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Injectable Cucurbit[8]uril-Based Supramolecular Gelatin Hydrogels for Cell Encapsulation

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Abstract

Recent efforts to develop hydrogel biomaterials have focused on better recapitulating the dynamic properties of the native extracellular matrix. In hydrogel biomaterials, binding thermodynamics and cross-link kinetics directly affect numerous bulk dynamic properties such as strength, stress relaxation, and material clearance. However, despite the broad range of bulk dynamic properties observed in biological tissues, present strategies to incorporate dynamic linkages in cell-encapsulating hydrogels rely on a relatively small number of dynamic covalent chemical reactions and host–guest interactions. To expand this toolkit, we report the preparation of supramolecular gelatin hydrogels with cucurbit[8]uril (CB[8])-based cross-links that form on demand via thiol–ene reactions between preassembled CB[8]-FGGC peptide ternary complexes and grafted norbornenes. Human fibroblast cells encapsulated within these optically transparent, shear thinning, injectable hydrogels remained highly viable and exhibited a well-spread morphology in culture. These CB[8]-based gelatin hydrogels are anticipated to be useful in applications ranging from bioprinting to cell and drug delivery.

Graphical Abstract

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The authors declare the following competing financial interest(s): A.C.M. and D.M. are co-inventors on a patent application related to the technology described in this Letter.

Supporting Information

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Experimental details and supporting figures (¹H NMR for gelatin and gelatin-norbornene, fluorescamine assay analysis of gelatin-norbornene functionalization, Ellman's assay characterization for free thiol content of gelatin and gelatin-norbornene, representative frequency sweeps, strain sweeps, and complex viscosity traces for supramolecular gelatin hydrogels with different weight percentages of gelatin-norbornene and degrees of FGGC peptide modification, stability/mass swelling characterization, cell density, and adjusted cell count characterization) (PDF)

Hydrogels are high water content, biocompatible materials commonly used to mimic the native extracellular matrix (ECM) for 3D cell encapsulation, tissue engineering, and drug delivery applications.^{1–3} While the native ECM is highly dynamic, with an ability to relax and dissipate stresses by reorganizing physical cross-links, commonly used hydrogel materials rely on covalent cross-links that yield static, brittle network architectures, hindering cell infiltration, migration, and remodeling.⁴ To overcome these limitations, recent efforts to develop adaptable hydrogel biomaterials have incorporated reversible cross-links that better recapitulate the cellular microenvironment.^{5,6} Because dynamic linkages can break and reform in response to cellular forces, adaptable hydrogels facilitate complex cellular functions, enabling more rigorous studies of cell–matrix interactions and better functional restoration in therapeutic applications.

In adaptable hydrogels, cell-instructive bulk properties such as stiffness, viscoelasticity, and macromolecule transport are directly dictated by the binding thermodynamics and kinetics of the dynamic linkages.^{7,8} However, present strategies to incorporate reversible cross-links in biomimetic scaffolds utilize a relatively small number of dynamic covalent chemical reactions and host–guest interactions.⁴ Although adaptable hydrogels comprising these dynamic linkages have shown great promise for cell encapsulation and drug delivery, the cross-link affinity range accessible using these chemistries is limited. Illustratively, while supramolecular hydrogels made from polymers with pendant macrocyclic host groups and pendant guest molecules are a promising class of soft materials that exhibit shear-thinning flow, self-healing, and stress relaxation, the commonly exploited cyclodextrin–adamantane motif exhibits a binding affinity constant on the order of 10^5 M^{-1} , which, though relatively modest, is among the highest obtainable with cyclodextrin cavitands.⁹ Binding affinity constants are estimated under idealized conditions, typically as free guest molecules and free host groups in water or phosphate buffered saline (PBS) using isothermal titration calorimetry or nuclear magnetic resonance spectroscopy, which enable isolation of the inclusion interaction,^{10–12} and likely overestimate cross-link affinity in hydrogels, where interactions with media components and the polymeric backbone reduce the thermodynamic

driving forces associated with guest inclusion.^{13,14} Accordingly, 10^5 M^{-1} currently serves as an upper bound on binding affinity for most supramolecular biomaterials.

To expand the toolkit of host–guest interactions for use in cell-encapsulating hydrogels, we developed gelatin hydrogels that cross-link through cucurbit[8]uril (CB[8]) homoternary complexes formed by simultaneous inclusion of two guest molecules within one CB[8] cavitand. Cucurbit[*n*]urils are rigid, symmetric cavitands composed of *n* methylene-linked glycouril oligomers that form a hydrophobic cavity lined with ureido-carbonyl oxygens possessing very high quadrupole moments.¹⁵ Due to a combination of hydrophobic and ion-dipole interactions, CB[8] ternary complexes are highly selective with very high association rates and binding equilibrium constants (up to 10^{14} M^{-2} under idealized conditions).¹⁶ CB[8] ternary complexes have previously been used to form synthetic and polysaccharide hydrogels for drug delivery and cell injection vehicle applications.^{17–21} However, previously reported CB[8]-based hydrogel materials are not suitable for long-term cell culture due to their slow gelation and short retention times, as well as the lack of cell-adhesive domains within the utilized polymer backbones. In this work, light-initiated thiol–ene reactions between preassembled CB[8]-FGGC peptide ternary complexes and grafted norbornenes were used to rapidly cross-link the cell-adhesive biopolymer gelatin into a supramolecular hydrogel, overcoming the limitations of prior systems to yield stable hydrogels that promote the uniform distribution, viability, and spreading of encapsulated human fibroblasts over 7 days in culture. Notably, while light is used here to form the network, the gel is thereafter injectable, shear-thinning, and self-healing and, thus, can be applied directly from a syringe to another site. For therapeutic applications, this means that the gel can be administered from a syringe to a wound site without the need for an additional trigger such as light, heat, or catalyst that may adversely affect surrounding tissue.

While hydrogel networks can form when CB[8] cavitands are added to aqueous solutions of guest-functionalized polymers with mechanical agitation, gelation kinetics are typically on the order of hours due to the relatively low aqueous solubility of CB[8] ($<0.01 \text{ mM}$ in water), which serves as a rate-limiting step for cross-link formation.¹⁵ In situ photopolymerization of vinyl monomers and vinyl-functionalized guest groups precomplexed with CB[8] partially overcomes this solubility limitation by increasing the solubility of CB[8] in the precursor solution, enabling higher CB[8] loading and cross-link density.^{19,22–24} However, the in situ polymerization approach still relies on continuous stirring to inhibit CB[8] precipitation and maintain network homogeneity, and reported photoreaction times range from 30 min to 6 h under oxygen-depleted conditions. Here we develop a three-step gelation approach to exploit the enhanced aqueous solubility of CB[8] ternary complexes relative to uncomplexed CB[8] while enabling homogeneous, user-triggered gelation under cell-compatible conditions (Figure 1).

First, FGGC peptides with an N-terminal phenylalanine and a C-terminal cysteine are precomplexed with CB[8] in HEPES buffered physiological saline (Figure 1A). N-terminally charged phenylalanines can form 2:1 complexes with CB[8] with exceptionally high equilibrium binding affinities (up to 10^{11} M^{-2}),¹⁶ and FGG has previously been utilized as a guest group in hydrogels cross-linked by CB[8] homoternary complexes.¹⁹ Second, norbornene-functionalized gelatin (GelNB), prepared according to a modified literature

protocol²⁵ (Figures 1B, S1, and S2, see SI for details), is added to the mixture and fully dissolved at 37 °C. Gelatin is an abundant biopolymer derived from denatured collagen that is commonly used in 3D cell culture and regenerative medicine applications due to its high water solubility and biocompatibility, intrinsic bioactivity, and biodegradability.^{26,27} The addition of GelNB up to at least 10% w/v does not induce CB[8] precipitation or phase separation. Moreover, the precursor solution exhibits low viscosity at 37 °C, permitting facile, homogeneous incorporation of therapeutic cargo. Third, gelation is rapidly triggered by adding the water-soluble, cytocompatible photoinitiator lithium phenyl2,4,6-trimethylbenzoylphosphinate (LAP) to the solution, equilibrating to room temperature, and exposing the mixture to 365 nm light (Figure 1C), cytocompatible conditions similar to those previously reported for several different cell-encapsulating hydrogel systems in which complete gelation was observed in 10 min (the exposure time used here) or less.^{25,28–33}

Upon light exposure, step-growth photoreactions between the C-terminal cysteines in the preassembled CB[8]·FGGC peptide ternary complexes and the pendant norbornene groups on the gelatin backbone form supramolecular cross-links between gelatin chains. Although no free thiol was detected in gelatin or gelNB using Ellman's assay (Figure S3 and Table S1), consistent with previous reports,^{34–36} some covalent bonds may also form upon light exposure, complementing CB[8]-based cross-links, gelatin physical interactions,³⁷ and additional entanglements introduced by peptide branch points³⁸ to yield mechanically robust hydrogels that are stable at physiological temperature in serum-containing cell culture medium for at least 1 week. In contrast to other reported thiol-based cross-linking chemistries (e.g., thiol-vinylsulfone, thiolmaleimide, and thiol-methylsulfone),³⁹ which either occur rapidly upon component mixing or require the addition of a base catalyst that reduces cucurbit solubility and binding affinity,⁴⁰ thiol-norbornene photoreactions occur on demand without inducing CB[8] precipitation, enabling homogeneous CB[8] homoternary complex distribution prior to peptide grafting. Moreover, unlike the random chain-growth polymerization chemistries previously exploited to prepare CB[8]-based hydrogels, as well as gelatin-polyacrylate hybrid hydrogels with supramolecular cross-links between naturally occurring gelatin aromatic amino acids and pendant cyclodextrins on the in situ polymerized polyacrylate backbone,^{32,41} thiol-norbornene photoreactions are not oxygen inhibited, require relatively low radical concentrations, produce single polymer hydrogel networks, and present a reduced risk of radical-mediated damage for sensitive cell types.^{42,43}

Phenylalanine is naturally present in gelatin.³² Although CB[*n*] affinity is considerably lower for midchain aromatic amino acids that lack N-terminal ammonium groups capable of interacting with the carbonyls on the CB[*n*] portal,⁴⁴ we performed inverted vial tests at 37 °C to confirm that mixing CB[8] with gelatin or gelNB alone did not produce a temperature-stable hydrogel (Figure 2A). Additionally, vial inversions at 37 °C validated that gelatin and gelNB do not form temperature-stable hydrogels and that gelNB mixed with preassembled ternary complexes yields a low viscosity liquid prior to light exposure (Figure 2A). Further, the reduced turbidity observed when gelNB is mixed with CB[8]·FGGC complexes instead of uncomplexed CB[8] indicates that preassembly increases CB[8] solubility. Adding photoinitiator and exposing the mixture to low dose UV light for 10 min produced a temperature-stable hydrogel (Figure 2B).

The frequency and strain-dependent mechanical properties of 9% w/v gelNB hydrogels containing ~3.9 mM CB[8] and ~7.7 mM FGGC peptide (1 mol CB[8] per 2 mol peptide), 30% theoretical lysine modification) were observed at 37 °C using oscillatory shear rheology (Figures 2C, 2D). Supramolecular hydrogels are promising materials for applications involving injection or extrusion because they exhibit frequency-dependent mechanical properties, flow under applied shear, and reform rapidly upon cessation of shear.⁵ As expected, the storage (G') and loss (G'') moduli of the gels varied with frequency, with the hydrogels acting as viscoelastic solids ($G' > G''$) over the investigated frequency range (0.1 to 10 Hz at 1% strain). The storage moduli of the gels at 37 °C could be tuned from ~200 to ~400 Pa by altering either the gelNB weight percentage or the supramolecular cross-link density, while still maintaining shear-thinning behavior in a dynamic frequency sweep (Figures S4 and S5). While the reported CB[8]-based hydrogels may not be suitable for cell culture and regenerative medicine applications that require stiffness values in the kPa or higher range to achieve physiological phenotypes due to their limited modulus tunability range, soft supramolecular hydrogels with comparable storage moduli have proven useful in a range of therapeutic applications, including neural cell expansion, controlled drug release, and hard tissue regeneration.⁵ The 9% w/v supramolecular gelatin hydrogels with storage moduli of approximately 200 Pa at 37 °C were used in further studies to characterize the CB[8]-based hydrogels.

The shear-thinning and self-healing capabilities of the supramolecular gelatin hydrogels at 37 °C were confirmed using oscillatory shear rheology over multiple rounds of strain cycling (sequential shear with 0.1% strain for 120 s followed by 1000% strain for 60 s; Figure 2E). The hydrogels' ability to dissipate stress under applied strain was also examined. As expected, the supramolecular gelatin hydrogels were able to rapidly reorganize cross-links, fully relaxing to dissipate stress in under 100 s (Figure 2F). To assess utility for applications involving injection or extrusion, an injection study was performed using a supramolecular gelatin hydrogel gelled inside a 1 mL disposable plastic syringe and then equilibrated to 37 °C. The warm gel could be injected through an 18G needle into a mold and rapidly reformed on a 37 °C stage (Figure 3A). Furthermore, human fetal lung fibroblasts (MRC-5s) encapsulated in a CB[8]-based hydrogel and then injected through an 18G needle in the gelled state remained homogeneously distributed and highly viable, with greater than 94% viability observed 24 h after injection (Figure 3B). This rapid injection molding behavior makes supramolecular gelatin hydrogels attractive materials for bioprinting, regenerative medicine, and cell transplantation applications in which uniform cargo distribution and precise control over material placement are highly desirable.

Poor light transmittance can impede the observation of encapsulated cell morphology and behavior during 3D cell culture. It can also be an impediment for certain regenerative medicine applications where transparency is critical, such as materials for the eye. Previously reported CB[8] hydrogels commonly used 1:1:1 heteroternary complexes to form cross-links through simultaneous inclusion of one electron-rich guest and one electron-poor guest in the CB[8] cavitand.^{45,46} However, the resulting hydrogels are highly colored due to the formation of charge-transfer complexes between the guest groups, and uncapped charged guest groups (e.g., methyl viologen) raise cytotoxicity concerns in long-term cell culture applications.⁴⁷ FGGC guest groups, which do not undergo charge transfer when complexed

with CB[8], were selected in part to promote light transmittance through the hydrogels and to minimize interference during fluorescence imaging. Additionally, FGGC peptides composed of natural amino acids were expected to be biocompatible in both the capped and uncapped state. Further, in contrast to previous CB[8]-based hydrogels prepared using PBS,^{18,48} we used HEPES buffered saline to dissolve the hydrogel components. Similar to previous reports, dissolving the hydrogel components in PBS produced turbid gels (data not shown), likely due to charge and ionic screening interactions associated with the phosphate anions. Using the cell-compatible zwitterion HEPES as a buffering agent instead of phosphate yielded optically clear hydrogels when the gels were cast in a 0.5 mm thick mold (Figure 4A).

To further characterize the hydrogels' optical properties, 100 μ L of gel solution containing LAP was pipetted into 96-well plates and exposed to 365 nm light for 10 min, resulting in gels with thicknesses of approximately 3 mm. Absorbance values were measured over the soft UV and visible light range (350 to 800 nm) using a SpectraMax M2 microplate reader and converted to transmittance through the Beer–Lambert Law, which assumes that poor light transmittance in the samples results from light scattering rather than absorbance (Figure 4B,C). Hydrogel samples were equilibrated at either ambient temperature or 37 °C prior to measuring absorbance at the same temperature. Transmittance in 1 mm thick hydrogels was estimated by assuming that gel thickness is approximately equal to the optical path length in the Beer–Lambert Law. Light transmittance properties appeared to be independent of temperature over the tested range. While thick hydrogels exhibited relatively poor transmittance over the visible range (380–700 nm), with only $39.2 \pm 1.6\%$ and $38.6 \pm 1.6\%$ of visible light transmitted through the gels at ambient temperature and 37 °C, respectively, hydrogels with a more representative thickness for in vitro studies (1 mm) are expected to have significantly improved light transmittance properties (>70%).

To confirm that the light transmittance properties are sufficient for light microscopy observation of cell behavior during long-term 3D cell culture, we encapsulated MRC-5 cells in supramolecular gelatin hydrogels. The cell-encapsulating hydrogels were immersed in serum-containing cell culture medium for up to 1 week prior to Live/Dead staining. 3D image reconstruction showed that viable MRC-5s could be observed in the hydrogels up to a Z-axis depth of approximately 500 μ m (the upper bound on imaging depth for the microscope used) over 7 days in culture (Figure 4D–F). Moreover, the 3D reconstructions confirmed that cells encapsulated in the gels did not settle prior to the onset of gelation and remained homogeneously distributed for at least 1 week in culture.

Acute viability following light exposure was quantified using a Live/Dead cytotoxicity assay to further establish that the thiol–ene photoreactions are not cytotoxic. Two hours after encapsulation, the fibroblasts remained rounded but highly viable, with a live cell fraction of 88.7% (Figures 4D and 5A,D). To verify that the CB[8]-based hydrogels support longterm culture of encapsulated cells, MRC-5 cells were cultured within the gels for an additional 2–7 days. After 2 days in culture, the fibroblasts exhibited a well-spread morphology with distinct actin stress fibers (Figures 4E and 5B,E). After 1 week in culture, the fibroblasts remained highly viable, with a live cell fraction of 95.9%, and formed large cell clusters composed of spread cells with well-defined actin stress fibers (Figures 4F and 5C,F). While

cell-free CB[8]-based hydrogels swelled approximately 2-fold without degrading over the course of 1 week in serum-containing cell culture medium at physiological conditions (Figure S6A,B), cell density remained relatively constant during the culture period (Figure S7A), indicating that the encapsulated fibroblasts proliferate in spite of the soft microenvironment (Figure S7B). Future work will focus on tuning hydrogel swelling and retention properties through the use of tandem covalent and supramolecular crosslinks.^{41,49,50} Taken together, these results indicate that CB[8]-based gelatin hydrogels prepared using light-initiated thiol–ene reactions are biocompatible and support long-term 3D cell culture.

In conclusion, we have developed a supramolecular gelatin hydrogel system that forms via user-triggered thiol–ene reactions between grafted norbornenes and preassembled CB[8]-FGGC peptide ternary complexes. In situ conjugation of the preassembled CB[8]-guest complexes to the gelatin backbone overcomes three major limitations of previous CB[8]-based hydrogels and supramolecular gelatin materials by eliminating the need for a second synthetic, chain-growth polymer network while still enabling rapid, on-demand gelation and long-term cell culture. These hydrogels exhibited favorable optical properties and were injectable in the gelled state via an 18G syringe needle. Moreover, human fibroblasts encapsulated in CB[8]-based gelatin hydrogels remained highly viable and uniformly distributed throughout the gels for 7 days in culture. Looking forward, CB[8]-based supramolecular gelatin hydrogels represent a promising new material for use in bioprinting, regenerative medicine, cell transplantation, and fundamental studies of cell–matrix interactions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Idealized schematic of rapid, user-triggered cross-linking of CB[8]-based gelatin hydrogels under cell-compatible conditions. (A) 2:1 FGGC peptide:CB[8] homoternary complexes are formed by simultaneous inclusion of two FGGC peptides in one CB[8] cavitand, increasing the solubility of CB[8] in physiological medium. (B) Gelatin-norbornene (GelNB) is prepared by reacting carbic anhydride with primary amines in gelatin, yielding amide-linked norbornenes. (C) Supramolecular hydrogels are formed via user-triggered reactions between grafted norbornenes and preassembled CB[8]·FGGC peptide ternary complexes.

Figure 2.

CB[8]-based supramolecular gelatin hydrogels are stable, shear-thinning, and self-healing at a physiological temperature. (A) Images of gelatin, gelatin mixed with CB[8], gelNB, gelNB mixed with CB[8], and gelNB mixed with preassembled CB[8]-FGGC peptide ternary complexes at 37 °C. Inverted vials demonstrate that none of these systems form temperature-stable hydrogels without a further stimulus. (B) Temperature-stable CB[8]-based supramolecular gelatin hydrogels form following photoinitiator addition and 10 min of exposure to 365 nm light. These hydrogels exhibit (C) frequency-dependent (data presented as mean $\pm$ SD, $n = 3$) and (D) strain-dependent ($\omega = 10$ rad/s, data presented as mean $\pm$ SD, $n = 3$) mechanical properties in oscillatory shear rheology at 37 °C. Additionally, the gels can shear-thin and self-heal over multiple rounds of strain cycling (sequential shear with 0.1% strain for 120 s (white region), followed by 1000% strain for 60 s (purple region); $\omega = 10$ rad/s; data presented as mean, $n = 3$) (E) and rapidly dissipate stress under a 2% strain at 37 °C (F).

Figure 3.

(A) Preformed CB[8]-based supramolecular gelatin hydrogels can be injected through an 18G syringe needle and rapidly reform at 37 °C. (B) Cells encapsulated in preformed gels are highly viable after injection (scale bar: 200 μm). Viability data are mean $\pm$ SD, determined using four hydrogel replicates (three z-stacks per replicate), 24 h after injection.

Figure 4.

CB[8]-based supramolecular gelatin hydrogels permit light-based observation of the morphology and behavior of encapsulated MRC-5s over 7 days in culture. (A) Photograph of a 0.5 mm thick freestanding hydrogel under ambient conditions. Transmittance spectra of supramolecular gelatin hydrogels (1 mm thick (predicted), 3 mm thick (actual)) from 350 to 800 nm at ambient temperature (B) and 37 °C (C). Data are presented as error envelopes (mean $\pm$ SD, $n = 3$). Confocal 3D reconstructions of encapsulated, viable human fibroblasts, stained by calcein AM, in CB[8]-based supramolecular gelatin hydrogels after (D) 2 h, (E) 2 days, and (F) 7 days.

Figure 5. Supramolecular CB[8]-based gelatin hydrogels support the culture of MRC-5 human lung fibroblasts for 7 days in culture. Viability of MRC-5s encapsulated in the hydrogels after (A) 2 h, (B) 2 days, and (C) 7 days, as measured by a Live/Dead cytotoxicity assay. Viability data are mean $\pm$ SD, determined using 9 z-stacks (2–3 independent hydrogel replicates per condition). Confocal fluorescence micrographs showing spreading of MRC-5s cultured in CB[8]-based supramolecular gelatin hydrogels for (D) 2 h, (E) 2 days, and (F) 7 days. The actin cytoskeleton was visualized using a blue fluorescent protein (BFP) reporter (LifeAct-mTagBFP2).