Rapid Diels-Alder Crosslinking of Cell Encapsulating Hydrogels

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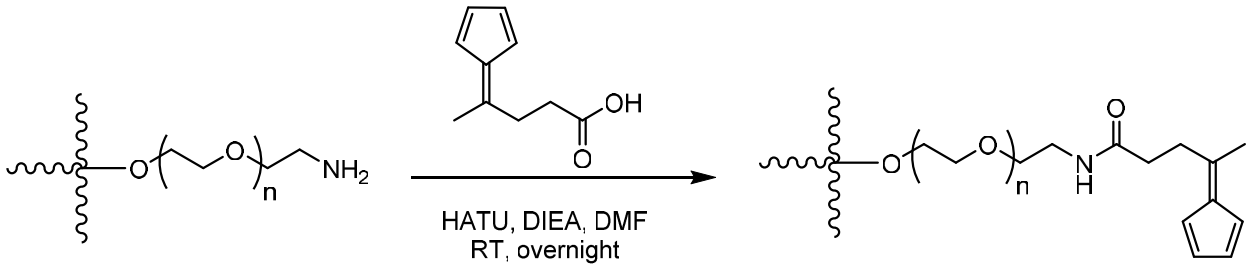
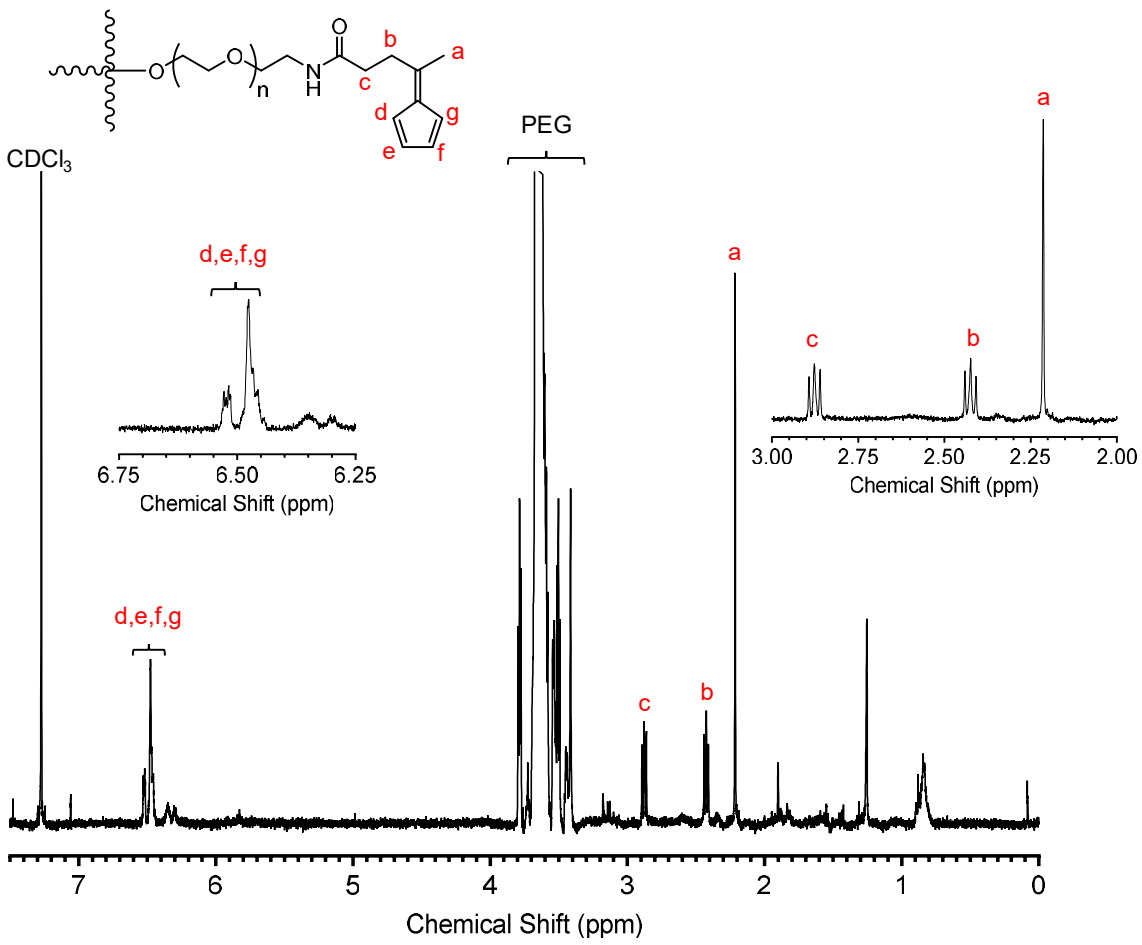
Supporting Information

1. Experimental Protocols

A. Synthesis of 4-arm PEG-fulvene

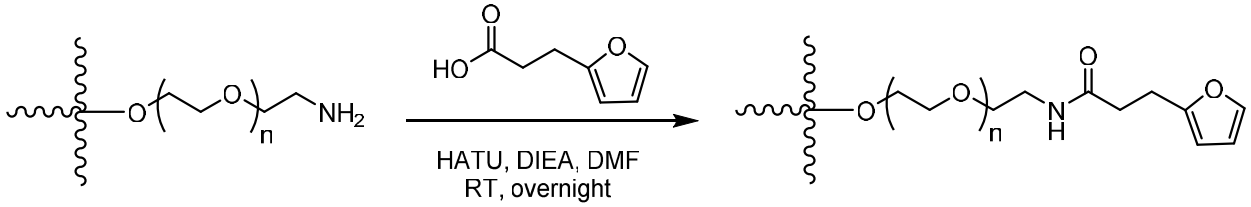


4-arm PEG-amine (200 mg, MW ~ 20 kDa) was added to a 25 mL round bottom flask and dissolved in anhydrous DMF (3 mL). In a separate vial, 4-(cyclopenta-2,4-dien-1-ylidene)pentanoic acid (26.3 mg, 4 eq relative to amines) and HATU (91.3 mg, 6 eq) were dissolved in anhydrous DMF (2 mL). To this solution, *N,N*-diisopropylethylamine (83.6 µL, 12 eq) was added, and the mixture was stirred for 10 minutes at room temperature. The mixture was then added dropwise to the stirring PEG solution, and the reaction was allowed to proceed overnight at room temperature. The fulvene-modified PEG was precipitated by dropwise addition to ice cold diethyl ether and collected by centrifugation. The pellet was washed with ice cold diethyl ether (2×25 mL) and dried under a stream of compressed air. The resulting yellow solid was redissolved in MilliQ water (5 mL) and dialyzed against MilliQ water (MWCO 2 kDa, 4°C, 4×4 L). The resulting solution was frozen at -80°C and lyophilized to afford the 4-arm PEG-fulvene as a yellow solid (181 mg, 88% yield). ^{1}H NMR characterization is presented below. The degree of substitution was estimated to be 4 fulvenes per PEG molecule by comparing the integrated intensity of the fulvene peaks (4H, 6.55-6.45 ppm) with the integrated intensity of a peak corresponding to the end groups of the PEG arms (2H, 3.44 ppm).

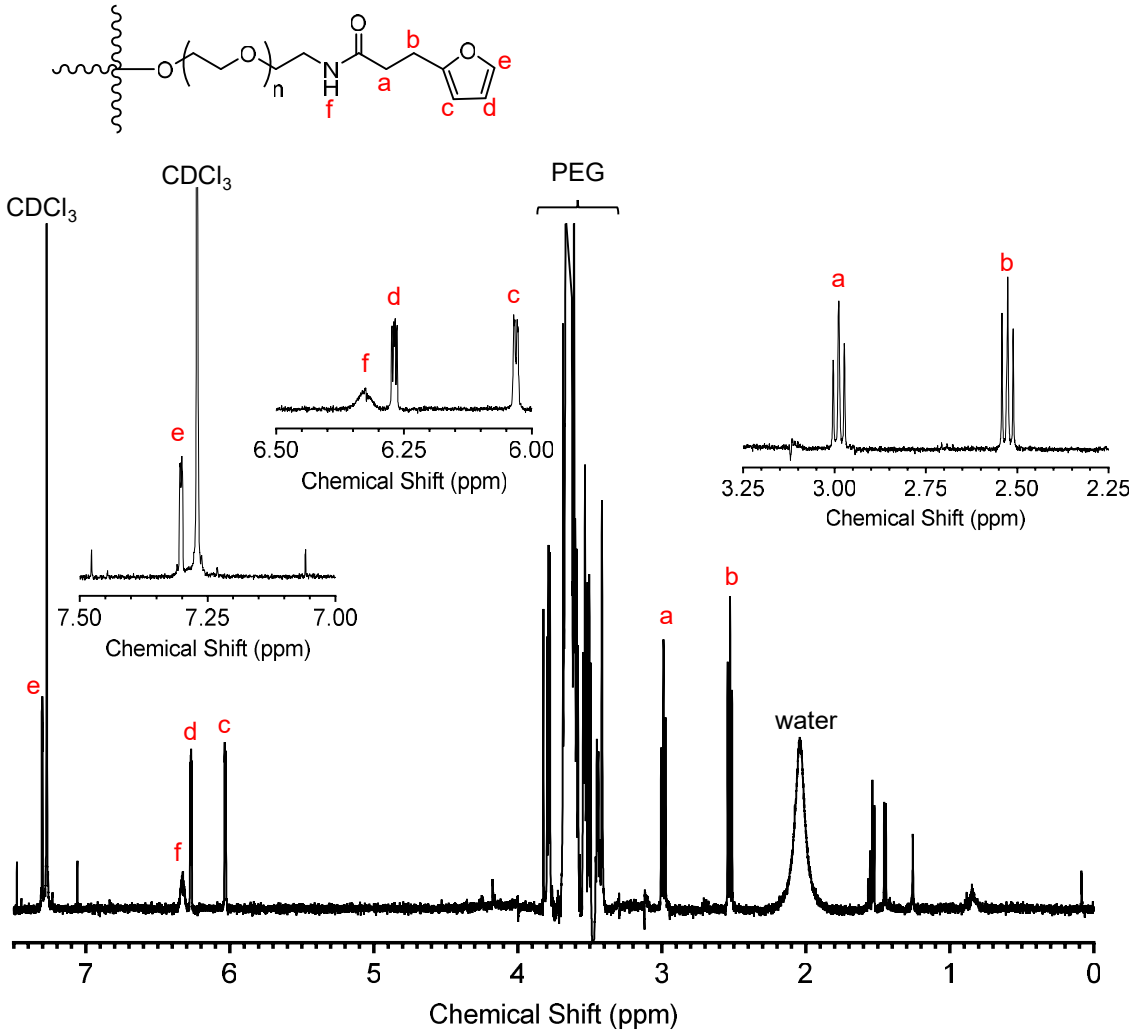


^{1}H NMR characterization of 4-arm PEG-fulvene (500 MHz, $CDCl_3$).

B. Synthesis of 4-arm PEG-furan

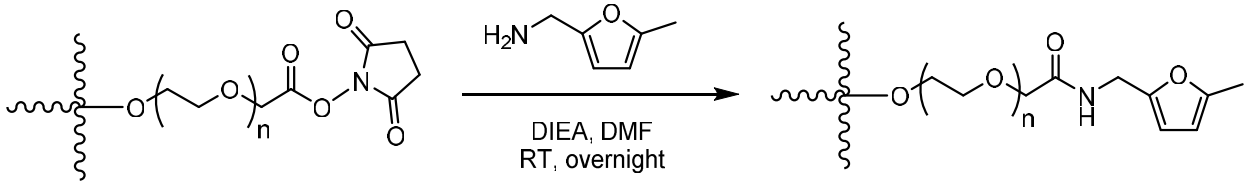


4-arm PEG-amine (200 mg, MW ~ 20 kDa) was added to a 25 mL round bottom flask and dissolved in anhydrous DMF (3 mL). In a separate vial, 3-(2-furyl)propionic acid (22.4 mg, 4 eq relative to amines) and HATU (91.3 mg, 6 eq) were dissolved in anhydrous DMF (2 mL). To this solution, *N,N*-diisopropylethylamine (83.6 μL, 12 eq) was added, and the mixture was stirred for 10 minutes at room temperature. The mixture was then added dropwise to the stirring PEG solution, and the reaction was allowed to proceed overnight at room temperature. The furan-modified PEG was precipitated by dropwise addition to ice cold diethyl ether and collected by centrifugation. The pellet was washed with ice cold diethyl ether (2×25 mL) and dried under a stream of compressed air. The resulting light brown solid was redissolved in MilliQ water (5 mL) and dialyzed against MilliQ water (MWCO 2 kDa, 4°C, 4×4 L). The resulting solution was frozen at -80°C and lyophilized to afford the 4-arm PEG-furan as a light brown solid (203 mg, 99% yield). ^{1}H NMR characterization is presented below. The degree of substitution was estimated to be 4 furans per PEG molecule by comparing the integrated intensity of the furan peaks (1H, 7.30 ppm; 1H, 6.27 ppm; 1H, 6.03 ppm) with the integrated intensity of a peak corresponding to the end groups of the PEG arms (2H, 3.44 ppm).

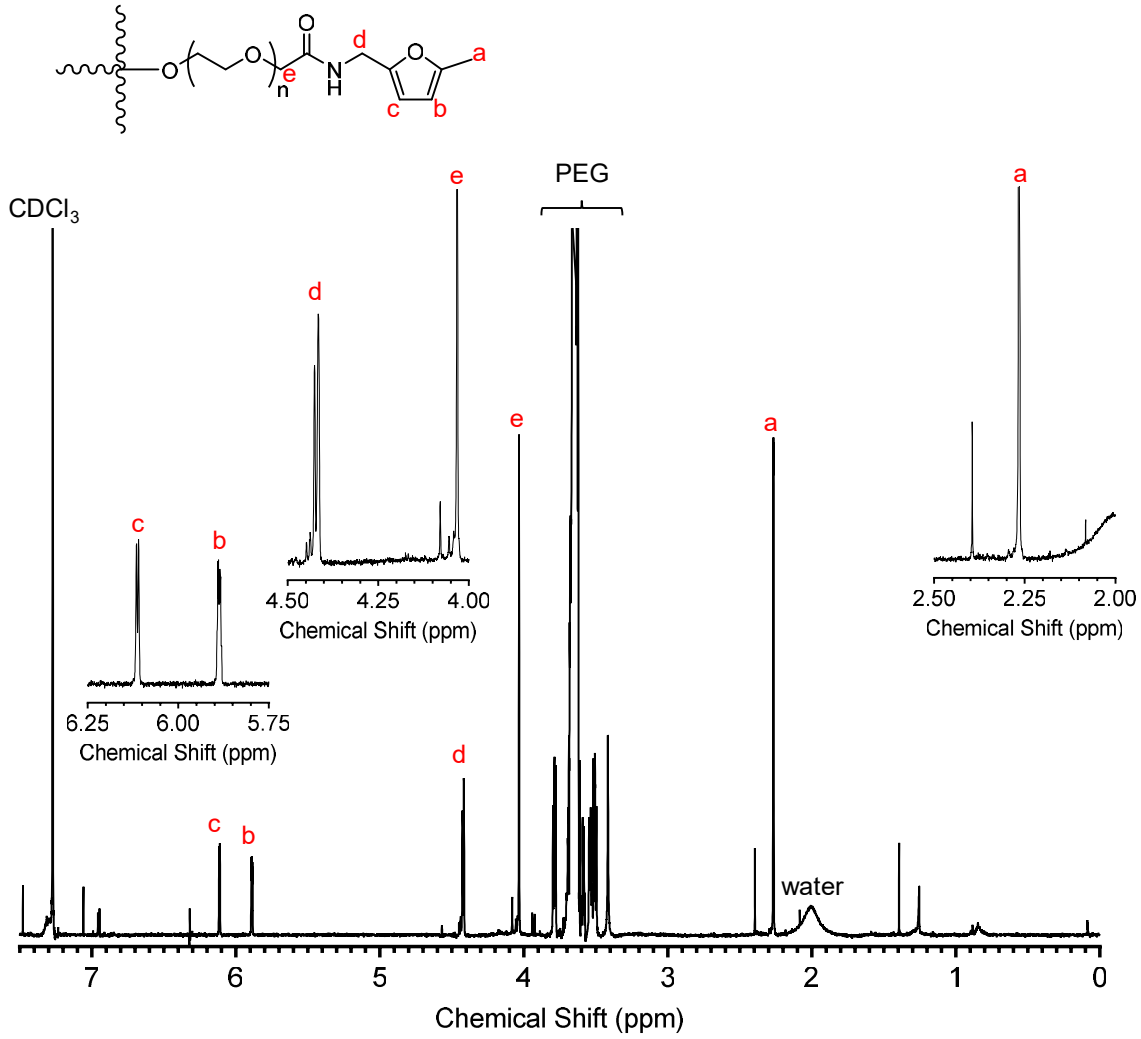


^{1}H NMR characterization of 4-arm PEG-furan (500 MHz, $CDCl_3$).

C. Synthesis of 4-arm PEG-methylfuran

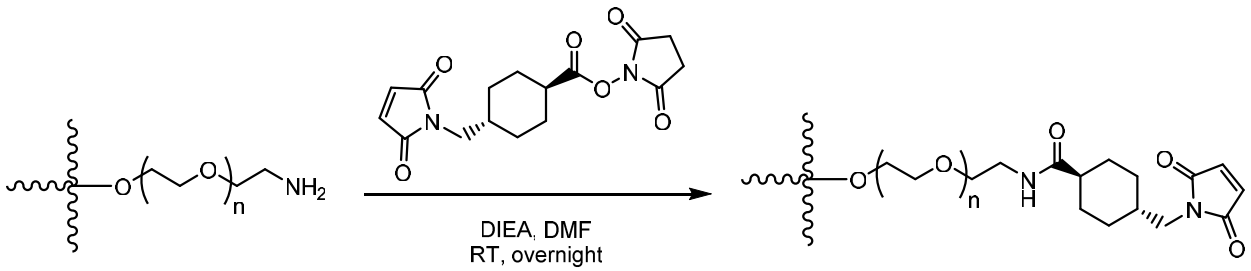


4-arm PEG succinimidyl carboxymethyl ester (200 mg, MW ~ 20 kDa) was added to a 25 mL round bottom flask and dissolved in anhydrous DMF (4 mL). To the stirring PEG solution, 5-methylfurfurylamine (TCI; 17.9 μL, 4 eq relative to NHS esters) and *N,N*-diisopropylethylamine (27.9 μL, 4 eq) were added sequentially. The reaction was allowed to proceed overnight at room temperature. The reaction mixture was diluted into MilliQ water (20 mL) and dialyzed against MilliQ water (MWCO 2 kDa, 4°C, 4×4 L). The resulting solution was frozen at -80°C and lyophilized to afford the 4-arm PEG-methylfuran as a white solid (184 mg, 92% yield). ^{1}H NMR characterization is presented below. The degree of substitution was estimated to be 3.4 methylfurans per PEG molecule by comparing the integrated intensity of the methylfuran peaks (1H, 6.11 ppm; 1H, 5.88 ppm) with the integrated intensity of a peak corresponding to the end groups of the PEG arms (2H, 3.50 ppm).

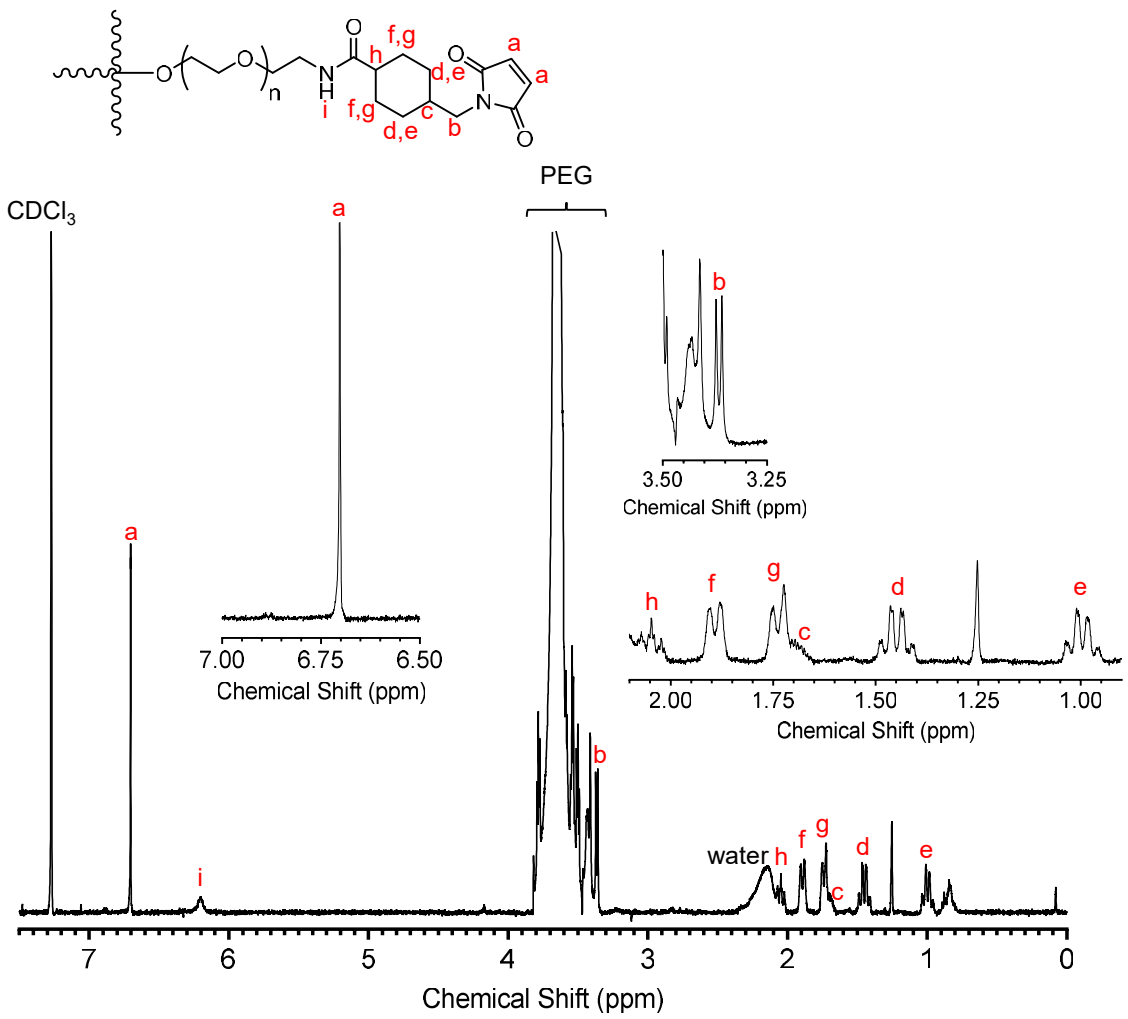


^{1}H NMR characterization of 4-arm PEG-methylfuran (500 MHz, $CDCl_3$).

D. Synthesis of 4-arm PEG-maleimide

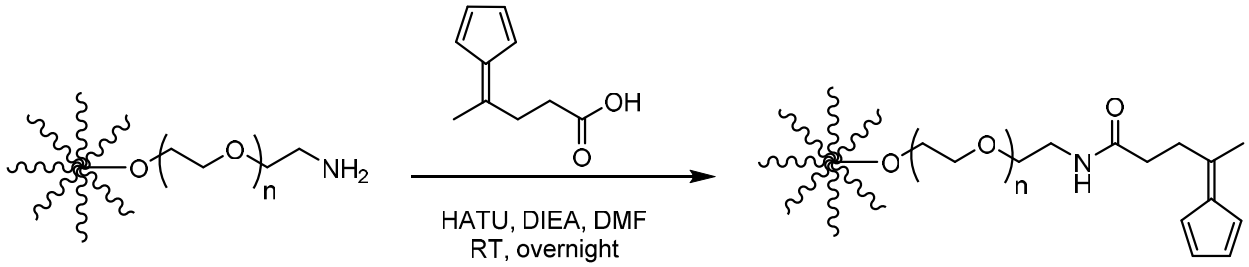


4-arm PEG-amine (200 mg, MW ~ 20 kDa) and SMCC (20.1 mg, 1.5 eq relative to amines) were added to a 25 mL round bottom flask and dissolved in anhydrous DMF (4 mL). *N,N*-diisopropylethylamine (20.9 μL, 3 eq) was added, and the reaction was allowed to proceed overnight at room temperature. The maleimide-modified PEG was precipitated by dropwise addition to ice cold diethyl ether and collected by centrifugation. The pellet was washed with ice cold diethyl ether (2×25 mL) and dried under a stream of compressed air. The resulting white solid was redissolved in MilliQ water (5 mL) and dialyzed against MilliQ water (MWCO 2 kDa, 4°C, 4×4 L). The resulting solution was frozen at -80°C and lyophilized to afford the 4-arm PEG-maleimide as a white solid (174 mg, 83% yield). ^{1}H NMR characterization is presented below. The degree of substitution was estimated to be 4 maleimides per PEG molecule by comparing the integrated intensity of the maleimide peak (2H, 6.70 ppm) with the integrated intensity of a peak corresponding to the end groups of the PEG arms (2H, 3.44 ppm).

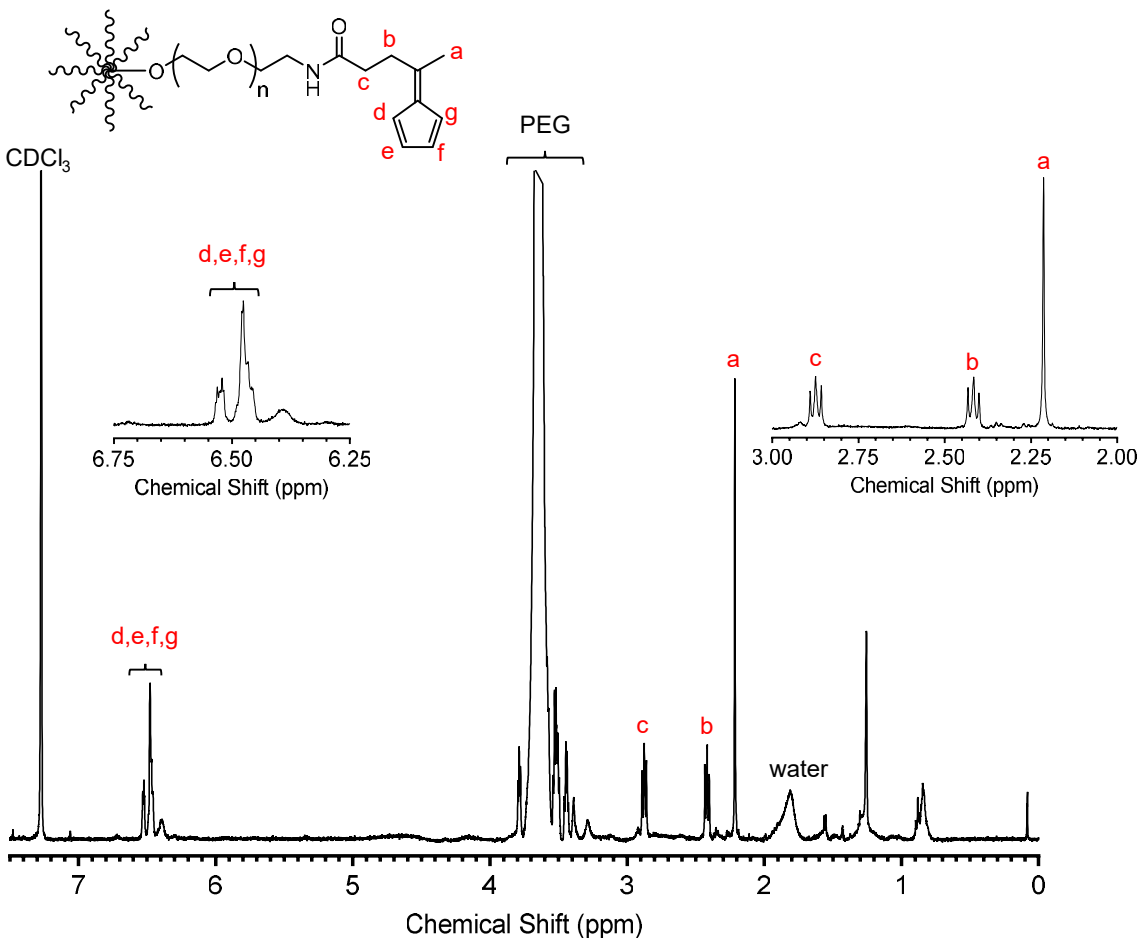


^{1}H NMR characterization of 4-arm PEG-maleimide (500 MHz, $CDCl_3$).

E. Synthesis of 8-arm PEG-fulvene

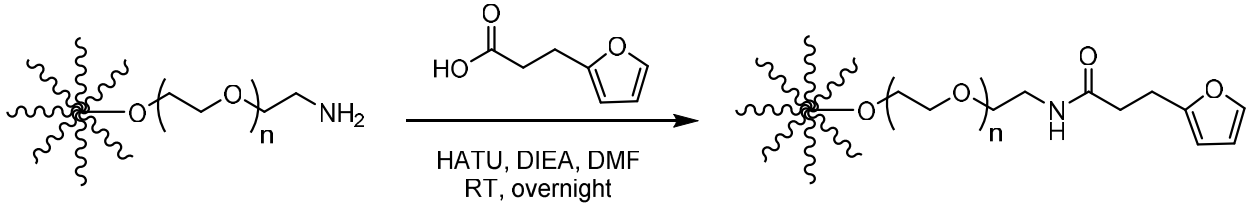


8-arm PEG-amine (100 mg, MW ~ 20 kDa) was added to a 25 mL round bottom flask and dissolved in anhydrous DMF (3 mL). In a separate vial, 4-(cyclopenta-2,4-dien-1-ylidene)pentanoic acid (26.3 mg, 4 eq relative to amines) and HATU (91.3 mg, 6 eq) were dissolved in anhydrous DMF (2 mL). To this solution, *N,N*-diisopropylethylamine (83.6 µL, 12 eq) was added, and the mixture was stirred for 10 minutes at room temperature. The mixture was then added dropwise to the stirring PEG solution, and the reaction was allowed to proceed overnight at room temperature. The fulvene-modified PEG was precipitated by dropwise addition to ice cold diethyl ether and collected by centrifugation. The pellet was washed with ice cold diethyl ether (2×25 mL) and dried under a stream of compressed air. The resulting yellow solid was redissolved in MilliQ water (5 mL) and dialyzed against MilliQ water (MWCO 2 kDa, 4°C, 4×4 L). The resulting solution was frozen at -80°C and lyophilized to afford the 8-arm PEG-fulvene as a yellow solid (70.3 mg, 66.4% yield). ^{1}H NMR characterization is presented below. The degree of substitution was estimated to be 8 fulvenes per PEG molecule by comparing the integrated intensity of the fulvene peaks (4H, 6.55-6.45 ppm) with the integrated intensity of a peak corresponding to the end groups of the PEG arms (2H, 3.44 ppm).

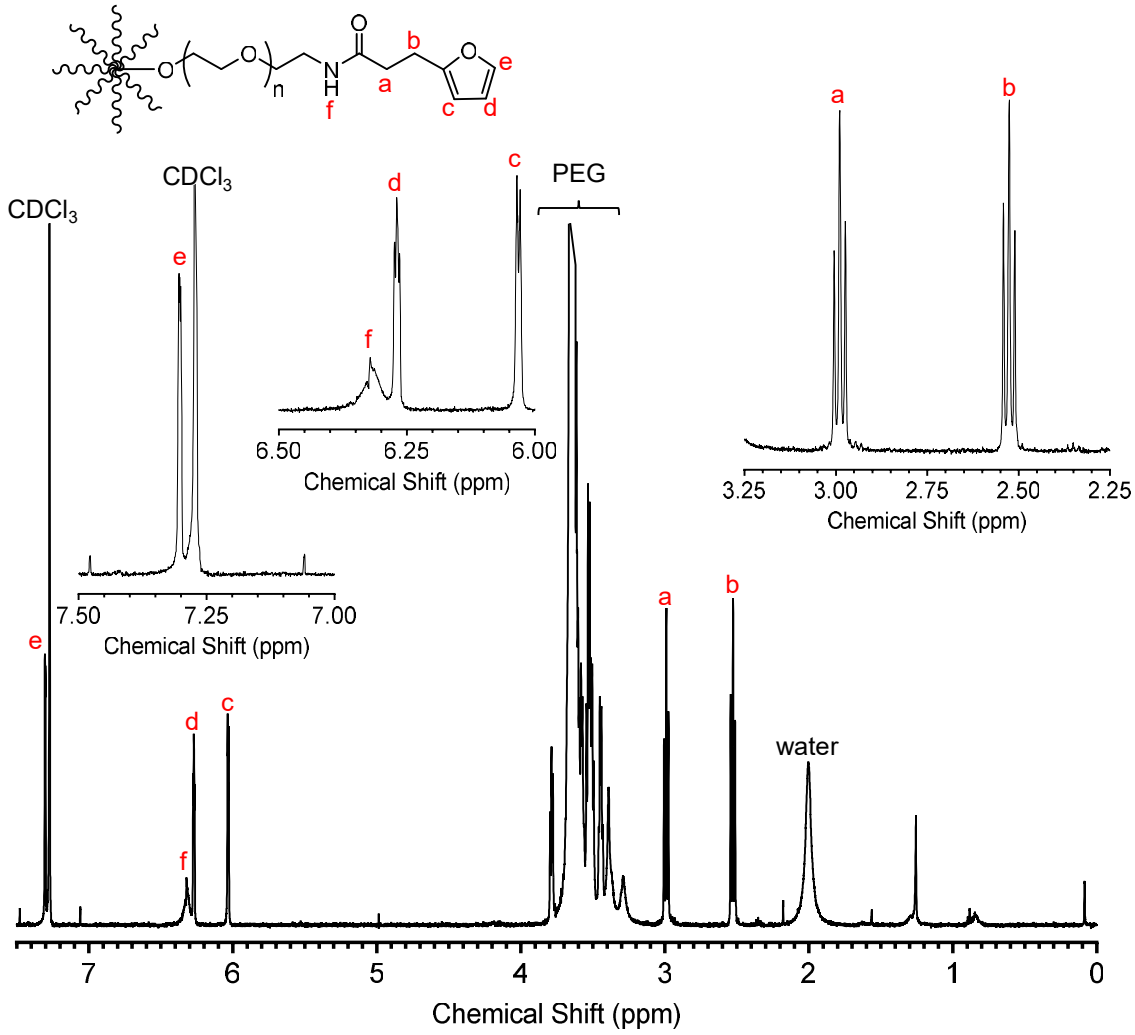


^{1}H NMR characterization of 8-arm PEG-fulvene (500 MHz, $CDCl_3$).

F. Synthesis of 8-arm PEG-furan

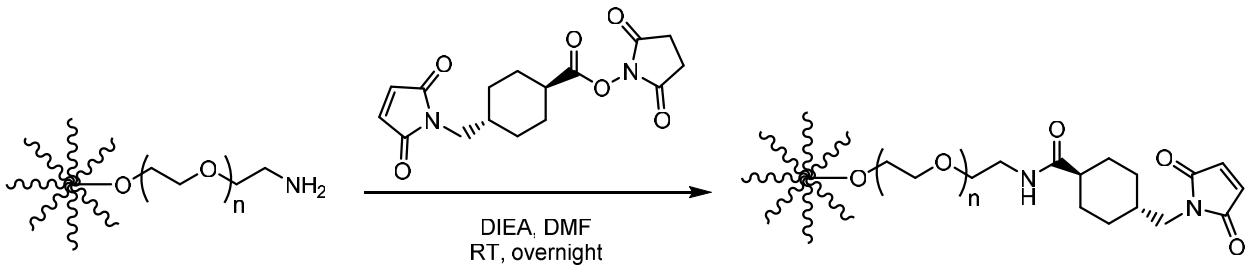


8-arm PEG-amine (100 mg, MW ~ 20 kDa) was added to a 25 mL round bottom flask and dissolved in anhydrous DMF (3 mL). In a separate vial, 3-(2-furyl)propionic acid (22.4 mg, 4 eq relative to amines) and HATU (91.3 mg, 6 eq) were dissolved in anhydrous DMF (2 mL). To this solution, *N,N*-diisopropylethylamine (83.6 μL, 12 eq) was added, and the mixture was stirred for 10 minutes at room temperature. The mixture was then added dropwise to the stirring PEG solution, and the reaction was allowed to proceed overnight at room temperature. The furan-modified PEG was precipitated by dropwise addition to ice cold diethyl ether and collected by centrifugation. The pellet was washed with ice cold diethyl ether (2×25 mL) and dried under a stream of compressed air. The resulting light brown solid was redissolved in MilliQ water (5 mL) and dialyzed against MilliQ water (MWCO 2 kDa, 4°C, 4×4 L). The resulting solution was frozen at -80°C and lyophilized to afford the 8-arm PEG-furan as a light brown solid (65.4 mg, 62.4% yield). ^{1}H NMR characterization is presented below. The degree of substitution was estimated to be 8 furans per PEG molecule by comparing the integrated intensity of the furan peaks (1H, 7.30 ppm; 1H, 6.27 ppm; 1H, 6.03 ppm) with the integrated intensity of a peak corresponding to the end groups of the PEG arms (2H, 3.44 ppm).

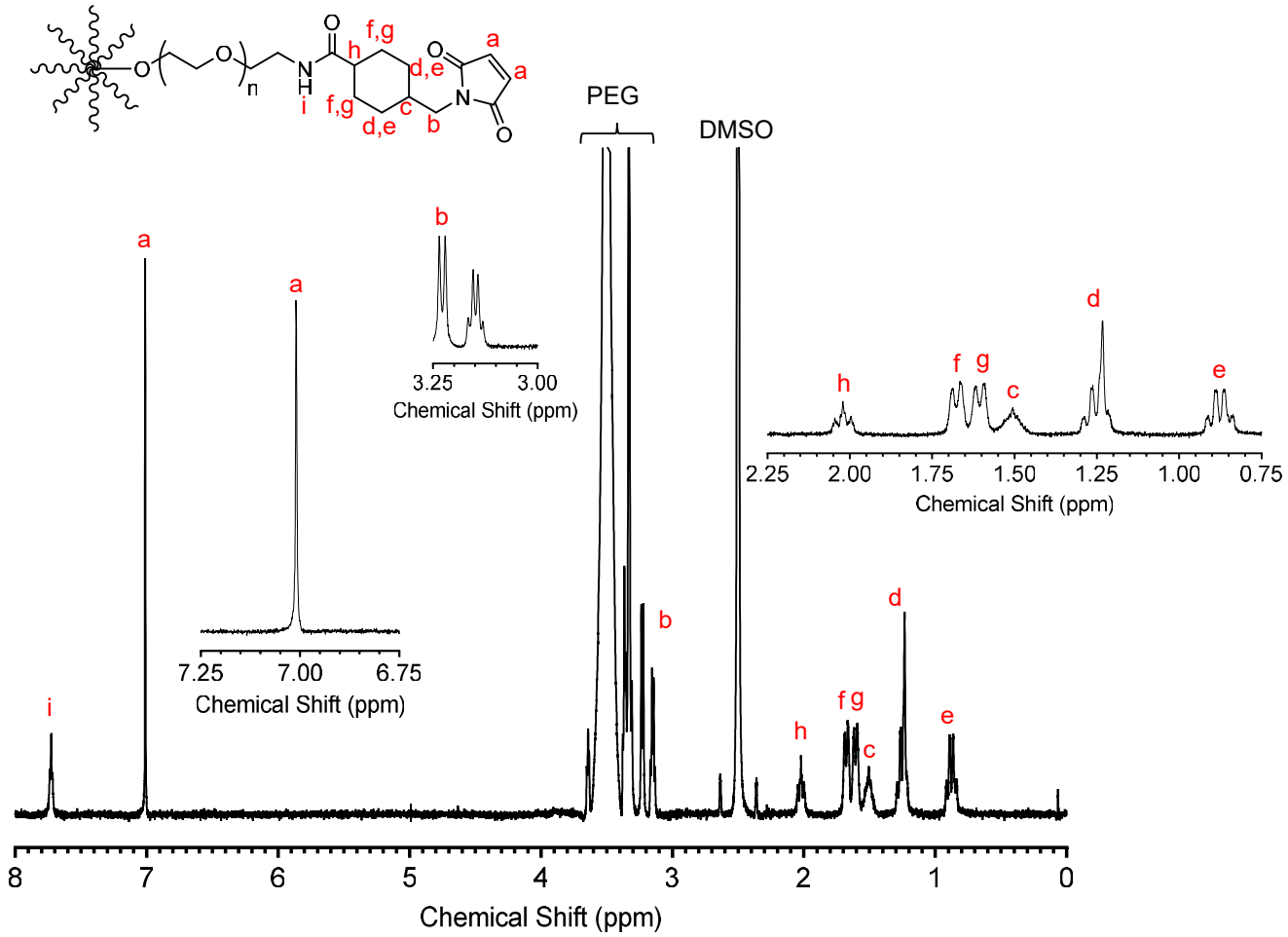


^{1}H NMR characterization of 8-arm PEG-furan (500 MHz, $CDCl_3$).

G. Synthesis of 8-arm PEG-maleimide

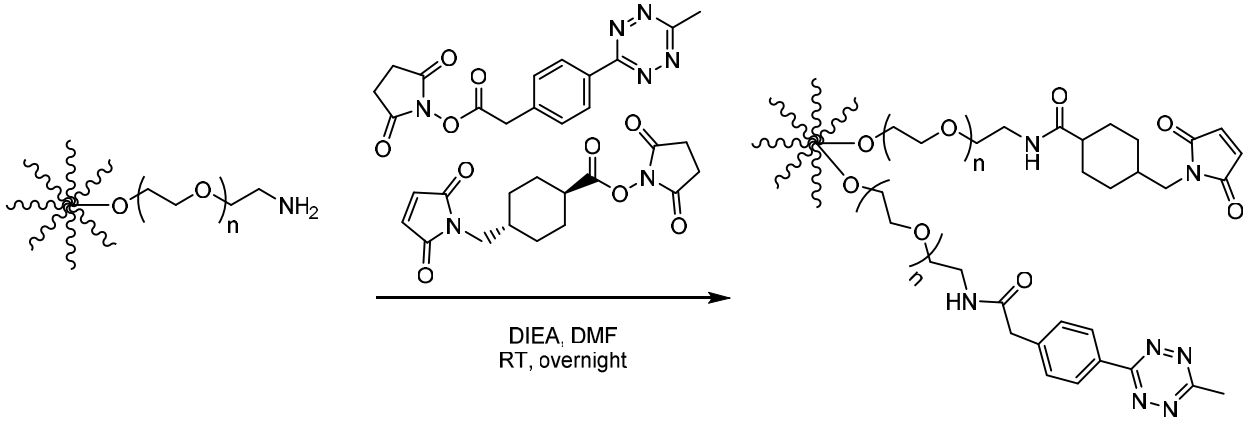


8-arm PEG-amine (100 mg, MW ~ 20 kDa) and SMCC (16.1 mg, 1.2 eq relative to amines) were added to a 25 mL round bottom flask and dissolved in anhydrous DMF (4 mL). *N,N*-diisopropylethylamine (16.7 μL, 2.4 eq) was added, and the reaction was allowed to proceed overnight at room temperature. The maleimide-modified PEG was precipitated by dropwise addition to ice cold diethyl ether and collected by centrifugation. The pellet was washed with ice cold diethyl ether (2×25 mL) and dried under a stream of compressed air. The resulting white solid was redissolved in MilliQ water (5 mL) and dialyzed against MilliQ water (MWCO 2 kDa, 4°C, 4×4 L). The resulting solution was frozen at -80°C and lyophilized to afford the 8-arm PEG-maleimide as a white solid (77.3 mg, 71.1% yield). ^{1}H NMR characterization is presented below. The degree of substitution was estimated to be 8 maleimides per PEG molecule by comparing the integrated intensity of the maleimide peak (2H, 7.01 ppm) with the integrated intensity of a peak corresponding to the end groups of the PEG arms (2H, 3.15 ppm).

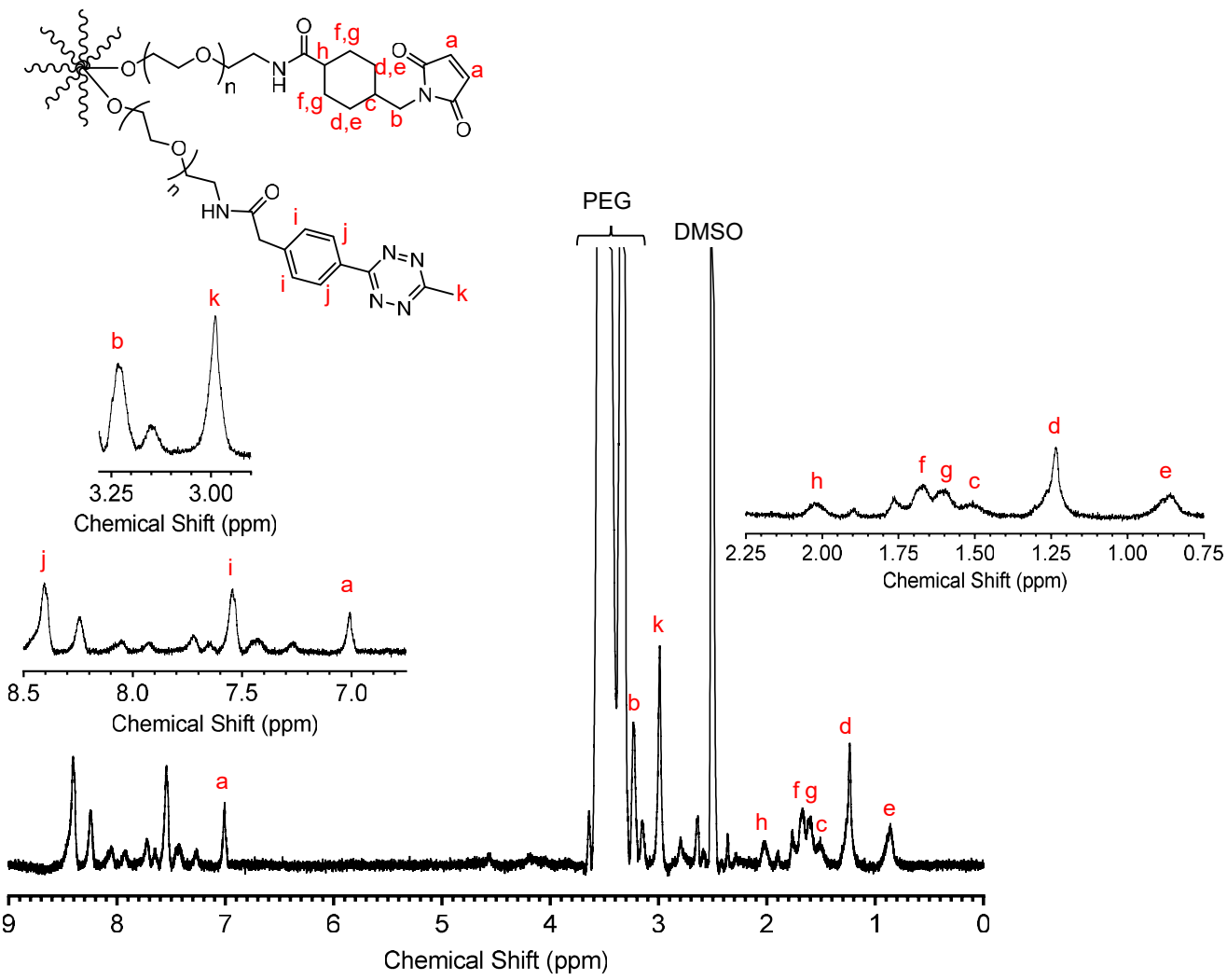


[1]H NMR characterization of 8-arm PEG-maleimide (500 MHz, DMSO-d6).

H. Synthesis of 8-arm PEG-[maleimide/tetrazine]



8-arm PEG-amine (200 mg, MW ~ 20 kDa), methyltetrazine-NHS (Click Chemistry Tools; 16.4 mg, 0.63 eq relative to amines), and SMCC (20.1 mg, 0.75 eq) were added to a 25 mL round bottom flask and dissolved in anhydrous DMF (3 mL). *N,N*-diisopropylethylamine (41.8 µL, 3 eq) was added, and the reaction was allowed to proceed overnight at room temperature. The maleimide-modified PEG was precipitated by dropwise addition to ice cold diethyl ether and collected by centrifugation. The pellet was washed with ice cold diethyl ether (25 mL) and dried under a stream of compressed air. The resulting pink solid was redissolved in dichloromethane (5 mL), washed with water (2.5 mL) and brine (2.5 mL), and concentrated to ~ 2 mL. The PEG was precipitated by dropwise addition to ice cold diethyl ether and collected by centrifugation. The pellet was washed with ice cold diethyl ether (25 mL) and dried under a stream of compressed air. The solid was redissolved in 10 mL MilliQ water, frozen in liquid nitrogen, and lyophilized to afford the 8-arm PEG-[maleimide/tetrazine] as a pink solid (178 mg, 75.9% yield). ^{1}H NMR characterization is presented below. The degree of substitution was estimated to be 3.8 maleimides and 3.6 tetrazines per PEG molecule by comparing the integrated intensity of the maleimide peak (2H, 7.01 ppm) and the aromatic peaks from the tetrazine linker (2H, 8.41 ppm; 2H, 7.55 ppm) with the integrated intensity of a peak corresponding to the end groups of the PEG arms (2H, 3.15 ppm).

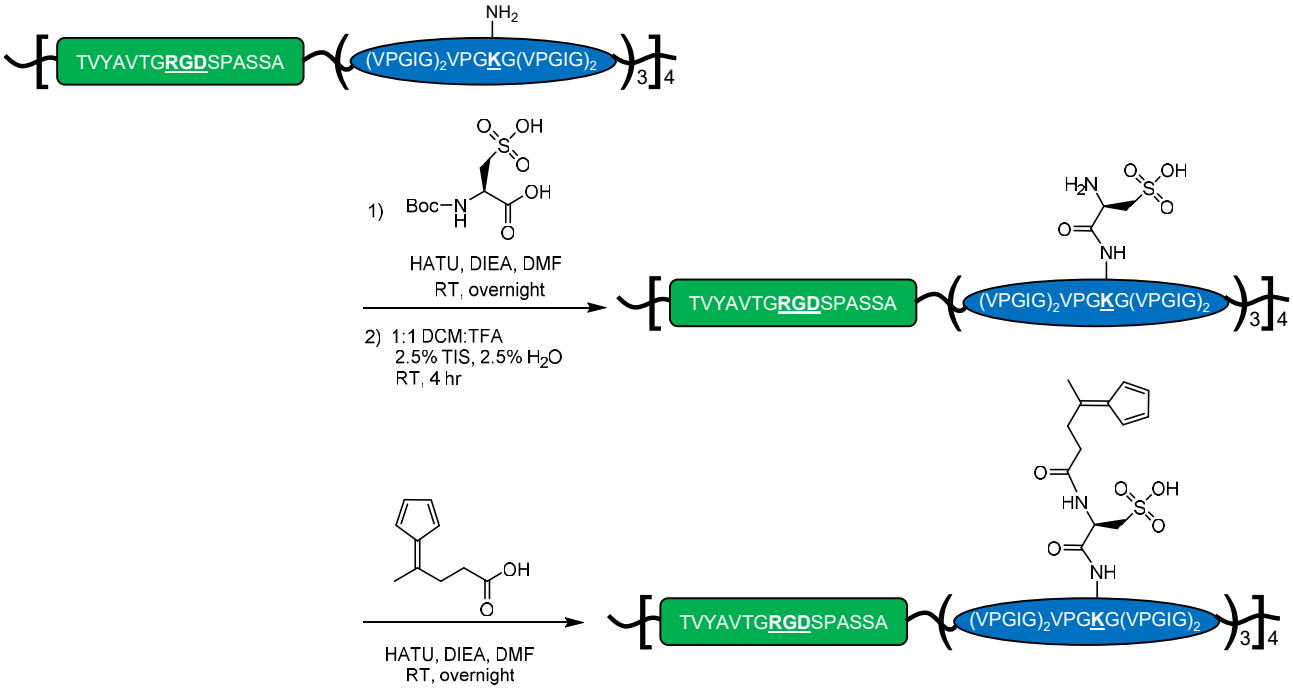


[1]H NMR characterization of 8-arm PEG-[maleimide/tetrazine] (500 MHz, DMSO-d6).

I. Expression and purification of elastin-like protein (ELP)

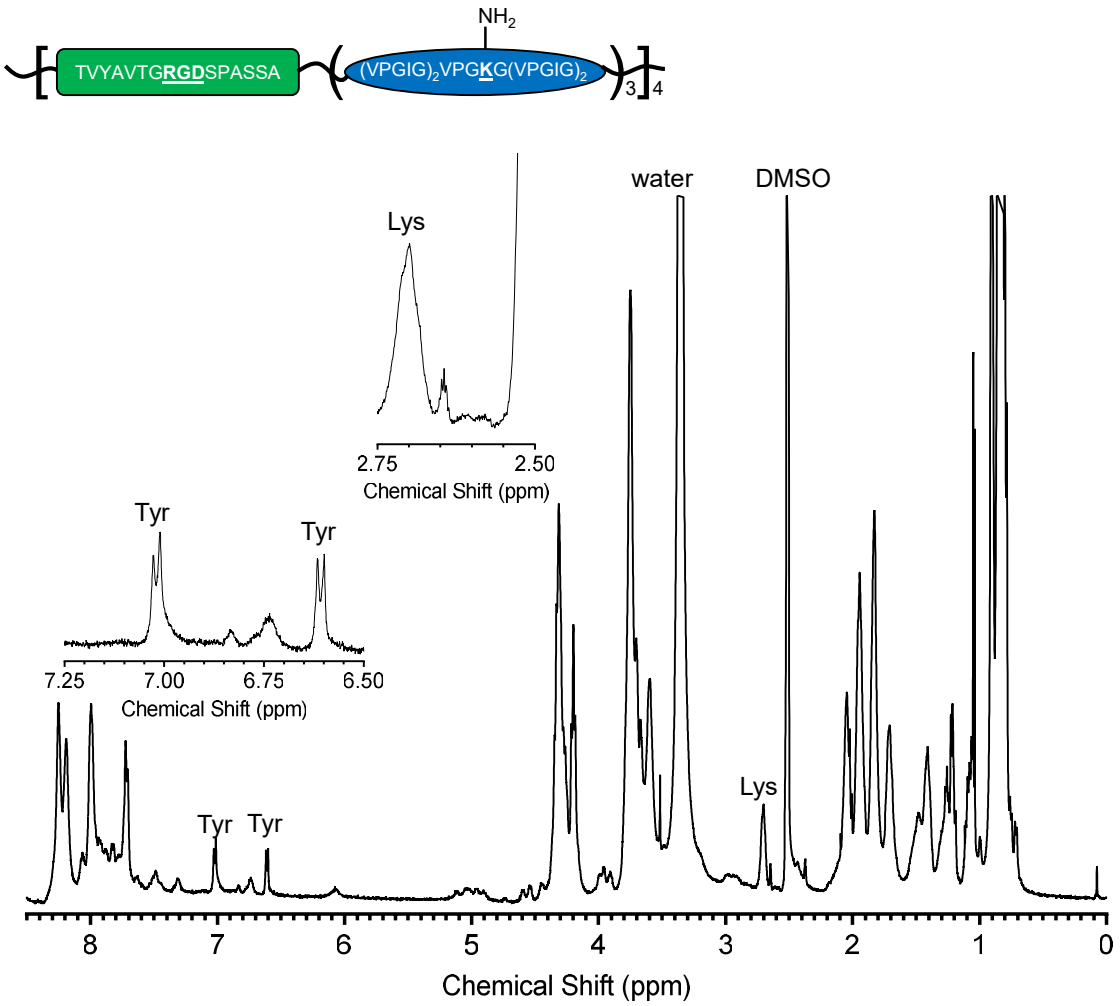
Briefly, pET-15b plasmids encoding the ELP construct were transformed into BL21(DE3)pLysS *Escherichia coli*, and ELP expression was induced by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to cultures of the bacteria in Terrific Broth. After 7 hours of expression, the bacteria were collected by centrifugation, treated with protease inhibitors, and lysed by repetitive freeze-thaw cycles. The RGD-ELP was purified from the crude lysate by inverse temperature cycling, followed by dialysis against MilliQ water (MWCO 2 kDa, 4°C, 6×4 L) and lyophilization to afford the product as a white solid. A typical yield from 12 L of expression culture is approximately 1 g. ELP purity was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot.

L. Synthesis of ELP-fulvene

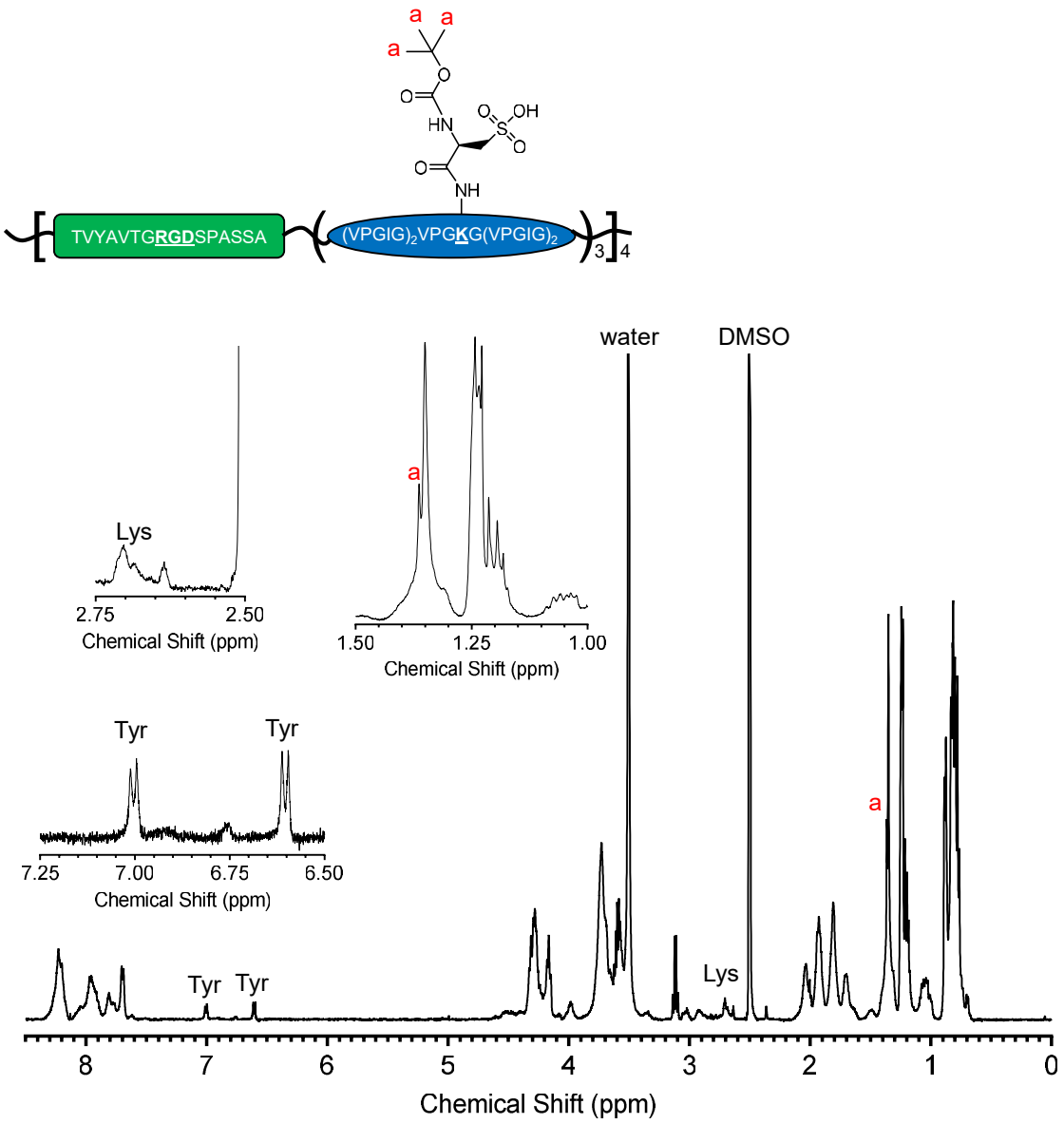


RGD-ELP (100 mg, MW ~ 38 kDa) was added to a 25 mL round bottom flask and dissolved in 1.5 mL anhydrous DMSO. After dissolution, 1.5 mL anhydrous DMF was added. In a separate vial, Boc-L-cysteic acid (AnaSpec; 39.7 mg, 4 eq relative to primary amines) and HATU (55.9 mg, 4 eq) were dissolved in anhydrous DMF (1.5 mL). To this solution, *N,N*-diisopropylethylamine (64 µL, 10 eq) was added, and the mixture was stirred for 10 minutes at room temperature. The mixture was then added dropwise to the stirring ELP solution, and the

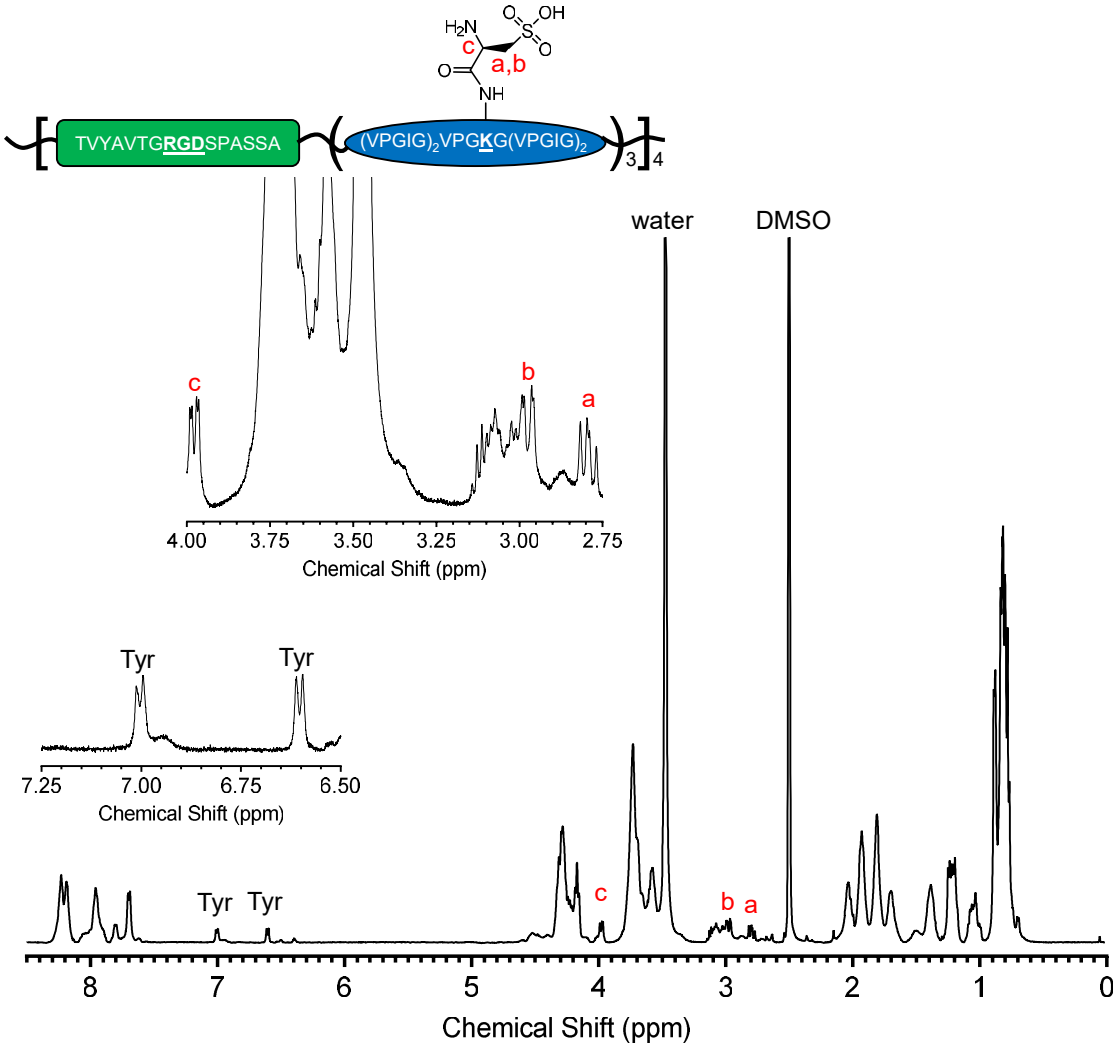
reaction was allowed to proceed overnight at room temperature. The reaction mixture was diluted into MilliQ water (15 mL) on ice and dialyzed against MilliQ water (MWCO 2 kDa, 4°C, 4×4 L). The resulting solution was frozen and lyophilized to afford the ELP-Sulfo-Boc as an off-white solid. The Boc-protected sulfated ELP was then added to a 25 mL round bottom flask and dissolved in a mixture of dichloromethane (1.5 mL), trifluoroacetic acid (1.5 mL), triisopropylsilane (75 µL), and water (75 µL). The Boc deprotection reaction was allowed to proceed at room temperature for 4 hours. The ELP was precipitated by dropwise addition to ice cold diethyl ether (40 mL) and collected by centrifugation. The pellet was redissolved in 2:1 DMF:DMSO (3 mL) and re-precipitated by dropwise addition to ice cold diethyl ether. After centrifugation, the supernatant was decanted, and the pellet was dried briefly in air. The pellet was dissolved in MilliQ water (20 mL) and dialyzed against MilliQ water (MWCO 2 kDa, 4°C, 4×4 L). The resulting solution was frozen and lyophilized. The solid was dissolved in 2:1 DMF:DMSO (3 mL). In a separate vial, 4-(cyclopenta-2,4-dien-1-ylidene)pentanoic acid (6.1 mg, 1 eq relative to primary amines) and HATU (14.0 mg, 1 eq) were dissolved in anhydrous DMF (150 µL). To this solution, *N,N*-diisopropylethylamine (16.0 µL, 2.5 eq) was added, and the mixture was agitated for 10 minutes at room temperature. The mixture was then added dropwise to the stirring sulfated ELP solution, and the reaction was allowed to proceed overnight at room temperature. The reaction mixture was diluted into MilliQ water (20 mL) on ice and dialyzed against MilliQ water (MWCO 2 kDa, 4°C, 4×4 L). The resulting solution was frozen and lyophilized to afford the ELP-fulvene as a light yellow solid (90 mg, 81% yield over three steps). ^{1}H NMR characterization is presented below. Analysis of ^{1}H NMR data indicates that all free amines (14 per ELP) were sulfated and that ~8 fulvenes were incorporated per ELP molecule.



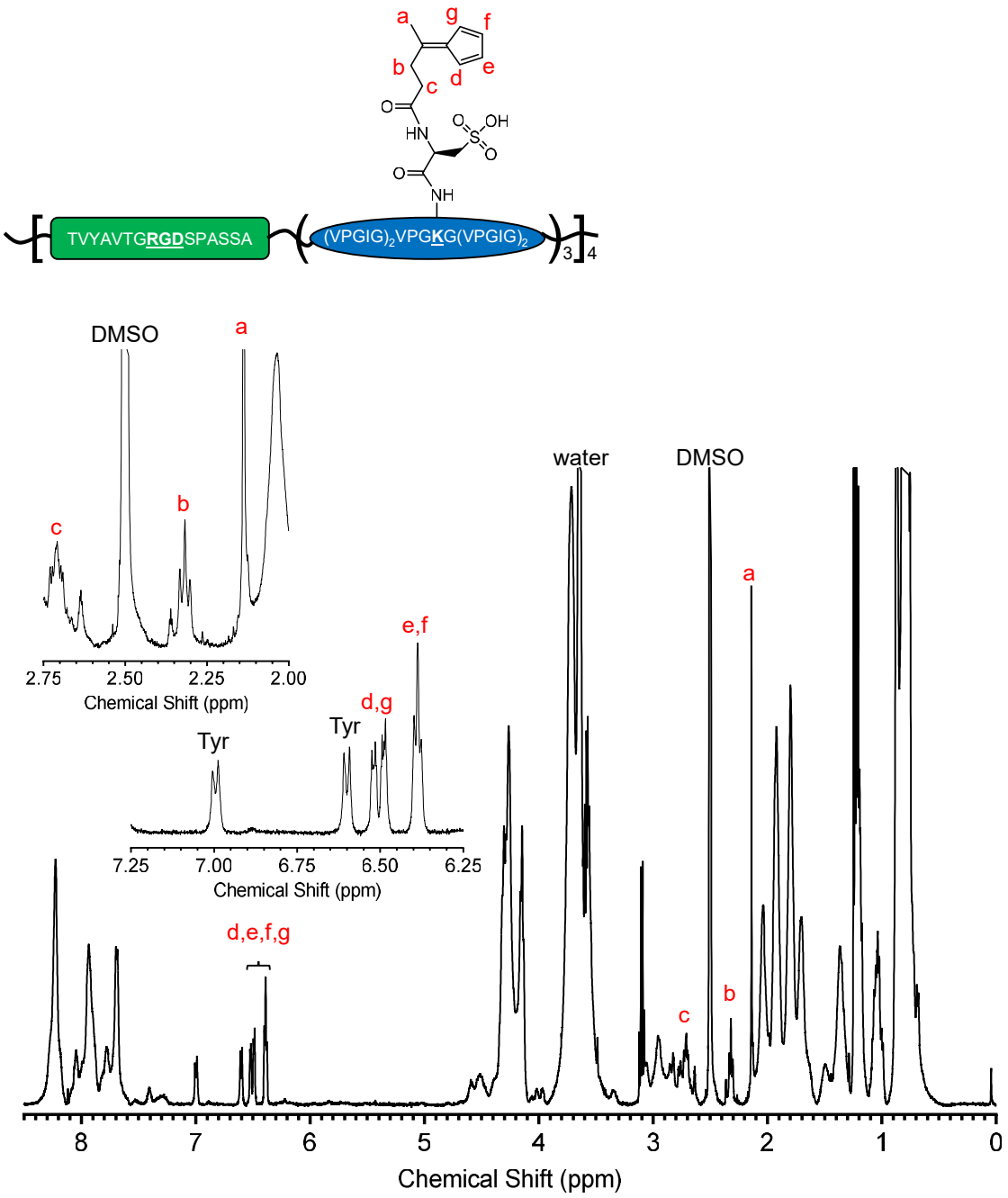
^{1}H NMR characterization of unmodified RGD-ELP (500 MHz, DMSO-d6).



^{1}H NMR characterization of Boc-protected sulfated ELP (500 MHz, DMSO-d6 + D_2O).



^{1}H NMR characterization of sulfated ELP (500 MHz, DMSO-d6 + D_2O).



[1]H NMR characterization of ELP-Fulvene (500 MHz, DMSO-d6 + D_2O).

2. Supporting Figures

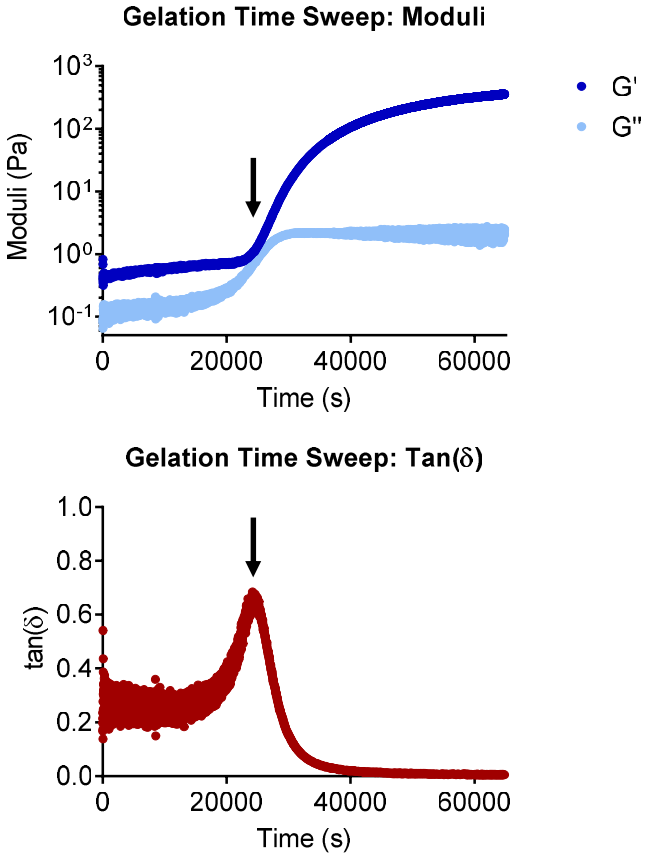


Figure S1. Representative gelation time sweep of 4-arm PEG-methylfuran hydrogels presenting the storage (G') and loss (G") moduli and tan(δ) as a function of time. The black arrows denote the gelation point.

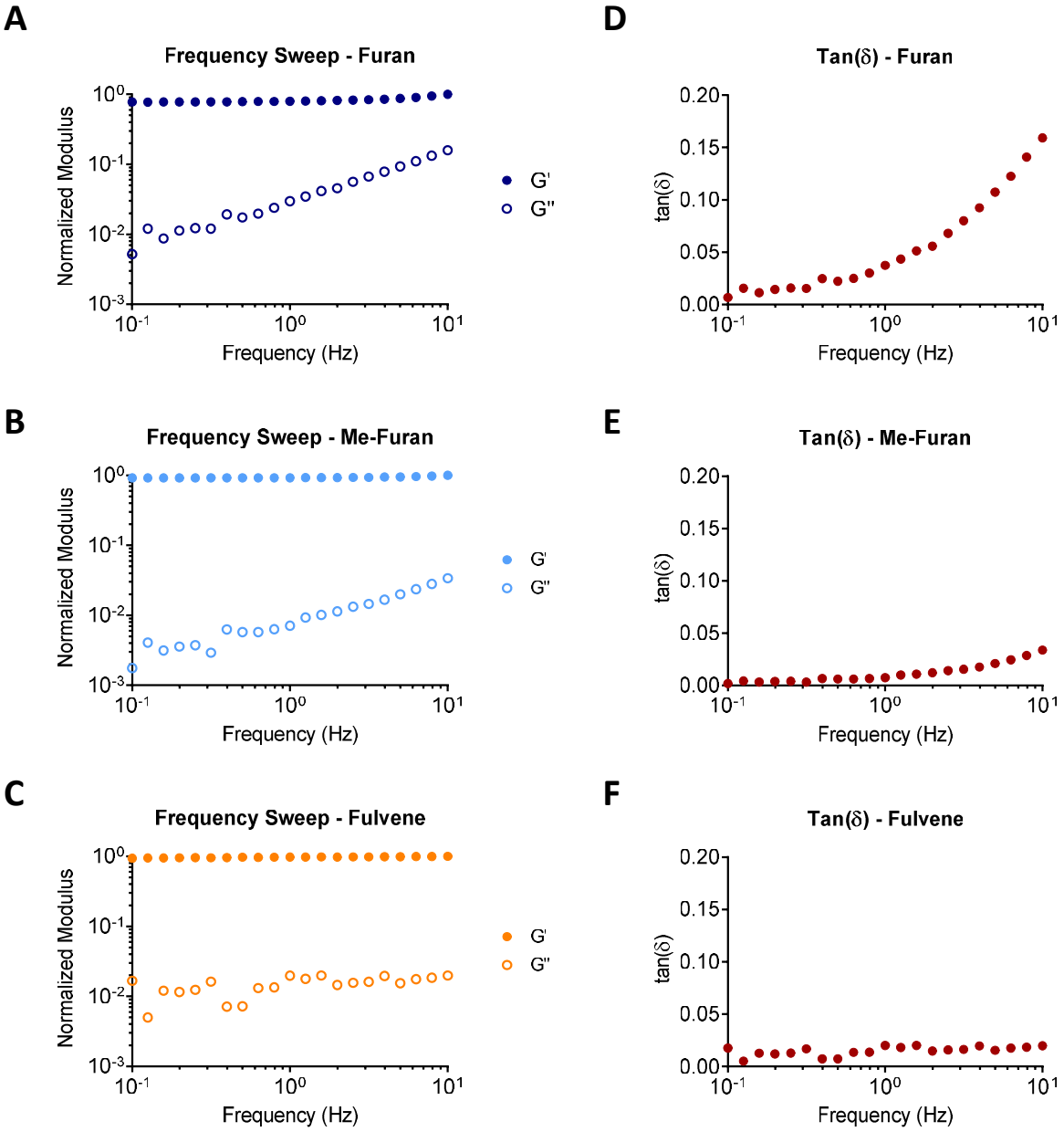


Figure S2. Representative frequency sweeps reporting storage (G') and loss (G") moduli for 4-arm PEG gels with **(A)** furan, **(B)** methylfuran, and **(C)** fulvene groups as the dienes. Loss tangent as a function of frequency for **(D)** furan, **(E)** methylfuran, and **(F)** fulvene gels demonstrates greater frequency-dependent viscoelastic character in furan and methylfuran gels compared to fulvene gels, suggesting decreased reversibility in the fulvene Diels-Alder reaction.

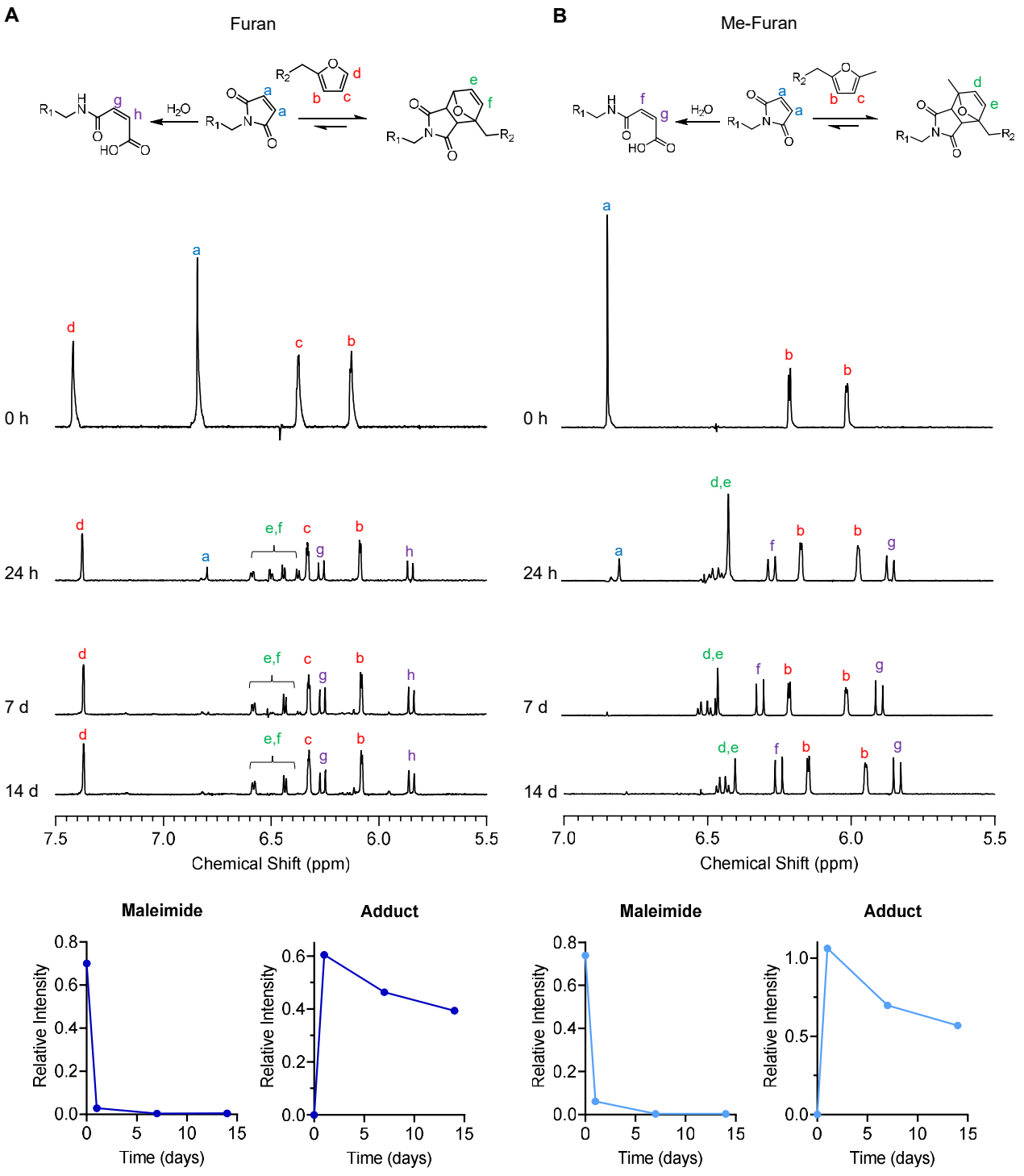


Figure S3. ^{1}H NMR analysis of hydrogel crosslinking and degradation for **(A)** furan and **(B)** methylfuran-based gels. The disappearance of the maleimide signal and corresponding increase in the adduct signals between 0 and 24 hours indicate the crosslinking reaction is complete. Subsequent decrease in intensity of the adduct signals and increase in intensity of the maleimide hydrolysis peaks are consistent with degradation by a retro-DA reaction followed by hydrolysis.

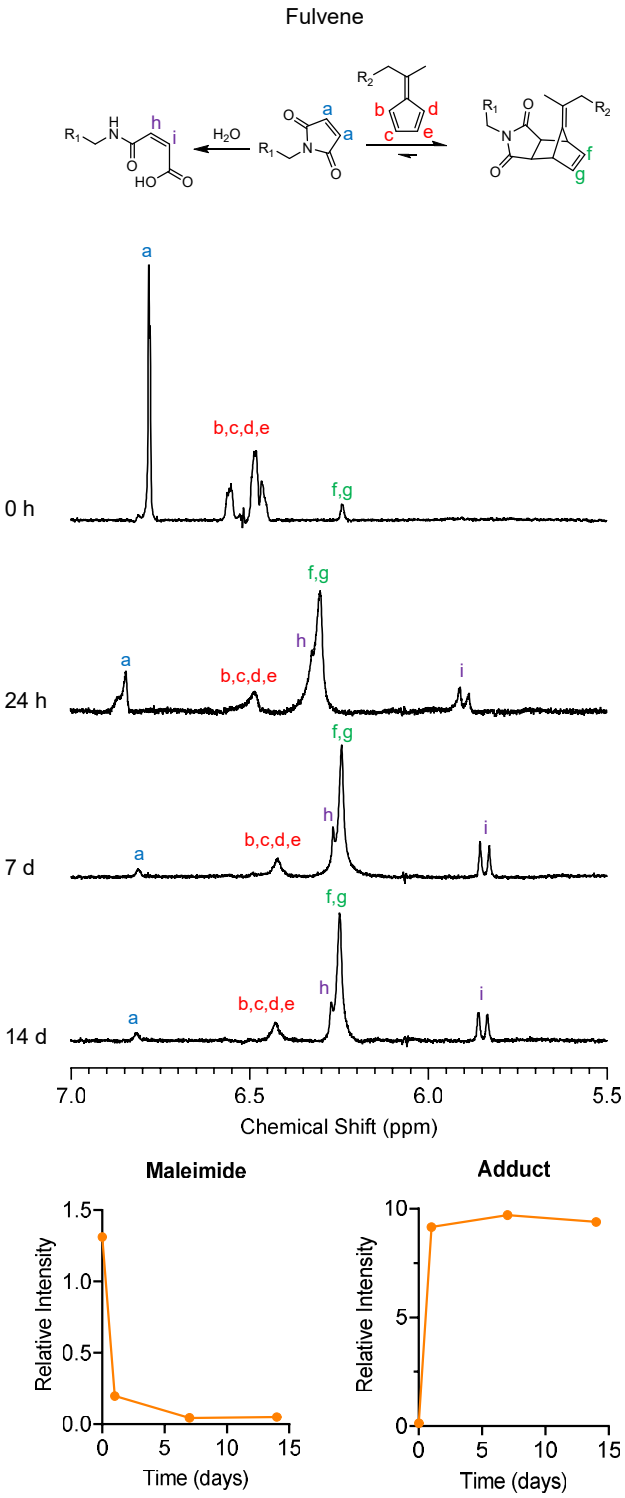


Figure S4. [1]H NMR analysis of hydrogel crosslinking and degradation for fulvene-based gels. The disappearance of the maleimide signal and corresponding increase in the adduct signal between 0 and 24 hours indicate the crosslinking reaction is complete. The adduct signal intensity remains consistent over time, indicating that the fulvene-based crosslinks remain stable.

3. Supporting Table

Diene	PEG Macromer	Plateau Storage Modulus (mean ± s.d.)
Furan	4-arm	71.6 ± 41.2 Pa
Methylfuran	4-arm	622 ± 396 Pa
Fulvene	4-arm	1640 ± 377 Pa
Furan	8-arm	5490 ± 257 Pa
Fulvene	8-arm	2050 ± 85.3 Pa

Table S1. Summary of plateau storage moduli (1 Hz, 10% strain) for PEG-based Diels-Alder hydrogels. Data represent 3-4 independent replicates per condition.