NMR Characterization of Polyethylene Glycol Conjugates for Nanoparticle Functionalization

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Supplemental Information

Experimental

Materials and Methods

Unless otherwise noted, starting materials were obtained from commercial suppliers and used without further purification. Pd-10 column G25 sephadex media were purchased from Global Life Sciences solutions USA LLC.

General characterization

Standard ^{1}H NMR spectra were recorded on a Bruker Avance III HD nanobay AX-400 at 400 MHz. Effect of ^{1}H-^{13}C coupling was studied using a Bruker Avance NEO 600-MHz NMR with TCI cryoprobe at 600 MHz, and on Bruker Avance II+ 400-MHz NMR with BBFO SmartProbe at 400 MHz. ^{1}H-^{13}C decoupling was achieved use the ^{1}H-^{13}C decoupling program WALTZ16 composite pulse decoupling on the Bruker Avance II+ 400-MHz NMR with BBFO SmartProbe. Chemical shifts were referenced to appropriate internal standards. Data for ^{1}H is recorded as follows: chemical shift (d, ppm), multiplicity (s, singlet; d, doublet; t, triplet; m, multiplet) integration, coupling constant. Matrix Assisted Laser Desorption Ionization mass spectra (MALDI-MS) were recorded on an Applied Biosystems-Sciex 5800 MALDI/TOF/TOF-MS at the LeClaire-DOW instrumentation facility of the Department of Chemistry of the University of Minnesota using a matrix of α-cyano-4-hydroxycinnamic acid (CCA). Data was processed using Polymerix version 3.01 (Sierre Analytics, Inc., Modesto, Ca, USA). Size exclusion chromatography (SEC): THF-SEC was performed on an Agilent 1260 series instrument (1.0 mL/min, 25 °C). Separation was obtained by a Tosoh Styragel guard column and three successive Tosoh Styragel columns (G6000, G4000, and G2000) packed with 5 μm styrene-divinylbenzene particles. Number-average molecular weight (Mn), were determined using a Wyatt OPTILAB T-rEX refractive index detector (based on a 10-point calibration curve with polystyrene standards).

Bioconjugation of mPEG

The ester coupling to mPEG were synthesized based our small molecule phosphonate linkage method from previous work.[1] Copied with permission: **"(PEG-4-oxobutyl)phosphonic acid:** ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.27 (t, 2H), 3.66 (m, 180H), 3.57 (t, 2H), 3.40 (s, 2H), 2.51 (t, J = 7.1 Hz, 2H), 2.07 – 1.92 (m, 2H), 1.90 – 1.76 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.20 (s, 1C), 71.94 (s, 1C), 70.57 (s, 1C), 69.11 (s, 1C), 63.59 (s, 1C), 59.04 (s, 1C), 34.17 (d, J = 15.9 Hz, 1C), 24.93 (d, J = 141.8 Hz, 1C), 18.12 (d, J = 4.8 Hz, 1C). ^{31}P NMR (162 MHz, $CDCl_3$) δ 31.15 (s, 1P)."

Dilution of coupled mPEG with free mPEG

Two stock solutions were made using 3.75 mL of $CDCl_3$ added to coupled mPEG2000 (**(PEG-4-oxobutyl)phosphonic acid)** (57 mg, 0.026 mmol) or free mPEG2000 (57 mg, 0.028 mmol). Dilutions were created by adding 0, 300, 600, 900 or 1200 µL of coupled PEG solution to 1200, 900, 600, 300, or 0 µL free PEG solution, to make 0, 25, 50, 75 and 100% coupled PEG solutions, respectively.

Full NMR spectra:

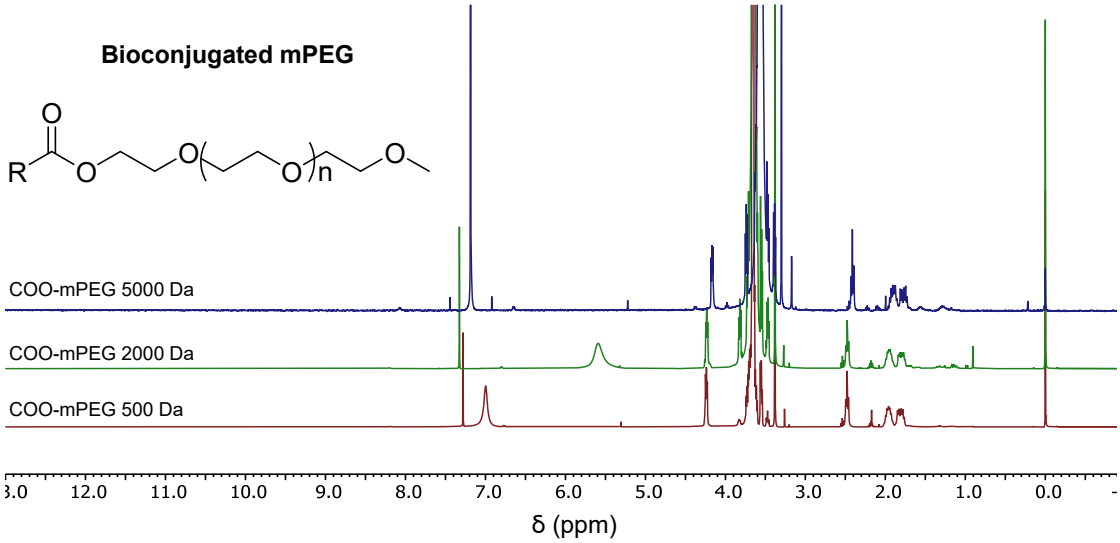

Figure S1. Full ^{1}H NMR spectra of ester mPEG stack.

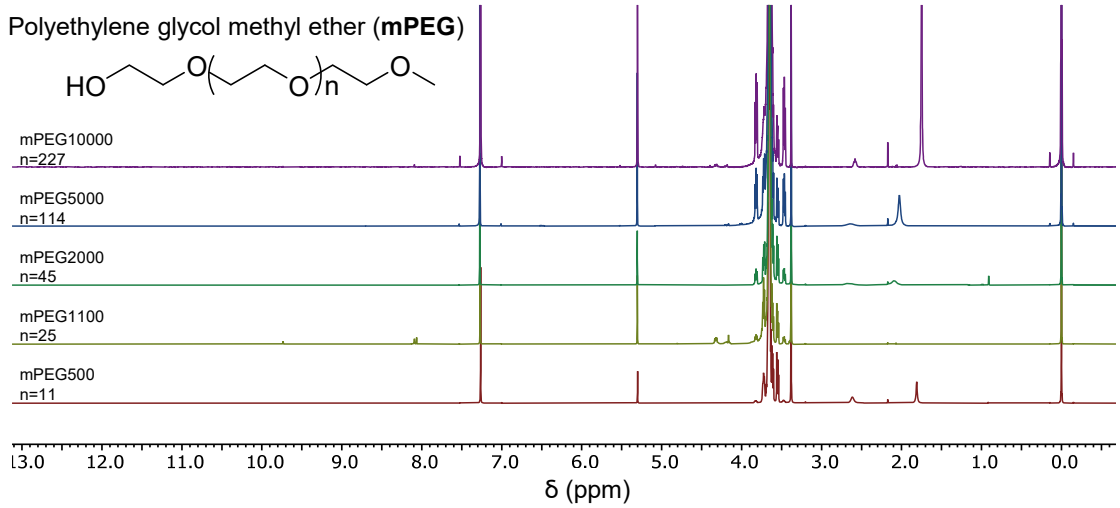

Figure S2. Full ^{1}H NMR spectra of mPEG MW stack.

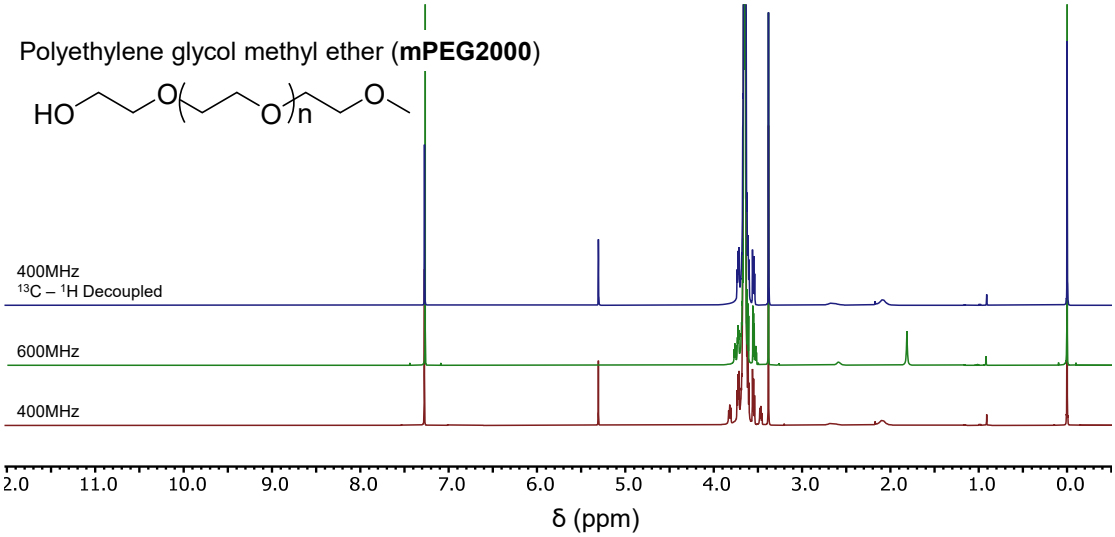

Figure S3. Full ^{1}H NMR spectra of mPEG ^{1}H-^{13}C decoupling determination.

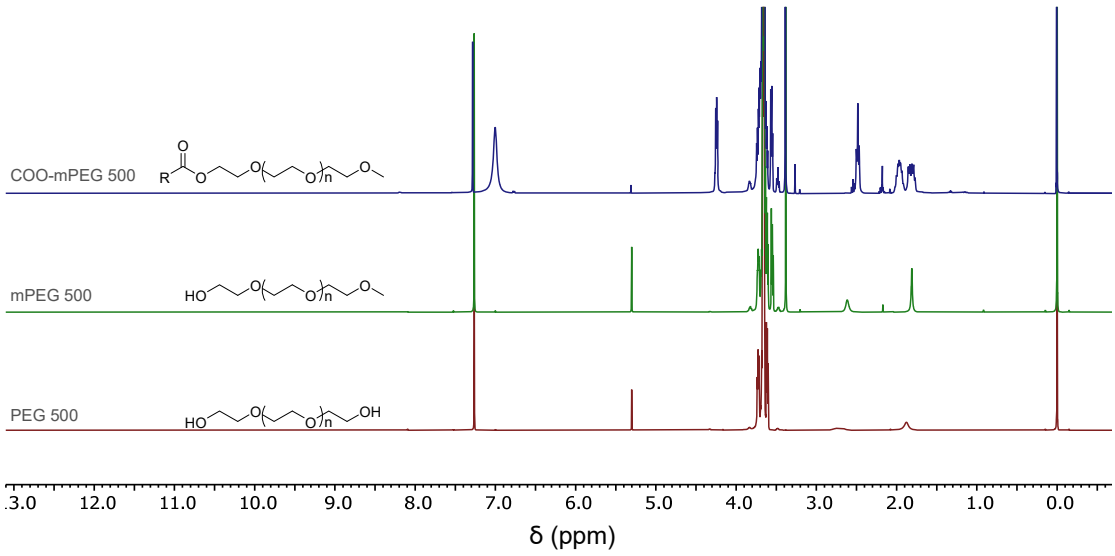

Figure S4. Full ^{1}H NMR spectra of mPEG peak identification.

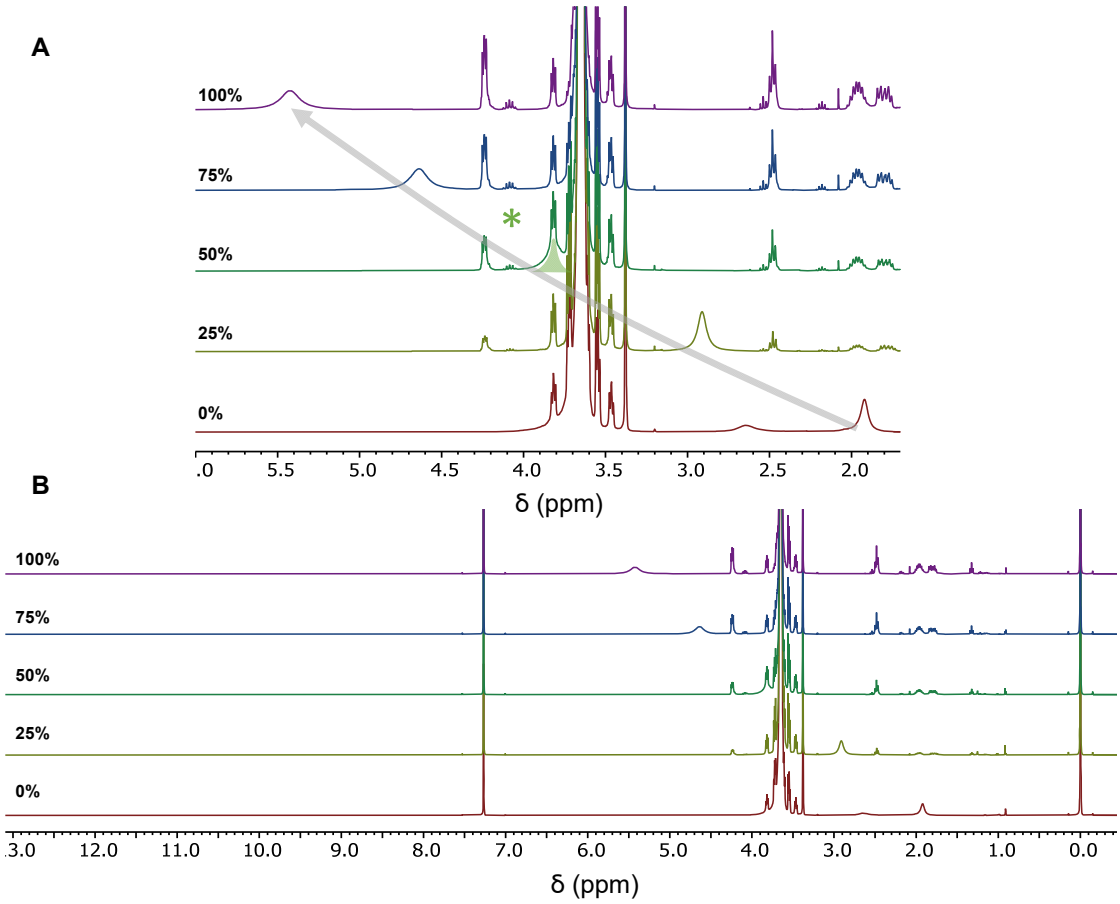

Figure S5. ^{1}H NMR spectra of ester mPEG dilution with free PEG. 100% is purified coupled mPEG2000, 0% is commercial mPEG2000. **A.** Zoomed in ^{1}H NMR spectra of dilutions, grey arrow depicts movement of mPEG alcohol peak with increased coupling. Green star and green shading depict overlap of PEG alcohol peak with c* peak. The alcohol peak may overlap with PEG peaks depending on concentration and coupling percentage. **B.** Full spectra of A.

MW comparison

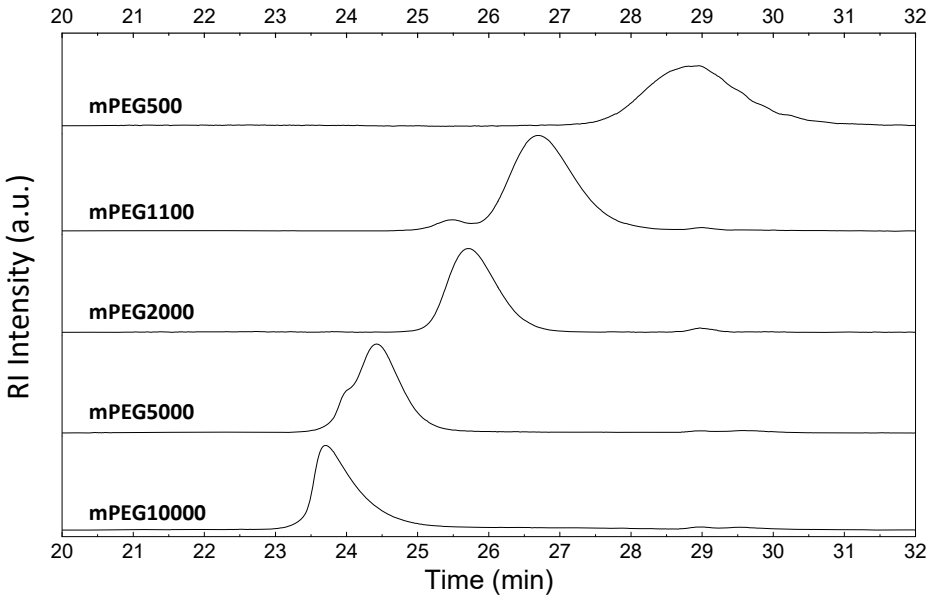

Figure S6. SEC chromatograms of mPEG (THF).

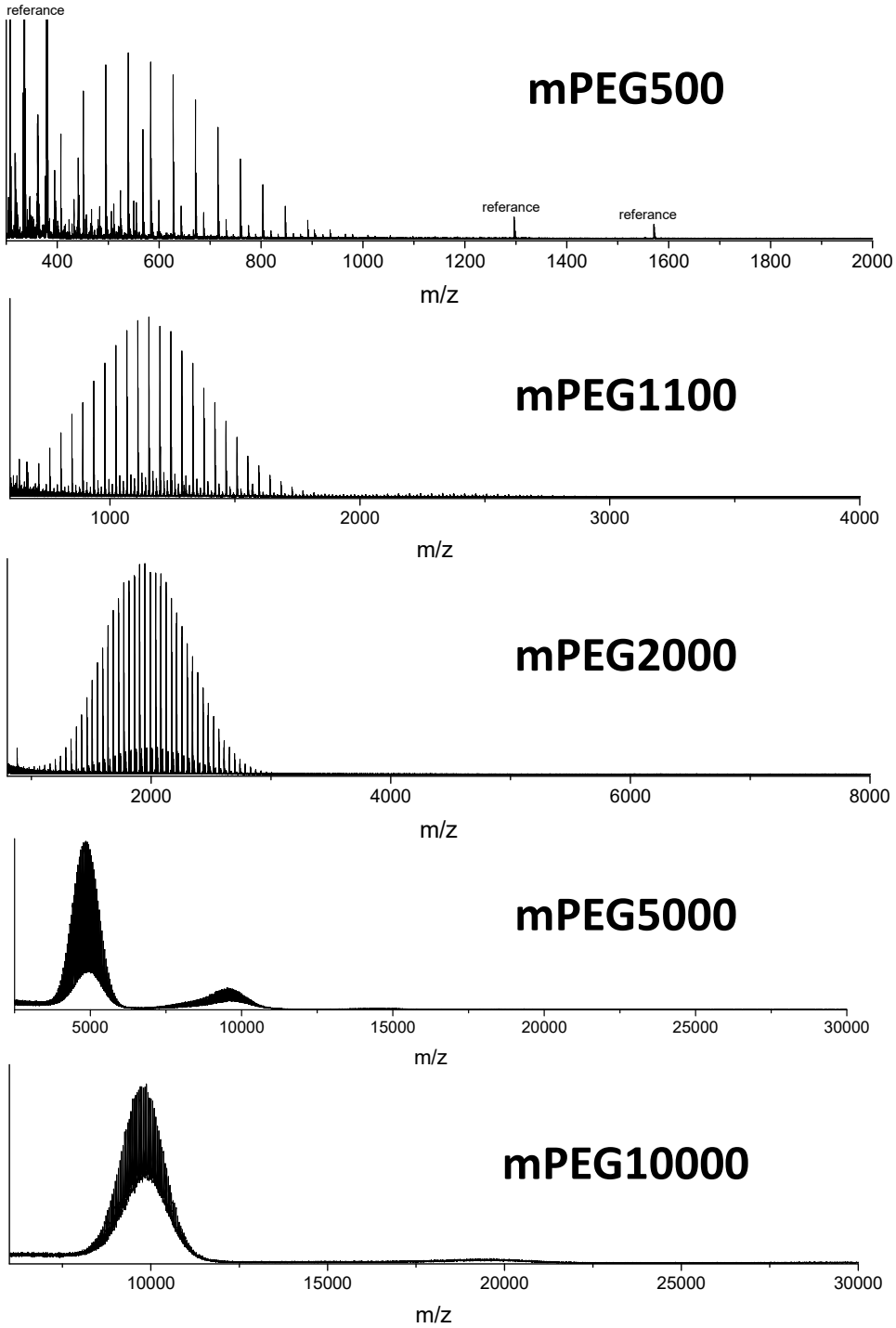

Figure S7. MALDI-MS spectra of mPEG. Internal reference peaks at 307 m/z, 379 m/z, 1297 m/z and 1571 m/z were not included in M_n calculations.

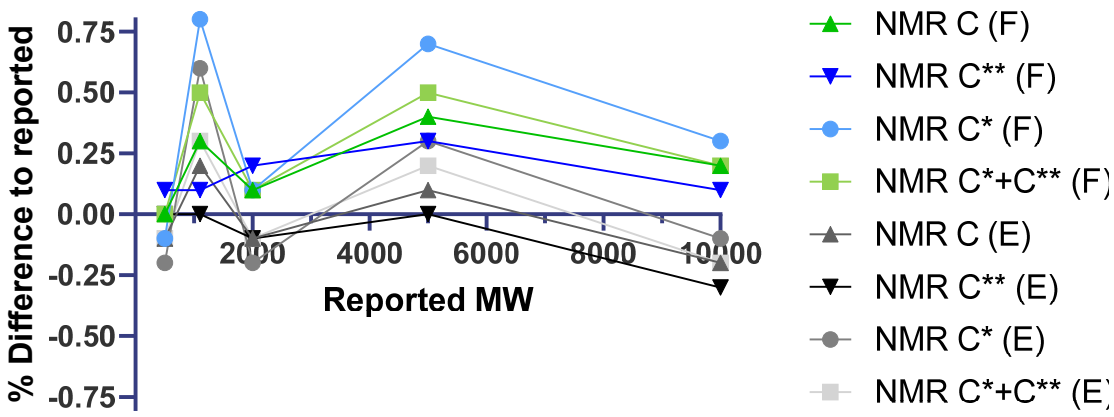

Figure S8. [1]H NMR mPEG MW determination normalizing to various peaks and calculating MW from c or c* peaks. There is a large discrepancy between normalizing with either the "f" or "e" peak and the reported amount. The "f" peak may overestimate the MW of mPEG if there is some PEG diol as an impurity. The "e" peak would have similar issue except that at larger MW the main peak starts to overlap and increase the integral, resulting in an under estimation of the MW. Additionally, the c* and c** method agreed with the normalization methods closely.

Coupling Dilution comparison

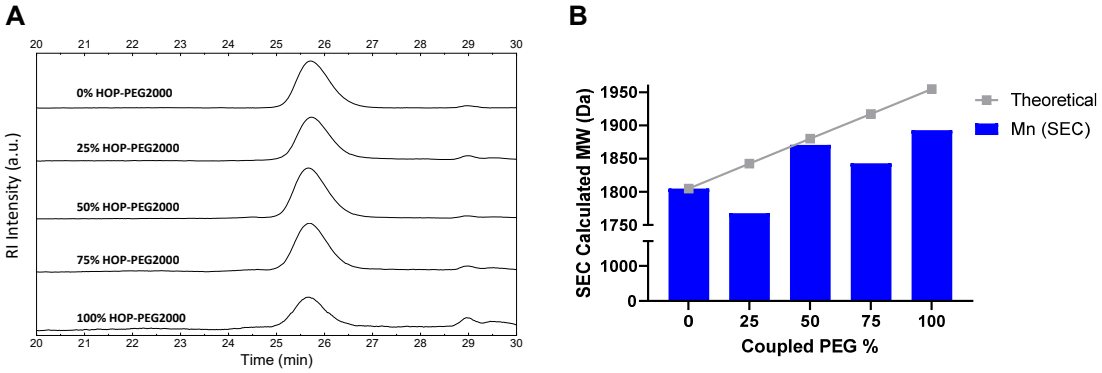

Figure S9. A. SEC chromatograms of mPEG2000 diluted with HOP-PEG2000 (in THF). **B**. MW of coupled PEG dilution using SEC, blue bars showing the calculated values, grey line showing theoretical MW based on phosphate linker of 150 Da and the percentage added.

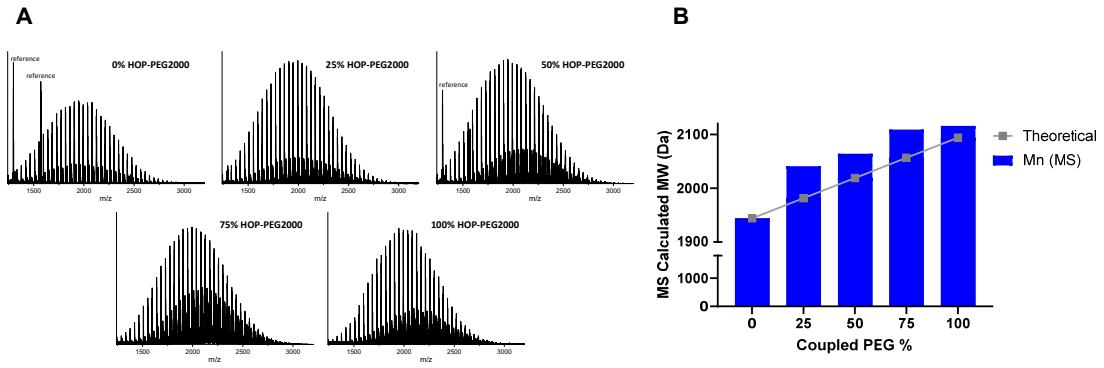

Figure S10. A. MALDI-MS spectra for samples of mPEG2000 diluted with HOP-PEG2000. Internal reference peaks at 1297 m/z, 1571 m/z and 2466 m/z were not included in M_n calculations. **B**. MW of coupled PEG dilution using MS, blue bars showing the calculated values, grey line showing theoretical MW based on phosphate linker of 150 Da and the percentage added.

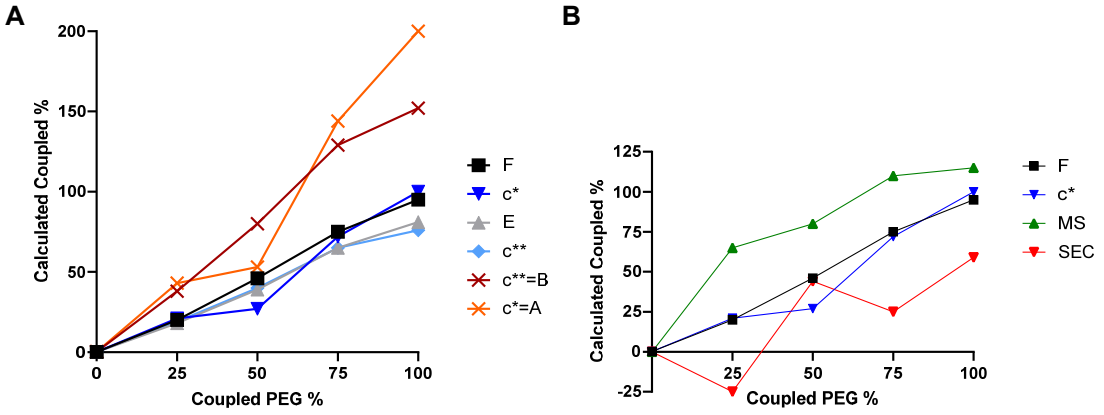

Figure S11. Comparison of NMR, SEC, and MS of coupling. **A.** Coupling calculated by ^{1}H NMR by using various peaks to normalize the spectra, compared to misidentifying c* and c** peaks as end groups. **B.** Coupling calculated based on MW determined by SEC (Figure s9) or MS (Figure s10) compared to using ^{1}H NMR normalized by either methyl peak or c* peak.

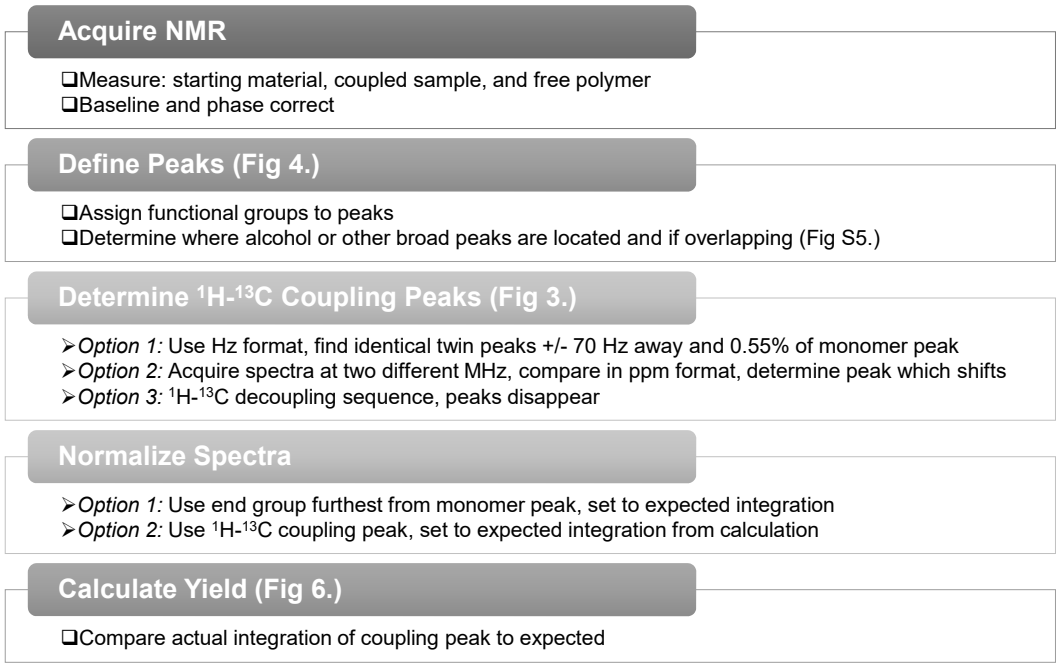

Figure S12. Flow chart to correctly find coupling yield.

Discussion on determining yield coupled PEG

Taking ^{1}H NMR using approximately 20 mg /mL of sample on each starting material, coupled sample, and free polymer will give a starting point understanding of which peaks are created or removed during coupling, similar to Figure S5 0% and 100%. This comparison can also be used to understand the left over terminal groups of the polymer, which are not affected by coupling. If a broad peak is observed in the free polymer sample but not in coupled sample, it is possible the peak is overlapping with another peak, as in Figure S5 50%. Increasing or decreasing the concentration of the material may change the position of the peak. Alternatively, one can analytically (ensuring total polymer concentration remains constant) dilute the coupled sample with known amounts of free polymer, such as in Figure S5, and trace the overlap back to the approximate location in

the initial sample. The large polymer peaks can be identified by the magnitude and chemical shift. ^{1}H-^{13}C coupling peaks need to be identified to not be misconstrued as terminal monomers, as they both mimic the monomer peak shape and are close to the polymer shifts, but do not integrate as such. This can be done in various ways: as in Figure 3 and, if supplies are available, using increasing MW as in Figure 2, whichever is most accessible onsite. The simplest way is to view spectra in absolute frequency (Hz) where the ^{1}H-^{13}C coupling peaks will be exactly +/- 70 Hz from main polymer peak with integration of 0.0055 of it. One can use the same sample on an instrument with a different frequency and compare the two spectra in ppm format. The ^{1}H-^{13}C coupling peaks will shift based on the MHz of the instrument (70Hz/instrument MHz = ppm shift). A ^{1}H-^{13}C decoupling pulse sequence after the initial scan can also be used, if available, and the twin peaks will be removed. Finally, if a sample of the polymer with a larger MW is available, the ^{1}H NMR spectra can be compared to view which peaks are increasing compared to the spectra of the original sample. After successfully identifying all peaks, the next step is to normalize the spectra.

The ^{1}H NMR spectra can be normalized to any peak by identifying an integration range and setting it to the expected values or number of protons. To determine coupling yield of a polymer to another compound, it is best to normalize with a peak related to the polymer but with the least interference from the main polymer peak. Generally, this will be the end group peak furthest from the main polymer peak unless it is overlapping with an alcohol or other broad peak. Any peak that has overlap interference from a broad peak, such as in Figure S5 50%, should not be used to normalize. The absolute integration of the peak will

be increased from the addition of the broad peak, resulting in lowered integration (and therefore calculated yield) of all other peaks. If the next furthest peak is a ^{1}H-^{13}C coupling peak, set integration to 0.0055 of the main polymer peak (equation below). $Mw_{polymer}$ can be determined by GPC (MW above 2000), MS, or NMR.

$$\int c = \#protons_{monomer} * \frac{MWpolymer - MWendgroup}{MWmonomer}$$

Finally coupling yield can be determined by the resulting integration of the coupling peak normalized to the chosen peak. The integration divided by the expected number of protons is the yield.

$$Yield = \frac{coupling\ Integration}{\#\ of\ protons}$$

References:

1. Pasek-Allen, J. W., Randall Gao, Zhe Gao; Pierre, V. C.; Bischof, J., Phosphonate Coating of Commercial Iron Oxide Nanoparticles for Nanowarming Cryopreserved Samples *J. Mat. Chem. B*. **2022**, *10*, 3734-3746.