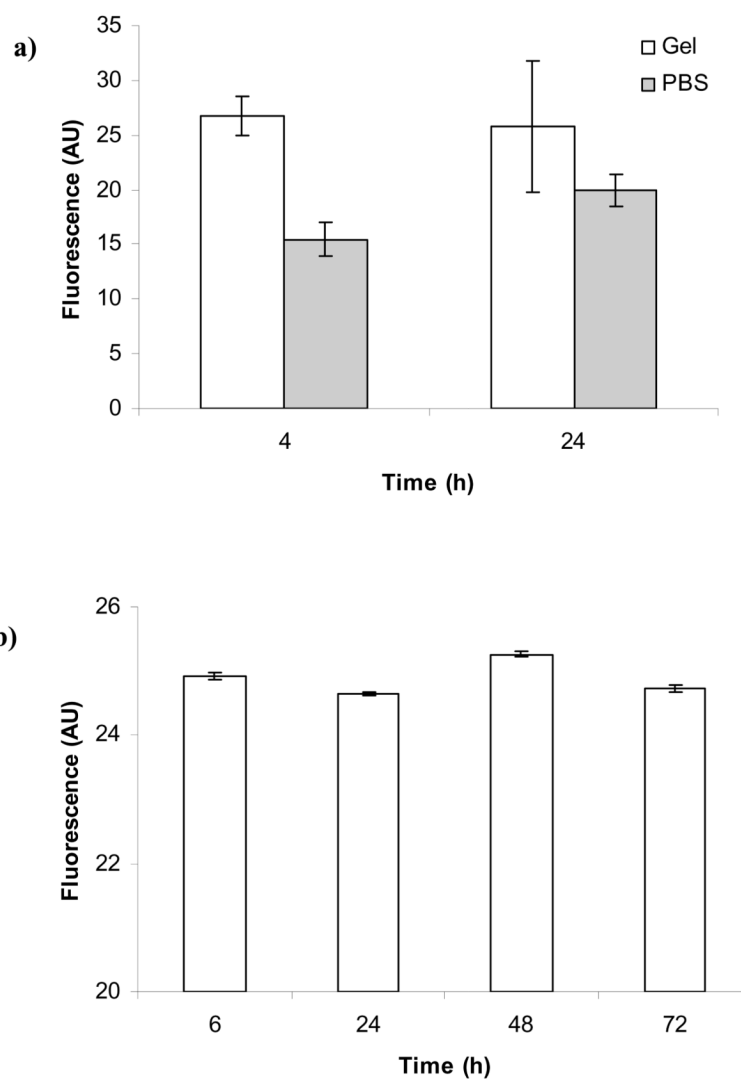


Figure 2. PEG hydrogel with covalently incorporated fluorescent ligand was used to determine that unbound ligand: a) was released in the supernatant PBS initially prior to 4 h, but b) was not released from the hydrogel over time (i.e., differences between sample fluorescence at time points up to 72 h are not statistically distinct).

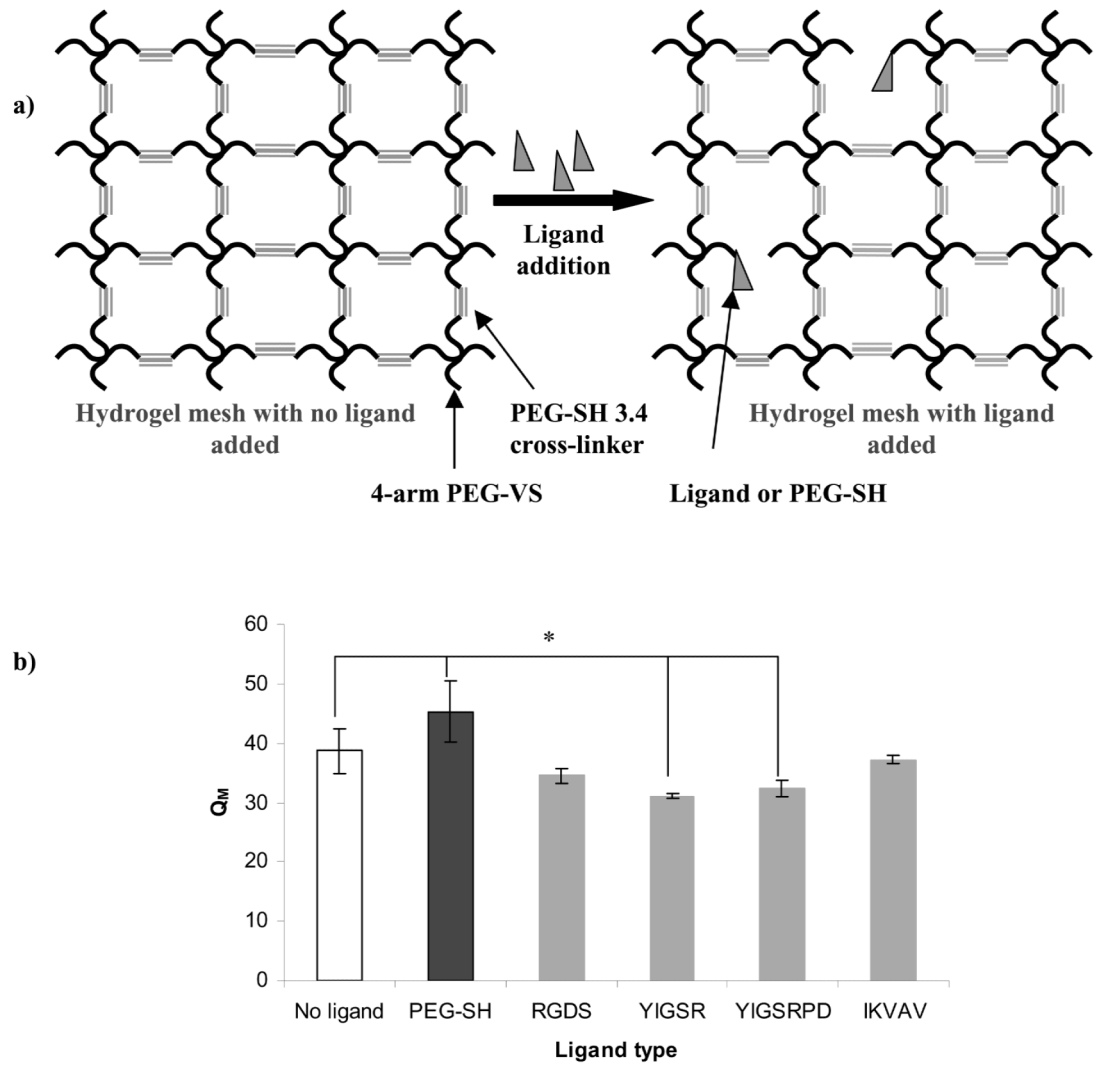


Figure 3.

a) Schematic representation of hydrogel mesh disruption upon covalent binding of ligand or PEG-SH to the 4-arm PEG-VS; b) Influence of ligand type on PEG hydrogel swelling ratio (Q_M). All hydrogels were prepared as 10% w/v polymer with 100 μ M ligand. Asterisks designate significant differences.

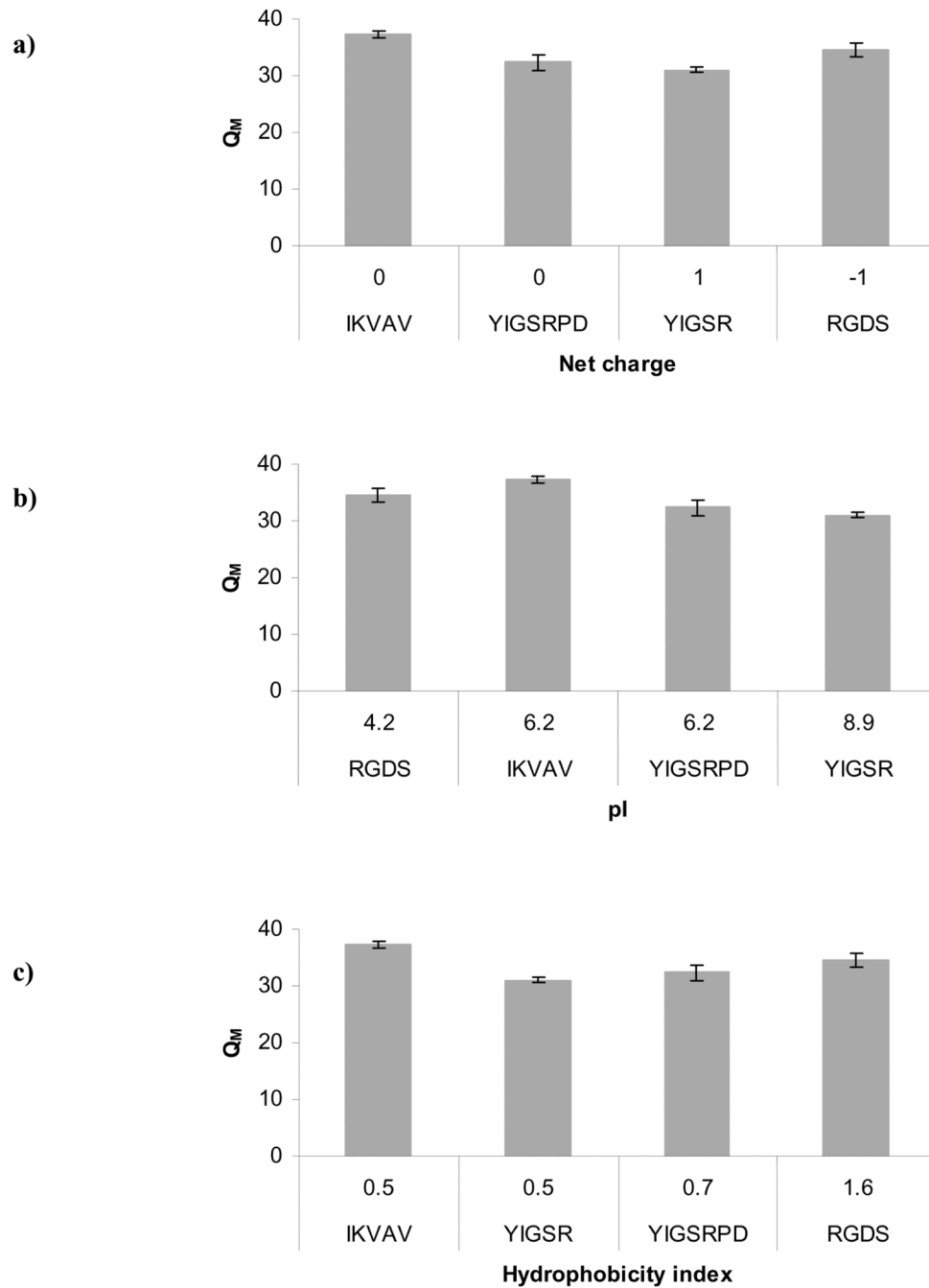


Figure 4. Influence of ligand type on PEG hydrogel swelling ratio (Q_M). Each plot highlights a different ligand property: a) net charge; b) hydrophobicity index; c) pI. All hydrogels were prepared as 10% w/v polymer with 100 μ M ligand.

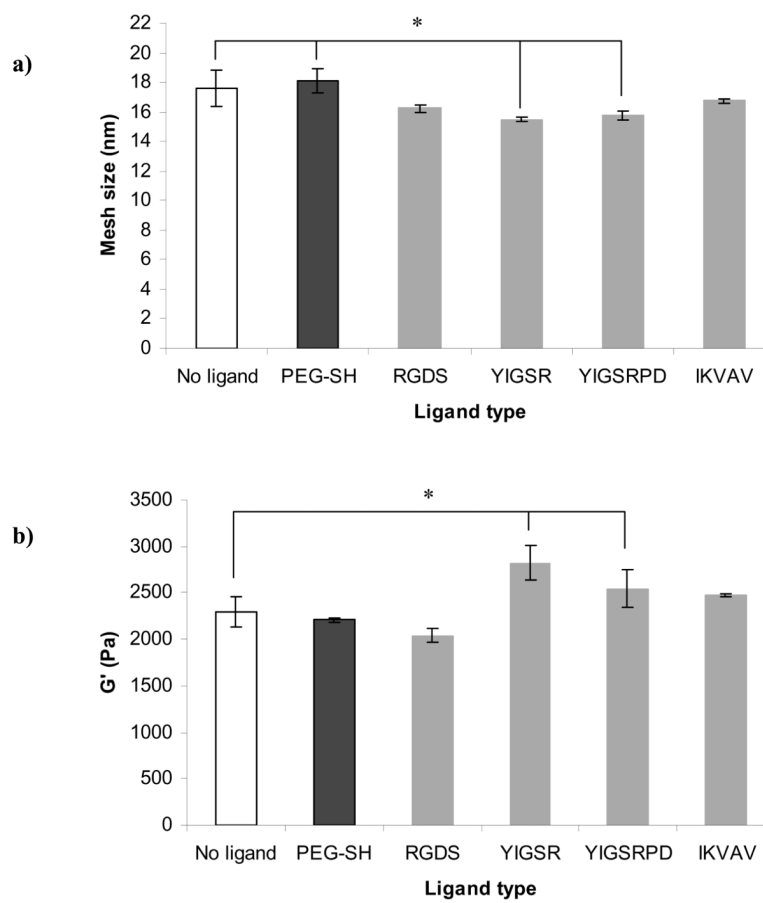


Figure 5. a) Influence of ligand type on PEG hydrogel mesh size; b) Influence of ligand type on PEG hydrogel storage modulus (G'). All hydrogels were prepared as 10% w/v polymer with 100 μM ligand. Asterisks designate significant differences.

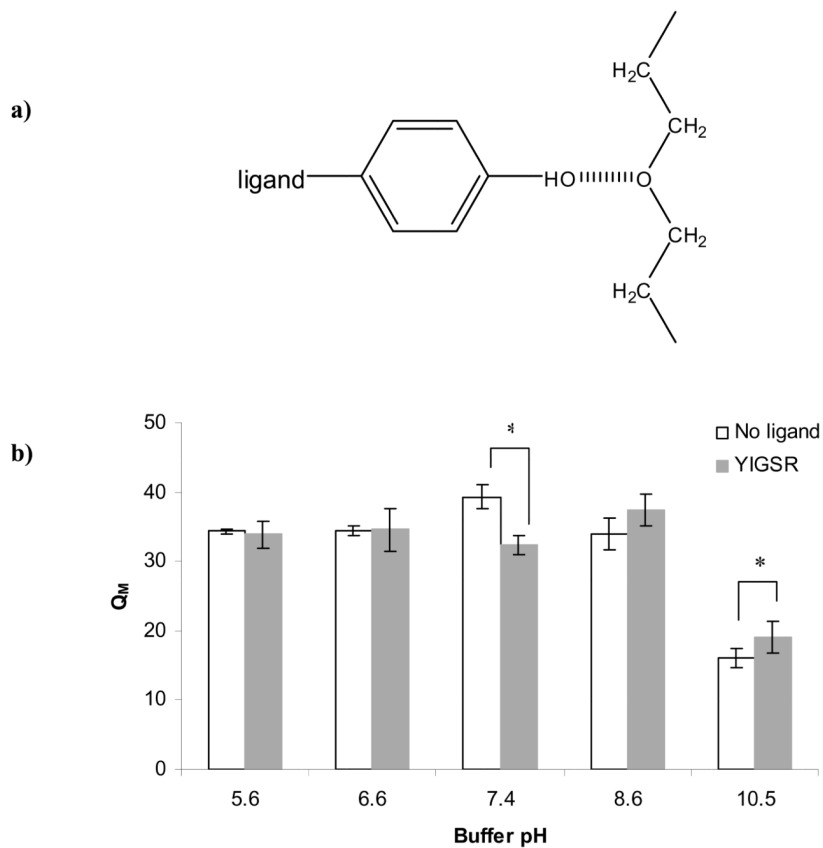


Figure 6.

a) Schematic representation of hydrogen bonding between the phenolic OH group of the tyrosine (Y) amino acid of the YIGSR and YIGSRPD ligands and the ether oxygen of the PEG polymer; b) Influence of buffer pH on the swelling ratios of PEG hydrogels containing no ligand or 100 μ M YIGSR. All hydrogels were 10% w/v. Asterisks designate significant differences.

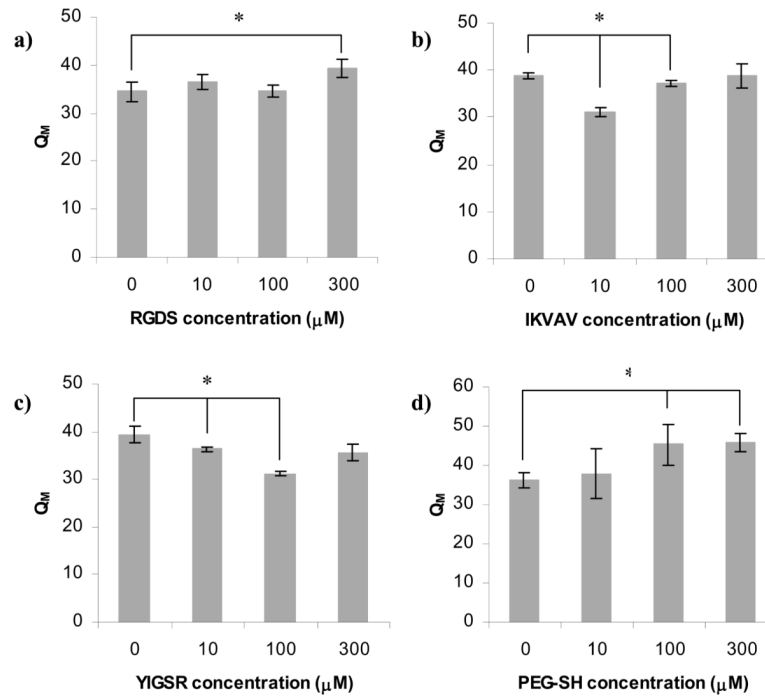


Figure 7. Influence of ligand concentration on swelling ratio (Q_M) of PEG hydrogels: a) RGDS ligand; b) IKVAV ligand; c) YIGSR ligand; d) PEG-SH control. All hydrogels were prepared as 10% w/v polymer. Asterisks designate significant differences.

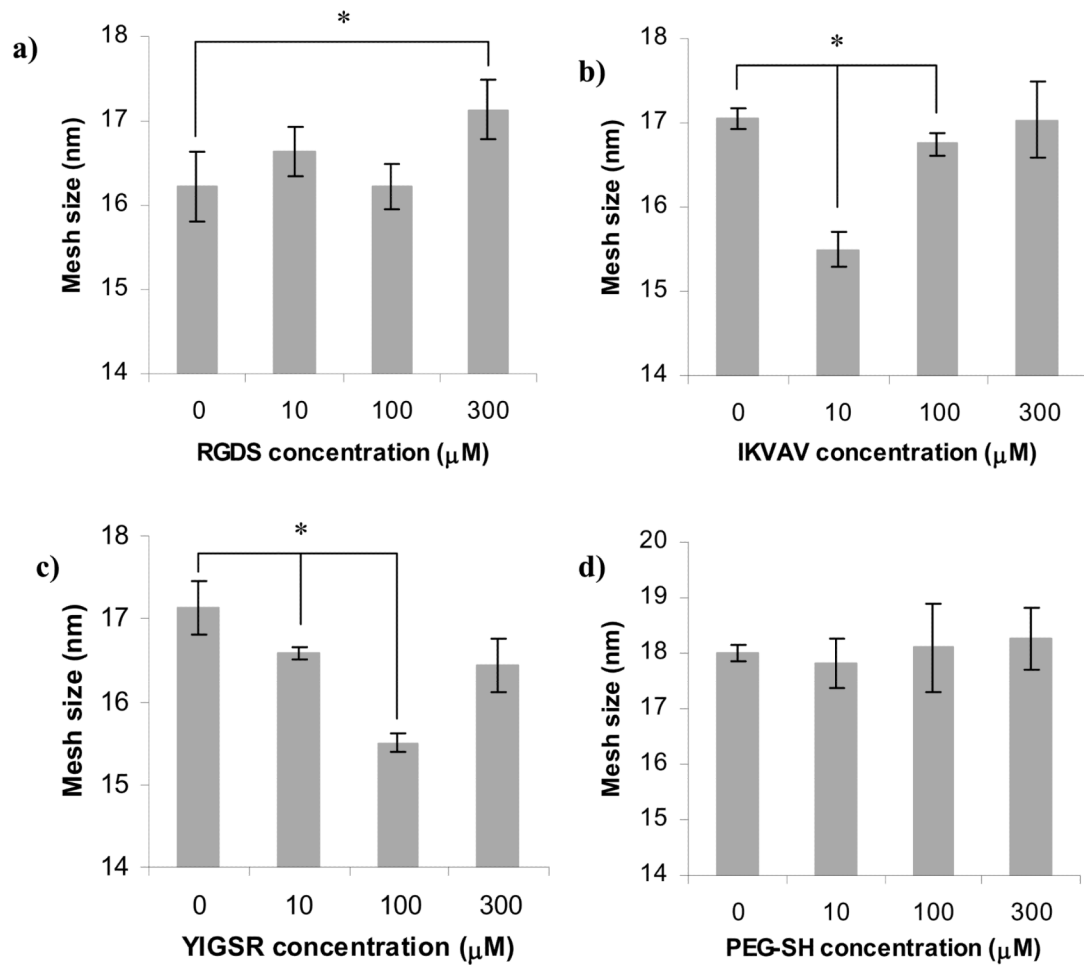


Figure 8. Influence of ligand concentration on mesh size of PEG hydrogels: a) RGDS ligand; b) IKVAV ligand; c) YIGSR ligand; d) PEG-SH control. All hydrogels were prepared as 10% w/v polymer. Asterisks designate significant differences.

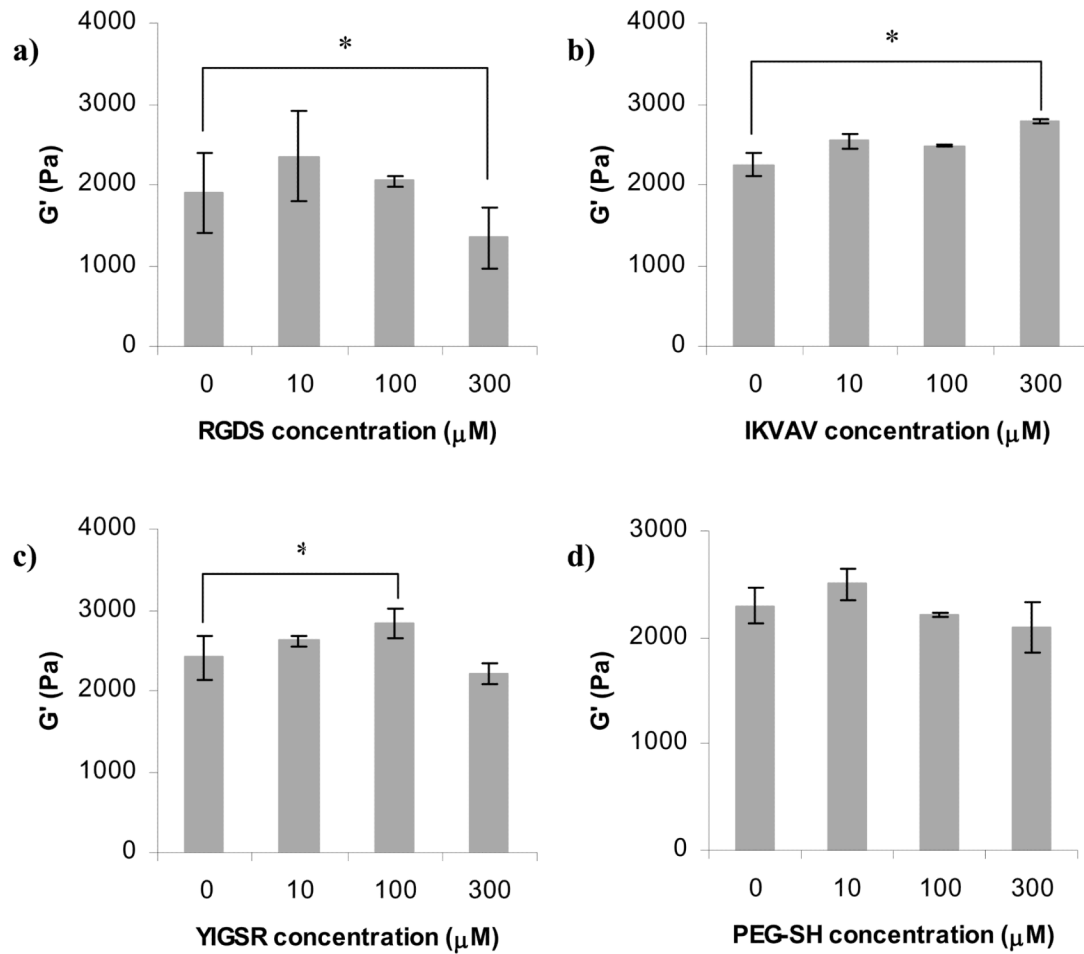


Figure 9. Influence of ligand concentration on storage modulus (G') of PEG hydrogels: a) RGDS ligand; b) IKVAV ligand; c) YIGSR ligand; d) PEG-SH control. All hydrogels were prepared as 10% w/v polymer. Asterisks designate significant differences.

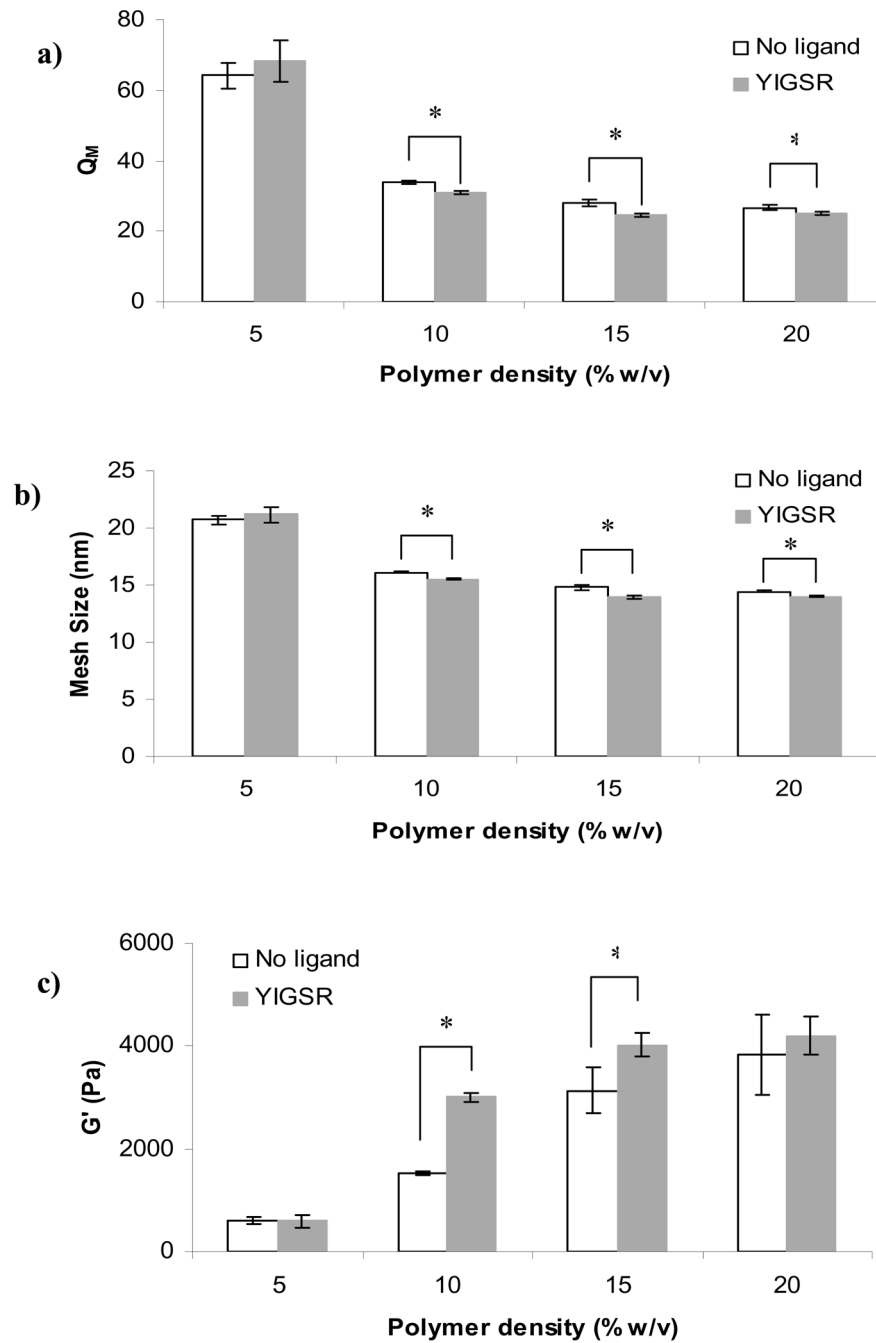


Figure 10. Comparison between PEG hydrogels with varying polymer density prepared with 100 μ M YIGSR ligand or no ligand and the effects on: a) swelling ratio, b) mesh size, c) and storage modulus. Asterisks designate significant differences.

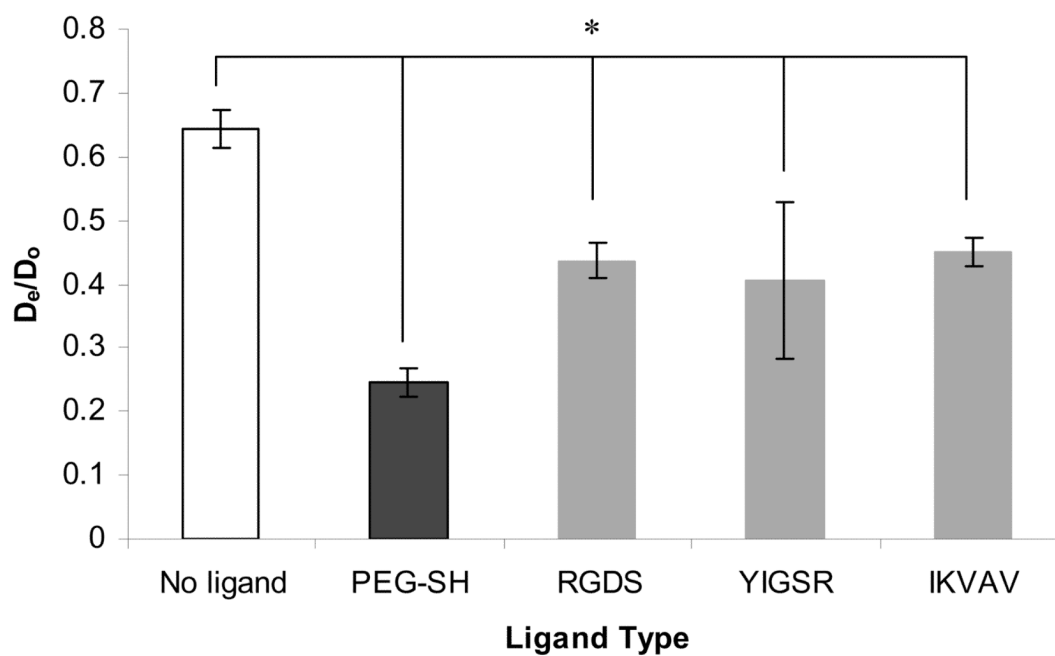


Figure 11. Effect of ligand type on BSA diffusivity (D_e) in PEG hydrogels. All hydrogels were prepared as 10% w/v polymer with 100 μ M ligand. Asterisks designate significant differences. The BSA diffusivity in the hydrogel is normalized by that of BSA diffusivity in water ($D_0 = 0.941 \times 10^{-5}$ mm²/s).

Table 1

Properties of adhesive ligands and PEG-SH control.

Peptide Sequence	Net Charge	pI	Hydrophobicity	Molecular Weight, Da	Abbreviation
GRCD- <i>YIGSR</i>	+1	8.9	0.5	1026	YIGSR
GRCD- <i>RGDS</i> -PD	-1	4.2	1.6	1077	RGDS
GRCD- <i>YIGSR</i> -PD	0	6.2	0.7	1238	YIGSRPD
GRCD- <i>IKVAV</i> -PD	0	6.2	0.5	1172	IKVAV
PEG-SH	0	N/A	N/A	1000	PEG-SH