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3D cell entrapment in crosslinked thiolated gelatin-poly(ethylene glycol) diacrylate hydrogels

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Abstract

The combined use of natural ECM components and synthetic materials offers an attractive alternative to fabricate hydrogel-based tissue engineering scaffolds to study cell-matrix interactions in three-dimensions (3D). A facile method was developed to modify gelatin with cysteine *via* a bifunctional PEG linker, thus introducing free thiol groups to gelatin chains. A covalently crosslinked gelatin hydrogel was fabricated using thiolated gelatin and poly(ethylene glycol) diacrylate (PEGdA) *via* thiol-ene reaction. Unmodified gelatin was physically incorporated in a PEGdA-only matrix for comparison. We sought to understand the effect of crosslinking modality on hydrogel physicochemical properties and the impact on 3D cell entrapment. Compared to physically incorporated gelatin hydrogels, covalently crosslinked gelatin hydrogels displayed higher maximum weight swelling ratio (Q_{max}), higher water content, significantly lower cumulative gelatin dissolution up to 7 days, and lower gel stiffness. Furthermore, fibroblasts encapsulated within covalently crosslinked gelatin hydrogels showed extensive cytoplasmic spreading and the formation of cellular networks over 28 days. In contrast, fibroblasts encapsulated in the physically incorporated gelatin hydrogels remained spheroidal. Hence, crosslinking ECM protein with synthetic matrix creates a stable scaffold with tunable mechanical properties and with long-term cell anchorage points, thus supporting cell attachment and growth in the 3D environment.

Keywords

ECM protein; crosslinking modality; gelatin; PEG; 3D cell entrapment

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1. Introduction

Tissue engineering has emerged as a viable alternative in improving and restoring biological function of tissues and organs. One common approach involves culturing therapeutic cells in temporary three-dimensional (3D) scaffolds [1]. Studies suggest that an appropriate scaffold for encapsulating cells should not only permit cell attachment, and promote both cell growth and differentiation, but will also be biocompatible, biodegradable and mechanically strong for processing [1,2]. It is known that the process of cell division, proliferation, migration, and apoptosis relies on both the spatial and temporal organization. Hence, the 3D culture model is more relevant to the physiological condition to explore cell-cell and cell-matrix interaction. Crosslinked hydrogel networks are known to possess material characteristics such as high water content, physical properties emulating the native extracellular matrix (ECM), and diffusion-driven solute transport [3,4]. As such, hydrogels are used extensively as an effective matrix platform to encapsulate cells in 3D cell cultures [5,6]. Currently, synthetic polymer and naturally-derived matrices are the two major types of hydrogels that are extensively studied. In general, synthetic gel matrices are characterized by well controlled mechanical strength and degradation profiles. However, synthetic polymer scaffolds lack biofunctionality and may not actively interact with cells. In contrast, naturally-derived biomaterials such as collagen, fibrin, hyaluronic acid, and alginate are capable of providing potential cell interaction sites, but are proven to have less controllable mechanical properties and less predictable degradation profiles [7-10]. For instance, collagen-based gel matrix may lose mechanical strength due to cell contraction [11-13]. Immunogenicity and pathogen transmission associated with natural materials has always been a concern [14]. Additionally, degraded natural components may induce undesirable biological responses including excessive inflammatory and growth inhibitory effects. A recent finding illustrated that a collagen hydrolytic peptide proline-glycine-proline (PGP), a well-known chemotactic factor for neutrophils, has a profound inhibitory effect on keratinocyte migration and proliferation [15]. Therefore, crosslinking biomolecules with a synthetic matrix can create a stable scaffold with tunable mechanical properties, and thus has received increasing attention in biomaterial development.

Gelatin, the denatured product of collagen, is easily available, degradable, and demonstrates good biocompatibility *in vivo*. Additionally, gelatin retains cell binding motifs such as RGD and MMP-sensitive degradation sites, which is critical in successful cell encapsulation [2,16]. A semi-interpenetrating network (sIPN) has been developed as an effective drug delivery and tissue engineering scaffold which consists of photocrosslinked PEG matrices and physically entrapped gelatin. Primary human monocyte adhesion and protein expression on sIPN surfaces was found to depend on bioactive ECM peptide and integrin identities [17]. Physically incorporated gelatin underwent rapid dissolution at physiological temperature such that more than 70% gelatin loss was observed within the first 48 h *in vitro* [18]. Thus, different gelatin crosslinking modalities may result in different matrix structures and different gelatin chain relaxation behaviors at physiological temperature, which may further affect bulk physicochemical matrix properties such as those controlling swelling, degradation and viscoelasticity. Moreover, it is hypothesized that by covalently crosslinking gelatin into the PEG matrix, the resulting network should retain labile gelatin chains, provide effective cell anchorage point, and support cell attachment and growth in the 3D environment.

Various gelatin-based hydrogels had been explored for 3D cell entrapment. Photopolymerized methacrylate- and aldehyde-bifunctionalized dextran and gelatin hydrogel was developed for 3D smooth muscle cell culture in which gelatin was crosslinked *via* Schiff base reaction [19]. The observed immiscibility of gelatin and aldehyde modified dextran at high aldehyde density was most likely due to phase separation [19,20].

Photopolymerized styrenated gelatin hydrogel was used for chondrocytes encapsulation [21]. Although embedded chondrocytes maintained viability for up to 21 d, cells did not proliferate within the gel matrix. An enzyme-catalyzed gelatin crosslinking method was also developed for cell encapsulation [22]. However, the use of exogenous chemicals such as hydrogen peroxide may lead to irreversible cell damage [23]. Thus, it is necessary to develop a method to crosslink gelatin as a model for other ECM molecules without introducing harmful chemicals or decreasing gelatin solubility. Currently, two distinct reaction mechanisms have been developed for step-growth polymerization of cell instructive PEG-peptide hydrogel network, *i.e.* Michael-type addition polymerization or thiol-ene photopolymerization. Thiol-ene photopolymerization reactions can be conducted at neutral pH, and allow both spatial and temporal gelation process control [24]. In this study, a facile method has been developed to modify gelatin with cysteine by grafting homobifunctional PEG linker to the gelatin backbone thus introducing free thiol groups to gelatin. The linear PEG chains conjugated onto the gelatin backbone render gelatin chains more hydrophilic thus achieving good miscibility with bifunctional PEG crosslinkers. The thiolated gelatin chains were then photocrosslinked with PEG-diacrylate *via* thiol-ene reaction (Fig. 1). These crosslinked gelatin-based hydrogels allow easy mechanical property modulation through varying precursor concentration and ratios. Cell morphology and proliferation study demonstrated that gelatin crosslinking modality is crucial to providing long-term integrin binding sites and supporting cell attachment and proliferation in a 3D environment.

2. Materials and methods

2.1 Materials

Poly(ethylene glycol) (PEG)-diol with average molecular weight of 2000 Da and 3400 Da, gelatin (type A, 300 bloom, from porcine skin), PEG-diacrylate (PEGdA, Mw 575 Da), *N,N'*-disuccinimidyl carbonate (DSC), 4-(dimethylamino)pyridine (DMAP), *L*-cysteine (Cys), *N,N'*-diisopropylethylamine (DIPEA), acryloyl chloride, triethylamine (TEA), *dimethylformamide* (DMF), and dioxane were obtained from Sigma-Aldrich (USA) and used without further purification. Irgacure® 2959 (I-2959) was provided by Ciba (NY, USA). I-2959 is water soluble and well tolerated by most mammalian cell types over a wide concentration range [25,26]. All other chemical reagents were of analytical grade and used as received.

2.2 Synthesis of cysteine grafted gelatin (Gel-PEG-Cys)

N-hydroxysuccinimide functionalized PEG (bis-NHS-PEG) was synthesized using an established method with minor modifications (see Fig. 2) [27]. PEG-diol (Mw, 2000 Da) (5.0 g, 2.5 mmol) was dissolved in 20 mL of dry dioxane. DSC powder (6.4 g, 25 mmol) was suspended in another 20 mL of dry dioxane and added to the solution. DMAP (3.05 g, 25 mmol) was dissolved in 50 mL of acetone and added dropwise to the above solution under stirring. The reaction was kept at room temperature for 6 h under argon protection. The activated PEG products were directly precipitated in diethyl ether and dried in vacuum overnight. The raw product (~5.0 g) was dissolved in 50 mL of dichloromethane (DCM), and washed with 0.5 N HCl solution four times to remove side products. The DCM phase was further precipitated in diethyl ether and dried in vacuum (with a yield of 80%). ¹H-NMR (CDCl₃) confirmed the final product: δ2.8, s, 4Hs from succinimidyl group; δ3.65, m, -CH₂- from PEG backbone; δ4.45, t, 2Hs from -CH₂OCO-NHS. The HPLC system consisted of Model 306 pumps (Gilson) and an ELSD-LT2 detector (Shimadzu). Separations were performed on a reverse phase column (C₁₈-DVB, 500 Å, 5 μm pore size, 4.6 × 150 mm, Jordi). A gradient elution was carried out as follows: 30% (v/v) acetonitrile (ACN) was used for 0 – 5 min, then linearly increased to 70% (v/v) ACN for 5 – 15 min and maintained

for another 10 min. A single retention peak was observed, indicating the high purity of the modified PEG derivative.

Gelatin modification with *L*-cysteine *via* bis-NHS-PEG was conducted per previously described procedure (Fig. 2) [12]. Briefly, bis-NHS-PEG (0.5 g, 0.25 mmol) was dissolved in 2 mL of anhydrous DMF, *L*-cysteine (0.036 g, 0.3 mmol) was suspended in 2 mL of DMF and added dropwise to the bis-NHS-PEG solution followed by the addition of 1.2 eq of DIPEA. The reaction was kept at room temperature under argon for 40 min. The remaining NHS- on NHS-PEG-Cys was grafted with gelatin. In general, 1 eq mol of NHS-PEG-Cys was added to 1% gelatin in PBS adjusted to pH 8.0 and the reaction was stirred for 1 h at room temperature. The raw products were subjected to dialysis against ddH₂O (MWCO 6000 – 8000) for 2 d to remove unreacted PEG derivatives and other side products. The purified products were sterile-filtered through 0.22 μ m membrane filter, frozen at -80 °C and lyophilized. Gel-PEG-Cys was characterized by ¹H-NMR and gel permeation chromatography (GPC). The chromatographic system consisted of a single phase HPLC pump (Waters 1515) with a column heater and a refractive index detector (Waters 2414). Separation was accomplished using three TSK-GEL columns (Sigma-Aldrich) in series: G5000PW_{XL} (pore size = 1000 μ m, 30 cm $\times$ 7.8 mm), G3000PW_{XL} (pore size = 200 μ m, 30 cm $\times$ 7.8 mm), G2500PW_{XL} (pore size < 200 μ m, 30 cm $\times$ 7.8 mm). The system was calibrated using a series of PEO/PEG standards (232 – 932,000 Da). Samples were diluted with the eluent to about 10 mg/mL and filtered through 0.22 μ m membrane filter (Millipore, USA) before analysis. All samples were run at 0.5 mL/min at 37 °C in a mobile phase consisting of 80% (v/v) 0.1 M NaNO₃ with 20% (v/v) acetonitrile. Chromatogram analysis was performed using PeakFit software (v4.12, SeaSolve Software, Inc.) to fit Gaussian peaks based on features within the chromatogram [18,28]. The ¹H-NMR spectrum of Gel-PEG-Cys with D₂O as solvent shows: δ 1.3, d, 2Hs, -CH₂SH; δ 2.89, t, 1H, -CHCH₂SH; δ 3.65, m, -CH₂- from PEG backbone; broad peaks composed of many overlapping small peaks at 1.7, 1.75, 1.92, 3.18, 4.19, and 4.2 ppm were characteristic gelatin peaks, consistent with previous findings [17]. GPC chromatogram (Fig 3) showed a broad distribution of Gel-PEG-Cys product with retention time from 30 min – 52 min, indicating products of a wide molecular weight distribution. A retention shift of the bulk gelatin peak from 41 min in unmodified gelatin to 36 min in Gel-PEG-Cys was observed. The gelatin lysyl modification ratio was defined as the percentage of free -NH₂ from lysyl residue that has been modified, based on the average 300 bloom gelatin molecular weights and the average lysine content of the gelatin [29]. The lysyl modification ratio was evaluated *via* the trinitrobenzenesulfonic acid assay method (TNBS) to be approximately 70%, *i.e.* approximately 17 lysyl residues have been modified per each gelatin chain. The free thiol concentration was determined using Ellman's test to be (550 $\pm$ 29.5) μ M for 10 mg/mL Gel-PEG-Cys sample solution.

2.3 Hydrogel fabrication

Covalently-crosslinked gelatin hydrogels were prepared by photo-crosslinking Gel-PEG-Cys with PEGdA Mw 575 Da or 3400 Da (Fig. 1). PEGdA (Mw 3400 Da) was synthesized following a previously established method [30]. In brief, 20% w/v Gel-PEG-Cys stock solution was prepared in pH 7.4 1 $\times$ PBS at 37 °C. PEGdA was accurately weighed, and dissolved in 0.5% (w/v) I-2959 solution. Gel-PEG-Cys solution was mixed with PEGdA solution *via* vortexing to give various formulations as shown in Table 1. 100 μ L of hydrogel precursor solution was transferred to glass bottom petri dish molds (8 mm in diameter, 0.8 mm in thickness, In Vitro Scientific, USA) and subjected to photo-crosslinking with LED long-wavelength UV (λ_{max} = 365 nm, intensity at 100 mW/cm²) for 2 min (Clearstone technology Inc., USA). Hydrogels with physically entrapped gelatin were prepared using PEGdA with unmodified gelatin (type A, 300 bloom) instead of Gel-PEG-Cys as shown in Fig. 1 (Table 1). PEGdA-only hydrogel (15% w/w) was prepared using PEGdA (Mw 3400)

macromer in the same way without the addition of gelatin. Hydrogel nomenclature is defined as follows: for covalently crosslinked hydrogel, $G_{cys}XPYZ$, X is the Gel-PEG-Cys weight percentage, Y is the molecular weight of PEGdA, Z is the PEGdA weight percentage; for physically entrapped gelatin gels, $GWPYZ$, W is the gelatin weight percentage.

2.4 Hydrogel characterization: swelling, degradation and gelatin dissolution, storage and loss moduli

Covalently crosslinked gelatin-based hydrogels and physically incorporated gelatin hydrogels were synthesized (Table 1) and accurately weighed prior to the swelling study. The gel disks were then immersed in 2.5 mL of pH 7.4 1× PBS at 37 °C. At predetermined times, hydrogels were carefully removed from the petri dish, blotted with Kim-wipes® to remove excess water from the hydrogel surfaces, and then weighed. Equilibrium weight swelling ratio (Q_s) is defined as:

$$Q_s = \frac{W_{(t)} - W_{(d)}}{W_d} \quad \text{Eq 1}$$

where $W_{(t)}$ is the gel weight at time t , and W_d is the dried hydrogel weight. Moreover, 2.5 mL of swelling medium was collected at each time and this was then replaced with fresh medium. The gelatin concentration in the medium was determined *via* BCA assay (Bicinchoninic acid kit for protein, Pierce, USA). The standard curve was established using gelatin solution in 1× PBS with concentrations of 1500, 1000, 500, 250, 125, 62.5 µg/mL ($Y = 0.0003X + 0.0242$, $R^2 = 0.994$).

We measured the complex shear modulus, $G^* \equiv G' + iG''$, in small amplitude oscillatory shear with an ARES-LS2 2000ex rheometer (TA Instruments, USA) using the parallel disk geometry with a 8 mm diameter disks at 25 °C $\pm$ 0.1 °C and 37 °C $\pm$ 0.1 °C, over a frequency range of 0.1 - 10 Hz, and with a constant shear strain amplitude of 5%. The real part of the complex shear modulus is called the shear storage modulus (G'), and the imaginary part, the shear loss modulus (G'') [31]. The magnitude of the complex shear modulus is thus given by Eq 2.

$$|G^*| \equiv \sqrt{G'^2 + G''^2} \quad \text{Eq 2}$$

Hydrogel samples were prepared to yield disks of 8 mm diameter and ~0.8 mm thickness. Hydrogel samples were covered with a thin layer of silicone oil to minimize water evaporation. The complex shear modulus $|G^*|$ provided insight into the gel stiffness under dynamic conditions [32]. The viscous dissipation (often unfortunately called the “lost work”) per unit volume per cycle:

$$\int \tau_{yx} d\gamma = \pi \gamma_0^2 G'' \quad \text{Eq 3}$$

This is the cyclic integral of the shear stress response to small amplitude oscillatory shear, with respect to the shear strain. The mechanical loss angle, δ , is defined by Eq 4 that governs the viscous dissipation (see Eq. (176) in [31]; also Eq. (177) in [33]).

$$\tan \delta \equiv \frac{G''}{G'} \quad \text{Eq 4}$$

The mechanical loss angle is confined to $0 \leq \delta \leq \pi/2\text{rad}$ where 0 corresponds to the behavior of a simple rubber (purely elastic), and $\pi/2$ 2rad, to the behavior of a Newtonian liquid

(purely viscous). So the dimensionless trigonometric function, $\tan \delta$, is confined to $0 \leq \tan \delta \leq 1$, and this also reflects the type of gel behavior, and specifically, the proximity of gel behavior to the purely elastic or purely viscous extremes.

2.5 In vitro cell culture and assay

Neonatal human dermal fibroblasts (NHDF) were obtained from Lonza (NJ, USA) and cultured in 75 cm² T-flask using fibroblast basic medium-2 (FBM-2, Lonza) supplemented with 10% fetal bovine serum (FBS, Gibco, Invitrogen, USA). Fibroblasts with passage 5 - 10 were used in the following experiments. All cell cultures were maintained at 37 °C and 5% CO₂. For 2D cell adhesion assays, G0P₃₄₀₀15, G10P₃₄₀₀15, and G_{cys}10P₃₄₀₀15 hydrogel surfaces were statically seeded with fibroblasts at a concentration of 4×10^4 cells/mL. After 24 h, samples were washed twice with respective culture medium to remove non-adherent cells. Adherent cells on all surfaces were stained with Live/dead® stain (2 μM calcein AM / 4 μM EthD-1, Invitrogen, USA) and imaged under an inverted microscope (Nikon, Eclipse TE300, Japan).

To compare the effect of gelatin crosslinking modality on the behaviors of 3D encapsulated cells, G_{cys}10P₃₄₀₀15, and G10P₃₄₀₀15 were employed to entrap fibroblasts. PEGdA-only hydrogel with 15% (w/w) PEGdA 3400 served as a control (G0P₃₄₀₀15). Stock solutions of PEGdA (Mw 3400 Da), Gel-PEG-Cys, and unmodified gelatin were prepared in sterile 1× *Dulbecco's Phosphate Buffered Saline (DPBS, Fisher, USA)* containing 0.5% I-2959, respectively. Fibroblasts in culture flasks were trypsinized, centrifuged and resuspended with 1× DPBS containing 0.5% I-2959. Single cell suspension was then added to hydrogel precursor solutions with gentle agitation to yield a final concentration of 2×10^6 cell/mL. 100 μL of cell encapsulated precursor solution was transferred to glass bottom petri dish molds and subject to photopolymerization with long-wavelength UV light (100 mW/cm²) for 2 min. Cell entrapped hydrogels were then transferred to TCPS culture plates and submerged in additional culture medium. The viability of fibroblasts encapsulated in hydrogel samples was determined after 1, 7 and 28 days of incubation *via* Live/dead® stain. Three images per sample were taken at random fields of view at 10× magnification. Live and dead cell numbers were analyzed using ImageJ (NIH, USA). The *cell viability* was presented as the number fraction of live cells. Due to the plane of focus, the results are represented as a ratio of live to total cells and these results are not normalized to material area or volume. Fibroblast proliferation within 3D hydrogels was evaluated using CellTiter-Blue® kit (Promega, USA). Cell encapsulated samples were prepared as described above. On day 1 and 7, 200 μL of CellTiter-Blue® reagent was added directly to the culture medium and samples were incubated for 4 h in the incubator. After incubation, supernatant was collected and transferred to a clear 96-well plate. The fluorescence intensity ($\lambda_{\text{ex}} = 545$ nm, $\lambda_{\text{em}} = 590$ nm) was analyzed by fluorescence spectrometer (FLUOstar Omega, BMG Labtech, USA). The fluorescence intensity of chemically crosslinked gelatin gel and physically incorporated gelatin gel samples were normalized to the intensity of PEGdA-only hydrogel for further comparison. To evaluate cell morphology, fibroblasts cultured within different hydrogel disks were stained for F-actin, integrin $\alpha 5 \beta 1$ and vinculin. After 2 weeks culture in 3D environment, the cell entrapped hydrogels were fixed in 4% paraformaldehyde. Fixed cells were permeabilized with 0.25% triton X-100 (Sigma, USA) for 30 min, and nonspecific bindings were blocked with 1% BSA. The samples were next incubated with the primary antibody (mouse monoclonal anti-Integrin $\alpha 5 \beta 1$ (1:500, Sigma, USA) or mouse monoclonal anti-vinculin (1:500, Sigma, USA)) at 4 °C for 24 h respectively followed by the secondary antibody (TRITC-conjugated goat anti-mouse IgG (1:1000, Sigma, USA)) for 2 h at room temperature. Cytoskeletal F-actin fibers were stained for 1 h with 1 U/mL Alexfluor488 conjugated Phalloidin (Molecular probes, USA). After staining, samples were washed extensively with 1× PBS prior to fluorescence microscopy.

Microscopic images of cells cultured within hydrogels with F-actin/vinculin and F-actin/Integrin $\alpha 5\beta 1$ co-stain were recorded using confocal microscope (F1000, Olympus, Japan).

2.6 Statistics

All data are represented as a mean plus or minus ($\pm$) a standard deviation (S.D.) of samples in at least three independent experiments. Swelling, gelatin dissolution, linear viscoelastic behavior, and cell proliferation data were analyzed by unpaired Student-*t* test. A value of $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1 Effect of crosslinking modality on hydrogel physical properties

Both the covalently crosslinked Gel-PEG-Cys hydrogel and the physically incorporated gelatin hydrogel displayed rapid water absorption and swelling to reach maximum or near maximum weight swelling ratio (Q_{max}) within 1 – 2 h (Fig. 4). Higher PEGdA content showed lower Q_{max} , lower water content, and longer time (T_{max}) to reach Q_{max} (Table 2). PEGdA 3400 based hydrogels showed higher Q_s than PEGdA 575 crosslinked hydrogels (Fig. 4). The linear chain length of PEGdA 3400 is larger than PEGdA 575, and therefore, crosslinking Gel-PEG-Cys *via* PEGdA 3400 would be expected to result in a more relaxed polymer network after swelling. A higher degree of swelling of Gel-PEG-Cys hydrogel is most likely due to the larger mesh size in the covalently crosslinked gelatin hydrogel than PEG network. Gelatin solution is known to display thermal reversible sol-gel transition behavior resulting from the coil-to-triple helix conformation change [34]. Hence, physical gelatin gels will form at R.T. when gelatin concentration is above a critical level [35]. At R.T., unmodified gelatin (10% w/w) in solution forms a physical gel, while at 37 °C, gelatin chains undergo relaxation, becoming labile and gradually diffuse out of the PEG network. In contrast, covalently crosslinking gelatin chains helped retard gelatin dissolution. Moreover, gelatin that is covalently crosslinked with PEGdA may retain the coil-to-helix transition which affects hydrogel bulk properties such as swelling, degradation, and viscoelasticity. Physically entrapped gelatin hydrogels showed rapid gelatin dissolution profile with 30 - 50% gelatin loss for up to 24 h (Fig. 5A, 5C). In contrast, both Gel-PEG-Cys groups with PEGdA 575 and 3400 as crosslinker showed much less cumulative gelatin release from 10% to 20% at 24 h ($p < 0.05$) (Fig. 5B, 5D).

At both R.T. and 37 °C, the bulk property of covalently crosslinked hydrogels exhibited a substantial elastic response with G' significantly higher than G'' (Fig. 6). For example, $G_{cys10P340010}$ displayed frequency-independent G' values about an order of magnitude higher than its G'' values (Fig. 6). G' increased from 1409 ± 16 Pa with 10% (w/w) PEGdA to 5641 ± 173 Pa with 20% (w/w) PEGdA. G'' increased from 140 ± 41 Pa with 10% (w/w) PEGdA to 1488 ± 351 Pa with 20% (w/w) PEGdA. Similar results were observed in the physically crosslinked gelatin hydrogels, with G' increasing from 2447 ± 38 Pa to 10740 ± 171 Pa. At the same PEGdA concentration level, higher G' was observed with physically incorporated gelatin gels. For instance, $G_{10P340020}$ displayed an average G' of 10740 ± 171 Pa, a factor of 1.9 over $G_{cys10P340020}$. The differences in G' values between covalently and physically crosslinked gelatin hydrogels indicated differences in their network structure. Specifically, PEGdA-based network in physically crosslinked system resulted in lower mesh size, higher crosslinking density, and thus higher G' . The PEGdA content in both covalently crosslinked and physically incorporated gelatin hydrogels showed a positive correlation with G' values. For example, covalently crosslinked Gel-PEG-Cys hydrogels showed G' increased from 472 ± 78 Pa with 10% (w/w) PEGdA to 3659 ± 514 with 20% (w/w) PEGdA. Temperature effects were investigated *via* a dynamic frequency sweep at 37 °C and results showed G' values at 37 °C were lower than values obtained at R.T for both

covalently and physically crosslinked gelatin hydrogels. The decrease of shear storage modulus at higher temperature may be explained by the sol-gel transition of gelatin, which involves triple helix to protein coil transition [36]. The unfolding of triple helices in the gelatin backbone may result in the gelatin chain relaxation at higher temperature and hence, in decreased hydrogel mechanical strength. Comparing $|G^*|$ across formulation groups showed a positive correlation between PEGdA concentration and $|G^*|$ values. As shown in Fig. 7, the average $|G^*|$ was determined as 1416.7, 4261.3, and 5845.7 Pa for w/w ratio of Gel-PEG-Cys to PEGdA 3400 of 10:10, 10:15, and 10:20, respectively. All three formulations showed $0 \leq \tan \delta \leq 1$, exhibiting more solid-like behavior at R.T. Similarly, $|G^*|$ increased significantly with increasing PEGdA concentration in the physically incorporated gelatin gels (Fig. 7). Swelling and degradation studies revealed that the molecular weight of crosslinker PEGdA was another critical factor that affects the bulk physical properties of such hydrogels. Therefore, covalently crosslinked and physically incorporated gelatin hydrogels prepared with PEGdA 575 were also investigated. Similar bulk rheological properties and trends with PEGdA 575 were observed. For example, higher PEGdA 575 content resulted in higher G' and G'' , and higher temperature resulted in lower G' values. Compared to PEGdA 3400 group, significantly higher G' and G'' values were observed for both covalently and physically crosslinked hydrogels (Fig. 6), mainly due to the higher crosslinking density resulted from the lower molecular weight of PEGdA crosslinkers.

The bulk property also depends on the hydrogel swollen state and this has a direct impact on cell encapsulation. For example, cell encapsulation was conducted during hydrogel formation, *i.e.* in the non-swollen state, but the subsequent culture was performed in excess of culture medium at which the gel matrix will be in the fully swollen state. Fully swollen hydrogels of both covalently crosslinked and physically incorporated gelatin hydrogels displayed much lower G' and G'' values than nonswollen hydrogels (Fig. 7A, 7C). A comparison between nonswollen and swollen gels showed significantly lower $|G^*|$ with swollen gels, mainly due to the higher water content and more relaxed state of polymer chains in the swollen state (Fig. 7B, 7D). Covalently crosslinked gelatin hydrogels showed lower $|G^*|$ than physically incorporated gelatin hydrogels at the same polymer concentration level ($p < 0.05$), indicating polymer chains between crosslinks were in a more relaxed state in the covalently crosslinked gelatin hydrogels. This finding is in good agreement with swelling results in which covalently crosslinked gelatin hydrogels may have higher mesh size thus higher water absorption. Based on the swelling study, physically incorporated gelatin hydrogels reach Q_{max} after about 1 h and while the majority of gelatin still retains in the gel matrix. Thus, both G10P₃₄₀₀10 and G_{cys}10P₃₄₀₀10 were subjected to swelling for 60 min prior to the measurement. Over the frequency range from 0.1 – 10 Hz, G' and G'' of G10P₃₄₀₀10 no longer displayed frequency-independent profiles, and instead, G' and G'' crossed over at frequency 0.2797 Hz (Fig. 8A). In the lower frequency range, shear storage modulus values were lower than loss modulus values ($G' < G''$), displaying a more liquid-like viscous behavior at room temperature. The non-interacting high molecular macromolecule, *i.e.* gelatin, would be below the percolation threshold thus exhibiting relaxation to viscous-like properties at low frequencies [37]. When in the nonswollen state, gelatin will be in the form of a physical gel and not in the relaxed state and thus G10P₃₄₀₀10 displayed normal gel-like viscoelastic response over the frequency ranges of 0.1 – 10 Hz (Fig. 6). G_{cys}10P₃₄₀₀10 swollen gel displayed a frequency-independent shear storage modulus profile with a much lower average G' (Fig. 8B). Unlike G10P₃₄₀₀10 swollen gels, no crossover of G' with G'' was observed in the G_{cys}10P₃₄₀₀10 swollen gel, also demonstrating the absence of non-interacting high molecular weight component such as gelatin within the matrix. Additionally, others had showed higher gel stiffness ($G' > 1200$ Pa) acts as a barrier for cells cultured in 3D. It is suggested by Bott K et al. [2] that cell proliferation, migration, and differentiation profile may achieve best performance with G'

ranging from 10 – 1000 Pa. Our results showed that by varying PEGdA concentration, the gel stiffness can be tuned. In general, higher PEGdA concentration and lower PEGdA molecular weight will lead to the formation of higher crosslinking density thus higher matrix stiffness. Moreover, the significant differences in gel stiffness between nonswollen and swollen gels should be accounted for in cell entrapment studies.

3.2 Effect of crosslinking modality on 2D and 3D cell behaviors

By covalently crosslinking Gel-PEG-Cys with PEGdA, it is hypothesized that the crosslinked gelatin would provide long-term cell anchorage to support cell attachment and growth in the 3D environment. Human dermal fibroblasts were encapsulated in three hydrogel scaffolds including $G_{cys}10P_{3400}15$, $G10P_{3400}15$, and $G0P_{3400}15$. Fibroblasts in all three scaffolds showed high viability for up to 28 days with only few dead cells (red) observed on day 28 in $G0P_{3400}15$ and $G10P_{3400}15$. However, extensive cell spreading and the formation of intercellular contacts were only observed in the covalently crosslinked Gel-PEG-Cys hydrogel group (Fig. 9A). Fibroblasts encapsulated within PEGdA-only hydrogel and physically incorporated gelatin gel remained rounded with no discernible cell-spreading (Fig. 9A). The live cell percentage was determined as the number of live cells over total cell number. As shown in Fig. 9B, significant differences were observed only on Day 28 samples with significantly higher live cell percentage in covalently crosslinked Gel-PEG-Cys hydrogel sample ($p < 0.05$). All three formulations maintained live cell percentage over 75% from day 1 to day 28. Cells encapsulated in $G10P_{3400}15$, and $G_{cys}10P_{3400}15$ hydrogels continued to proliferate over the 7-day study period, as indicated by the increase in relative fluorescence intensity in CellTiter-Blue® assay (Fig. 9C). Among all groups, cell viability of $G_{cys}10P_{3400}15$ hydrogel increased the most. Furthermore, the fluorescence of $G_{cys}10P_{3400}15$ is two times higher than $G10P_{3400}15$, indicating stronger proliferation capability of fibroblasts in the covalently crosslinked gelatin hydrogel.

To further explore cell-material interaction, a 2D cell adhesion study was conducted to investigate cell behavior on three hydrogel surfaces. Both fibroblasts and keratinocytes adhered to all three surfaces after 24 h (Fig. 10) with fibroblasts adhered on $G_{cys}10P_{3400}15$ and $G10P_{3400}15$ displaying elongated and spindle like morphology at day 1. Fibroblasts adhered on $G0P_{3400}15$ gel surface remained rounded after 24 h. In comparison, very few adherent fibroblasts on $G0P_{3400}15$ and $G10P_{3400}15$ surfaces with significantly smaller size were observed by day 7. There were much more adherent fibroblasts observed on $G_{cys}10P_{3400}15$ than on $G0P_{3400}15$ and $G10P_{3400}15$ surfaces at day 7. Hence, 2D cell adhesion displayed a pattern that was significantly different from that in 3D culture model. The decreasing adherent cell density over time in 2D may be due to the loss of functional cell attachment sites on the gel surface, which has also been observed in previous studies [38]. It is also possible that non-specific cell adhesion resulted in cell detachment over time. By covalently crosslinking gelatin, the labile gelatin chains can be better retained in the gel matrix at 37 °C and help induce cell attachment, and adhesion.

Fibroblasts are anchorage-dependent cells (ADCs) and the survival of these cells requires attachment to a substrate. The interaction of ADCs within a 3D environment begins with the binding of integrin receptors with specific motifs in the ECM [39]. To explore cell behavior under 3D environment, fibroblasts encapsulated in physically incorporated and covalently crosslinked gelatin hydrogels were investigated using immunofluorescence stain. Phalloidin-staining of the cytoskeleton F-actin showed well-spread cells with assembled stress fibers when entrapped within $G_{cys}10P_{3400}15$ (Fig. 11). However, no cell spreading and evidence of F-actin bundle could be observed in cells entrapped within $G10P_{3400}15$ and $G0P_{3400}15$. The significant cytoplasmic spreading and F-actin bundling in $G_{cys}10P_{3400}15$ indicated a more active interaction between the material and fibroblasts. A study by Chen *et al.* indicated that spheroidal ADCs were prone to apoptosis but stretched cells survive and proliferate [40].

Thus, cell adhesion and spreading are two essential factors determining the fate of cells entrapped in new microenvironments. The immunofluorescence staining of vinculin and integrin $\alpha 5 \beta 1$ also confirmed the formation of focal adhesion in cells entrapped within $G_{cys}10P_{3400}15$ (Fig. 11). Higher expression and more aggregated vinculin and integrin $\alpha 5 \beta 1$ distribution were observed in cells within $G_{cys}10P_{3400}15$. The presence of vinculin or integrin $\alpha 5 \beta 1$ rich focal adhesion-like structure was not detected in $G10P_{3400}15$ and $G0P_{3400}15$. This further confirms the crucial role of gelatin in providing effective cell anchorage points to support cell attachment and growth in 3D environment. More specifically, the long-term presence of gelatin rather than the free cell-binding motifs within gel scaffolds at 37 °C is of great significance to 3D cell entrapment. As a result, covalently crosslinking gelatin chains offers an effective way to retain labile gelatin chains at 37 °C within the hydrogel matrix thus supporting sustained cell function in a 3D environment.

4. Conclusion

Due to the sol-gel transition property of gelatin, physically incorporated gelatin gel vs. covalently crosslinked gelatin hydrogels showed significantly different physicochemical properties and as a result, encapsulated cells displayed vastly different behaviors in 3D environment. Covalently crosslinking gelatin *via* PEGdA provides long-term biofunctionality to support cell attachment, adhesion, and proliferation in a 3D environment.

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Figure 1. Scaffold structure of covalently crosslinked Gel-PEG-Cys hydrogel *via* thiol-ene photopolymerization (A) and crosslinked PEGdA hydrogel with physically incorporated gelatin (B).

Step 1: Synthesis of Bis-NHS-PEG via carbonate linkage

Step 2: Synthesis of Cysteine modified gelatin (Gel-PEG-Cys)

Figure 2.
Synthesis scheme of Gel-PEG-Cys

Figure 3. GPC Chromatogram of Gel-PEG-Cys (dark grey solid line), unmodified gelatin (light grey solid line), and PEG-diol 3.4 KDa (black dash line).

Figure 4. Swelling and degradation profiles of physically incorporated gelatin hydrogels (A and C) versus covalently crosslinked Gel-PEG-Cys hydrogels (B and D) at 37 °C. (n=3)

Figure 5. Gelatin dissolution behavior over time. Physically incorporated gelatin hydrogels (A, PEGdA 575 Da; C, PEGdA 3400 Da) versus covalently crosslinked Gel-PEG-Cys hydrogels (B, PEGdA 575 Da; D, PEGdA 3400 Da) at 37 °C. Data represented as mean ± S.D. (n=3).

Figure 6.

Bulk rheology of covalently crosslinked gelatin-based hydrogels $G_{\text{cys}}10P_{3400}10$, $G_{\text{cys}}10P_{3400}15$, $G_{\text{cys}}10P_{3400}20$, $G_{\text{cys}}10P_{575}10$, $G_{\text{cys}}10P_{575}15$, $G_{\text{cys}}10P_{575}20$ and physically incorporated gelatin hydrogels $G10P_{3400}10$, $G10P_{3400}15$, $G10P_{3400}20$, $G10P_{575}10$, $G10P_{575}15$, $G10P_{575}20$ at R.T. and 37 °C. Shear storage modulus G' at R.T. (●), 37 °C (●); loss modulus G'' at R.T. (○), 37 °C (◇). *, significantly different compared to corresponding nonswollen formulation with $p < 0.05$.

Figure 7.

Bulk rheology of covalently crosslinked gelatin-based hydrogels (A) and physically incorporated gelatin hydrogels (C) at swollen state. Shear storage modulus G' of $G_{\text{cys}}10P_{3400}10$ and $G10P_{3400}10$ (●), $G_{\text{cys}}10P_{3400}15$ and $G10P_{3400}15$ (●), $G_{\text{cys}}10P_{3400}20$ and $G10P_{3400}20$ (◆); loss modulus G'' of $G_{\text{cys}}10P_{3400}10$ and $G10P_{3400}10$ (◇), $G_{\text{cys}}10P_{3400}15$ and $G10P_{3400}15$ (○), $G_{\text{cys}}10P_{3400}20$ and $G10P_{3400}20$ (□). Complex shear moduli of covalently crosslinked Gel-PEG-Cys hydrogels (B) and physically incorporated gelatin hydrogels (D). *, significantly different compared to corresponding nonswollen formulation with $p < 0.05$.

Figure 8. Bulk rheology of covalently crosslinked gelatin hydrogel $G_{cys}10P_{3400}10$ (A) and physically incorporated gelatin hydrogel $G10P_{3400}10$ (B) with 60 min of swelling in PBS at R.T. Shear storage modulus G' and loss modulus G'' .

Figure 9. (A) Viability and morphology of human dermal fibroblasts encapsulated in hydrogels. Covalently crosslinked gelatin hydrogels increase cell cytoplasmic spreading in 3D environment. Fibroblasts were stained with calcein-AM for live cell (green) and EthD for dead cell (red). (Magnification 10×) (B) Normalized live cell percentages of encapsulated fibroblasts in G0P₃₄₀₀15 (■), G10P₃₄₀₀15 (□), G_{cys}10P₃₄₀₀15 (◆) at various culture times. (C) Fibroblasts proliferation in 3D conditions at day 1 (■) and day 7 (□) of culture. Fluorescence intensities of cells entrapped in covalently crosslinked G_{cys}10P₃₄₀₀15 hydrogels and physically incorporated G10P₃₄₀₀15 hydrogels were normalized to that of PEGdA-only hydrogel G0P₃₄₀₀15. *, significantly different with $p < 0.05$.

Figure 10. 2D adherent fibroblasts on hydrogel surfaces fabricated using different crosslinking modalities, including G0P₃₄₀₀15, G10P₃₄₀₀15, and G_{cys}10P₃₄₀₀15. (Magnification 10×)

Figure 11. Morphology of fibroblasts encapsulated within G_{cys}10P₃₄₀₀₁₅, G10P₃₄₀₀₁₅, and G0P₃₄₀₀₁₅ after 14 d. A: F-actin (green) and vinculin (red) were stained and imaged with CLSM. B: co-localization of F-actin and Integrin $\alpha 5 \beta 1$.

Table 1

Formulation table

	Formula	Gel-PEG-Cys wt%	Gelatin wt%	PEGdA 3400 wt%	PEGdA 575 wt%
Covalently crosslinked gelatin hydrogel	G _{cys} 10P ₃₄₀₀ 10	10	0	10	0
	G _{cys} 10P ₃₄₀₀ 15	10	0	15	0
	G _{cys} 10P ₃₄₀₀ 20	10	0	20	0
	G _{cys} 10P ₅₇₅ 10	10	0	0	10
	G _{cys} 10P ₅₇₅ 15	10	0	0	15
	G _{cys} 10P ₅₇₅ 20	10	0	0	20
Physically incorporated gelatin hydrogel	G10P ₃₄₀₀ 10	0	10	10	0
	G10P ₃₄₀₀ 15	0	10	15	0
	G10P ₃₄₀₀ 20	0	10	20	0
	G10P ₅₇₅ 10	0	10	0	10
	G10P ₅₇₅ 15	0	10	0	15
	G10P ₅₇₅ 20	0	10	0	20

Table 2

Parameters related with weight swelling ratio time curve.

Formulations	Q_{max}	T_{max} (h)
G10P ₅₇₅ 10	11.6±0.8	2
G10P ₅₇₅ 15	8.2±0.7	1
G10P ₅₇₅ 20	6.7±0.5	1
G _{cys} 10P ₅₇₅ 10	8.4±2.0*	2
G _{cys} 10P ₅₇₅ 15	6.5±2.0*	2
G _{cys} 10P ₅₇₅ 20	5.3±0.6*	2
G10P ₃₄₀₀ 10	15.4±1.0	2
G10P ₃₄₀₀ 15	10.0±1.0	6
G10P ₃₄₀₀ 20	8.3±1.0	8
G _{cys} 10P ₃₄₀₀ 10	18.3±1.8*	4
G _{cys} 10P ₃₄₀₀ 15	12.7±1.0*	6
G _{cys} 10P ₃₄₀₀ 20	10.1±0.2*	8

* $p < 0.05$, significantly different compared to corresponding PEG hydrogel formulations with physically incorporated gelatin *via* Student's *t*-test.