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***In Situ* forming poly(ethylene glycol)-based hydrogels *via* thiol-maleimide Michael-type addition**

Yao Fu¹ and Weiyuan John Kao^{1,2,3,*}

¹School of Pharmacy, University of Wisconsin-Madison, 777 Highland Ave., Madison, WI 53705, USA

²Department of Biomedical Engineering, College of Engineering, University of Wisconsin-Madison, Madison, WI 53705, USA

³Department of Surgery, School of Medicine and Public Health, University of Wisconsin-Madison, Madison, WI 53705, USA

Abstract

The incorporation of cells and sensitive compounds can be better facilitated without the presence of UV or other energy sources that are common in the formation of biomedical hydrogels such as poly(ethylene glycol) hydrogels. The formation of hydrogels by the step-growth polymerization of maleimide- and thiol-terminated poly(ethylene glycol) macromers *via* Michael-type addition is described. The effects of macromer concentration, pH, temperature, and the presence of biomolecule gelatin on gel formation were investigated. Reaction kinetics between maleimide and thiol functional groups were found to be rapid. Molecular weight increase over time was characterized via gel permeation chromatography during step-growth polymerization. Swelling and degradation results showed incorporating gelatin enhanced swelling and accelerated degradation. Increasing gelatin content resulted in the decreased storage modulus (G'). The *in vitro* release kinetics of FITC-labeled dextran from the resulting matrices demonstrated the potential in the development of novel *in situ* gel-forming drug delivery systems. Moreover, the resulting networks were minimally adhesive to primary human monocytes, fibroblasts and keratinocytes thus providing an ideal platform for further biofunctionalizations to direct specific biological response.

Keywords

poly(ethylene glycol) hydrogel; gelatin; semi-interpenetrating network; Michael-type addition; cell adhesion

INTRODUCTION

Synthetic hydrogels are an important type of biomaterials with a wide range of applications in controlled drug delivery, implants, wound dressings, biosensors, and tissue scaffolds. In general, *in situ* forming hydrogels are prepared through various gelation methods, such as

*Corresponding author. Tel.: (608)263-2998, Fax: (608)262-5345 wjkao@pharmacy.wisc.edu (W.J. Kao).

photopolymerization, ionic interaction, and chemical crosslinking.¹⁻³ Among these methods, photopolymerization is one of the most widely used techniques, for example to fabricate poly(ethylene glycol) (PEG)-based hydrogels. However, photopolymerization requires the addition of highly toxic photoinitiators and the usage of UV light, which is clinically unfavorable. Thus, attentions have been focused on the chemical crosslinking method. The Michael addition is a facile reaction between nucleophiles and activated olefins and alkynes.⁴

The thiol-Michael addition to electron-deficient carbon-carbon double bonds carries many advantages such as requiring small amount of catalysts, displaying rapid reaction rates in benign reaction conditions (no heat or light needed)⁵, and the absence of byproducts. Functional groups that carry the reactive unsaturated structure include (meth)acrylates, maleimides, α,β -unsaturated ketones, fumarate esters, acrylonitrile, cinnamates, and crotonates.⁶ The thiol-Michael reaction has been extensively studied in small molecule synthesis, polymer modification, protein derivatization, tissue engineering scaffolds, and drug delivery devices.^{4,5} Hubbell and colleagues developed a series of degradable hydrogels for protein delivery formed *via* Michael-type addition reaction between acrylated star PEG polymer and dithiol.⁷⁻⁹ Yuan and Kopeček *et al* reported an enzyme-based hybrid hydrogel synthesized from maleimide terminated four-arm PEG and adenylate kinase *via* thiol-maleimide addition.¹⁰ Hyaluronic acid based hydrogels were also synthesized *via* thiol-Michael reaction for stem cell differentiation^{11,12}, cartilage repair¹³, and bone regeneration¹⁴. The mild reaction condition, the rapid cure and high conversions under physiological environment are of great significance for biological applications such as the incorporation of bioactive macromolecules and cells.

In this study, we demonstrated a step-growth polymerization *via* thiol-maleimide (SH-Mal) Michael-type addition in the formation of a physically entangled PEG network under mild aqueous conditions. The reaction is conducted between two linear PEG polymers modified with different end-functional groups. When the polymerization degree increases and the polymer chain concentration reaches a critical value, the polymer coils diffuse in each other and get tied into each other.¹⁵ Therefore, the gel formation is not due to the formation of crosslinks but rather the physical chain entanglements, which makes it an interesting system different from classical crosslinked PEG hydrogels. The effects of macromer concentration, pH, temperature, and the presence of biomolecule gelatin on gel formation were investigated. The storage and loss moduli (G' and G'') were evaluated by small strain oscillatory shear experiments to gain insight into the structure and mechanical properties of the newly formed hydrogel network. Reaction kinetics between macromer terminated maleimide and thiol functional groups were characterized. In our previous studies, gelatin was incorporated into the growing PEG networks to form a semi-interpenetrating network (sIPN) and provided a substrate for biofunctionalization, *i.e.* the gelatin backbone was conjugated with bioadhesive peptide sequences such as Arg-Gly-Asp and Pro-His-Arg-Ser-Asn tethered by a heterbifunctional PEG linker.¹⁶ The presence of labile gelatin also provides a system to explore the extent of macromolecular thiol-maleimide reaction in a heterogeneous mixture of biomolecules.

MATERIALS AND METHODS

Materials

Bihomofunctional poly(ethylene glycol)-maleimide (PEG-(Mal)₂, Mw ~2000) was purchased from Nanocs (NY, USA). Poly(ethylene glycol)-diol (Mw, ~2000), Fluorescein isothiocyanatedextran (FD-40, Mw ~40,000), gelatin (Type A, ~175 bloom), triethylamine (TEA), triethanolamine (TEOA), *p*-toluenesulfonyl chloride (TsCl), lithium aluminum hydride (LiAlH₄), and sodium hydrosulfide (NaSH) were obtained from Sigma-Aldrich (USA). Dichloromethane (CH₂Cl₂), tetrahydrofuran (THF), toluene, diethyl ether, and anhydrous magnesium sulfate (MgSO₄) were purchased from Fisher Scientific (USA). Polyethylene glycol diacrylate (PEGdA) was synthesized from polyethylene glycol (PEG; MW 2000; Sigma-Adrich) following an established procedure.¹⁷ All chemicals were used as received unless otherwise specified.

Synthesis of PEG-dithiol

PEG dithiol was synthesized *via* a two-step reaction per a previously described procedure with modifications (**Fig. 1**).¹⁸ 10.0 g of PEG diol (Mw 2000, 5 mM) was dissolved in 50 mL of dry CH₂Cl₂. 7.2 g of TsCl was dissolved in 20 mL of CH₂Cl₂ and added dropwise to the PEG-diol solution while stirring. 1.4 mL of TEA (10 mM) was subsequently added to the mixed solution. The reaction was kept at room temperature and stirred overnight. The crude mixture was filtered twice, concentrated *via* rotavap, dissolved in 50 mL of toluene, and filtered. Filtrates were collected and precipitated in cold diethyl ether while stirring. The precipitates were collected and dried under vacuum. PEG-(Tos)₂: 9.6 g, yield 96%. ¹H NMR (CDCl₃): δ 2.45, s, CH₃-aromatic; δ 3.65, m, PEG backbone; δ 7.5, d, 2Hs ortho to sulfonate; δ 7.8, d, 2Hs meta to sulfonate.

PEG-(Tos)₂ (5 g, 2.3 mM) dissolved in 100 mL of ddH₂O was treated with NaSH hydrate (1.4 g, ~ 25 mM). The reaction was stirred for 5 h at room temperature and then 1 h at 60 °C. The crude mixture was neutralized with concentrated H₂SO₄, and extracted with CH₂Cl₂. The organic layer was collected and dried over anhydrous MgSO₄ and precipitated in dry cold diethyl ether. The precipitated product was collected and dried in vacuum overnight (2.8 g, yield 56%). ¹H NMR (D₂O): δ 2.68, t, -SH; δ 2.76 -OCH₂CH₂SH; δ 2.92, t, -OCH₂CH₂SH; δ 3.65, m, PEG backbone. The products obtained from the above reaction were treated with LiAlH₄ in 20 mL of dry THF for 1 h and then 20 mL of ddH₂O was added to stop the reaction. Reduced PEG-(SH)₂ was extracted with CH₂Cl₂, dried with anhydrous MgSO₄, and precipitated in cold diethyl ether. The final products were dried in vacuum overnight and stored in argon at -20 °C. (**WARNING:** this reaction is extremely dangerous to perform due to the generation of H₂S during neutralization. Alternative synthesis method is to be used in future work using potassium thioacetate instead of NaSH.¹⁹)

PEG hydrogel and sIPN formation

For the preparation of gels, PEG-(SH)₂ and PEG-(Mal)₂ were dissolved in various buffer solutions in separate glass vials. Polymer weight percent (wt%) was varied by varying the total amount of solvents (**Table I**). Then, the two solutions were quickly mixed *via* vortexing (~30 s) to form the network (**Fig. 2**). The time of gelation was determined by the

test tube inverting method, *i.e.* observation of the stop of flow when inverting the vial horizontally.²⁰ pH effect was investigated using pH6.5 TEOA PBS, pH7.5 TEOA PBS, and pH8.0 TEOA PBS as the solvent for PEG-(SH)₂, while pH7.4 PBS was used to solvate PEG-(Mal)₂ (**Table II**). Temperature effect on formulation with 18.4% total polymer was also investigated at 25 °C and 37 °C. Gelatin solutions of various concentrations were prepared at 37 °C with PEG-(SH)₂ and PEG-(Mal)₂ (**Table III**). Prior to mixing, all solutions were placed in the 37 °C water bath. The gelatin solution was quickly mixed with PEG-(SH)₂ solution and subsequently mixed with PEG-(Mal)₂ solution *via* vortexing. The fully mixed prepolymer solution was placed in a 37 °C incubator to cure.

Fourier transform infrared spectroscopy (FTIR)

FTIR spectra were recorded on a Bruker Equinox 55 ATR-FTIR spectrometer (single reflection, ZnSe crystal, 20 scans per spectrum) in a N₂ atmosphere. Samples were dissolved in THF to minimize the influence of water on the absorption spectrum and spectra were recorded at predetermined time points up to 20 min. A decrease in the IR absorption peak at 695 cm⁻¹ was observed and attributed to the =C-H bending vibration of the maleimide group. The conversion of C=C bonds (=C-H bend, 695 cm⁻¹) on the maleimide group was determined using C=O stretch (1724 cm⁻¹) as the internal reference. Absorption peaks at 695 cm⁻¹ and 1724 cm⁻¹ were integrated using OPUS version 4.2 software provided with the spectrometer. The degree of reacted maleimide groups at a specific time was calculated from the peak area, *A*. Thus, the conversion of unsaturated maleimide groups, α , was calculated by the following equation:

$$\alpha = 1 - \left(\frac{(A_{695\text{cm}^{-1}}/A_{1724\text{cm}^{-1}})_t}{(A_{695\text{cm}^{-1}}/A_{1724\text{cm}^{-1}})_{t=0}} \right) \quad \text{Eq 1}$$

where A_i is the area of the vibration band at time t .²¹

Gel permeation chromatography (GPC)

The GPC analysis was performed to characterize the step-growth polymerization over time during hydrogel formation. 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 μm , 30 cm $\times$ 7.8 mm), G3000PW_{XL} (pore size = 200 μm , 30 cm $\times$ 7.8 mm), G2500PW_{XL} (pore size < 200 μm , 30 cm $\times$ 7.8 mm). The system was calibrated using a series of PEO/PEG standards (232 – 932,000 Da). All samples were run at 0.7 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. The integral area of each peak was determined and the percent area under the curve was then calculated. Samples subject to GPC analysis were prepared in a diluted condition, *i.e.* 0.05 g PEG-(SH)₂ and 0.042 g PEG-(Mal)₂ were dissolved in 2 mL of 1X PBS and allowed to react for 20 h, 2 d and one month. The reactants were diluted with the eluent to about 2 mg/mL and filtered through 0.22 μm membrane filter (Millipore, USA) before analysis.

Physical characterization: swelling, surface hydrophilicity and rheological characterization

The hydrogel and sIPN samples *via* thiol-maleimide addition were synthesized and dried in vacuum overnight. Photopolymerized PEG hydrogel and sIPN with 10% gelatin were prepared as previously described¹⁶ and tested for comparison. The dried gels were accurately weighed prior to the swelling study. The gel disks were then immersed in 10 mL of pH 7.4 PBS at 37 °C. At predetermined time points, hydrogels were carefully removed from the petri dishes, blotted with Kim-wipes® to remove the excess water on the hydrogel surfaces, and weighed. Equilibrium weight swelling ratio (Q_s) was defined as follows:

$$Q_s = \frac{(w_t - w_d)}{w_d} \quad \text{Eq 2}$$

where w_t is the gel weight at the time t , and w_d is the dried gel weight. The hydrogel surface hydrophilicity was quantified with a modified, computerized, underwater air-captured surface contact angle system (VCA2500; AST products, Inc. Billerica, MA). Fully swollen hydrogels were secured to a glass slide with Superglue® and placed in a water chamber. Air bubbles were placed on the exposed and submerged surface of the hydrogel *via* syringe. Two angles per bubble for three bubbles were measured for each sample to obtain the static contact angle.

Storage modulus (G') and loss modulus (G'') on nonswollen and swollen gels were measured with an ARES-LS2 2000ex rheometer (TA Instruments) in parallel plate geometry with a 8 mm diameter plate at 25 °C ± 0.1 °C, a frequency range of 0.1 - 10 Hz, and a constant 5% strain. Hydrogel samples were prepared to yield discs of 8 mm diameter and 1 mm thickness. Swollen samples were equilibrated in 1× PBS overnight and cut with razor blade to yield discs of 8 mm diameter and 1 mm thickness. The water from the hydrogel surface was carefully blotted before measurement and each sample was covered with a thin layer of silicone oil to minimize water evaporation effect. Samples include PEG SH-Mal hydrogel and PEG SH-Mal sIPN (Table IV).

In vitro controlled release

FD-40 (Mw, ~40 KDa) was selected as the model solute for *in vitro* release study. The stock solution was prepared by dissolving FD-40 in pH7.4 PBS at a concentration of 2 mg/mL. FD-40 was loaded prior to gelation, and two PEG SH-Mal formulations were included, *i.e.* 13% total polymer and sIPN with 20% total polymer. After complete polymerization, each disk was placed in 5 mL of pH7.4 phosphate buffer saline at 37 °C. At given time points, 1 mL of release sample was collected and replaced with 1 mL of fresh media. The concentration of FD-40 release samples were determined by fluorescence spectroscopy at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. FD-40 showed a linearity range between 3.9 – 62.5 µg/mL ($Y=468.86X+1617.3$, $R^2 = 0.9916$).

Cell culture and adhesion assay

Primary monocytes were isolated from healthy human volunteers using an established density gradient non-adhesion method with modifications.²² Cells were statically seeded on various hydrogel samples in tissue culture polystyrene (TCPS, Becton Dickinson Labware,

NJ) wells at a concentration of 10^6 cells/mL suspended in RPMI 1640 media with 10% autologous serum. Neonatal human dermal fibroblasts were obtained from Lonza (NJ, USA) and cultured in fibroblast growth medium-2 (FGM-2, Lonza) containing FBM, 0.1% insulin, 0.1% recombinant human fibroblast growth factor-B, 0.1% GA-1000, and 2% fetal bovine serum (FBS; Atlantic Biologicals, Miami, FL, USA). Fibroblasts for adhesion assay were derived from the same cell stock at passage 3 at a seeding density of 4×10^4 cells/mL. Normal human epidermal keratinocytes at passage 3 from neonatal foreskin (NHEK; Lonza, Allendale, NJ, USA) were cultured in keratinocyte growth medium (KGM; Lonza) supplemented with 5% FBS and seeded at a concentration of 4×10^4 cells/mL. All cell cultures were maintained at 37 °C and 5% CO₂.

Samples were cold-sterilized with 75% ethanol per standard procedures and equilibrated in culture medium prior to cell seeding. At 2, 24, 96, and 168 h thereafter, samples were washed twice with respective culture medium to remove non-adherent cells, and resupplied with fresh culture medium and allowed to further incubate. TCPS was employed as a positive surface control, and photopolymerized PEG hydrogel was used as an additional surface comparison. Adherent cells on all surfaces were imaged using a computer-assisted video analysis system (Metamorph v4.1) coupled to an inverted microscope (Nikon, Eclipse TE300). The adherent cells were quantified and expressed as number of cells/mm² surface area. Five images per sample were taken at random fields of view. Adherent cell density was determined per mm² surface area using the image analysis software ImageJ (NIH). Additionally, Live/dead[®] stain (4 μM calcein AM/4 μM EthD-1, Invitrogen) was used to evaluate fibroblast and keratinocyte viability and morphology.

Cytotoxicity via MTT assay

Hydrogel extracts were prepared by adding PEG SH-Mal hydrogel fragments to specific culture medium at a concentration of 10 mg/mL and incubated at 37 °C for 24 h.²³ After this period, the hydrogel extract was sterilized via filtration (0.22 μm, Millipore, USA). Primary human monocytes, neonatal human dermal fibroblasts, and normal human epidermal keratinocytes were used to evaluate the cytotoxic effects of hydrogel extracts. 100 μL of the cell suspension (Monocytes, 5×10^4 cells/well; Fibroblasts, 3000 cells/well; Keratinocytes, 8000 cells/well), were seeded in flat-bottomed 96-well micro-culture plates (Costar, USA). The 96-well plates were incubated at 37 °C in a 5% CO₂ humidified incubator. After reaching 70 - 80% confluence, the medium was discarded and replaced with 100 μL of hydrogel extract. The serum free culture medium was used as negative control. Samples and controls were tested in sextuplet. These plates were then incubated for 24 and 48 h, respectively, before the viability was determined via standard MTT assay. At the end of culturing time, 20 μL of MTT solution (5 mg/mL) was added to each well. After 4 h incubation at 37 °C in a 5% CO₂ humidified incubator, supernatant was removed carefully and 100 μL of dimethyl sulfoxide (DMSO) was added into each well to dissolve the intracellular blue formazan product. The optical density of each well was read using a microplate reader (Bio-Tek Elx800) at 570 nm with a reference wavelength of 650 nm. The percentage cell viability of samples compared to the control was calculated from the following equation:

$$viability (\%) = \frac{[OD]_{sample} - [OD]_{blank}}{[OD]_{negativecontrol} - [OD]_{blank}} \times 100$$

Statistical analysis

All data are represented as mean $\pm$ standard deviation (S.D.) of samples in at least three independent experiments. Cell density data were analyzed by unpaired Student-*t* test using SigmaStat version 2.03. A value of $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSIONS

Formation of PEG hydrogel and sIPN via thiol-maleimide Michael-type addition

Combining aqueous solutions of PEG-(Mal)₂ and PEG-(SH)₂ resulted in the formation of an entangled hydrogel system (**Fig. 2**). The effects of buffer system, macromer concentration, and reaction temperature were explored. Gelation time ranged from minutes to days (**Table I and II**) and was significantly affected by pH (**Table II**). More rapid gel formation was observed under basic conditions, most likely due to the favored condition for thiol groups to form nucleophiles.²⁴

However, the rates of thiol-Michael reaction and oxidation, *i.e.* the formation of disulfide products, were both sensitive to pH.²⁵ With an increase in pH value, oxidization was accelerated and the content of free -SH decreased.²⁶ Therefore, pH7.4 phosphate buffer saline and 0.3M TEOA were selected as the solvent for further studies on the macromer concentration effect and sIPN formation studies. Increasing macromer concentration led to a significant decrease in the gelation time, which might create more polymer entanglements during gel formation. While macromer concentration had a significant influence on gelation time, temperature effect was less significant between room temperature and 37 °C (data not shown). The presence of gelatin perturbed the macromeric thiol-maleimide reaction and the subsequent gelation time (**Table III**). Particularly, the presence of 3.3% w/v gelatin completely abrogated gelation after several days at 37 °C and room temperature. As shown in **Table II**, PEG hydrogel of the same macromer concentration (13% w/w) showed complete gelation after 20 h. 3% w/v gelatin solution gelled within minutes at room temperature.²⁷ However, the incorporation of gelatin into PEG networks at the concentration of 3.3% w/v displayed sol-gel properties different from either single component, implying that the formation of semi-interpenetrating network created a dynamic gel network at room temperature. Though gelatin solutions with concentrations ranging from 3% - 10% w/v remained in a solution form at 37 °C, more physical entanglements might be created within PEG networks during sIPN formation that led to the gel formation at 37 °C. Thus, a higher gelatin concentration may contribute to the complete gelation of sIPN at 37 °C.

PEG-maleimide and PEG-thiol reaction kinetics and network physical properties

The conversion of PEG terminal maleimide groups was monitored by the characteristic absorption bands at 1724 cm⁻¹ (ν, C=O in maleimide) and 695 cm⁻¹ (ν, =C-H in maleimide) (**Fig. 3**). The decrease of the latter band was due to the addition reaction between -SH and -

C=C– group. In the present study, the area under peaks was integrated and the 1724 cm^{-1} peak was used as the internal reference, which provided a kinetic conversion profile for the PEG terminal thiol- maleimide addition reaction. As shown in **Fig. 3**, the conversion of the C=C group reached steady state around 10 min, indicating a near complete conversion of the C=C group.

GPC chromatograms (**Fig. 4A**) showed a wide molecular weight distribution of the polymer products *via* thiol-maleimide addition over the time range from 20 h to 1 month. Peak integration analysis and molecular weight calibration revealed a major peak shift towards the higher molecular weight (**Table V**). In addition, the higher polymer concentration will also likely result in a higher degree of conversion further increasing the step-growth molecular weight increase. The hydrogel samples subject to swelling study were proven to degrade to small molecular weight products after about six months (**Fig. 4B**). The degradation is mostly likely due to the hydrolysis of maleimide- in the polymer. Taken together, the network formation that was observed in the study is due to the step-growth of PEG polymer chains that resulted in chain entanglement and hydrogel formation.

PEG SH-Mal hydrogels synthesized *via* thiol-maleimide addition displayed consistent and increasing swelling through 7 days, with a maximum average weight swelling ratio of (23 ± 3) (**Fig. 5**). In comparison, photopolymerized PEG hydrogels displayed a significantly lower maximum average weight swelling ratio of (5.4 ± 0.7) ($p < 0.05$). No significant mass loss of PEG SH-Mal hydrogel was observed during a 7-day period, indicating the relative robustness of the hydrogel structure. However, the amide linkage is likely to undergo hydrolysis over a long time period, and complete degradation was observed during six-month period as mentioned previously. The weight swelling ratio of PEG SH-Mal hydrogel reached a steady state around 48 h, which indicated an equilibrium swelling state of the hydrogel. However, photopolymerized PEG hydrogel reached the steady state at a more rapid swelling rate around 2 h. Compared to photopolymerized PEG hydrogels, the greater swelling ratio and more hydrophilic surface properties of PEG SH-Mal hydrogels are probably due to the less rigid polymer chain entanglements formed *via* thiol-maleimide addition. The incorporation of gelatin in gels resulted in different swelling and degradation properties. sIPN SH-Mal with 10% gelatin showed a maximum average swelling weight ratio of (18 ± 1) around 4 h (**Fig. 5**), while SH-Mal sIPN with 5% gelatin reached maximum average swelling weight ratio of (15 ± 3.4) in about 24 h (**Fig. 5**). Thus, increasing gelatin concentration may potentially increase the swelling ratio. However, the maximum weight swelling ratio is not significantly different between SH-Mal sIPN and PEG SH-Mal hydrogel ($p > 0.05$). The photopolymerized sIPN showed significantly smaller maximum weight swelling ratio than photopolymerized hydrogel ($p < 0.05$). Gelatin has such sol-gel transformation properties related to temperature changes, and thus it is expected that gelatin incorporated in the semi-interpenetrating network may undergo dissolution at 37°C thus creating more free volumes within the polymeric matrices that would contribute to the enhanced swelling effect. As shown in **Fig. 5**, sIPNs synthesized *via* either thiol-maleimide addition or photopolymerization showed mass loss after 24 h, and the PEG SH-Mal sIPNs became physically unstable to handle for measurement after 48 h.

In the underwater air-captured surface contact angle analysis, a larger surface contact angle indicates a higher surface hydrophilicity.^{28,29} PEG hydrogels synthesized *via* click chemistry showed a significantly larger surface contact angle than photopolymerized PEG hydrogels ($p < 0.05$), indicating a higher degree of hydrophilicity (**Table VI**). The surface hydrophilic nature of the material is associated with the surface structure. As is discussed in the previous section, hydrogels synthesized *via* thiol-maleimide addition may result in more dangling ends compared to crosslinked gel system. Thus, less stiff surface structure is expected compared to photopolymerized PEG hydrogel. The observation is in good agreement with empirical rules, *i.e.* the more stiff the gel, the higher the hydrophobicity, and the less degree of the permeability and gel swelling.^{30,31}

PEG hydrogels and sIPNs were subject to dynamic frequency sweep to gain insight into the mechanical characteristics and structure of the physically entangled network. The storage modulus (G') and loss modulus (G'') were monitored over frequency ranges from 0.1 to 10 Hz at 25 °C. PEG SH-Mal hydrogels with G' values independent of the tested frequencies were proven to have the characteristic viscoelastic properties of gels (**Fig. 6A**). Results also showed significantly higher storage modulus (G') than loss modulus (G'') for all formulations, indicating that these hydrogels are highly elastic. Non-swollen PEG SH-Mal hydrogel showed much higher G' (~5.8 KPa) and G'' (~2.6 KPa) at the polymer concentration of 48% than at 40%, indicating higher polymer concentration corresponds to higher storage modulus (**Fig. 6**). However, there are no significant differences in storage modulus (G') between different formulations in the swollen hydrogels (**Fig. 6A**). The similar storage modulus values may be due to the high water capacity of these hydrogels, which could be a specific characteristic of such non-crosslinked physical entanglements. Additionally, the storage modulus (G') value was decreased by up to 10-fold after equilibrium swelling. Increasing gelatin content resulted in the lower storage modulus (G') and lower loss modulus (G'') (**Fig. 6C**). Unlike swollen PEG SH-Mal hydrogels that displayed no significant differences in storage modulus (G') (**Fig. 6B**), swollen PEG SH-Mal sIPNs with lower gelatin content showed slightly higher storage modulus (G') (**Fig. 6D**), indicating incorporating gelatin into the PEG matrix creates more structural complexities to the current system. In summary, the storage modulus of PEG SH-Mal hydrogels/sIPNs can be adjusted by varying total polymer concentration and gelatin concentration.

***In vitro* controlled release kinetics**

The release kinetics of FD-40 showed a biphasic release profile (**Fig. 7**). FD-40 release from PEG SH-Mal hydrogel displayed a significant burst release with near 80% cumulative release at the 1-h point, which may be due to the great swelling effect of click hydrogels and the rapid release of surface associated solutes. In contrast, PEG SH-Mal sIPN showed a less significant burst effect. A near zero-order release was observed from 0 to 4 h, which corresponded well with previous release studies on the release kinetics of photopolymerized sIPN-based delivery systems.³² Although swelling results showed comparable maximum weight swelling ratio between click hydrogel and sIPN, the different release kinetics of FD-40 from both formulations indicated that the incorporation of gelatin greatly delayed the FD-40 release by potentially creating more physical entanglements thus more hydrodynamic

hindrance to solute diffusion. It is expected that the release kinetics can be tailored by varying the amount of gelatin incorporated in the network as well as the PEG-to-gelatin weight ratio.

Cell adhesion and cytotoxicity

Adherent monocyte density was significantly different amongst material surfaces. The monocyte morphology on TCPS remained relatively round up through 96 h but was larger with more cytoplasmic spreading by 168 h (**Fig. 8**). Monocytes on PEG SH-Mal hydrogels remained round through 168 h with a slightly noticeable increase in size by the end of the culture. Additionally, adherent monocytes on the PEG SH-Mal hydrogel were in small clusters throughout the culture duration. Monocytes on the photopolymerized PEG hydrogels increased most dramatically in size by 96 h and had been shown in an earlier study to be most phenotypically different than TCPS.¹⁷ Adherent density on TCPS decreased most dramatically with the largest decrease between 24 and 96 h (**Fig. 9A**). Adherent density on PEG SH-Mal hydrogels remained relatively constant through the culture with a slight decrease between 24 and 96 h (**Fig. 9A**). Adherent density on photopolymerized PEG hydrogels decreased over time but to a lesser degree than on TCPS (**Fig. 9A**), which was consistent to our previous studies.¹⁷ By 24 h, TCPS and photopolymerized PEG hydrogels supported similar levels of adhesion ($p > 0.05$) whereas at 96 h and 168 h photopolymerized PEG hydrogels had significantly higher levels of adhesion than either of the other two materials tested ($p < 0.05$). This sustained adhesion may be due to the limited and selective protein adsorption mediated by the hydrophilic hydrogel. Extensive studies had shown that protein molecules selectively adsorb onto hydrophobic surfaces *via* hydrophobic interactions³³ and as a result, the adsorption of adhesion mediating proteins can potentially promote cell adhesion onto more hydrophobic surfaces. Thus, the adhesion results might imply the relative surface hydrophilicity in the following order: TCPS < Photopolymerized PEG hydrogel < PEG SH-Mal hydrogel, which is in good agreement with the contact angle measurement.

Adherent keratinocyte and fibroblast densities on TCPS increased between the 2, 24, and 96-h time points (**Fig. 9B, 9C**). In contrast, adherent cell density on photopolymerized PEG hydrogels and PEG SH-Mal hydrogels remained significantly lower than on TCPS through the course of the experiment. At 96 h, photopolymerized PEG hydrogels showed a significantly higher adherent cell density than PEG SH-Mal hydrogels ($p < 0.05$). The differences in cell adhesion pattern may also be due to the differences in protein adhesion as discussed above. Photopolymerized PEG hydrogels may have more rigid and robust structure, while PEG SH-Mal hydrogels seem to have a more flexible and dynamic structure.

Though PEG SH-Mal hydrogels showed a significantly lower adherent cell density compared to TCPS, cells were shown to have normal morphology on the bottom of the TCPS surfaces that contained the PEG SH-Mal hydrogel sample (**Fig. 10**), indicating the material leachables and any potential degradation products were not cytotoxic. Additionally, cytotoxicity evaluation *via* MTT assay was conducted on three cell types that were used in the cell adhesion study, including primary human monocytes, fibroblasts, and keratinocytes. The results showed fibroblasts and monocytes treated with PEG SH-Mal hydrogel extract

maintained 90 – 100% viability compared to control groups at both 24 and 48 h in a serum-starved condition (**Fig. 11**). Keratinocytes at 24 h showed around 70% viability compared to the control and above 75% normalized viability at 48 h (**Fig. 11**). Both the presence of potential keratinocyte toxic material and the serum-depleted condition could contribute to this decrease. According to GB/T 16886.5-2003 (ISO 10993-5:1999), samples with cell viability larger than 75% can be considered as noncytotoxic. Thus, the hydrogel extract was of low toxicity to primary monocytes and fibroblasts.

CONCLUSIONS

A noncrosslinked PEG-based hydrogel was successfully synthesized *via* a metal-free thiolmaleimide reaction. Buffer pH, macromer concentration, and the presence of biomolecule gelatin showed significant effects on gel formation. Release studies using FD-40 indicated that the hydrogels has the potential as an *in situ* forming drug delivery matrix. Preliminary cell adhesion and cytotoxicity studies demonstrated that the networks were minimally adhesive to primary human monocytes, fibroblasts, keratinocytes and the material leachables were of low cytotoxicity thus providing an ideal platform for further biofunctionalization to direct specific biological response.

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FIGURE 1.

Synthetic scheme of PEG dithiol. Adapted from Ref 18.

FIGURE 2.
Synthetic scheme of poly(ethylene glycol) hydrogel *via* macromeric thiol-maleimide addition.

FIGURE 3.
PEG terminal maleimide conversion over time in THF *via* thiol-maleimide addition.

A. Molecular weight increase over time **B. Degraded PEG-Mal-SH 6 months** **FIGURE 4.**

GPC Chromatograms of PEG polymers via thiol-maleimide addition obtained at 20 h, 2 d, and 1 month (A) and degraded PEG SH-Mal hydrogels (B).

FIGURE 5.

Equilibrium weight swelling ratio of PEG hydrogels and sIPNs *via* thiol-maleimide addition and photopolymerization over time. (n=3)

FIGURE 6.

Storage modulus (G') and loss modulus (G'') at 25 °C as a function of frequency for non-swollen PEG SH-Mal hydrogels (A), swollen PEG SH-Mal hydrogels (B), non-swollen PEG SH-Mal sIPNs (C), swollen PEG SH-Mal sIPNs (D).

FIGURE 7.

In vitro release kinetics of FD-40 from PEG SH-Mal hydrogel and PEG SH-Mal sIPN with 10% gelatin at 37 °C.

FIGURE 8.

Optical micrographs of adherent monocytes on PEG SH-Mal hydrogel (A, B), photopolymerized PEG hydrogel (C, D), and TCPS (E, F) at 24 h (A, C, E) and 7 d (B, D, F) (20 $\times$ magnification).

FIGURE 9.

Adherent cell density on TCPS, photopolymerized PEG hydrogel and PEG SH-Mal hydrogel. Three cell types were included, primary human monocyte (A), keratinocyte (B), and fibroblast (C). Cells were observed at 10× magnification. Five images per sample were taken at random fields of view. †, $p < 0.05$ vs. the cell adhesion density on TCPS at the same time point; ‡, $p < 0.05$ vs. the cell adhesion density on photopolymerized PEG hydrogel at the same time point. All data presented as average $\pm$ standard deviation ($n=3$).

FIGURE 10.

Microscopic images of adherent fibroblasts and keratinocytes on TCPS surface in the presence of PEG SH-Mal hydrogels at 24, 48 and 96 h (20× Magnification). **(A)** Fibroblast optical microscopy images (left-hand side) and fluorescent microscopy images (right-hand side, green, live stained cells; red, dead stained cells); **(B)** Keratinocyte optical microscopy images (left-hand side) and fluorescent microscopy images (right-hand side, green, live stained cells; red, dead stained cells).

FIGURE 11.

Normalized cell viability of primary human monocytes, fibroblasts, and keratinocytes treated with hydrogel extracts on 24 and 48 h. Control: cells treated with serum-depleted medium only. (n=6)

Table I

Various macromer concentrations employed in PEG hydrogel formulation

Formulation #	PEG-(SH) ₂ (g)	PEG-(Mal) ₂ (g)	pH 7.5 PBS w/0.3M TEOA	pH 7.4 PBS (μl)	Total polymer % w/w	Gelation time
1	0.05	0.042	100	100	31	~ 40 min
2	0.05	0.042	200	200	18.4	~ 90 min
3	0.05	0.042	300	300	13	> 20 h

Table II

pH effect on gelation time

pH	PEG-(SH) ₂ (g)	PEG-(Mal) ₂ (g)	PBS w/ 0.3M TEOA	Gelation time
6.5	0.05	0.042	400	> 24 h
7.5	0.05	0.042	400	~ 70 min
8.0	0.05	0.042	400	~ 90 min

Table III

Various gelatin concentration employed in the sIPN formation

#	PEG-(SH) ₂ (g)	PEG-(Mal) ₂ (g)	Gelatin (g)	1× PBS (ml)	pH 7.5 TEOA (ml)	Total polymer w/w%	Gelation Time
1	0.05	0.042	0.02	0.4	0.2	15.7	– ([*])
2	0.05	0.042	0.03	0.4	0.2	17	~ 1 d
3	0.05	0.042	0.06	0.4	0.2	20	~ 1 d

* formulation #1 showed slight fluidity after 3 days of curation.

Table IV

(i) PEG SH-Mal hydrogel formulations for rheological characterization

Formula	PEG-(Mal) ₂ (g)	PEG-(SH) ₂ (g)	0.3M TEOA/PBS (μL)	Total polymer weight (w/w%)
1	0.042	0.05	100	48
2	0.042	0.05	138	40

(ii) PEG SH-Mal sIPN formulations rheological characterization

Formula	PEG-(Mal) ₂ (g)	PEG-(SH) ₂ (g)	Gelatin (g)	0.3M TEOA in PBS (μL)	Total polymer weight (w/w%)
1	0.042	0.05	0.002	140	40.5
2	0.042	0.05	0.004	180	35.3

Table V

Estimated molecular weight of major species in the chromatogram (Fig. 4)

Reaction time	Peak retention time	%AUC	Mw (approx)
20 h	30.00	18.1	25911
	31.80	21.4	11319
	34.20	23.5	4085
2 d	26.00	13.1	247753
	30.20	11.9	23540
	35.20	7.4	2714
1 month	25.01	13.6	499950
	29.95	74.2	26545
	35.09	11.2	2839

Table VI

Underwater air-captured surface contact angle

Formulation	Contact angle (Degree, mean $\pm$ S.D.)	
	Left	Right
PEG Mal-SH hydrogel (18%, w/w)	152 $\pm$ 1.9	152 $\pm$ 2.6
PEG Mal-SH + gelatin sIPN (17%, w/w)	159 $\pm$ 4.2	160 $\pm$ 3.5
Photopolymerized PEG hydrogel (10%, w/w)	141 $\pm$ 4.4	141 $\pm$ 4.9

n=3, with three air bubbles on each sample with two angles per air bubble.