Poly-epsilon-lysine hydrogels with dynamic crosslinking facilitates cell proliferation

1 *Article*

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4 **Nestor Lopez Mora,[1,] * Matthew Owens,[1] Sara Schmidt,[1] Andreia F. Silva,[2] and Mark Bradley.[1,] ***

5 [1] EaStCHEM School of Chemistry, The University of Edinburgh, Edinburgh EH9 3FJ, UK.
6 [2] School of Physics and Astronomy, The University of Edinburgh, Edinburgh EH9 3FD, UK.
7 * Correspondence: nesfab@gmail.com (N.L.M.); mark.bradley@ed.ac.uk (M.B.); Tel.: +44-0131-650-4820 (M.B.).

9 **Abstract:** The extracellular matrix (ECM) is a three-dimensional network within which fundamental
10 cell processes such as cell attachment, proliferation, and differentiation occur driven by its inherent
11 biological and structural cues. Hydrogels have been used as biomaterials as they possess many of
12 the ECM characteristics that control cellular processes. However, the permanent crosslinking often
13 found in hydrogels fails to recapitulate the dynamic nature of the natural ECM. This not only
14 hinders natural cellular migration but must also limit cellular expansion and growth. Moreover,
15 there is an increased interest in the use of new biopolymers to create biomimetic materials that can
16 be used for biomedical applications. Here we report on the natural polymer poly-ε-lysine in forming
17 dynamic hydrogels *via* reversible imine bond formation, with cell attachment promoted by RGD
18 incorporation. Together, the mechanical properties, and cell behavior on the dynamic hydrogels
19 with low poly-ε-lysine quantities indicated good cell viability, and high metabolic activity.

21

22 1. Introduction

23 Hydrogels are highly hydrated three-dimensional (3D) polymer networks that have been used
24 for a broad range of biomedical applications that range from tissue engineering [1,2], and surgical
25 glues [3,4], to contact lenses [5,6], materials for drug delivery[7], and biosensors [8]. In large part this
26 is due to their tunable mechanical properties and their biocompatibility [9]. The high water content
27 of these 3D macromolecular networks and the ability to decorate them with ligands creates an ideal
28 environment for diffusion and transport of nutrients, while offering optimal characteristics for
29 generating a 3D cell culture matrix. However, the high degree of crosslinking in hydrogels, necessary
30 to provide stability and structural support to cells, creates a static polymer network that hinders cell
31 migration, a critical feature of the dynamic environment of the natural extracellular matrix (ECM)
32 [10]. Cell migration has been achieved by the introduction of hydrogel degradability with, for
33 example, the incorporation of hydrolytically degradable polylactide segments [11,12], or substrates
34 of degradative enzymes such as metalloproteases [13,14], but in these cases the material is
35 permanently broken down leading to material collapse over time. Recently, using dynamic covalent
36 chemistry [15], hydrogel networks with exchangeable, reversible, or adaptable linkages have been
37 formed through Diels-Alder [16], hydrazine [17], Schiff's base (imine) [18], oxime [19], and disulfide
38 [20], bond formation. The dynamic bond breakage and reformation generates a rearranging
39 molecular network that allows cells to move and spread throughout the 3D polymer network [21],
40 opening a new set of potential properties such as self-healing [22,23], shape memory [24], and stimuli-
41 responsiveness [25-27], enhancing their potential use in biomedical applications.
42 ECM mimics have been successfully generated using numerous synthetic polymers, creating
43 hydrogels with unique structural and mechanical properties for use as structural cellular supports
44 [28]. In the majority of cases the desired characteristics include controllable matrix stiffnesses and cell

45 biocompatibility, resulting in optimal cell adhesion and survival [29]. Despite the advances in
46 synthetic biomaterials, there is considerable interest in using naturally occurring polymers to create
47 biomimetic materials for biomedical applications. Biopolymers previously explored include chitosan
48 [30,31], alginate [32,33], gelatine [34], and hyaluronic acid [35], which have been functionalized and
49 used as biopolymer precursors to form dynamic hydrogels. This creates a responsive structure akin
50 to the natural ECM and allows for *in situ* cell encapsulation. The hydrogel/cell association can be
51 further enhanced with the incorporation of peptides [36], proteins such as growth factors [37],
52 polysaccharides and proteoglycans [38], or synthetic analogues, thus allowing biological properties
53 such as cell attachment, proliferation, and differentiation, to be introduced and modulated. The
54 peptide motif arginine-glycine-aspartic acid (RGD) is a peptide adhesion sequence found in many
55 ECM proteins such as fibronectin, fibrinogen, vitronectin, and laminin as a specific integrin ligand
56 [39-41]. Many aspects of the RGD motif such as structure [42,43], spacing [44], and density
57 distribution [45-47], have been studied, with synergism between the biomechanical properties of the
58 polymer matrix and the RGD motif modulating the cellular adhesive response. Additionally, plasma
59 protein binding onto RGD-functionalized biomaterials further passivates the surface and promotes
60 cellular binding.
61   Poly-ε-lysine is a natural homo-poly-amino acid used as an emulsifier and preservative in the
62 food industry (with FDA certification [48]) that is nontoxic towards humans [49,50], and has been
63 demonstrated to be biocompatible [51]. Herein, the naturally occurring poly-ε-lysine, without further
64 functionalization, was exploited in the design of arrays of dynamic hydrogels that were crosslinked
65 *via* reversible Schiff-base bond formation with a 4-armed PEG-aldehydes. There are a limited number
66 of studies with hydrogels including poly-ε-lysine [52], or modified versions of poly-ε-lysine [53], to
67 form conventional chemically crosslinked hydrogels, for example *via* amide bond crosslinking. To
68 the best of our knowledge, however, no designed dynamic poly-ε-lysine hydrogels have been
69 proposed as ECM mimics [54]. This is perhaps due to the relative weakness of the reversible
70 crosslinking that produces softer hydrogels in comparison to stiff static hydrogels produced with
71 conventional chemical crosslinking. Contrary to our initial hypothesis that the high amount of amine
72 groups distributed along the poly-ε-lysine backbone would be detrimental to cell viability, high cell
73 viability was observed on the poly-ε-lysine dynamic hydrogels (HG-PεK) formulated at low ratios of
74 poly-ε-lysine. Additionally, the linear poly-ε-lysine backbone led to a suitable network with the
75 required biomaterial stiffness to afford cell structural support and proliferation. To aid cellular
76 adhesion and binding specificity, the linear peptide H-Ahx-G<u>RGD</u>SK-NH2 (referred to here as RGD)
77 with primary amines at both the amino and carboxyl termini (*via* the lysine residue) was incorporated
78 during hydrogel formulation. An RGD linear structure was selected over the cyclic analogue based
79 on a previous report that showed improved long-term cellular adhesion [55]. A range of RGD
80 concentrations was explored to determine cellular adhesion and survival on HG-PεK. The HG-PεK
81 was compared to a similar dynamic hydrogel made of the non-fouling polymer poly(ethylene glycol)
82 (HG-PEG) to isolate the RGD contribution to cell binding properties, with higher performance found
83 for HG-PεK compared to HG-PEG.

2. Materials and Methods

85   *Materials.* 4-arm PEG-OH (10,000 Da), and H2N-PEG-NH2 (2,000 Da) were purchased from
86 JenKem Technologies. Poly-ε-lysine (3,500 – 4,000 Da) was purchased from Carbosynth. All amino
87 acids, aminomethyl polystyrene resin, and the Fmoc-Rink amide linker were purchased from GL
88 Biochem (Shanghai) Ltd. or NovaBiochem. All other chemicals were purchased from Sigma Aldrich
89 or Acros and used without further purification, unless otherwise indicated. Dulbecco's Modified
90 Eagle Medium (DMEM), LIVE/DEAD Cell Imaging Kit (488/570 nm) and AlamarBlue® cell viability
91 reagent were purchased from Thermo Fisher. Phosphate-buffered saline (PBS) was purchased from
92 Sigma Aldrich. DMEM was prepared with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100
93 μg/mL streptomycin and 2 mM glutamine (referred to as complete DMEM). 4-arm PEG-aldehydes
94 with > 90% end group functionalization was synthesized following a method reported elsewhere (see
95 Figure S1 and S2 in Supplementary Materials) [56]. HeLa cells were purchased from American Type

96 Culture Collection and cultured in 25 cm² flasks (Corning) using complete DMEM, with passage
97 every 2-3 days using Trypsin-EDTA (Sigma).
98   *RGD solid-phase synthesis.* Linear H-Ahx-GRGDSK-NH$_2$ with a 6-aminohexanoic acid (Ahx)
99 spacer at the N-terminus was synthesized on amino methyl polystyrene resin, functionalized with an
100 Fmoc-Rink linker, using Fmoc/*t*Bu solid-phase synthesis. The peptide was cleaved for 3 hours in a
101 cleavage cocktail of 95% trifluoroacetic acid (TFA), 2.5% triisopropylsilane and 2.5% water with
102 constant mixing at room temperature. The peptide was precipitated from the filtrate using cold
103 diethyl ether, collected by centrifugation, and dried under vacuum. The RGD peptide was dissolved
104 in 0.1% formic acid in water and purified by reversed-phase flash chromatography (Biotage Isolera)
105 with a SNAP Ultra C18 column (Biotage). The solvents consisted of a mixture of 95% water/5%
106 acetonitrile (0.1% formic acid; solvent A) and 100% acetonitrile (solvent B). A gradient of solvent B
107 from 0 to 20% in 20 min, 20 to 95% in 5 minutes and 95% for 1 minute was used for peptide
108 purification. The appropriate fractions were detected at λ = 214 nm, combined, concentrated, and
109 lyophilized. The peptide was characterized by analytical C18 reversed-phase HPLC (Agilent 1100
110 ChemStation, USA) with an RGD peptide purity of > 95%, and HRMS (RGD m/z calculated 730.4086,
111 m/z found 730.4127).
112   *Hydrogel formation*. Hydrogels were prepared by dynamic covalent crosslinking *via* Schiff's base
113 formation. Stock polymer solutions were prepared by dissolving the 4-arm PEG-aldehydes, poly-ε-
114 lysine or H$_2$N-PEG-NH$_2$ to complete dissolution in PBS (pH 7.4) at room temperature. The hydrogel
115 poly-ε-lysine (HG-PεK) was prepared in 500 μL batches varying the molar ratios of the 4-arm PEG-
116 aldehydes and the lysine unit (in the poly-ε-lysine) (1:2 and 1:10) with a final polymer precursor
117 weight of 10% w/v.
118   Thus, the HG-PεK with a molar ratio of 1:2 was prepared by mixing stock solutions of 4-arm
119 PEG-aldehydes (250 μL, 20% w/v) with poly-ε-lysine (100 μL, 5% w/v) and 150 μL PBS. The HG-PεK
120 (molar ratio 1:10) was prepared by mixing stock solutions of the 4-arm PEG-aldehydes (250 μL, 20%
121 w/v) with poly-ε-lysine (130 μL, 20% w/v) and 120 μL PBS.
122   In a similar manner a non-fouling hydrogel was prepared by mixing 4-arm PEG-aldehydes (250
123 μL 20% w/v) with H$_2$N-PEG-NH$_2$ (200 μL, 20% w/v) and 50 μL PBS. Following the same procedures,
124 hydrogel constructs incorporating RGD were prepared at final concentrations of 0, 0.2, 2, 4, 6 and 20
125 mM RGD with the addition of the stock RGD solution (100 mM in PBS) (see Table S1-S3 in
126 Supplementary Materials). The pH of the final hydrogel solutions was measured immediately after
127 mixing the precursor stock solutions with pH paper and before gelation. Gelation occurred within 2
128 to 6 hours at room temperature.
129   *Rheological characterization*. Small amplitude oscillatory shear (SAOS) measurements were
130 performed in duplicate using a strain-controlled ARES-G2 Rheometer (TA Instruments). A
131 sandblasted plate-plate geometry (40 mm, stainless steel) with a gap of 300 μm was used. Hydrogel
132 samples of 500 μL were measured. Time sweeps were performed at an angular frequency of 1 Hz and
133 constant strain of 1% at 37 °C. Amplitude sweep experiments were performed for strains (γ) between
134 0.01 and 100% at constant frequency (ω = 10 rad/s), and it was found that for a strain of 1% both
135 moduli were in the linear viscoelastic region (LVE). Frequency sweeps were performed with a
136 constant strain of 1% for angular frequencies between 0.1 and 100 rad/s at 37 °C.
137   *Cryo-Scanning Electron Microscopy (cryo-SEM)*. The internal structure of the hydrogel constructs
138 was imaged in a Gemini 2 FIB-cryo-SEM (Zeiss). The hydrogel construct was placed in the sample
139 holder and frozen with liquid nitrogen. The hydrogel was then freeze fractured with a scraper,
140 sputtered with platinum, and imaged at 3.0 kV.
141   *Cell culture conditions*. A 96-well plate was passivated with a stock solution of poly-L-lysine
142 (0.01% w/v), incubated for 10 minutes, drained, and dried overnight at room temperature. Hydrogel
143 arrays of HG-PεK and HG-PEG with RGD concentrations of 0, 0.2, 2, 4, 6 and 20 mM were made by
144 transferring 50 μL of each hydrogel construct into the previously functionalized 96-well plate or 10
145 μL on a Ibidi μ-Slide Angiogenesis well plate (without surface passivation). After hydrogel
146 formation, the 96-well plate or Ibidi μ-Slide Angiogenesis well plate holding the hydrogel array was
147 sterilized by UV light for 60 minutes, before rinsing the chambers with complete DMEM. HeLa cells

148   were seeded at a density of 2×10^4 cells per well in a 96-well plate or a density of $2.7x10^3$ HeLa cells
149   per well in the Ibidi µ-Slide Angiogenesis well plate, and incubated at 37 °C, 5% CO_2. Cell culture
150   was maintained with complete DMEM exchanges every 48 hours.
151   *Live/Dead cell viability* analysis was performed using calcein AM for a live stain ($\lambda_{ex}/\lambda_{em}$ = 488/515
152   nm) and propidium iodide as a dead stain ($\lambda_{ex}/\lambda_{em}$ = 570/602 nm) after 48 hours of *in vitro* cell culture
153   in HG-PεK or HG-PEG with and without RGD. Fluorescence imaging was performed on a Zeiss
154   AxioVert 200m fluorescence microscope for the 96-well plate hydrogel and laser scanning confocal
155   microscopy on a Leica TCS SP8 confocal (CALM facilities, Queen's Medical Research Institute,
156   Edinburgh, UK) for the Ibidi µ-Slide Angiogenesis well plate and analyzed using the software
157   ImageJ.
158   *AlamarBlue proliferation assays* were performed in 96-well plates holding the hydrogel construct
159   array with measurement of AlamarBlue fluorescence after HeLa cell culture. HeLa cells were seeded
160   in HG-PεK and HG-PEG with and without RGD at a cell density of 2.0×10^4 cells per well and
161   incubated with complete DMEM for 48 hours at 37 °C and 5% CO_2. Subsequently, DMEM was
162   removed and AlamarBlue (10% v/v, 200 µL) in medium was added. An immediate baseline reading
163   of fluorescence was taken at 590 nm using a BioTek HT Synergy multimode reader with Gen 5.2.06.10
164   software. The cell seeded gels were then incubated for 5 hours and remeasured for fluorescence, with
165   subtraction of the baseline measurement per well. Cell numbers were quantified using a calibration
166   curve displaying fluorescence as a function of cell number prepared with HeLa cells seeded in 96-
167   well tissue culture plastic plates at densities of $6.0x10^4$, $3.0x10^4$, $1.2x10^4$, $6.0x10^3$, $3.0x10^3$ and $0.6x10^3$
168   cells per well incubated at 37 °C, 5% CO_2 (Figure S3 – S5 in Supplementary Materials).
169   *Statistical analysis* was performed by one-way ANOVA followed by Bonferroni's post-test using
170   Graphpad Prism 5.0 (USA). A $p < 0.05$ was considered statistically significant. Results are presented
171   as mean ± standard deviation. Three independent biological experiments were prepared for each
172   experiment.

3. Results and discussion

174   The well-defined lengths, ready functionalization of the terminal sites, and varied architectures
175   made the multi-arm PEG architectures attractive scaffolds for the design and fabrication of a wide
176   variety of hydrogels with different crosslinking chemistries [57]. Herein, the hydrogel precursor 4-
177   armed PEG-aldehydes were prepared by nucleophilic substitution of commercially available 4-arm
178   PEG-OH with 2-bromo-1,1-diethoxyethane, followed by acid hydrolysis of the acetal to yield the
179   corresponding aliphatic 4-arm PEG-aldehydes.
180   4-arm PEG-aldehydes precursor was mixed with poly-ε-lysine at molar ratios 1:2 and 1:10,
181   resulting in reversible crosslinking *via* imine formation in PBS (see Figure 1). Hydrogels were formed
182   with concentrations of 4-armed PEG-aldehydes above 5% w/v at both molar ratios (see Figure S6 in
183   Supplementary Materials). Similarly, 4-armed PEG-aldehydes were mixed with diamino-PEG to
184   produce hydrogels at precursor concentrations above 8% w/v (see Figure S7 in Supplementary
185   Materials). Generally, a faster gel formation was observed with higher 4-armed PEG-aldehydes
186   precursor concentrations. The cell adhesive peptide motif RGD was synthesized in such a way that it
187   would be incorporated into the hydrogel network as an additional crosslinker *via* dynamic imine
188   bond formation with concentration levels between 0 and 20 mM. Gel formation was observed for all
189   RGD constructs, but slower gelation was observed for gels with higher levels of RGD (see Figure 3
190   A-C). For example, HG-PεK with a molar ratio of 1:2 with 0.2 mM RGD produced a gel after 2 hours,
191   while with 20 mM gelation took over 6 hours at room temperature.

Dynamic imine crosslinking for hydrogel formation
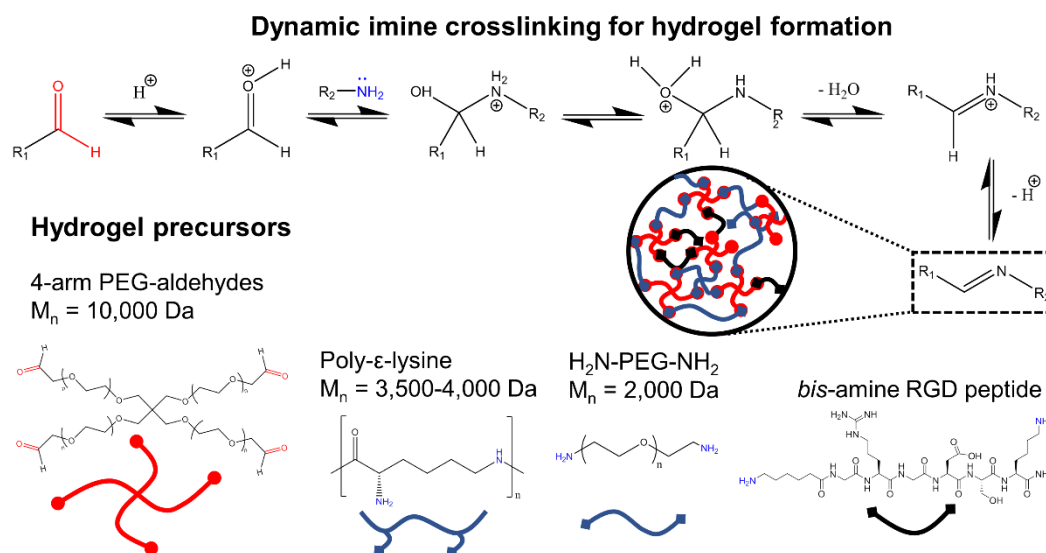
Figure 1. Hydrogels generated *via* dynamic imine crosslinking. The hydrogels were fabricated using either a 4-arm PEG each terminating in an aldehyde group and mixed with either poly-ε-lysine to give HG-PεK or a linear diamine PEG to give HG-PEG. The peptide RGD was synthesized as a *bis*-amine and was added at differing concentrations to promote cell binding.

The internal structure of the hydrogel was imaged by cryo-SEM, with the microstructure of HG-PεK and HG-PEG examined in the swollen state and compared to that of hydrogels containing the cell adhesive peptide RGD at 4 mM. Overall, all the cryo-dried hydrogels displayed a honeycomb-like 3D network structure with some notable differences in pore size. Smaller pores were observed for HG-PεK in comparison to HG-PEG for constructs with a molar ratio 1:2 (Figure 2A and 2C). The addition of RGD had no visible effect on the hydrogel microstructure of either HG-PεK or HG-PEG (Figure 2D and 2F). Increasing the PεK molar ratio to 1:10 in HG-PεK (Figure 2B), resulted in larger pore sizes than that of HG-PεK with a molar ratio of 1:2. Here, the addition of RGD had a clear effect on the microstructure of HG-PεK molar ratio 1:10, yielding a more compact microstructure with smaller pore sizes (Figure 2E).
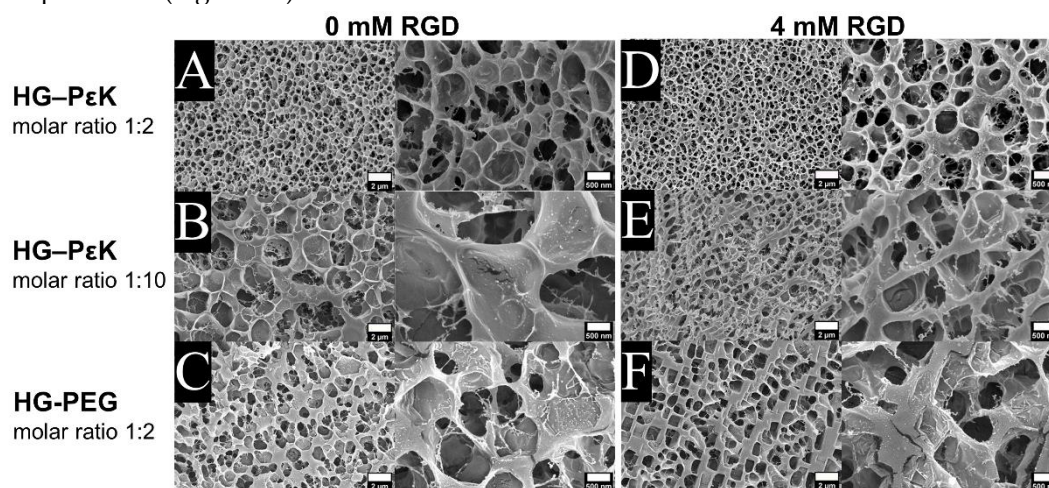
Figure 2. Hydrogel internal structures imaged by Cryo-SEM. Left panel: Hydrogel morphology without the RGD peptide for (A) HG-PεK molar ratio 1:2, (B) HG-PεK molar ratio 1:10, and (C) HG-PEG molar ratio 1:2. Right panel: Hydrogel morphology with 4 mM RGD peptide for (D) HG-PεK molar ratio 1:2, (E) HG-PεK molar ratio 1:10, and (F) HG-PEG molar ratio 1:2. For each panel the scale bars in the left micrographs are 2 μm and right micrographs are 500 nm.

Oscillatory rheology time and frequency sweeps were performed to provide insight into the gelation time and stiffness of HG-PεK and HG-PEG as a function of molar ratio and RGD

215 concentrations. HG-PεK with a molar ratio of 1:2 in the absence of RGD had a delay in gelation rate
216 compared to HG-PεK with a molar ratio of 1:10. This increase in gelation time presumably arises due
217 to fewer free poly-ε-lysine chains available during the reversible crosslinking. The sequential increase
218 of RGD concentration in HG-PεK at both molar ratios led to weaker network structures with slower
219 gelation rates. This behavior was more evident at molar ratios of 1:2 probably due to the competition
220 of amine groups from the RGD and the poly-ε-lysine chains for reversible imine bond formation
221 (Figure 3A and 3B). On the other hand, the opposite behavior was observed for the HG-PEG gels with
222 rapid gelation time upon RGD addition (Figure 3C).

223 The storage moduli was obtained from frequency sweeps with a fixed strain of 1% (within the
224 linear viscoelastic region) as a function of RGD concentration (Figure S8-S10 in Supplementary
225 Materials). RGD negligibly contributed to the storage moduli in HG-PεK with a molar ratio of 1:10
226 due to excess of poly-ε-lysine in the hydrogel formulation. In contrast, HG-PεK and HG-PEG with a
227 molar ratio of 1:2 showed distinct and opposing storage moduli trends as a function of RGD
228 concentration. While the storage moduli decreased in HG-PεK, the storage moduli in HG-PEG
229 increased with increases in RGD levels (Figure 3D). Overall the mechanical properties and cryo-SEM
230 analysis agreed with the higher storage moduli and the compact microstructure in HG-PεK with a
231 molar ratio of 1:2, compared to the lower storage moduli and larger pore sizes found in HG-PεK with
232 a molar ratio 1:10. On the other hand, the increase in the concentration of RGD led to significant
233 differences in HG-PEG storage moduli in both the absence and presence of RGD, despite similar
234 internal structure and pore size. Regardless of the opposite mechanical properties between HG-PεK
235 and HG-PEG, the constructs had the same storage moduli when formulated at 2 mM RGD, indicative
236 of similar material stiffnesses at this peptide concentration.
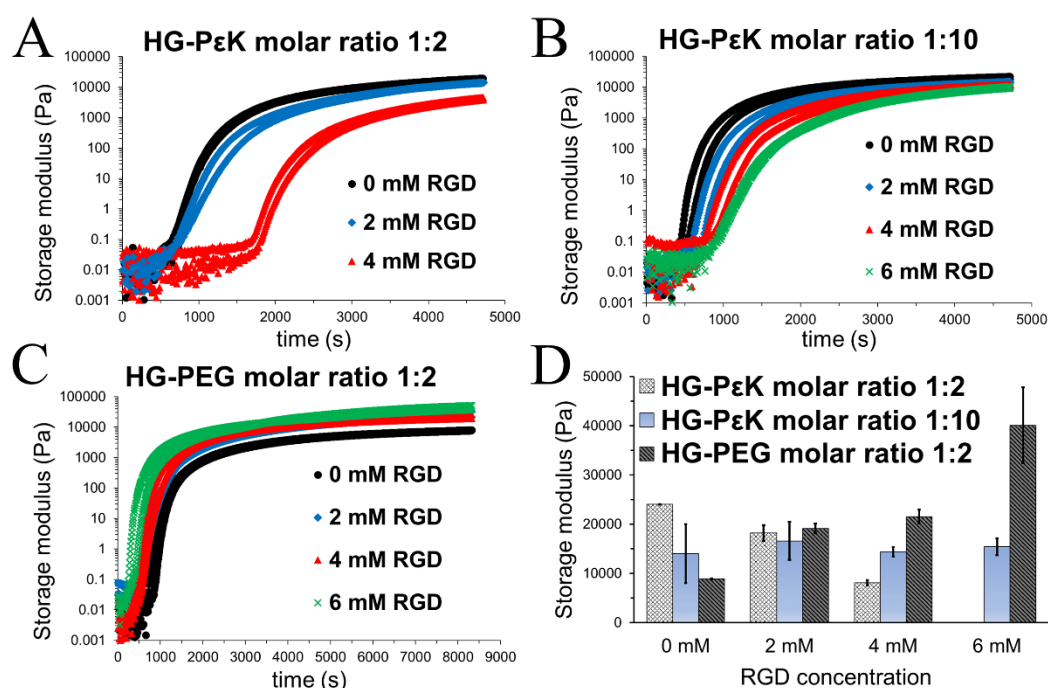

Figure 3. Hydrogel mechanical characterization by oscillatory rheology. Time sweep plots are displayed as two independent flow traces for each hydrogel construct as function of RGD concentration at 37 °C. (A) Time sweep for HG-PεK molar ratio 1:2 with 0 mM RGD (black circle), 2 mM RGD (blue diamond) and 4 mM RGD (red triangle). (B) Time sweep for HG-PεK molar ratio 1:10 with 0 mM RGD (black circle), 2 mM RGD (blue diamond), 4 mM RGD (red triangle) and 6 mM RGD (green cross). (C) Time sweep for HG-PEG molar ratio 1:2 with 0 mM RGD (black circle), 2 mM RGD (blue diamond), 4 mM RGD (red triangle) and 6 mM RGD (green cross). (D) Storage moduli as a function of RGD concentration at 37 °C. The storage moduli was obtained at 1 Hz from frequency sweeps in the linear viscoelastic region presented in Figure S8, Figure S9 and Figure S10 in Supplementary Materials. Data represents mean ± standard deviation (n=2).

248   HG-PεK and HG-PEG constructs were interrogated for their ability to allow cellular adhesion
249   and proliferation as a function of RGD concentration using confocal microscopy (Figure 4). HeLa
250   cells, a common and widely laboratory available cell system, were seeded on the preformed RGD
251   hydrogel constructs and incubated for 48 hours at 37 °C and 5% CO_2.

252   Imaging of cells on HG-PεK and HG-PEG at a 1:2 molar ratio in the absence of RGD showed
253   high cell viability with a higher number of cells observed in HG-PεK, indicative of the capability of
254   cells to interact with the hydrogel matrix. In contrast, higher numbers of dead cells were observed
255   for HG-PεK with a molar ratio of 1:10, with few cells surviving at higher concentrations of RGD. In
256   terms of cell morphology, ball-like cluster formations with high cell viability were observed in HG-
257   PεK with a molar ratio of 1:2 at all RGD concentrations, but with increasing numbers of dead cells at
258   higher RGD concentrations. Similarly, HG-PEG with a molar ratio of 1:2 gave ball-like cell cluster
259   formations with few dead cells at 0.2 mM RGD, while above this concentration string-like clusters
260   with high cell viability were observed along the strands of the hydrogel network structure, indicative
261   of the efficacy of RGD in promoting cell adhesion (Figure 4 and Figure S4 in Supplementary
262   Materials).
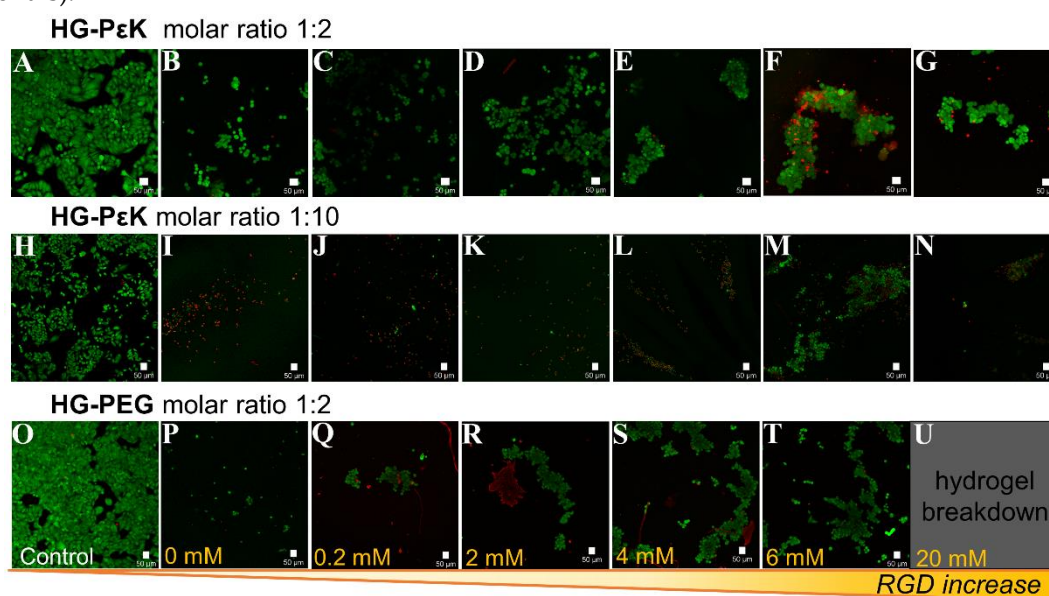
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264   **Figure 4.** Live/dead cell viability staining of HeLa cells seeded on a dynamic hydrogel array with
265   varying levels of the peptide RGD. HeLa cells were seeded and incubated with complete DMEM for
266   48 hours at 37 °C and 5% CO_2 before imaging on (A, H and O) tissue culture plate, (B) HG-PεK molar
267   ratio 1:2 with 0 mM RGD, (C) HG-PεK molar ratio 1:2 with 0.2 mM RGD, (D) HG-PεK molar ratio 1:2
268   with 2 mM RGD, (E) HG-PεK molar ratio 1:2 with 4 mM RGD, (F) HG-PεK molar ratio 1:2 with 6 mM
269   RGD, (G) HG-PεK molar ratio 1:2 with 20 mM RGD, (I) HG-PεK molar ratio 1:10 with 0 mM RGD, (J)
270   HG-PεK molar ratio 1:10 with 0.2 mM RGD, (K) HG-PεK molar ratio 1:10 with 2 mM RGD, (L) HG-
271   PεK molar ratio 1:10 with 4 mM RGD, (M) HG-PεK molar ratio 1:10 with 6 mM RGD, (N) HG-PεK
272   molar ratio 1:10 with 20 mM RGD, (P) HG-PEG molar ratio 1:2 with 0 mM RGD, (Q) HG-PEG molar
273   ratio 1:2 with 0.2 mM RGD, (R) HG-PEG molar ratio 1:2 with 2 mM RGD, (S) HG-PEG molar ratio 1:2
274   with 4 mM RGD, (T) HG-PEG molar ratio 1:2 with 6 mM RGD, and (U) HG-PEG molar ratio 1:2 with
275   20 mM RGD. Live cells are in green and dead cells are in red. Scale bars are 50 μm.

276   Additionally, HeLa cell viability/proliferation capability on the hydrogels was assessed using an
277   AlamarBlue assay (Figure 5). 2.0×10^4 HeLa cells per well were seeded on tissue culture plastic (TCP),
278   HG-PεK, and HG-PEG at a molar ratio of 1:2 with differing concentrations of RGD.

279   On TCP after 48 hours incubation time a three-fold cell increase was observed. A similar cell
280   increase was observed for HG-PεK with molar ratio 1:2 in the absence of RGD (no significant
281   difference, $p > 0.05$) while HG-PEG with a molar ratio of 1:2 (without RGD) showed the opposite
282   behavior with a three-fold decrease in cells (significantly different, $p \leq 0.0001$) compared to TCP and
283   HG-PεK in the absence of RGD. RGD levels in HG-PεK and HG-PEG resulted in significantly

284 different increases in cell number, with HG-PεK having a three-fold increase in cell number ($p \leq$
285 0.0001) at 0.2 and 2 mM RGD and a significantly different two-fold cell increase ($p \leq 0.0001$) at 4 and
286 6 mM RGD after 48 hours. No significant difference was observed between HG-PεK and HG-PEG at
287 20 mM RGD. These results were indicative of good cell viability and proliferation on HG-PεK within
288 the RGD concentration range analyzed from 0 to 6 mM. On the other hand, no cell proliferation was
289 detected on HG-PEG in line with cell viabilities reported in conventional chemically crosslinked PEG
290 hydrogels containing RGD [58].
291   Overall, hydrogels containing poly-ε-lysine were successfully formed using dynamic imine
292 crosslinking. High quantities of poly-ε-lysine in the hydrogel formulation improved the storage
293 moduli of gels and gelation time compared to low poly-ε-lysine levels (molar ratio 1:2 relative to
294 crosslinker). In contrast, low levels of poly-ε-lysine improved cell viability but a too high ratio of
295 poly-ε-lysine was detrimental to cell viability. This cytotoxicity at high levels of poly-ε-lysine (molar
296 ratio 1:10 relative to crosslinker) arises due to excesses of free amines in the hydrogel scaffold, in
297 agreement with reported cytotoxicity for polymeric biomaterials with high free amine concentrations
298 [51].
299   Oscillatory rheology and cryo-SEM characterization showed that HG-PεK with a molar ratio of
300 1:2 formed a stiffer construct compared to HG-PEG; a non-fouling hydrogel broadly used for 3D cell
301 culture. Interestingly, the incorporation of high levels of the *bis*-amine RGD peptide resulted in softer
302 HG-PεK constructs, while the expected crosslinking effect was detected in HG-PEG with increasing
303 levels of RGD leading to stiffer constructs. Despite this unexpected difference in mechanical
304 properties with the incorporation of RGD in the hydrogel formulation, HG-PεK constructs displayed
305 good cytocompatibility with higher metabolic activity/viability detected in HG-PεK with 0 and 0.2
306 mM of RGD leading to three-fold increase in cell number after 48 hours cell culture. Cell adhesion
307 and metabolic activity have been reported in chemically static poly-ε-lysine hydrogels with enhanced
308 stiffness (Young modulus of 0.11 MPa) [52]. Here, these investigations demonstrated that the
309 dynamic crosslinked HG-PεK with molar ratio of 1:2 (storage moduli of 0.02 MPa) formulated with
310 low RGD concentrations provided enough stability and structural support, like chemically static
311 poly-ε-lysine hydrogels, to afford cellular adhesion and proliferation.
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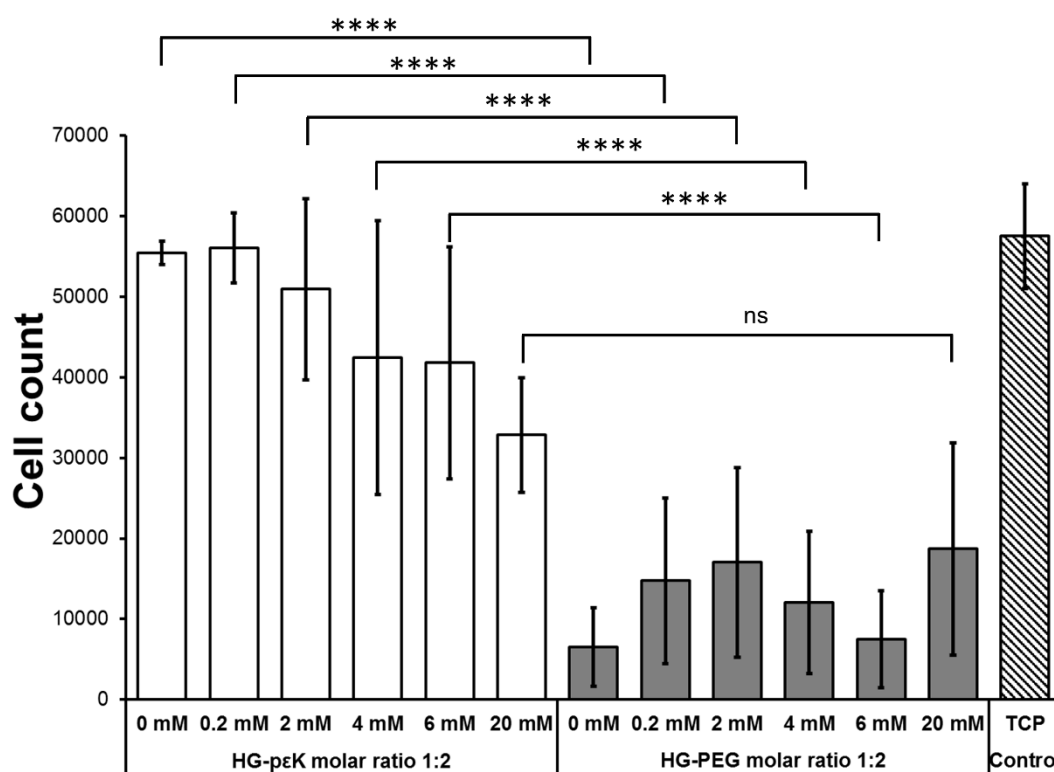
Figure 5. HeLa cell proliferation capability on HG-PεK and HG-PEG with molar ratios of 1:2 and varying levels of RGD. AlamarBlue metabolic activity assay after 48 hours cell incubation (see Figure S3, S4 and S5 in Supplementary Materials). TCP control experiment corresponds to cells seeded on tissue culture plate. Cell count on hydrogels confirmed live/dead cell viability experiments for the HG-PεK and HG-PEG gels. Cell number analysis indicated higher cell proliferation rates on HG-PεK after 48 hours compared to cells cultured on the HG-PEG constructs. One-way ANOVA with Bonferroni post-test (ns = no significant * $p \le 0.05$, ** $p \le 0.01$, *** $p \le 0.001$, and **** $p \le 0.0001$). Data represents mean ± standard deviation (n=3).

4. Conclusion

Using reversible Schiff-base bond formation, hydrogels containing poly-ε-lysine and PEG were prepared to evaluate their feasibility for cell culture. The use of poly-ε-lysine proved advantageous because of its simple formulation yielding gels *via* reversible imine formation using low quantities of material. This is in comparison to other natural polymer precursors that can require further functionalization to produce dynamic crosslinking. HG-PεK with low molar ratios of poly-ε-lysine resulted in suitable candidates for cell culture that displayed good cell adhesion and cytocompatibility. The cell binding RGD peptide was incorporated into the hydrogel network to enhance cell adhesion and biomechanical material properties. The metabolic activity of cells on the biomaterial was measured with the AlamarBlue assay, indicating higher metabolic activity in HG-PεK with low levels of RGD than in HG-PEG. In this study dynamic poly-ε-lysine hydrogels were generated with RGD but this approach is highly tunable, for example differing cell adhesive ligands such as laminin could be readily added for cell culture of other mammalian cells.

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