Supporting information

Anti-fibrotic effect of ketoprofen-grafted alginate microcapsules in the transplantation of insulin producing cells

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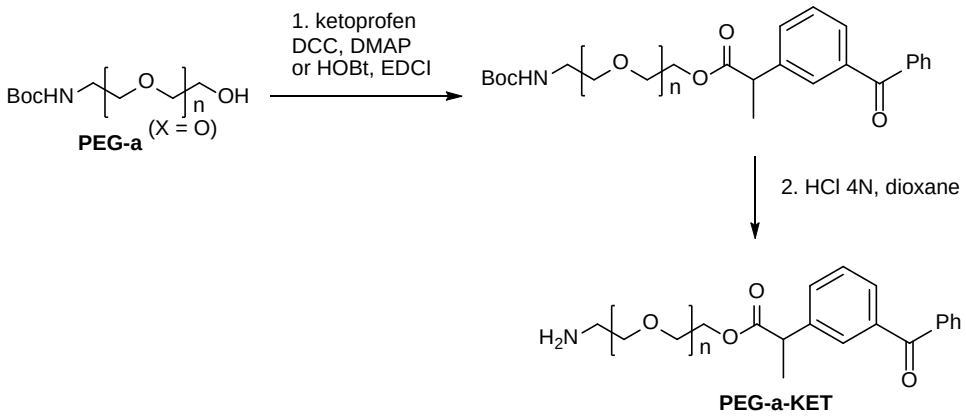
[‡]These authors contributed equally to the work.

Content

Preparation of PEG-a-KET



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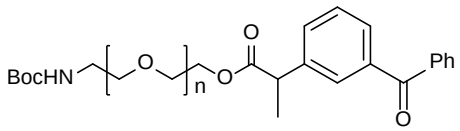
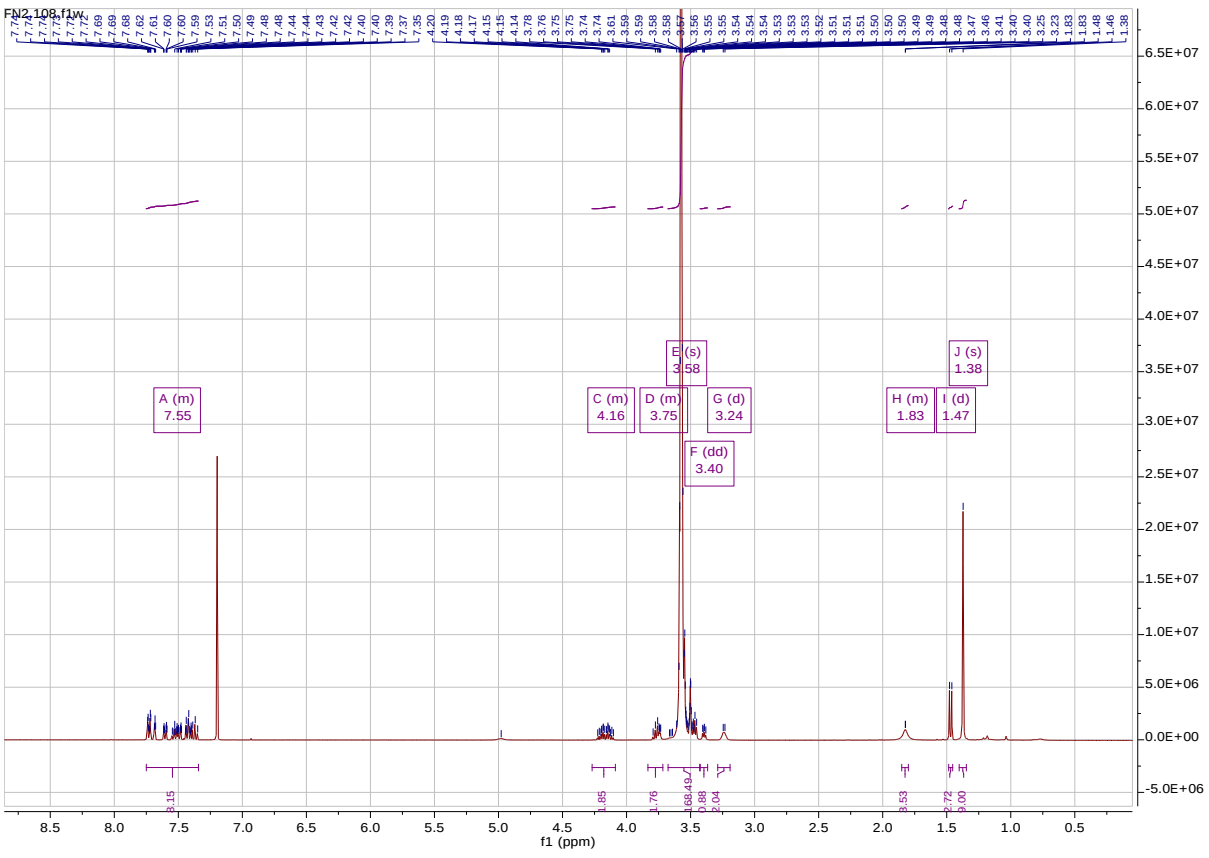


IR (neat): 2870, 1350, 1250, 1105 cm^{-1}.

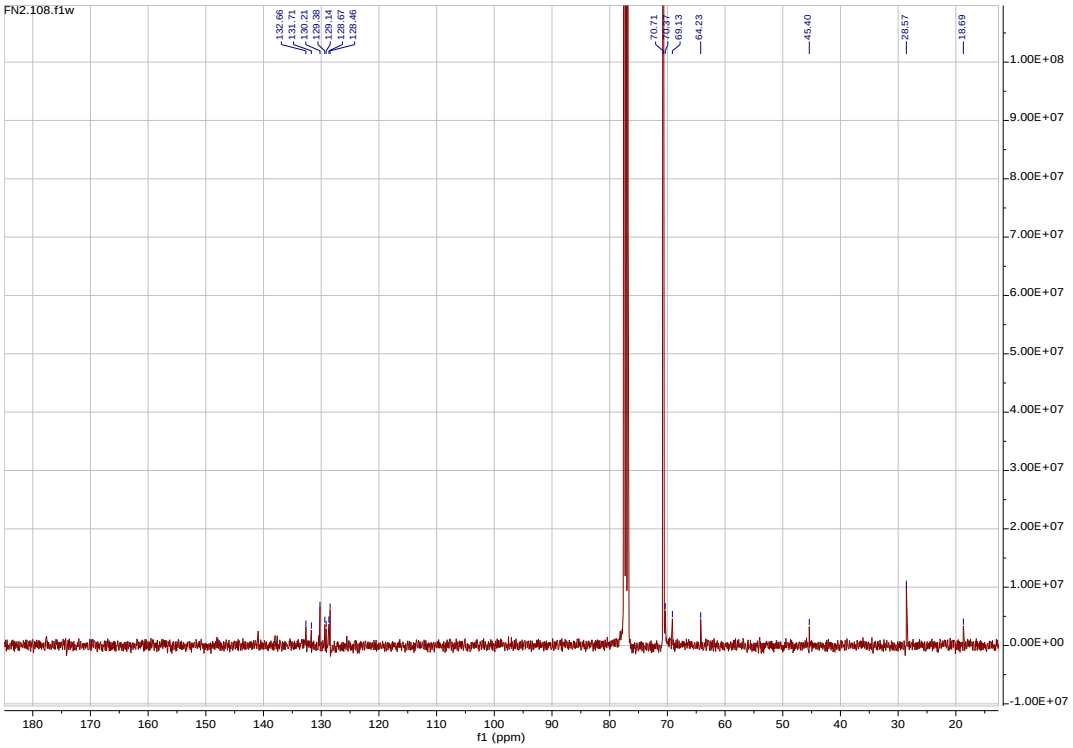
^{1}H-NMR spectrum (400 MHz, $CDCl_3$)

δ 7.75 – 7.35 (m, 9H, ar.), 4.27 – 4.09 (m, 2H, C*H*$_2$-NHBoc), 3.84 – 3.72 (m, 2H, CH_2-OC(O)), 3.58 (s, 174H, CH_2, PEG), 3.40 (dd, J = 5.8, 4.1 Hz, 1H, C*H*-Me), 3.24 (d, J = 5.4 Hz, 2H, C*H*$_2$-CH_2O), 1.89 – 1.78 (bs, 1H, N*H*), 1.47 (d, J = 7.2 Hz, 3H, CH_3), 1.38 (s, 9H, CH_3(^{t}Bu)).

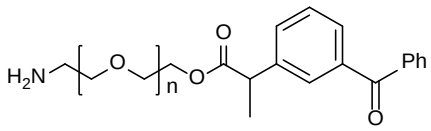


^{13}C-NMR spectrum (101 MHz, $CDCl_3$)

δ 132.66 (C ar.), 131.71 (C ar.), 130.21 (2 x C ar.), 129.38 (C ar.), 129.14 (C ar.), 128.67 (C ar.), 128.46 (2 x C ar.), 70.71 (CH_2), 70.37 (CH_2), 69.13 (CH_2), 64.23 (CH_2), 45.40 (CH), 28.57 (CH_2), 18.69 (3 x CH_3), 18.69 (CH_3).



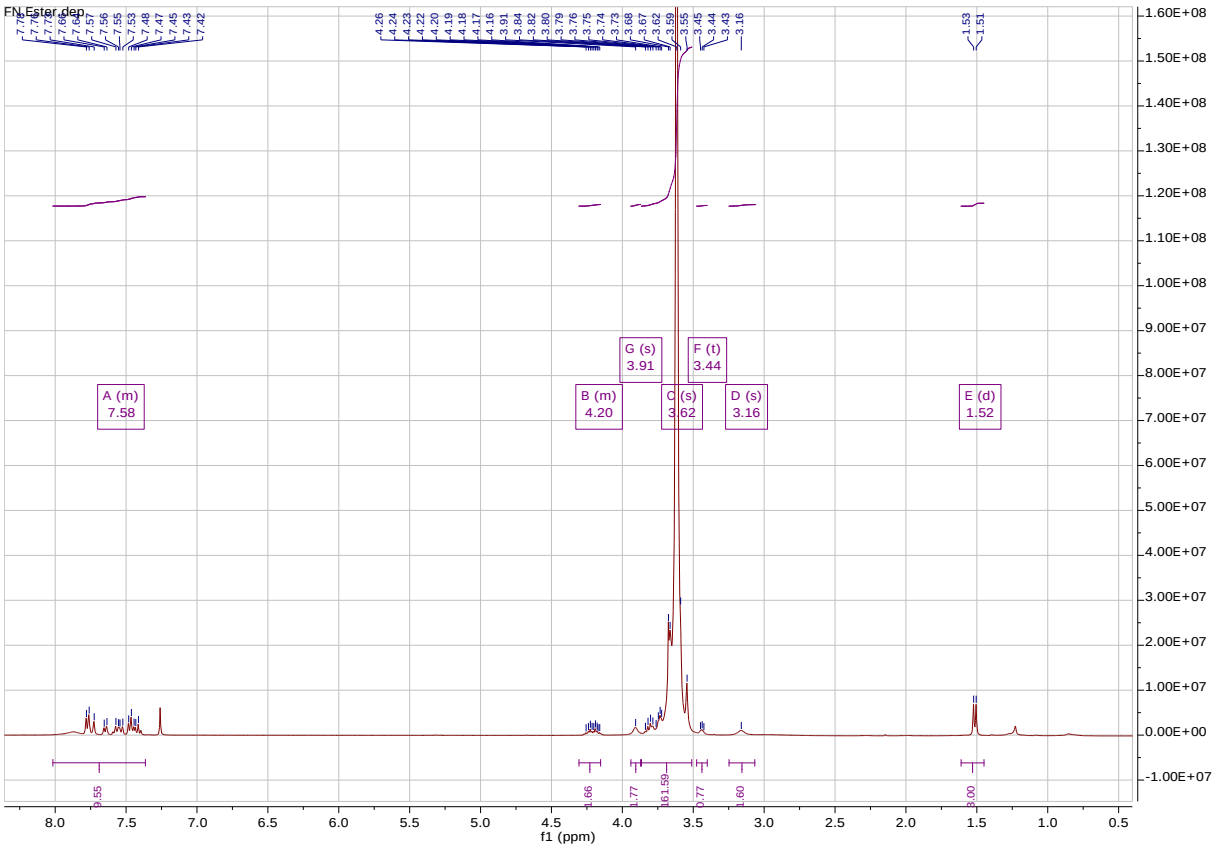
PEG-a-KET



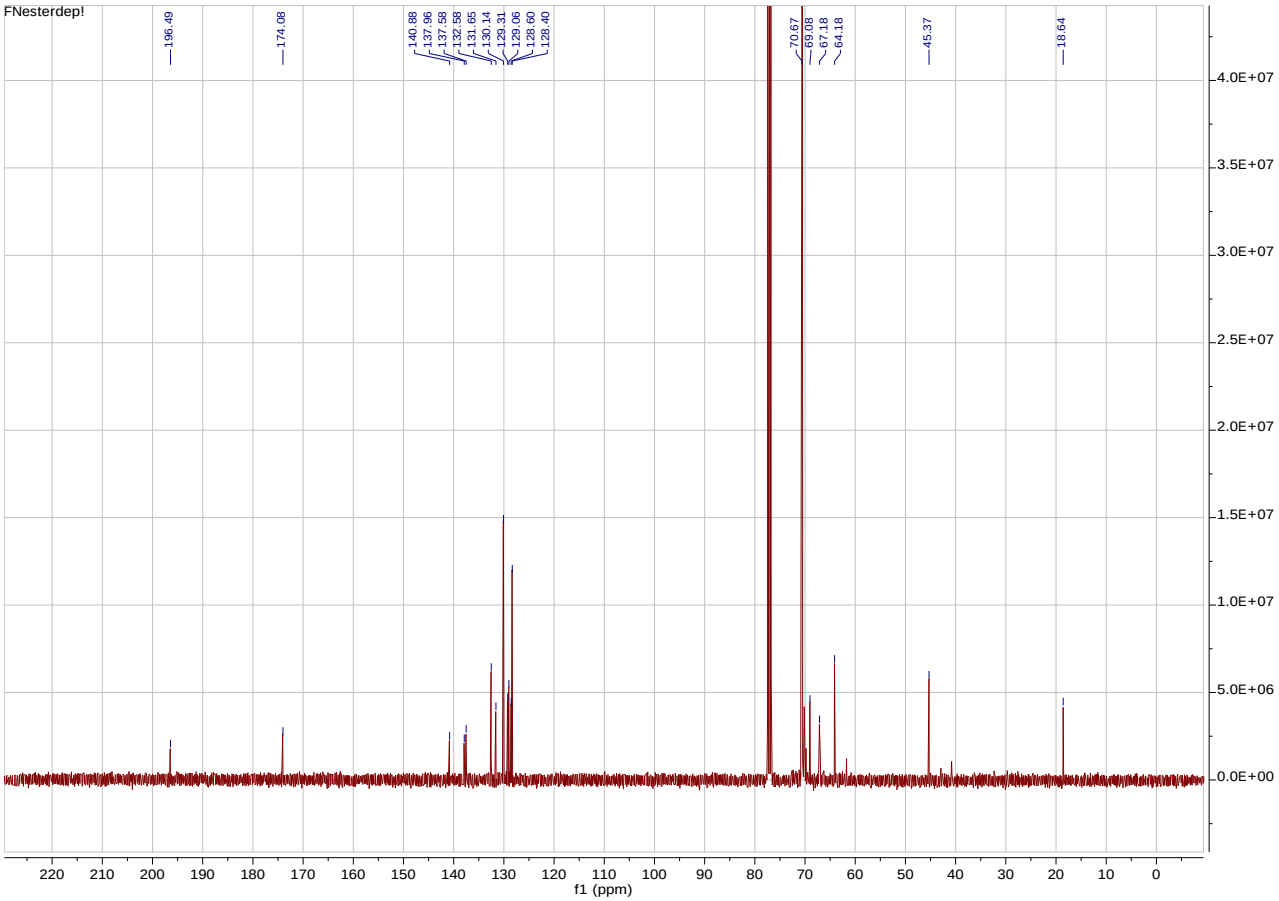
IR (neat): 2880, 1465, 1345, 1280, 1240, 1105, 960, 840 cm^{-1}.

^{1}H-NMR spectrum (400 MHz, $CDCl_3$)

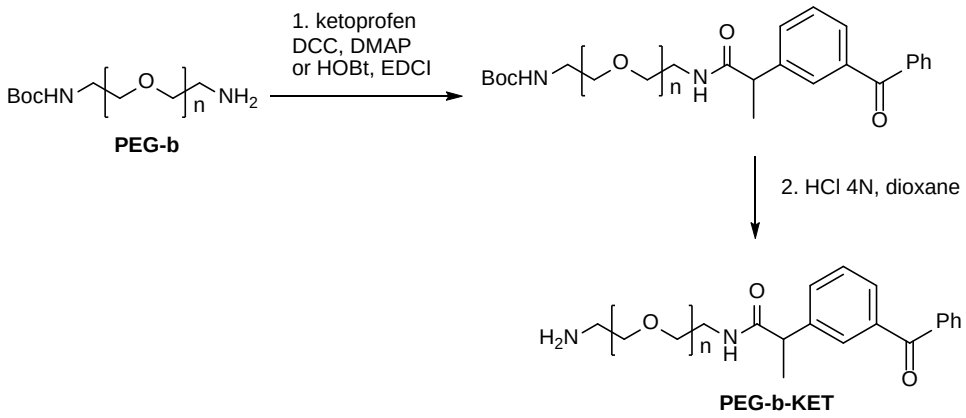
δ 78.02 – 7.37 (m, 9H, ar.), 4.31 – 4.16 (m, 2H, C*H*$_2$-NH_2), 3.91 (s, 2H, C*H*$_2$-OC(O)), 3.62 (s, 174H, PEG), 3.44 (t, J = 4.8 Hz, 1H, C*H*-Me), 3.16 (s, 2H, C*H*$_2$-CH_2O), 1.52 (d, J = 7.2 Hz, 3H, CH_3).



^{13}C NMR (101 MHz, $CDCl_3$) δ 196.49 (CO), 174.08 (CO), 140.88 (C ar.), 137.96 (C ar.), 137.58 (C ar.), 132.58(C ar.), 131.65 (C ar.), 130.14 (2 x C ar.), 129.31 (C ar.), 129.06 (C ar.), 128.60 (C ar.), 128.40 (2 x C ar.), 70.67 (n x CH_2), 69.08 (CH_2), 67.18 (CH_2), 64.18 (CH_2), 45.37 (CH), 18.64 (CH_3).



Preparation of PEG-b-KET



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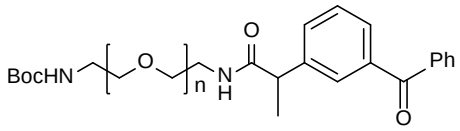
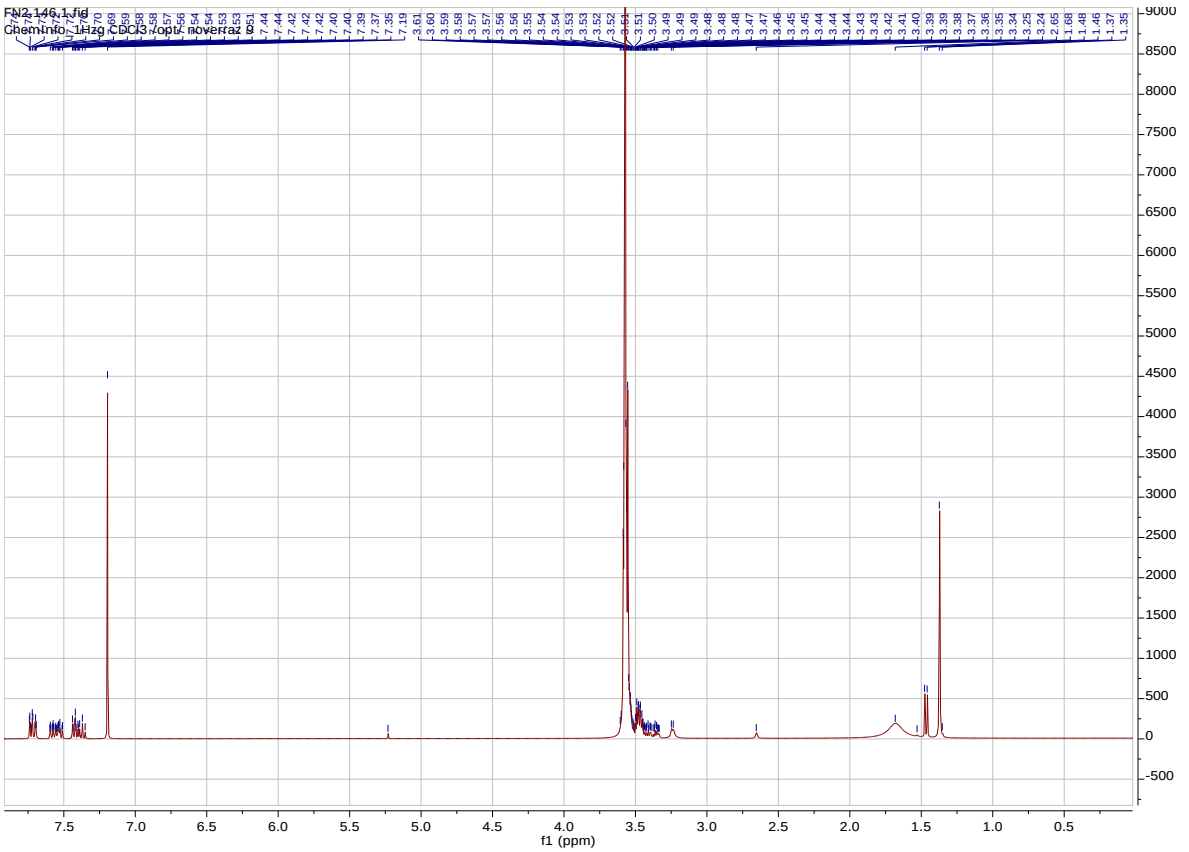
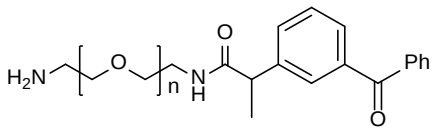


^{1}H NMR spectrum (400 MHz, $CDCl_3$)

δ 7.84 – 7.35 (m, 9H, ar.), 3.70 – 3.29 (m, 176H, CH_2, PEG), 1.53 (d, *J* = 7.1 Hz, 3H, CH_3), 1.44 (s, 9H, $CH_3(^tBu)$).



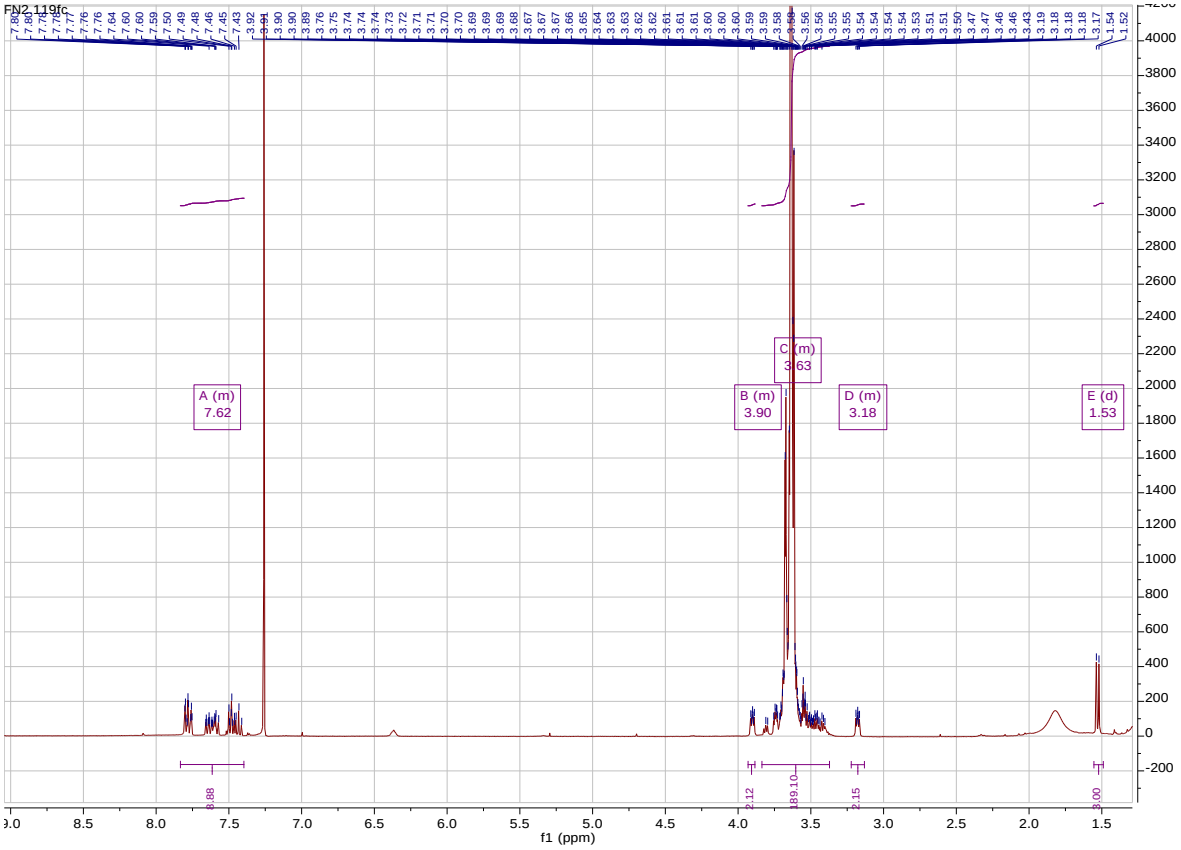
PEG-b-KET



IR (neat) : 2890, 1105 cm^{-1}.

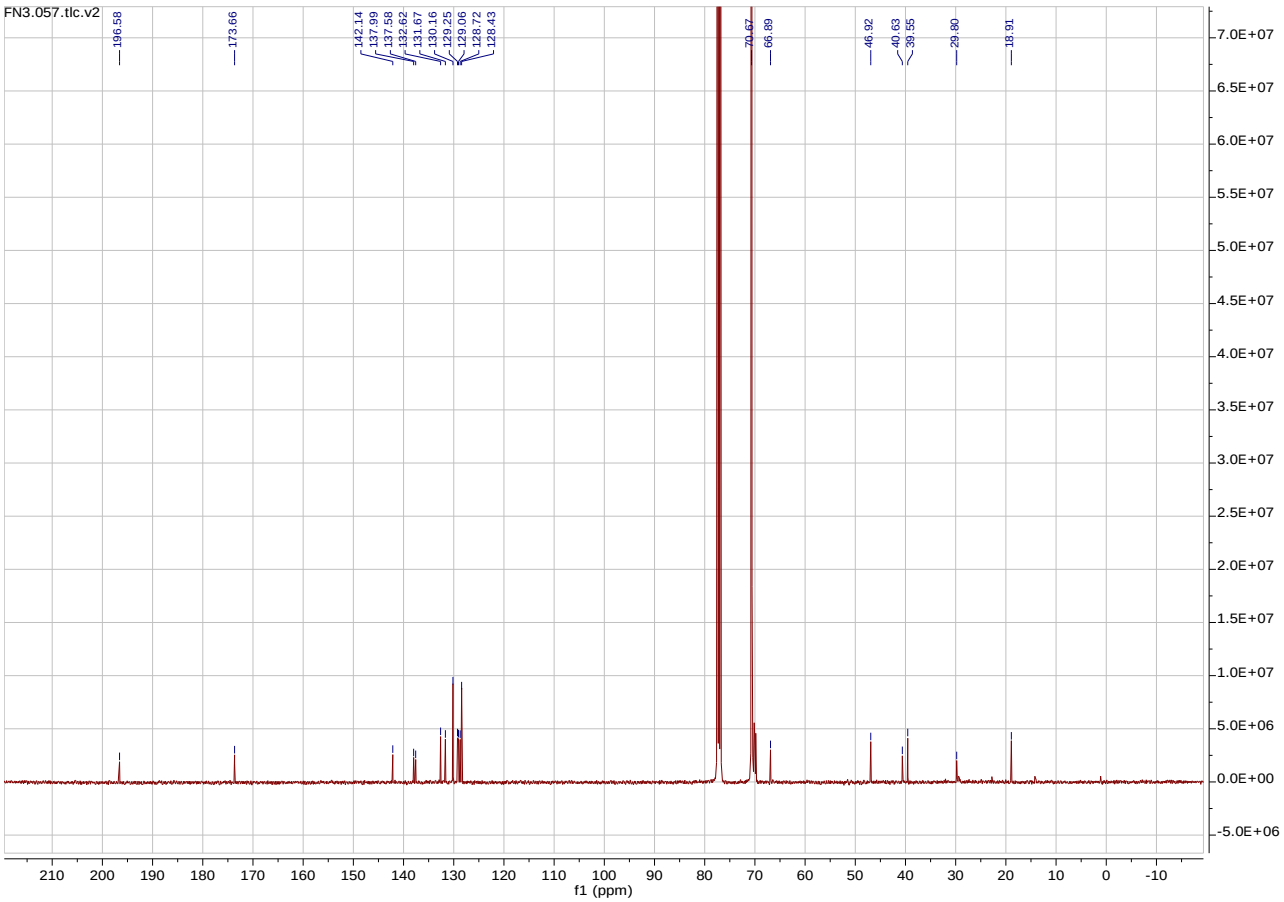
^{1}H NMR (400 MHz, $CDCl_3$)

δ 7.84 – 7.38 (m, 9H, ar.), 6.85 (t, J = 5.4 Hz, 1H, NH), 3.74 – 3.31 (m, 14H, CH_2-CH_2-O), 1.51 (d, J = 7.1 Hz, 3H, CH_3).



^{13}C NMR (101 MHz, $CDCl_3$)

δ 196.58(CO), 173.66(CO), 142.14 (C ar.), 137.99 (C ar.), 137.58 (C ar.), 132.62 (C ar.), 131.67 (C ar.), 130.16 (2 x C ar.), 129.25 (C ar.), 129.06 (C ar.), 128.72 (C ar.), 128.43 (2 x C ar.), 70.67 (n x CH_2), 66.89 (CH_2), 46.92 (CH), 40.63 (CH_2), 39.55 (CH_2), 29.80 (CH_2), 18.91 (CH_3).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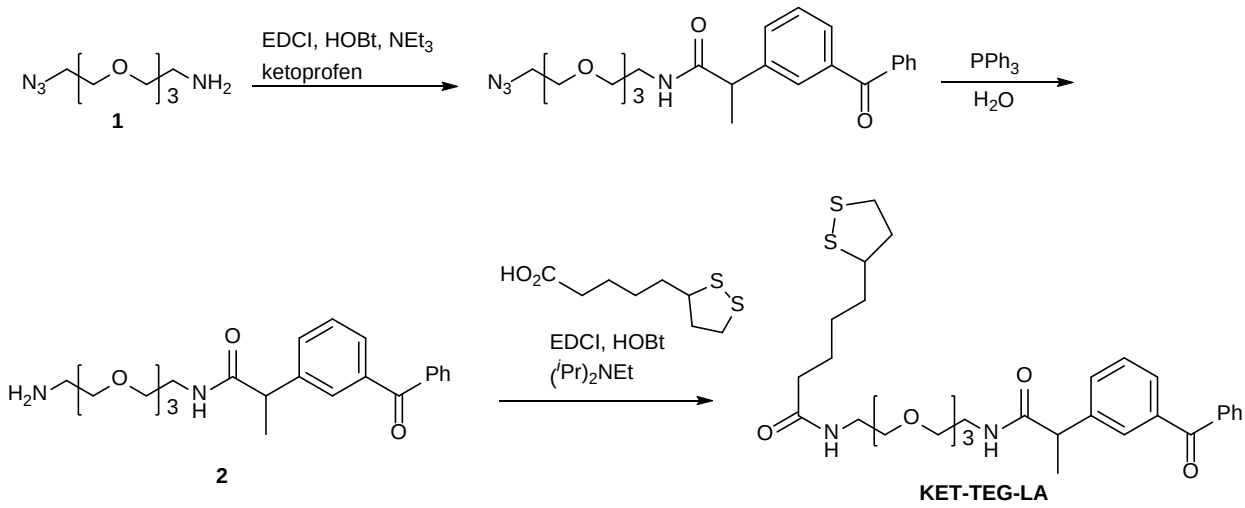
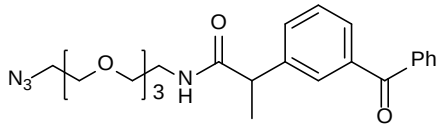
196.58
173.66
142.14
137.99
137.58
132.62
131.67
130.16
129.25
129.06
128.72
128.43
70.67
66.89
46.92
40.63
39.55
29.80
18.91
7.0E+07
6.5E+07
6.0E+07
5.5E+07
5.0E+07
4.5E+07
4.0E+07
3.5E+07
3.0E+07
2.5E+07
2.0E+07
1.5E+07
1.0E+07
5.0E+06
0.0E+00
-5.0E+06
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)

Preparation of KET-TEG-LA



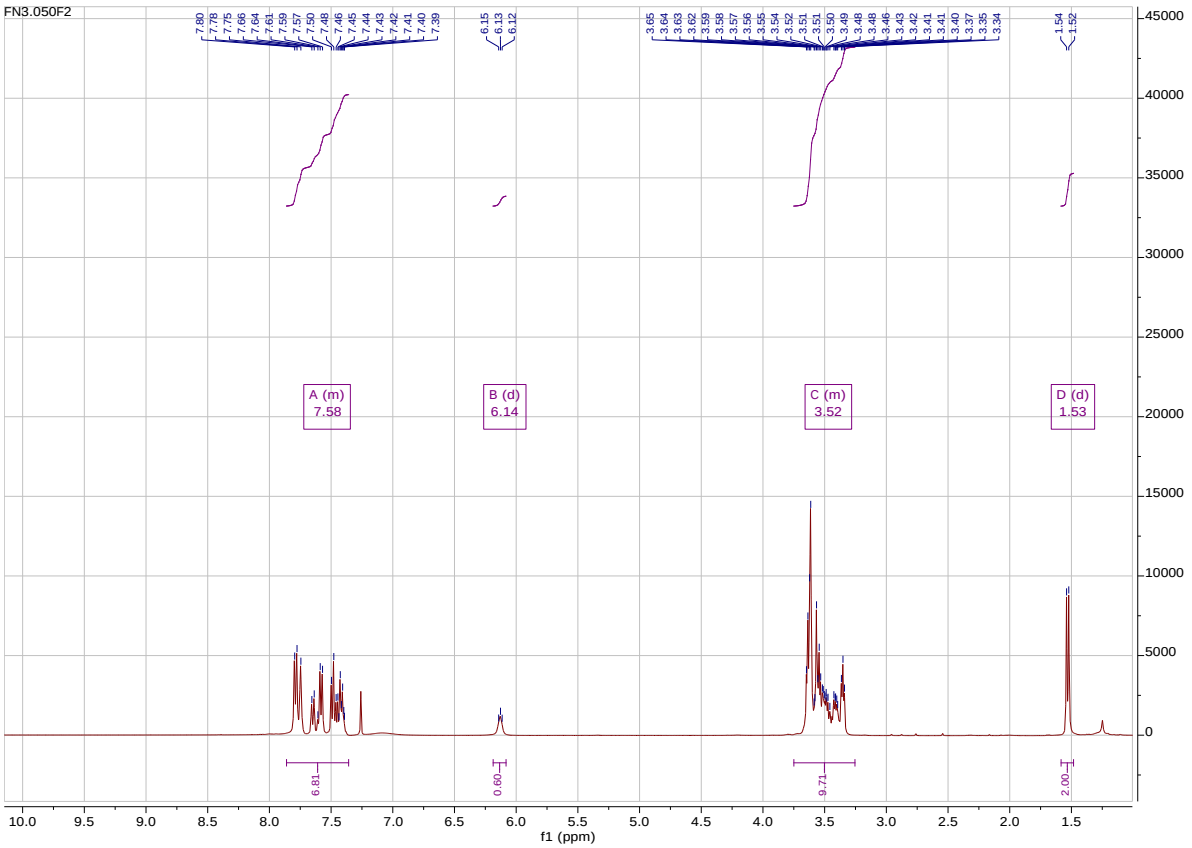
Intermediate



IR (neat): 2880, 2095, 1655, 1285, 1115 cm^{-1}.

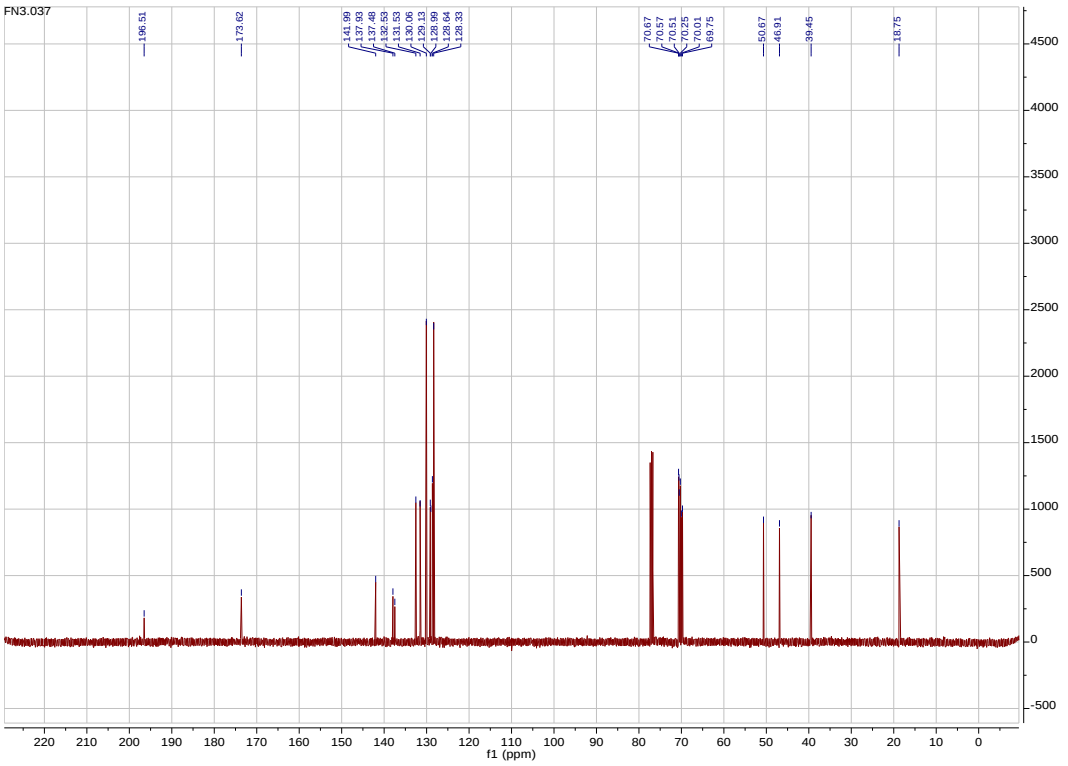
^{1}H NMR (400 MHz, $CDCl_3$)

δ 7.83 – 7.40 (m, 9H, ar.), 6.07 (d, J = 5.3 Hz, 1H, NH), 3.70 – 3.30 (m, 17H, CH_2-CH_2-O + CH), 1.54 (d, J = 7.1 Hz, 3H, CH_3).

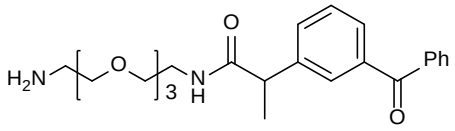


^{13}C NMR (101 MHz, $CDCl_3$)

δ 196.65 (CO), 173.79 (CO), 142.35 (C ar.), 137.91 (C ar), 137.60 (C ar), 132.58 (C ar), 131.62 (C ar), 130.14 (2 X C ar), 129.23 (C ar), 128.97 (C ar), 128.64 (C ar), 128.63 (C ar), 128.40 (2 x C ar), 70.55 (CH_2), 70.52 (CH_2), 70.29 (CH_2), 70.18 (CH_2), 70.01 (CH_2), 46.77 (CH-CH_3), 39.51 (CH_2), 18.85 (CH_3).



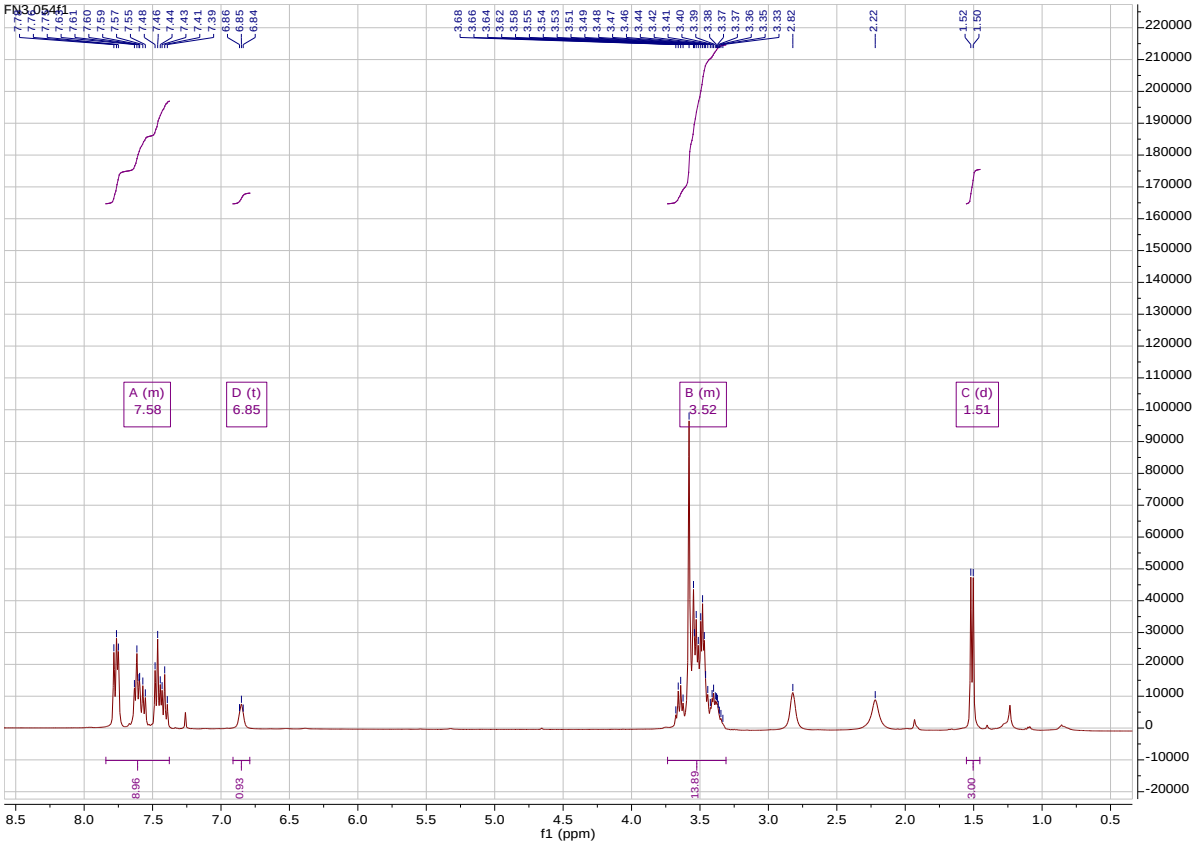
Compound 2



IR (neat): 2925, 1660, 1080 cm^{-1}.

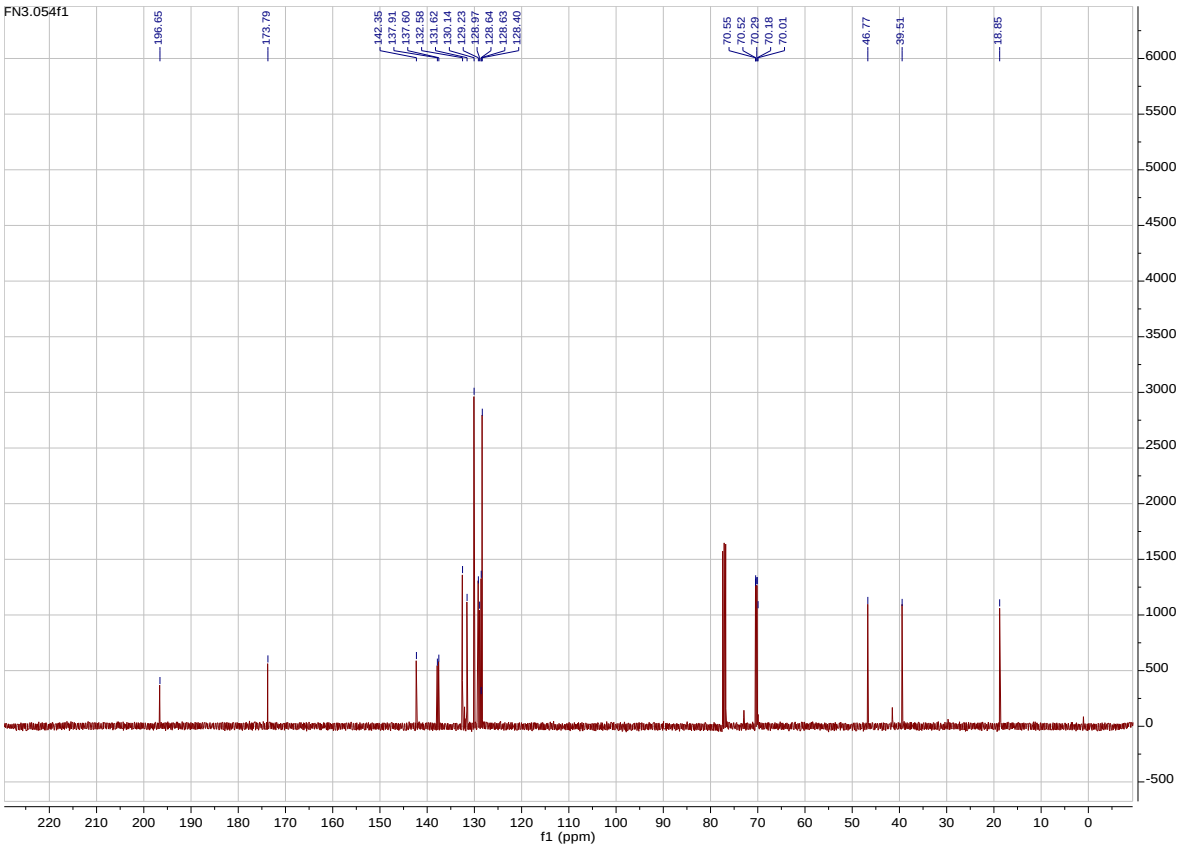
^{1}H NMR (400 MHz, $CDCl_3$)

δ 7.84 – 7.38 (m, 9H, ar.), 6.85 (t, J = 5.4 Hz, 1H, NH), 3.74 – 3.31 (m, 17H, CH_2-CH_2-O + CH), 1.51 (d, J = 7.1 Hz, 3H, CH_3).

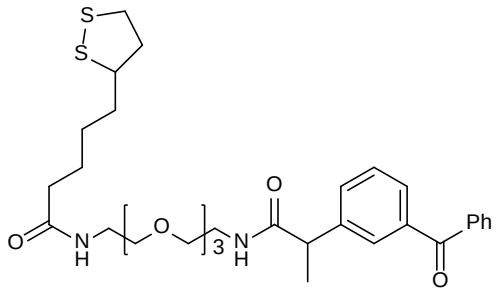


^{13}C NMR (101 MHz, $CDCl_3$)

δ 196.65 (CO), 173.79 (CO), 142.35 (C ar.), 137.91 (C ar), 137.60 (C ar), 132.58 (C ar), 131.62 (C ar), 130.14 (2 X C ar), 129.23 (C ar), 128.97 (C ar), 128.64 (C ar), 128.63 (C ar), 128.40 (2 x C ar), 70.55 (CH_2), 70.52 (CH_2), 70.29 (CH_2), 70.18 (CH_2), 70.01 (CH_2), 46.77 (CH-CH_3), 39.51 (CH_2), 18.85 (CH_3).



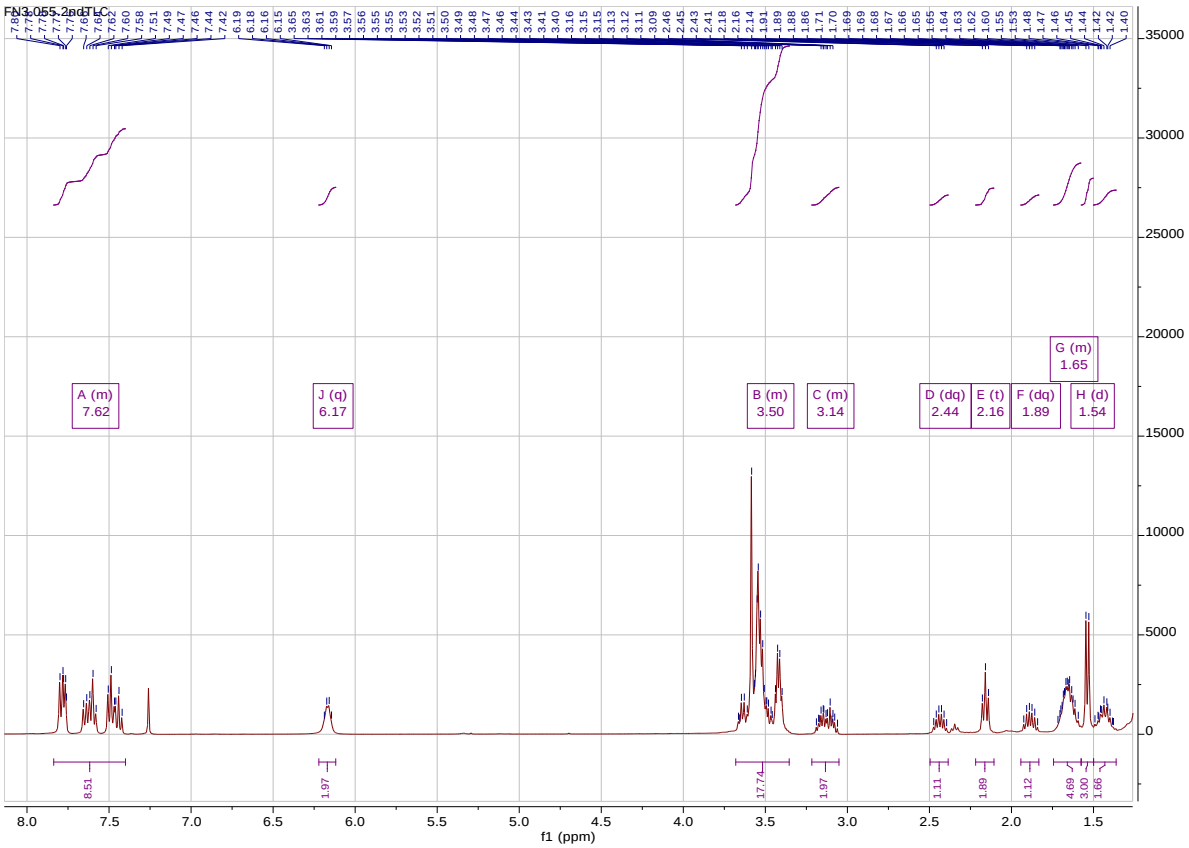
KET-TEG-LA



IR (neat): 3315, 2940, 1655, 1560, 1450, 1370, 1265, 1095 cm^{-1}.

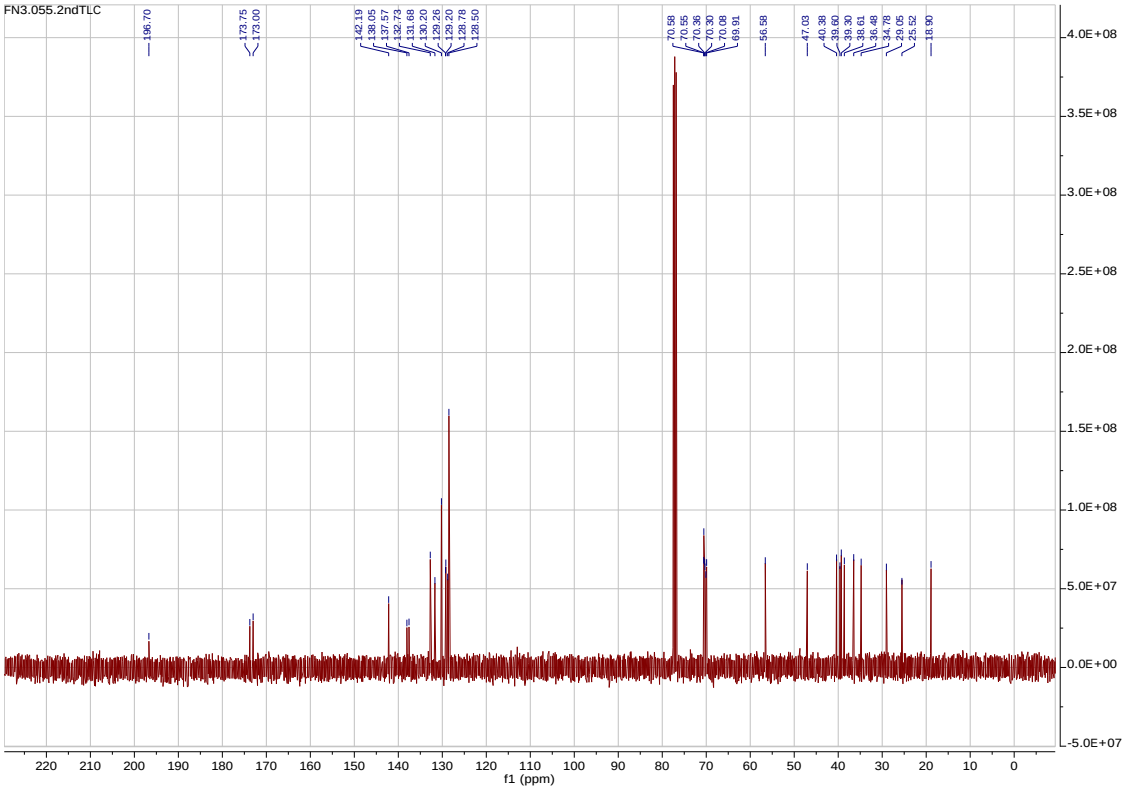
^{1}H NMR (400 MHz, $CDCl_3$)

δ 7.84 – 7.40 (m, 9H, ar.), 6.17 (2 bs, 2H, 2 x NH), 3.68 – 3.36 (m, 18H, CH_2-CH_2-O, C*H*-Me, C*H*-S), 3.22 – 3.05 (m, 2H, C*H*$_2$-S), 2.44 (dq, J = 12.5, 6.3 Hz, 1H, C*H*$_2$'-CH_2S), 2.16 (t, J = 7.5 Hz, 2H, C*H*$_2$-C(O)NH), 1.89 (dq, J = 13.7, 7.0 Hz, 1H, C*H*$_2$''-CH_2S), 1.75 – 1.58 (m, 4H,2 x CH_2), 1.54 (d, J = 7.1 Hz, 3H, CH_3), 1.44 (ddd, J = 14.8, 8.3, 5.8 Hz, 2H, CH_2).

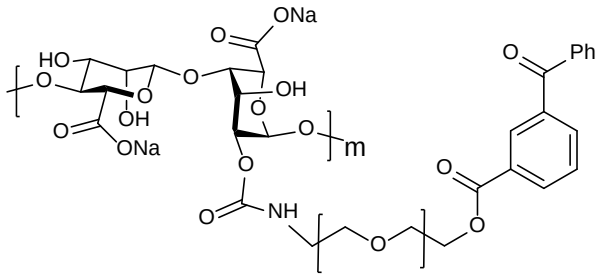


^{13}C NMR (101 MHz, $CDCl_3$)

δ 196.70 (CO), 173.75 (CO), 173.00 (CO), 142.19 (C ar.), 138.05 (C ar.), 137.57 (C ar.), 132.73 (C ar.), 131.68 (C ar.), 130.20 (2 x C ar.), 129.26 (C ar.), 129.20 (C ar.), 128.78 (C ar.), 128.50 (2 x C ar.), 70.58 (CH_2), 70.55 (CH_2), 70.36 (CH_2), 70.30 (CH_2), 70.08 (CH_2), 69.91 (CH_2), 56.58 (CH), 47.03 (CH), 40.38 (CH_2), 39.60 (CH_2), 39.30 (CH_2), 38.61 (CH_2), 36.48 (CH_2), 34.78 (CH_2), 29.05 (CH_2), 25.52 (CH_2), 18.90 (CH_3).

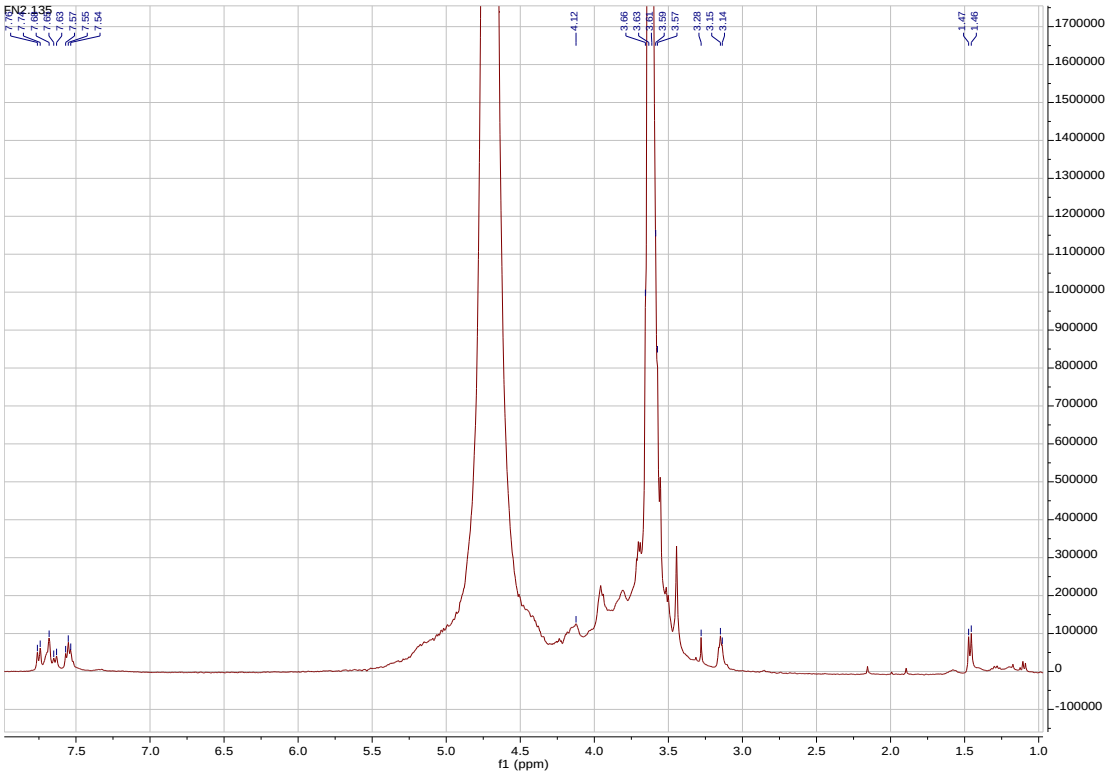


Alg-PEG-a-KET

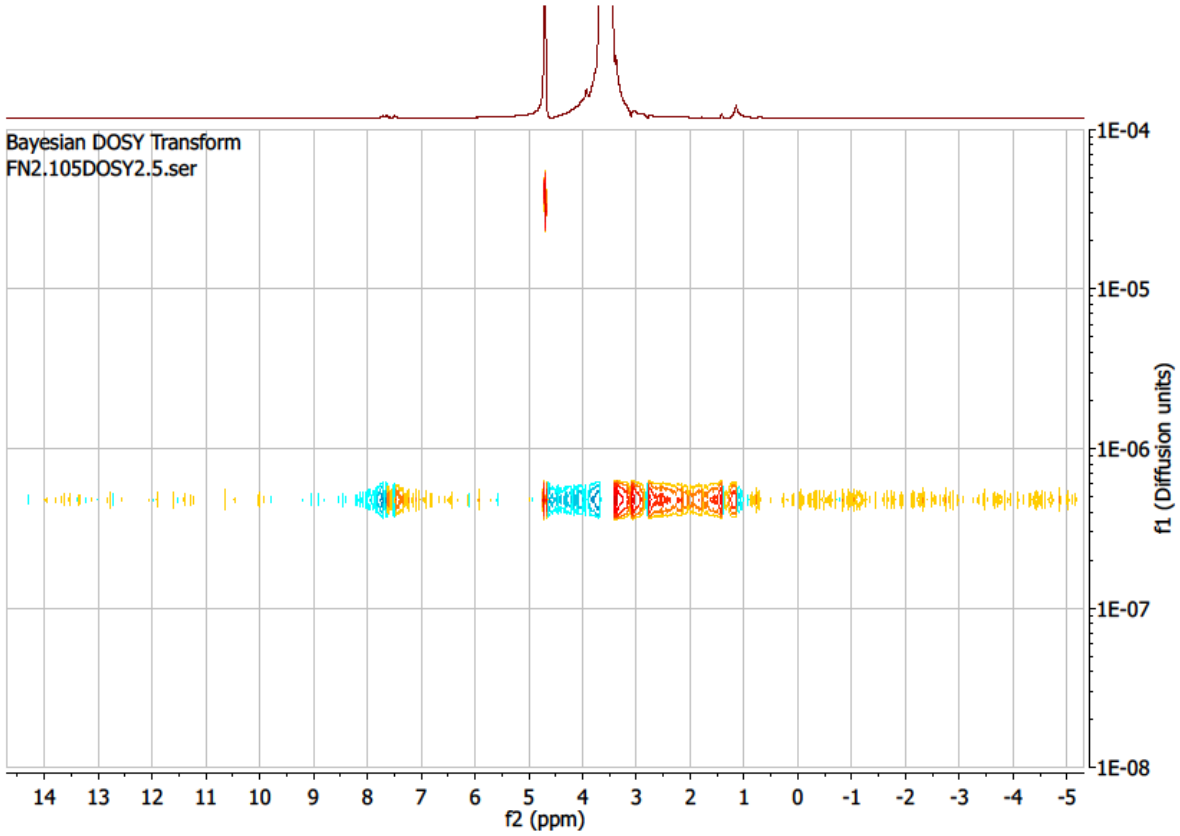


^{1}H NMR (400 MHz, D_2O)

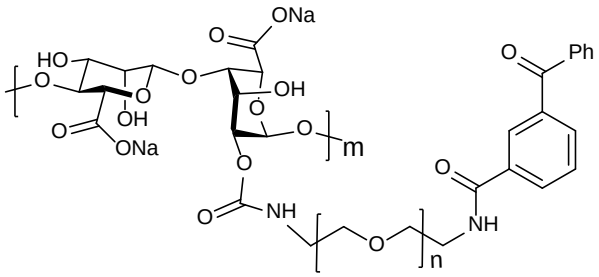
7.76 – 7.51 (m, ar.), 4.10 – 3.40 (m, C*H*-Alg + C*H*2-C*H*2-O), 3.14 (m, CH_2-NHC(O)), 1.46 (d, *J* = 7.1 Hz, CH_3).



DOSY-spectrum

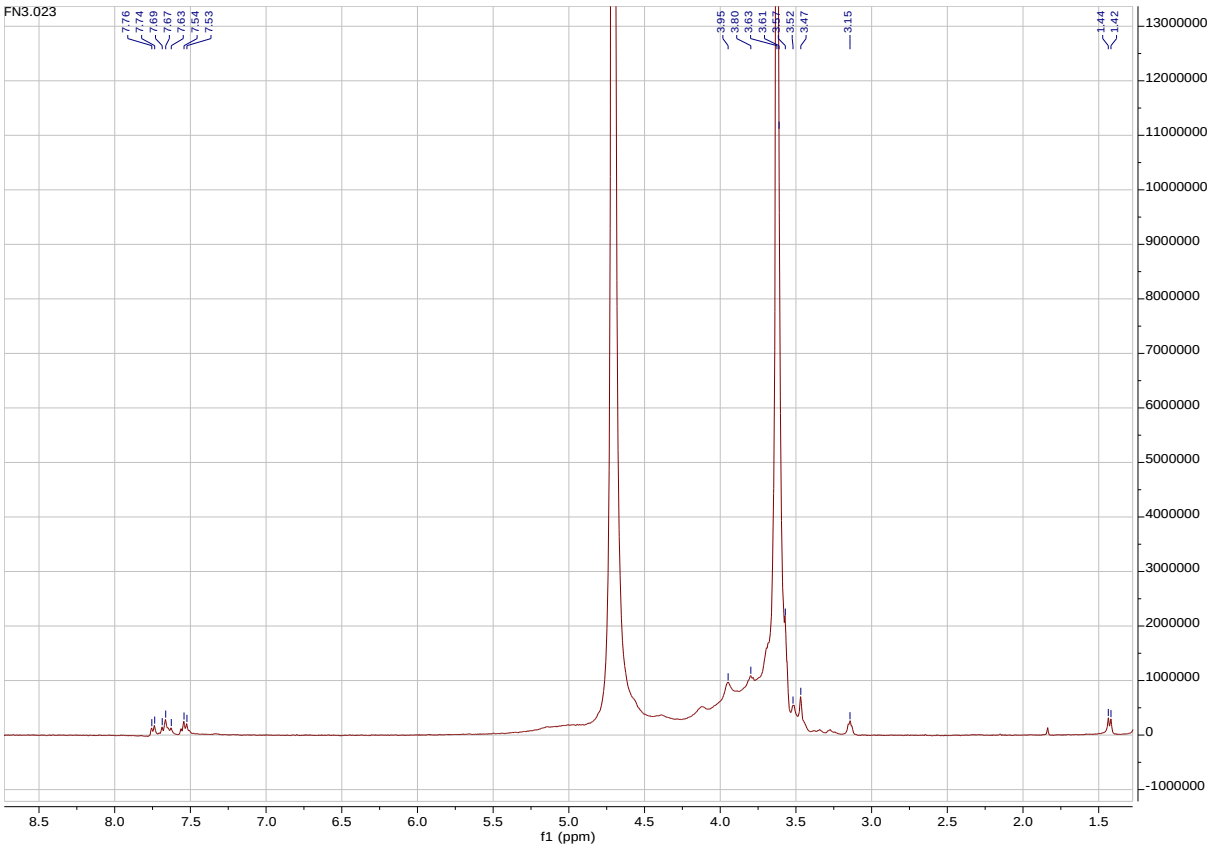


Alg-PEG-b-KET



^{1}H NMR (400 MHz, D_2O)

7.76 – 7.53 (m, ar.), 4.10 – 3.47 (m, C*H*-Alg + C*H*2-C*H*2-O), 3.15 (m, CH_2-NHC(O)), 1.43 (d, *J* = 7.1 Hz, CH_3).


FN3.023
7.76
7.74
7.69
7.67
7.63
7.54
7.53
3.95
3.80
3.63
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3.52
3.47
3.15
1.44
1.42
13000000
12000000
11000000
10000000
9000000
8000000
7000000
6000000
5000000
4000000
3000000
2000000
1000000
0
-1000000
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
f1 (ppm)

Determination of the degree of grafting

The procedure is described for Alg-PEG-a-KET. The same methodology was performed on Alg-PEG-b-KET.

1D ^{1}H-NMR of Alg-PEG-a-KET was run at 800 MHz with a repetition delay of 5s as ^{1}H longitudinal relaxation time constants (T_1) of PEG-a-KET and alginate were measured to be $T_1 < 1$s. Line broadening was set to 0.3 Hz. The baseline was automatically corrected with Bruker Topspin routine, using degree 5 polynomial. The percentage of grafting was estimated by comparison of integration of ^{1}H resonances of PEG fragment (176 protons) and alginate unit (5 protons). Due to overlap, separation between PEG and alginate resonances was performed by deconvolution with Lorentzian functions, using Bruker Topspin curve fitting and line shape analysis tool. The grafting degrees of Alg-PEG-a-KET are of 7% and 20% when using 0.1 and 0.2 equiv. of PEG-a-KET, respectively, in the coupling procedure.

Representative spectra

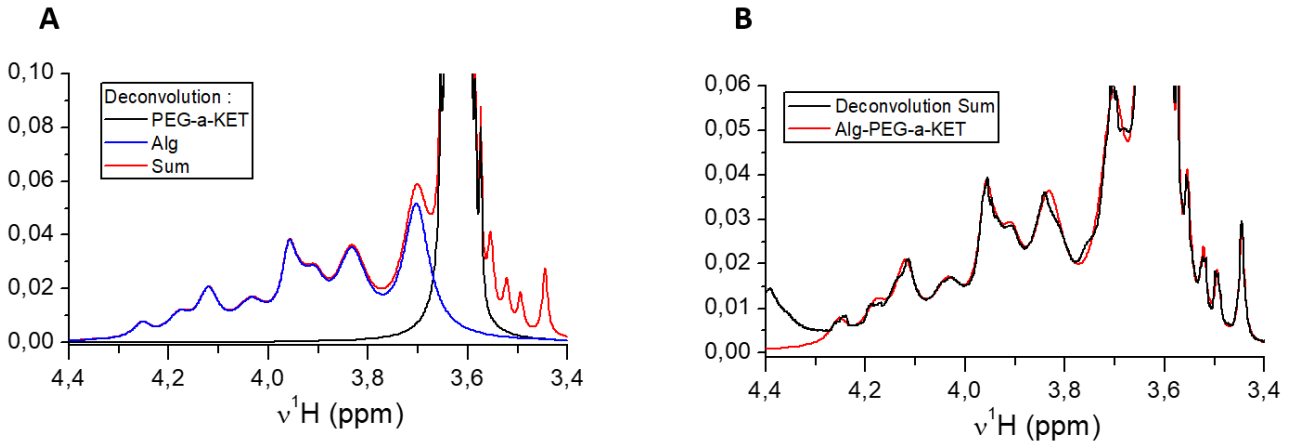


Figure S1. (A): Simulated spectra of PEG-a-KET, Alg, D_2O and their sum calculated from lorentzian functions. (B): The method was validated by superimposition of the recorded spectrum of Alg-PEG-a-KET with the simulated spectrum obtained from the sum of the lorentzian functions

Ketoprofen release from MS fabricated with pure Alg-PEG-KET polymers

MS were produced by extruding a solution of Alg-PEG-a-KET (7% grafting degree, 3 wt %, 1mL) or Alg-PEG-b-KET (7% grafting degree, 2.5 wt %, 1.2 mL) in MOPS (100 mM, pH = 7.4) into a gelation bath containing $CaCl_2$ as ionic crosslinker and tween 80 (diluted 1/10 000). The resulting MS (1.5 mL) were kept in 3 mL of gelation solution (100 mM $CaCl_2$ in 100 mM MOPS buffer, pH 7.4) under slow mechanical agitation. Aliquots of the supernatant were withdrawn after 0.5, 1, 3, 6, 24, 48, 72, 96 and 168 h, for quantitative MS analysis. At each time point, fresh supernatant was added to compensate for the withdrawn volume.

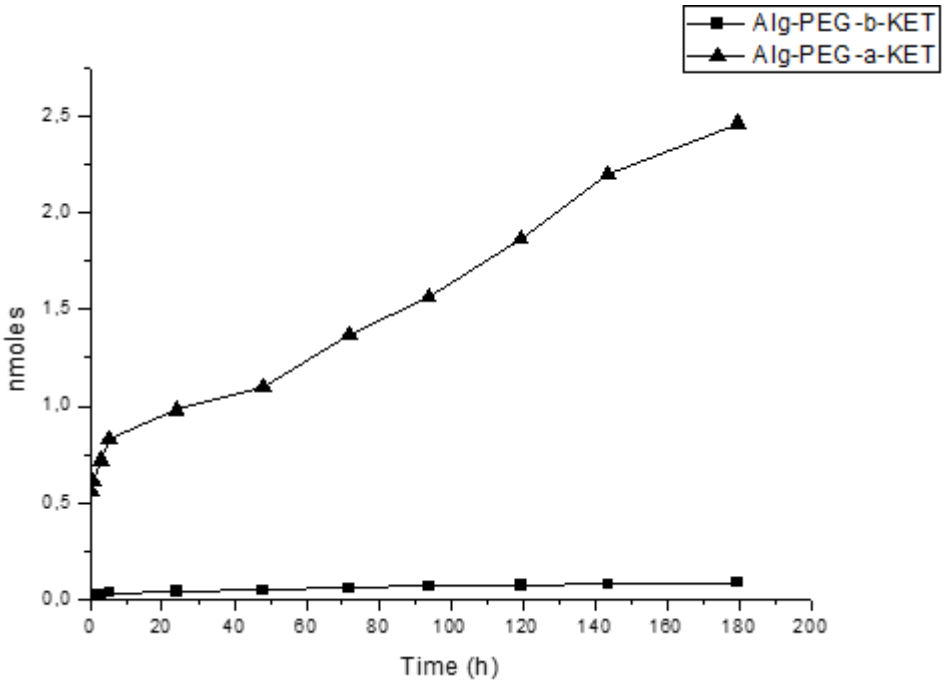


Figure S2. Cumulative release of KET from Alg-PEG-a-KET and Alg-PEG-b-KET MS at physiological pH (7.4)

Quantitative analysis by UHPLC-ESI-HRMS

MS quantitive analysis were performed on a Agilent 6530 Accurate Mass Q-TOF LCMS mass spectrometer coupled to an Agilent 1290 series UHPLC system (Agilent Technologies, USA). The separation was achieved using an ACQUITY UPLC® BEH C18 1.7µm column, 2.1 mm x 50 mm (Waters) heated at 30°C. Mobile phase consisted of 0.1% formic acid in water as eluent A and 0.1% formic acid in acetonitrile as eluent B. The separation was carried out at 0.4 mL/min over a 6 min total run time using the following program: 0-0.5 min, 1-5% B; 0.5-3 min, 5-95% B; 3-3.5 min, 95-1% B; 3.5-6 min, 1% B to re-equilibrate the system in initial conditions. The sample manager was system temperature was fixed at 10°C and the injection volume was 5µL. Mass spectrometer detection was operated in positive ionization using the Dual AJS Jet stream ESI Assembly. The QTOF instrument was operated in the 4 GHz High Resolution mode in profile mode. The Instrument was calibrated in positive full scan mode using ESI-L+ solution (Agilent Technologies, USA). The TOF mass spectra were acquired over the range of *m/z* 100-1000 at an acquisition rate of 3 spectra/s. ESI AJS settings were as follows RF drying gas flow, 8 L/min; drying gas temperature, 300°C; nebulizer pressure, 35psi; capillary voltage, 3500V; nozzle voltage, 1000V; fragmentor voltage, 175V; skimmer voltage, 65V; octopole 1 RF voltage, 750V; Sheath gas temperature, 350°C; Sheath gas low; 11L/min. Data was processed using the MassHunter Workstation (Agilent Technologies, USA). Extracted ions chromatograms (XIC) were based on a retention time (RT) window of ±0.2 min with a mass-extraction-window (MEW) of ±50 ppm centered on m/z_{theor}. Calibration curves were fitted with a polynomial order 2 equation, with R2 > 0.995.

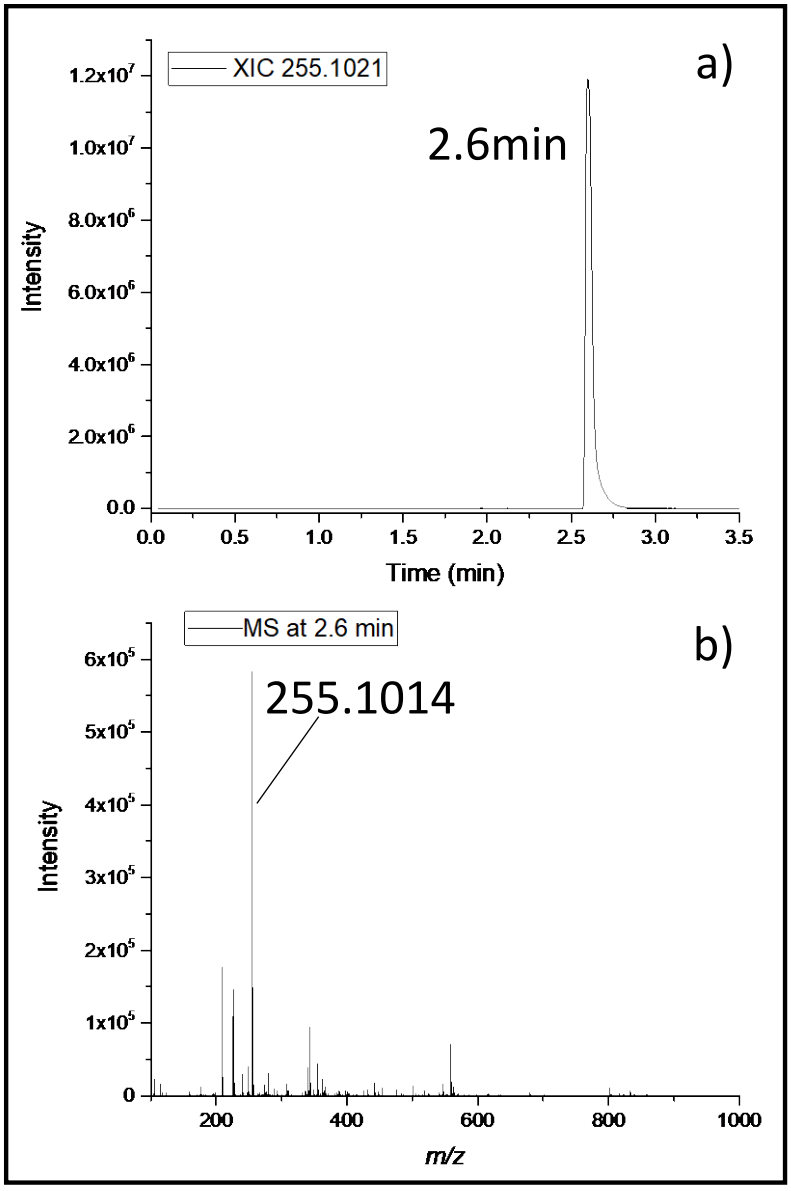


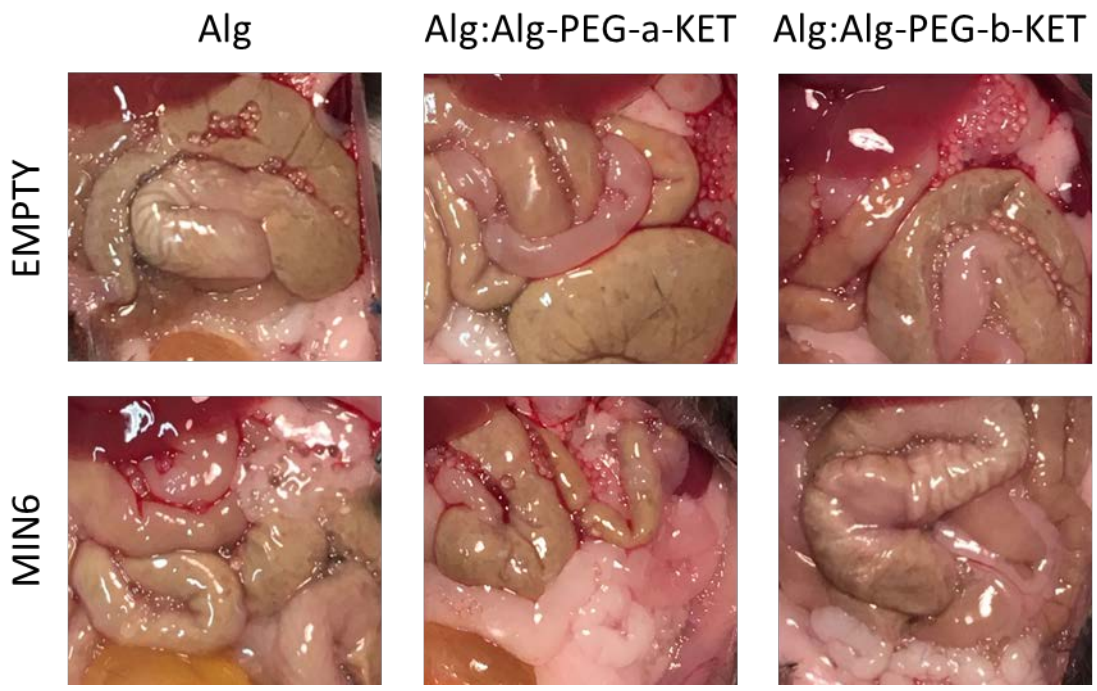
Figure S3. (a) Typical XIC (255.1021 MEW of ±50 ppm) of ketoprofen standard (b) Typical MS of ketoprofen standard at 2.6 min

Estimation of the number of MS for transplantation

We considered the average diameter of MS as 0.55 mm and used the formula $4/3\ \pi\ r^3$ to calculate the sphere volume. Hence, the sphere volume of one MS was determined as 0.087 mm^3. Total volume of transplanted MS is, 1500mm^3 (1.5 mL), adjusted by the Kepler conjecture 1500*0.74 resulting in 1110 mm^3. Therefore the total number of MS contained in 1.5 mL of solution is 1110 mm^3/0.087 mm^3 = 12'758.

Observation of free-floating MS in the peritoneum of transplanted mice

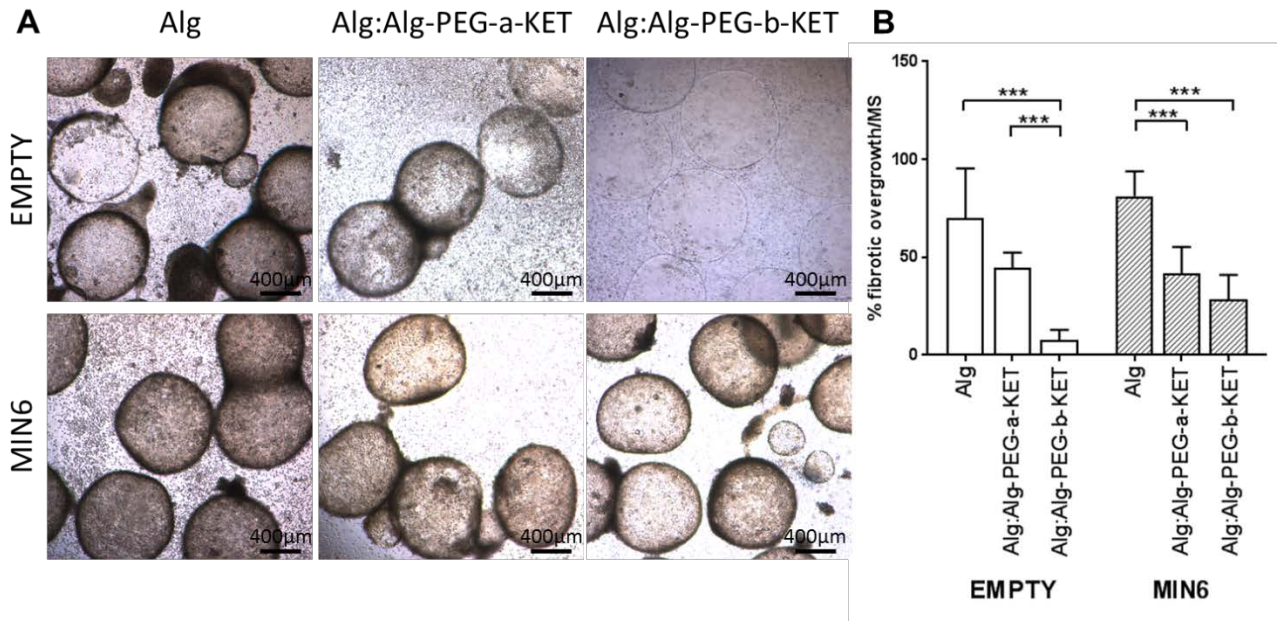
Figure S4. Non-adherent distribution of MS in the peritoneum 30 days after transplantation.



Images of empty and MIN6-containing MS, floating in the peritoneum. Na-alg (left rows), Alg:Alg-PEG-a-KET (middle rows) and Alg:Alg-PEG-b-KET (right rows); cell-free MS (upper panels) and MS containing MIN6 cells (lower panels).

Quantification of PFO on cell-free and MIN6 cells containing MS 15 days after transplantation

Figure S5. Quantification of pericapsular fibrotic overgrowth on cell-free and MIN6 cells containing MS 15 days after transplantation.

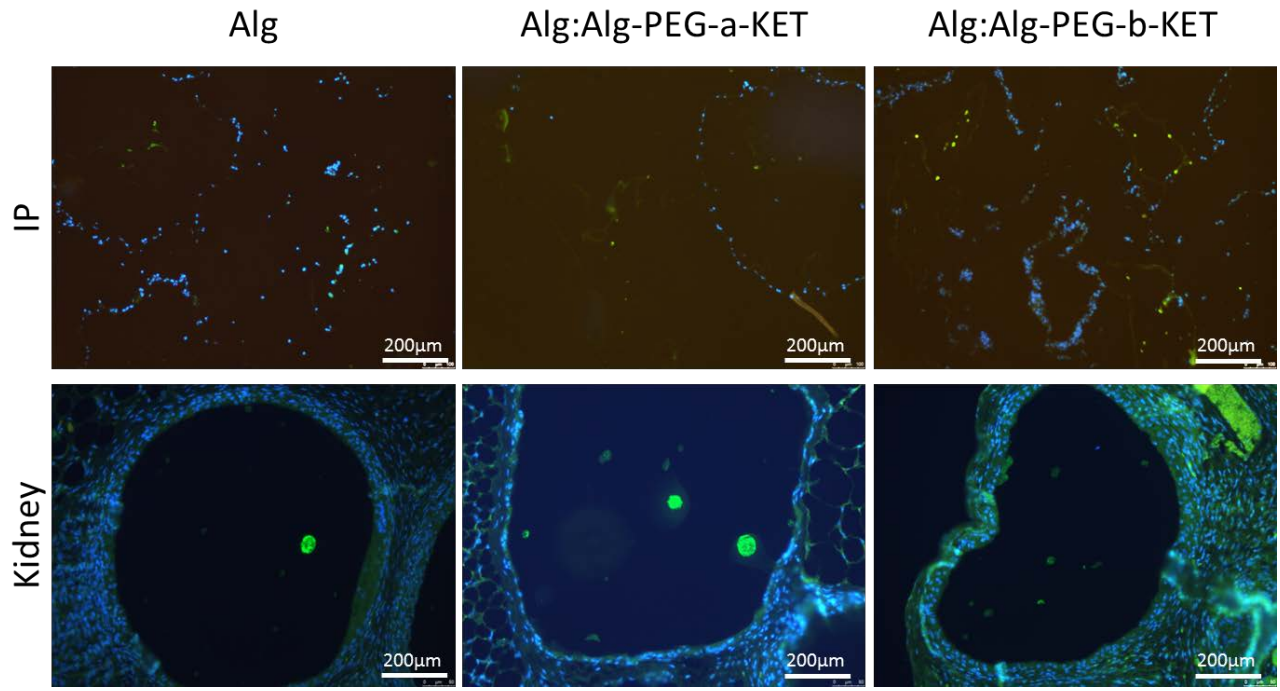


(A) Bright-field microscopy images of MS retrieved from the peritoneum following 15 days of transplantation. Na-alg (left rows), Alg:Alg-PEG-a-KET (middle rows) and Alg:Alg-PEG-b-KET (right rows); cell-free MS (upper panels) and MS with MIN6 cells (lower panels). (B) Pericapsular fibrotic overgrowth was quantified using ImageJ and expressed as a percentage of fibrotic overgrowth. For each MS, fibrotic overgrowth (grey levels) were normalized to the total area of MS measured. Fibrotic overgrowth for empty MS (white bars) and for MS with MIN6 cells (striped bars) are represented. All values are means ± standard deviation (SD) of at least 5 MS. ***$p<0.001$.

Encapsulated MIN6 cells remained viable until 30 days after transplantation

To assess whether encapsulated MIN6 cells remained viable 30 days after transplantation, MS retrieved from the peritoneum were cryopreserved in OCT and kidneys, containing MS under the kidney capsule, were collected and prepared for paraffin embedding. Both, crysections and paraffin-sections were stained for insulin as previously described (Montanari et al, stem cell research and therapy, 2017). Briefly, to demask epitopes on paraffin-sections, slides were heated with 0.01 mol/l citrate for 15 minutes in a microwave. Then, paraffin sections and air-dried cryosections were incubated with 0.5% FCS for 30 minutes to prevent non specific staining. Insulin were stained with a guinea pig anti-porcine insulin antibody, diluted 1/500 (Dako) and a secondary antibody anti-guinea pig Alexa 488, diluted 1/500 (ThermoFisher). Sections were mounted with Vectashield mounting medium, containing Dapi to stain cell nuclei (Vector Laboratories, CA,USA). Images were acquired using a fluorescence microscope and LAS V4.5 software (Leica Microsystem, Heerbrugg, Switzerland). All types of MS contained viable MIN6 cells at 30 days of transplantation.

Figure S6. Insulin staining of encapsulated MIN6 cells 30 days after transplantation



MS containing MIN6 cells, retrieved from the peritoneum (IP), were embedded in OCT and cryosections stained for insulin in green (upper panels). Kidneys, containing transplanted MS, were collected, embedded in paraffin and sections stained for insulin (lower panels). MIN6 cells encapsulated in Na-alg MS (left), Alg:Alg-PEG-a-KET MS (middle) and Alg:Alg-PEG-b-KET MS (right) are positively stained for insulin after 30 days of transplantation.

Table S1. Primers for real-time RT – PCR experiments

H-RPLP0	Fwd 5'-TGC ATC AGT ACC CCA TTC TAT CAT-3' Rev 5'-AAG GTG TAA TCC GTC TCC ACA GA-3'
H-αSMA	Fwd 5'-ACT GGG ACG ACA TGG AAA AG-3' Rev 5'-TAC ATG GCT GGG ACA TTG AA-3'
H-COL1A1	Fwd 5'-CCA GGC AGA GAT GGT GAA GA-3' Rev 5'-GCA GGT CCT TGG AAA CCT TG-3'
H-MMP-1	Fwd 5'-GGA GGA AAA GCA GCT CAA GAA-3' Rev 5'-TCC AGG GTG ACA CCA GTG ACT-3'

H-RPLP0, human ribosomal protein large P0; H-α-SMA, human α smooth muscle actine; H-COL1A1, human type 1 collagen; H-MMP-1, human metalloproteinase-1.