

Diels—Alder Click-Cross-Linked Hydrogels with Increased Reactivity Enable 3D Cell Encapsulation

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Supporting Information



ABSTRACT: Engineered hydrogels have been extensively used to direct cell function in 3D cell culture models, which are more representative of the native cellular microenvironment than conventional 2D cell culture. Previously, hyaluronan-furan and bis-maleimide polyethylene glycol hydrogels were synthesized via Diels–Alder chemistry at acidic pH, which did not allow encapsulation of viable cells. In order to enable gelation at physiological pH, the reaction kinetics were accelerated by replacing the hyaluronan-furan with the more electron-rich hyaluronan-methylfuran. These new click-cross-linked hydrogels gel faster and at physiological pH, enabling encapsulation of viable cells, as demonstrated with 3D culture of 5 different cancer cell lines. The methylfuran accelerates Diels–Alder cycloaddition yet also increases the retro Diels–Alder reaction. Using computational analysis, we gain insight into the mechanism of the increased Diels–Alder reactivity and uncover that transition state geometry and an unexpected hydrogen-bonding interaction are important contributors to the observed rate enhancement. This cross-linking strategy serves as a platform for bioconjugation and hydrogel synthesis for use in 3D cell culture and tissue engineering.

INTRODUCTION

It is broadly recognized that conventional 2-dimensional (2D) cell culture does not adequately represent the native cellular microenvironment, necessitating the design of 3-dimensional (3D) cell culture strategies.^{1,2} Hydrogels are used extensively to engineer 3D cellular microenvironments and direct cell function. These materials can be engineered to mimic different characteristics of the extracellular matrix (ECM) including: mechanical properties, biochemical composition, and architecture.³ These biomimetic strategies enable improved understanding of the role of the ECM in disease progression and allow for additional functional readouts in higher content drug screens.^{3,4} For the latter, 3D cell culture allows cell survival as well as cell invasion into the hydrogel to be probed, which is critical to understanding highly invasive diseases, yet impossible to ascertain in conventional, 2D cell culture.^{5,6}

Hydrogels for 3D cell culture comprise bioinert polymers, such as PEG, or bioactive polymers, such as polypeptides or glycosaminoglycans. Cross-linking strategies for these materials often rely on thiol-based chemistries including Michael-type addition and radical-mediated thiol—ene chemistry; however, thiols are sensitive to oxidation, which can complicate these reactions.⁷ Strain-promoted azide–alkyne cycloaddition (SPAAC) is a more reliable alternative, but is synthetically complex.⁸ Diels–Alder chemistry, such as the inverse electron demand tetrazine–norbornene system, has been effectively used to cross-link PEG hydrogels⁹ and provides a simpler alternative to SPAAC with the same advantages of long-term stability, efficient conversion due to favorable thermodynamics, and a clean reaction profile.¹⁰

Hyaluronan hydrogels have been formed by the Diels–Alder reaction between furan-modified hyaluronan with either bismaleimide-poly(ethylene glycol) or bismaleimide peptides.^{3,5,11–14} This reaction, while useful in the synthesis of preformed gels on which cells are cultured on the surface, is unsuitable for cell encapsulation because it is inefficient at physiological pH 7.4, requiring a low pH of 5.5 for a timely reaction; however, these conditions are too acidic and too slow

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Figure 1. (A) Covalently cross-linked hydrogels can be fabricated using furan-maleimide Diels–Alder (DA) chemistry. (B) Replacing furan with methylfuran accelerates the DA reaction at pH 7.4.

for live cell encapsulation.¹¹ With a view toward utilizing these hyaluronan hydrogels for 3D cell culture, a more efficient crosslinking reaction is required at physiological pH. Although reaction kinetics can be accelerated by increasing reaction temperature, polymer concentration, and the degree of bioorthogonal functional group substitution, changing these parameters alters the porous architecture of the system, which may lead to changes in cell mechanosensing, cell-confinement, and downstream pathways.¹⁵ An alternative approach to changing the reaction kinetics is to use more reactive coupling agents.

We developed HA-based hydrogels that are cross-linked at physiological pH 7.4, thereby enabling encapsulation and 3D culture of viable cells (Figure 1). The Diels–Alder (DA) reaction was accelerated at pH 7.4 by replacing furan with the more electron-rich diene, methylfuran, that couples with maleimide cross-linkers.^{10,16,17} Using both experimental and computational studies, we gain a thorough understanding of the chemistry of the cross-linked system and demonstrate its utility for 3D cell culture.

EXPERIMENTAL SECTION

Materials. Lyophilized sodium hyaluronate (HA; 289 kDa) was purchased from Lifecore Biomedical (Claska, MN, U.S.A.). Bismaleimido-poly(ethylene glycol) ((Mal)₂PEG) was purchased from RAPP Polymere GmbH (Tülbingen, Germany). 5-Methylfurfurylamine and 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) was purchased from TCI (Boston, MA, U.S.A.). Sodium chloride and 2-(*N*-morpholino)-ethanesulfonic acid (MES) were purchased from BioShop Canada Inc. (Burlington, ON, Canada). Hyaluronidase, deuterium oxide (D₂O), and Dulbecco's phosphate buffered saline (PBS) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Dialysis membranes were purchased from Spectrum Laboratories (Rancho Dominguez, CA, U.S.A.).

Synthesis and Characterization of Furan-Modified Hyaluronan (HA-FA) and Methylfuran-Modified Hyaluronan (HÁ-mF). HA-FA was prepared as previously reported.¹⁰ Methylfuran-modified HA (HA-mF) was synthesized following the same strategy. Briefly, HA was dissolved in MES buffer (100 mM, pH 5.5) at a concentration of 1.00% w/v. DMTMM was then added, followed by the dropwise addition of 5-methylfurfurylamine. The molar equivalencies of DMTMM and furfurylamine were varied to achieve different degrees of furan substitution. After 24 h, the reaction mixture was sequentially dialyzed against 0.1 M NaCl for 1 day then distilled water for 2 days $(M_{\rm w}$ cutoff 12–14 kDa). The contents of the dialysis bag were lyophilized to isolate the HA-mF as a white fibrous material. The degree of furan substitution was determined from ¹H NMR spectra by comparing the ratio of the integrations of the methylfuran proton signals (5.98, 6.04, and 6.28 ppm) to that of the N-acetyl glucosamine peak at 2.01 ppm. ¹H NMR spectra were measured in D₂O on an

Agilent DD2-500 MHz NMR spectrometer (Santa Clara, CA, U.S.A.). Hydrogels were then prepared by combining HA-mF or HA-FA with bis-maleimide PEG in either PBS buffer (0.1 M phosphate) at pH 7.4 or MES buffer (0.1 M) at pH 5.5. All hydrogels were synthesized using HA-mF or HA-F with DS between 54 and 60% at a final concentration of HA of 1.50% w/v with a 1:0.6 molar ratio of furan to maleimide.

Rheology. The viscoelastic properties of the HA-mF or HA-FA/ PEG hydrogels were determined using an AR-1000 rheometer fitted with a 20 mm 1° acrylic cone using parallel plate geometry and a solvent trap to reduce evaporation (TA Instruments, New Castle, DE, U.S.A.). The hydrogel solutions were loaded onto the rheometer and the upper plate was lowered to a gap size of 20 μ m. A time sweep was performed at 37 °C and 0.2% strain for 8 h for HA-FA and 3 h for HAmF to allow for complete gelation. The time at which G' (shear storage modulus) crossed G" (shear loss modulus) was taken as the gel time, and the average G' from the last 10 min of the time sweep was taken to be the final G' (Figure S1). Following the time sweep, a strain sweep was conducted to ensure the strain used was within the linear viscoelastic region for the hydrogels.

Swelling. To examine the equilibrium swelling properties of the hydrogels, 100 μ L of HA-FA or HA-mF hydrogels were prepared in either pH 5.5 or pH 7.4 buffer in preweighed vials and allowed to react overnight. Samples (n = 3) were then weighed to give M_0 and then incubated in 200 μ L of PBS at 37 °C. At select time points, the swelling ratio was determined by measuring the mass (M_t) following removal of the buffer and gentle drying with a Kim wipe. Hydrogels were then replenished with fresh buffer. Swelling ratios were calculated as M_t/M_0 .

¹**H NMR Determination of Coupling Efficiency.** To examine the Diels–Alder coupling efficiency in situ, ¹H NMR was used to quantify the amount of remaining unreacted furan and methylfuran on the HA backbone following gelation with (Mal)₂PEG. A total of 100 μ L of hydrogels were prepared as above. The Diels–Alder reaction was stopped at the selected time points by the addition of pH 9.0 phosphate buffer (100 mM), which quenched the maleimide partner via ring-opening hydrolysis^{16,17} (Figure S2). The quenched hydrogels were then digested by hyaluronidase (200 U/100 μ L gel) over 2 days, lyophilized, and resuspended in D₂O for ¹H NMR analysis of the unreacted furan peaks (at 6.28 ppm for HA-mF and 7.54 ppm for HA-FA). In order to calculate the percentage of furan (or methylfuran) groups that had reacted, and the number of furan groups available before ((DS_{initial}) and after (DS_{final}) gelation were compared (eq 1) and normalized to the equivalent maleimides present:

$$(\%DS_{\text{final}} - \%DS_{\text{initial}})/60\%_{\text{mal/furan}} \times 100\%$$
(1)

Computational Methodology. Density functional theory (DFT) calculations employing hybrid functional B3LYP/6-31G(d,p)¹¹ and the IEF-PCM solvation model¹⁸ were carried out using the Gaussian 09 package on Sharcnet (Shared Hierarchical Academic Research Computing Network). The optimized geometries were then confirmed by frequency computations as minima (zero imaginary frequencies) or transition states (one imaginary frequency). Single-point calculations

were performed at the M06-2X/6-311+G(d,p)^{19–21} levels using the B3LYP/6-31G(d) optimized geometries. The HOMO and LUMO molecular orbitals and their associated energies were calculated at this M06-2X/6-311+G(d,p) level of theory. Natural bond orbital analysis (NBO), version 3.1, as implemented in Gaussian 09, was applied. Structural visualizations were performed using GaussView v5.0.8.4. A topological analysis of the electron density was carried out with Bader's quantum theory of atoms in molecules (QTAIM) using the AIM2000 software.²² The absolute value of the reaction rate constant at 311 K in water was calculated from the computed Gibbs free energy of activation.

Maintenance of Cancer Cell Lines. Breast cancer cell lines (BT474, MCF7 and T47D) were purchased from ATCC (Manassas, VA, U.S.A.). Cell lines were maintained in tissue culture flasks in an incubator (37 °C, 5% CO₂, 95% humidity) using their corresponding growth media: BT474 and MCF7 in Dulbecco's modified Eagle's medium Nutrient Mixture F-12 Ham, and T47D in RPMI-1640. All cell culture media were supplemented with 10% FBS, penicillin (10 μ mL⁻¹) and streptomycin (10 μ g mL⁻¹). T-47D and MCF-7 growth media were also supplemented with insulin (10 μ g mL⁻¹).

Patient-derived glioma neural stem cell lines (G523, G411) were contributed by the laboratory of Dr. Peter Dirks in accordance with Research Ethics Board approvals at the Hospital for Sick Children, Toronto, and the University of Toronto. Cells were maintained in Corning Primiaria flasks coated with poly(L-ornithine) and laminin (Sigma-Aldrich, St. Louis, MO, U.S.A.) using serum-free media (NS-A media StemCell Technologies, Vancouver, BC, Canada) supplemented with N-2, B27 (Life Technologies, Carlsbad, CA, U.S.A.), EGF, FGF-2, and heparin (Sigma-Aldrich, St. Louis, MO, U.S.A.), as described previously for neural stem cells.^{23–25}

3D Cell Encapsulation. Hydrogels were prepared using HA-mF and (Mal)₂PEG dissolved in the appropriate cell culture medium at pH 7.4.²⁶ Cells were mixed at a concentration of 8000 cells per 15 μ L gel in wells of a 384-well plate. Gel solutions were mixed gently with a pipet, plated, and then allowed to gel for 2 h, after which 50 μ L of media was added on top of the gels. Media was changed every 2 days. For cell distribution analysis, cell-laden hydrogels were washed with PBS twice and then fixed with 4% paraformaldehyde (PFA) 24 h following preparation. Gels were washed twice more with PBS to remove excess PFA and then treated with a 1/500 solution of Hoechst nuclear counterstain in PBS (Cell Signaling Technologies, Danvers, MA, U.S.A.). The gels were imaged using an Olympus Fluoview FV1000 confocal microscope with xy scans every 20 μ m in the zdirection. The number of cells in each imaged volume of hydrogel was quantified using Imaris Bitplane software. For live/dead viability staining, a mixture of MitoTracker Red (1:3000) and Sytox Green (1:10⁶; Thermo Fisher Scientific, Waltham, MA, U.S.A.) was added to the media and allowed to equilibrate for 2 h, following which, the gels were imaged. The number of live and dead cells in each imaged volume of hydrogel was quantified using Imaris Bitplane software.

Statistical Analysis. Statistical analyses were performed using GraphPad Prism version 6 and 7 (GraphPad Software, San Diego, CA, U.S.A.). Statistical differences among two or more groups were assessed using one-way ANOVA, followed by a Tukey posthoc test with $\alpha = 0.05$ as the criterion for statistical significance. Graphs are annotated with p values represented as $*p \le 0.05$, $**p \le 0.01$, and $***p \le 0.001$. All data are presented as mean \pm standard deviation.

RESULTS

HA-mF/Mal₂PEG Hydrogel Synthesis and Characterization. HA-mF was prepared by activating the carboxylate of HA with DMTMM, followed by coupling with the primary amine of 5-methylfurfurlylamine (Figure 2A).²⁷ The degree of methyl furan substitution on HA was determined by ¹H NMR, where the ratios of the integrals of the *N*-acetyl glucosamine peak on the HA-backbone (Figure 2B, peak a) to the aromatic furan peaks give the degree of substitution (Figure 2B, peaks b



Figure 2. Synthesis and characterization of HA-mF: (A) Synthetic scheme of DMTMM-mediated coupling between HA-COOH and amine-functionalized furan; (B) ¹H NMR spectrum of HA-mF. Degree of substitution (DS) is determined by the ratio of integration of HA-N-acetyl glucosamine peak (a) with aromatic methylfuran peaks (b and c); (C) DS can be controlled by varying stoichiometric equivalents of DMTMM and 5-methyl furfurylamine and reaction time.

and c). The degree of substitution can be controlled by varying reagent stoichiometric ratios and reaction time (Figure 2C).

We compared the bulk physical properties of the HA-FA and HA-mF cross-linked hydrogels in terms of their rate of gelation, elastic storage modulus and equilibrium swelling. Rheological measurements at pH 7.4 demonstrated that methylfuranfunctionalized hydrogels increased the gelation rate (12 \pm 2 min) compared to furan-functionalized hydrogels $(32 \pm 9 \text{ min})$; Figure 3A). This was consistent with our hypothesis that the increased electron density of methylfuran versus furan would increase the rate of DA reaction and therefore gelation rate. The differences in gelation rate did not affect the bulk properties of the resulting network: both HA-FA and HA-mF cross-linked hydrogels exhibited similar final storage moduli (G') of 1000–1300 Pa (Figure 3B), as determined from the rheological time traces (Figure S1). For the equilibrium swelling studies, HA-FA and HA-mF hydrogels were crosslinked with (Mal)₂PEG at either acidic pH 5.5 (0.1 M MES) or physiological pH 7.4 (0.1 M PBS) and then monitored for change in mass over 16 days with incubation in PBS (pH 7.4) at 37 °C. Gels cross-linked at pH 7.4 swelled more than those cross-linked at pH 5.5 (Figure 3C,D), as there were likely fewer cross-links formed at pH 7.4 due to the hydrolysis of maleimides under slightly basic conditions (Figure S2). Interestingly, no differences were observed between HA-FA and HA-mF swelling at each time point whether cross-linked at pH 5.5 or 7.4 over the first 10 days. This is consistent with the



Figure 3. Comparison of bulk properties of HA-FA and HA-mF hydrogels cross-linked with $(Mal)_2$ PEG (1.5% w/v HA 1:0.6 furan/maleimide molar equivalents). (A) Rheological determination of time to gelation of HA-mF and HA-FA (n = 3, mean + standard deviation *p < 0.5, one-way ANOVA). (B) Final elastic storage moduli (stiffness) of HA-mF and HA-FA (n = 3 mean + standard deviation). Equilibrium swelling for gels prepared at (C) pH 5.5 and (D) pH 7.4. (E) Swelling of HA-mF gels cross-linked for different amounts of times (n = 3 mean ± standard deviation, *p < 0.5, ***p < 0.001, two-way ANOVA).

rheological data that show that the HA-mF and HA-FA hydrogels have similar initial bulk mechanical properties. Notably, after 10 days, the HA-mF hydrogels seemed to swell more than HA-FA hydrogels (Figure 3C,D).

We investigated the impact of gelation time on swelling and stability and found differences in neither swelling nor stability between HA-mF hydrogels that were allowed to cross-link at pH 7.4 for 45 min, 2 h, or 24 h before the addition of swelling buffer. This demonstrates that the cross-linked HA-mF gels are stable, even when cross-linked for short periods of time (Figure 3E).

To gain insight into conjugation efficiency, we examined the hydrogels by solution-state ¹H NMR where the yield of the cross-linking reaction was calculated from the amount of unreacted furan groups in the hydrogel as a function of time (eq 1). Hydrogels were formulated with either HA-mF or HA-FA, cross-linked with $Mal_2(PEG)$ cross-linker, then digested with hyaluronidase, an enzyme that cleaves the glycosidic linkages in the HA backbone, while leaving the Diels–Alder adduct intact. Following enzymatic digestion, unreacted furan peaks were quantified using solution state ¹H NMR (Figures 4A,B and S4).

Analysis of the methylfuran-maleimide coupling over time revealed that methylfuran Diels-Alder coupling is more efficient than furan-maleimide coupling (Figure 4C). Analysis of 24 h coupling efficiencies also showed that methylfuran Diels-Alder coupling results in a higher overall conversion than that with the monosubstituted furan at both pH 5.5 and 7.4 (Figure 4D, blue vs orange bars). As the reaction proceeds in water, background hydrolysis of the maleimide remains a competing reaction at pH 7.4 and provides an alternative, degradative pathway to the slow cyclization of the HA-FA system. Since the methylfuran cyclizes far faster, the degree of competing hydrolysis is limited, leading to the higher degree of cross-linking observed in the substituted methylfuran vs furan system. Notwithstanding the more efficient cross-linking observed for methylfuran-maleimide vs furan-maleimide, the percentage of furans reacted was reduced for HA-FA and HA-mF gels synthesized at pH 7.4 vs pH 5.5 (Figure 4D, striped vs solid bars) because maleimides are susceptible to greater hydrolysis at higher pH.²⁸

In order to elucidate whether the retro-Diels–Alder (rDA) reaction was occurring over time, HA-mF and HA-FA, crosslinked with PEG-bismaleimide at physiological pH 7.4, were subjected to swelling conditions over 2 weeks and analyzed by ¹H NMR for furan peaks. In this case, an increase in the percentage of furans available over time indicates the occurrence of the retro-Diels–Alder reaction, as this would liberate NMR-detectable free furans in the system. Interestingly, we found that the methylfuran-maleimide system underwent the rDA reaction faster than the furan-maleimide system (Figure 4E). This indicates that the methylfuran reacts more quickly than the furan in both forward and reverse Diels–Alder reactions.

Computational Analysis of Forward and Reverse HAmF and HA-FA Diels–Alder Reactions. We carried out a computational analysis to better understand the mechanism accounting for the differences in gelation rates and stability observed between the HA-mF and HA-FA cross-linked gels. Our model used the HA-FA disaccharide unit (*N*-(furanylmethyl)- β -D-glucopyranosiduronamido-(1–3)- β -D-*N*-acetylglucosamine) or the HA-mF disaccharide unit (*N*-(methylfuranylmethyl)- β -D-glucopyranosiduronamido-(1–3)- β -D-*N*-acetylglucosamine) as the diene component, and an *N*methoxyethyl maleimide as the dienophile partner (Figure 5; see Supporting Information). All computational measurements



Figure 4. ¹H NMR analysis of HA-FA and HA-mF hydrogels reveals that HA-mF gels form more efficient cross-links. (A) ¹H NMR spectrum of unreacted HA-mF and (B) hyaluronidase-digested HA-mF/PEG hydrogels. (C) Calculated %total furans reacted for HA-mF and HA-FA hydrogels synthesized at pH 7.4 and quenched at different time points by the addition of pH 9 phosphate buffer. (D) %Total furans reacted for HA-mF and HA-FA hydrogels allowed to react for 24 h at pH 5.5 and pH 7.4 (n = 3-6, mean + standard deviation, **p < 0.01, ***p < 0.001 by two-way ANOVA). (E) %Furans available for HA-mF and HA-FA hydrogels prepared at pH 7.4 then swollen for 0, 7, or 14 days prior to quenching and ¹H NMR analysis. Day 0 data is replicated from (D) (pH 7.4; HA-mF and HA-FA; n = 3-6, mean ± standard deviation, **p < 0.01, ***p < 0.001 by two-way ANOVA).

were carried out at physiological temperature (311 K) using an implicit water model.

As expected, our calculations indicated that the endo transition state (TS) is lower in energy than the *exo* transition state for both the furan and the methylfuran systems (Figure 6A), and thus, we reasoned that the relative energies of the endo pathways would be the most important factor in exploring the observed rate enhancement, as it appeared to be the major product based on the experimental results. Assigning ¹H NMR resonances to the endo and exo adducts is challenging; however, in studies on similar furan-maleimide systems, a single pattern of reactivity and spectroscopic behavior is universally observed for electron-rich diene systems: the endo cycloadduct predominates, and the resonances of its olefins are always found upfield of those of its *exo* isomer.^{17,29,30} Furthermore, the two exo olefin signals often form a multiplet AB spin system, while those of the endo always provide two clean AX doublets. The experimental pattern that was observed from the spectrum in Figure 4B, two clean doublets upfield of the other olefinic resonances, is completely consistent with the presence of the endo product, and the predominance of the endo cycloadduct is

consistent with both existing theory and our computational calculations. The minor downfield signals at 6.4 ppm are consistent with the *exo* cycloadduct. This physiological-temperature Diels—Alder clearly generates one cycloadduct as the major component, and all the data strongly suggests that it is the *endo* product. Accordingly, the following computational discussion is restricted to these *endo* pathways.

Introducing the methyl substituent to furan significantly changed the energetic profile of the reaction (Figure 6A). The activation barrier for the cycloaddition process was only 16.1 kcal/mol compared to 18.3 kcal/mol for the unsubstituted system. This $\Delta\Delta G^{\ddagger}$ (2.2 kcal/mol) implied a 35-fold difference in the calculated rate constant ($k_{\rm DA}$): 31.38 s⁻¹ for HA-mF and only 0.89 s⁻¹ for HA-FA. This was broadly consistent with the experimental results.

Detailed computational analysis indicated that this difference in rate originated mainly from the differences in the occupiedvacant orbital interactions, and from the polarization stabilization interactions of the two transition states. The calculations showed a considerable difference in the energy of the HOMOs of the two systems: substituting a methyl at the 4-



Figure 5. $B3LYP/6-31G^{**}$ -calculated electron distribution and energies (eV) of the frontier molecular orbitals and the estimated band gap between the HOMOs of the dienes and the low-lying LUMO of the dienophile. Note that the electron density of the HOMO orbital is located almost entirely on the furan (except for the small lobe on the C-6 hydroxyl of the glucosamide in the case of HA-mF).

position of the furan raised the energy of its HOMO relative to the unsubstituted system (-7.35 vs -7.68 eV, Figure 5). This made the substituted system the more reactive diene as it decreased the value of the HOMO_{furan}-LUMO_{maleimide} gap (E_g = 5.72 vs 6.05 eV), directly leading to a more beneficial interaction energy profile (ΔE^{\dagger}_{int} = -30.1 in HA-mF vs -27.1 kcal/mol in HA-FA).

In addition to these more favorable orbital interactions, methyl substitution also disturbed the geometry of the optimized transition state: the emerging σ bonds were much more asymmetric in the substituted system ($\Delta\Delta d = 0.09$ Å, Figure 6B). This implied a transient unequal charge distribution; in an ideal pericyclic reaction, bond formation is synchronous, but deviation from perfect symmetry, and the implied induction in charge transfer that accompanies this process, is associated with a lower activation barrier leading to a faster reaction.³¹ Although the $\Delta\Delta d$ of the processes and, thus, the larger charge transfer, was enough to lower the energy of the HA-mF pathway, it was insufficient to change the mechanism: both *endo* reactions proceeded via concerted pathways (Figure 6A).

A final key factor favoring HA-mF reaction was the subtle stabilizing nonclassical hydrogen bond, CH_{methyl} ···O₆, between the methyl C–H bond of HA-mF and the C6 hydroxyl of the *N*-acetyl glucosamine residue. This further increased the electron-rich nature of the methyl furan substituent, contributing to the increase in the energy of the HOMO, as can be seen in Figure 5.

These combined factors help to explain the decrease in the energy barrier observed for the substituted system: improved orbital overlap due to a lower frontier molecular orbital (FMO) gap, the induction of a more polar asymmetric transition state, and the presence of a stabilizing nonclassical hydrogen bond that also contributed to the lower FMO gap.

We investigated the retro-Diels-Alder (rDA) reactivity for HA-mF versus HA-FA gels computationally, deriving the potential energy surfaces for both systems (Figure 6A). Introducing the methyl substituent proved to be beneficial in this case as it increased the exergonicity of the reaction by 1.3 kcal/mol relative to the unsubstituted system ($\Delta G_r = -9.6$ vs -8.3 kcal/mol). Consequently, not only was the substituted furan kinetically preferred, it was also the thermodynamically more stable product. However, this improvement in product stability was not as large as the difference in the transition state energies between the two systems ($\Delta\Delta G^{\ddagger} > \Delta\Delta G_{\text{prod}}$), and consequently, the ΔG_{rDA}^{\dagger} is smaller for the substituted system (25.8 kcal/mol) than for the unsubstituted furan (26.6 kcal/ mol). This implied that the reverse reaction was slightly more facile for the methylfuran than the furan, reflecting our experimental observations and corroborating that both the forward and reverse Diels-Alder (rDA) reactions occurred more rapidly in the methylfuran than in the furan substituted system.

HA-mF Cross-Linked Hydrogels Enable 3D Cell Culture. One of the benefits of the HA-mF/(Mal)₂PEG system is the ability to rapidly form hydrogels at physiologically relevant pH 7.4 and the subsequent possibility of cell encapsulation during gelation. We investigated these crosslinked gels for 3D cell culture with a diversity of cells to gain insight into its utility as a platform technology. We tested five different cancer cell lines, three breast cancer cell lines, BT474, MCF7 and T47D, and two glioblastoma cells isolated and cultured from two primary glioblastoma biopsies, G523 and G411. We found that the cells remained viable and dispersed during the encapsulation and subsequent cell culture studies. Encapsulation of MCF-7 cells with the HA-mF gels yielded evenly distributed cells throughout the depth of the hydrogel whereas encapsulation in HA-FA gels resulted in 46% of the cells at the bottom 50 μ m of the well (Figure 7A). Furthermore, we observed that >80% of the breast cancer cells remained viable over a 7-day period (Figure 7B-E), as determined by live/dead staining with Mitotracker (red, live cells) and Sytox (green, dead cells). Moreover, MCF7 cells displayed characteristic spheroid formation in these hydrogels (Figure 7B), reflecting their typical in vivo morphology.³ Encapsulated patient-derived glioblastoma stem cells, G523 and G411, also remained viable in these hydrogels (Figure 7F,G).

DISCUSSION

Diels–Alder chemistry is widely used in polymer conjugation strategy and advantageous in biomacromolecule functionalization as it requires no initiators, creates no byproducts, and proceeds well in aqueous environments.³⁴ While the furan-maleimide chemistry is effective in the synthesis of cross-linked hydrogels,^{11,28,34,35} it is inherently limited to acidic aqueous pH environments and hence unsuitable for cell encapsulation. The use of methylfuran instead of furan has a profound influence on the use of physiologically relevant pH 7.4 for hydrogel formation and subsequent 3D cell culture.

By manipulating the electronics of the furan ring with the inclusion of a methyl group, the Diels–Alder reaction kinetics were adjusted such that we obtained a more rapid transformation. The rate of methylfuran–maleimide reactions have been previously predicted to be faster than furan-maleimide via computational methods as the presence of the methyl group in the furan ring lowers the free energy of the transition state;⁵ however, the calculations were done on simple 2-substituted



Figure 6. (A) M06-2X/6-311+G(d,p) calculated potential energy profiles for the DA cycloadditions and cycloreversions of truncated maleimide with HA-FA and HA-mF, with relative free energies shown in kcal/mol. The *endo*-pathways are in gray; the *exo*-pathways are in red. The route to the right described the reaction with HA-mF; the reaction to the left was with HA-FA. (B) Calculated optimized geometries of the *endo* transition states involved in the Diels–Alder reactions of furan-modified hyaluronan (HA-FA, left) and methylfuran-modified hyaluronan (HA-mF, right) with maleimide. The full structures are provided in the SI. This expansion portrays the reactive functionalities as tube models, and the remainder of the structure is portrayed as a ball and stick model. The difference in bond length (Δd) is defined as the difference between the C1–C4 and C2–C3 bonds in each transition state.

furans, not the 2,5-disubstituted furans used in the current carbohydrate-supported platform. Our calculations show that this trend was maintained in our system and that the energy difference between the *endo* and *exo* transition states was greater than that expected from the simple model system due to the cooperative effects between the furan and the hyaluronan backbone. This includes the nonclassical hydrogen bond between the methyl group of the methyl furan and the carbonyl of the maleimide. These weak interactions are increasingly being recognized for their important role in organizing and stabilizing transition states and preferred geometries in crystal structures.^{35–37} To better quantify the strength of this stabilizing interaction, we used natural bond

orbital (NBO) analysis³⁸ and estimated that the strength of the $nO_6 \rightarrow \sigma^* CH_{methyl}$ interaction E(NBO) was 1.9 kcal/mol; this is a strong, but by no means unprecedented, nonclassical hydrogen bond. Evidence for this interaction is further supported by a quantum theory of atoms-in-molecule^{35–37} analysis that indicates an elongation of the C–H bond, and the presence of a 2.5 Å CH_{methyl}···O₆ nonclassical hydrogen bond (Figure S3). This is longer than classical hydrogen bonds that are below 2.0 Å. This stabilizing nonclassical H-bond interaction was not available in the higher energy HA-FA transition state, as no atom can be so positioned. This effect is an important factor in favoring the substituted system and suggests that these types of noncovalent interactions in the



Figure 7. Encapsulated cells are well-distributed and remain viable. (A) Histograms for percentage of cells found at varying gel depths for MCF-7 cells encapsulated in 1.5 wt % HA-mF or HA-FA/PEG(Mal)₂ hydrogels (n = 8 technical replicates, mean + standard deviation). (B) 3D z-stack reconstruction of encapsulated MCF-7 breast cancer cells in HA-mF hydrogel at day 1 and day 7. Cells are stained with MitoTracker red (live stain) and Sytox Green (dead stain). (C–E) Quantification of cell viability from n = 3 biological replicates for three different breast cancer cell lines: (C) MCF7, (D) BT474, and (E) T47D and two patient-derived glioma neural stem cell lines (F) G523 and (G) G411.

transition states of pericyclic reactions need to be carefully considered along with the traditional electronic analyses.

Investigating the retro-Diels–Alder behavior intimated that the systems were thermally stable at ambient or physiological temperatures. Previous accounts have shown that cycloreversion in similar systems occurs at significant rates only at elevated temperatures, above 80 °C,^{22,23} which is far beyond the range that would be seen with biological systems. The methyl-substituted system was calculated to more readily undergo cycloreversion than the monosubstituted furan, corroborating the experimental results.

The major limitation of our computational model was that it did not account for the expected cooperativity effects that emerged as cross-linking progressed. The computationally derived rate enhancement only pertained to a single furanmaleimide, whereas the experimentally obtained gelation time required several cross-links in order to form the hydrogel. Notwithstanding this limitation, our computational findings provide an excellent model for initial rates of cycloadduct formation and elucidate that differences in the transition states of the two processes contributed to the observed rate enhancement.

A disadvantage of furan-maleimide chemistry is the hydrolytic susceptibility of the maleimide unit.^{12,14,39} The increased rate of cycloaddition with use of the methylfuran (vs furan) helped to alleviate this challenge, leading to a more cross-linked scaffold. However, the calculations and experiments suggest that, compared to furan-cross-linked hydrogels, the methylfuran-cross-linked hydrogels degraded more quickly due to a faster reverse reaction. While the HA-mF hydrogels started to degrade after 7 days, their cytocompatibility for cell encapsulation and culture demonstrated their benefit for studies where this time frame is suitable.

3D cell culture using chemically defined hydrogels provides an important platform for studying the effects of the cellular microenvironment on cell fate.¹⁶ With well-defined hydrogel matrices, the roles of different factors on cell fate can be decoupled. For example, the role of matrix mechanics, biochemical properties, and physical architecture have all been shown to influence cell fate.⁴⁰ The synthetic accessibility of the methylfuran/maleimide Diels–Alder reaction is promising for both hydrogel synthesis and biocompatible conjugation strategies, making it a suitable platform for broad applicability in, for example, drug screening assays of cancer cells in a biomimetic environment or in tissue engineering.

CONCLUSIONS

We demonstrated that methylfuran-maleimide click-crosslinked hydrogels can be synthesized efficiently at pH 7.4, enabling the encapsulation and 3D culture of cells. The rate of the conventional furan-maleimide reaction was increased by replacing furan with the more electron-rich methylfuran. Using both experimental and computational techniques, we demonstrated that both the forward and reverse Diels–Alder reactions of the methylfuran–hyaluronan cross-linked gel were faster than that of the furan-hyaluronan gel. The computational analyses not only corroborated the experimental data, but also provided insight into the structural detail of the mechanisms, which will assist with further development of improved crosslinking agents.

ASSOCIATED CONTENT

S Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bio-mac.7b01715.

Sample rheological time sweep for HAmF hydrogel; proposed mechanism of maleimide hydrolysis; selected DFT energy-minimized geometries of the transition states, precomplexes, products, and the related reactants; and the thermochemical data for the Diels–Alder reaction of furan-modified hyaluronan (HA-FA) and methyl furan-modified hyaluronan (HA-mF) with maleimide (PDF).

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ORCID [®] John F. Trant: 0000-0002-4780-4968 R.Y.T., A.E.G.B., and N.B.M. S.M.T. performed all computational calculations and analyses, guided by J.F.T. M.S.S. guided the experimental work and reviewed and revised the manuscript. The manuscript was written with contributions of all authors. All authors approve the final version of the manuscript. **Notes**

L.J.S. performed all experimental work, with contributions from

The authors declare no competing financial interest.

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Author Contributions

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ABBREVIATIONS

ECM, extracellular matrix; PEG, polyethylene glycol; SPAAC, strain-promoted azie-alkyne cycloaddition; HA, hyalyronan; HA-FA, hyaluronan-furan; HA-mF, hyaluronan methylfuran; DA, Diels–Alder; TS, transition state; rDA, reverse Diels–Alder

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