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Supporting information

Hemiacetal-linked pH-sensitive PEG-lipids for non-viral gene delivery

Filipe Coelho[1], Laura M. Salonen[1],* Bruno F. B. Silva[1,*]

[1]International Iberian Nanotechnology Laboratory (INL), Av. Mestre José Veiga, 4715-330 Braga, Portugal

laura.salonen@inl.int; bruno.silva@inl.int.

*corresponding authors:

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1. Materials and Methods

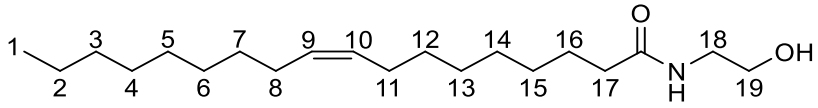
Triethylamine (Et_3N, 99%), *p*-toluenesulfonic acid (*p*-TsOH, 99%), chloroform (99%), diethyl ether (99%), 2-aminoethanol (99%), 3-aminopropanol (98%), 2-amino-2-hydroxymethyl-propane-1,3-diol (T, 99%), acetic acid (99%), sodium acetate, deuterium oxide (D_2O, 99%), acetonitrile, acetonitrile-*d* (99%), methoxy polyethylene glycon 2000 kDa (mPEG─OH), *N,N*'-dicyclohexylcarbodiimide (DCC, 99%), 4-(dimethylamino)pyridine (DMAP, 99%), and 5-formylbenzoic acid (97%) were purchased from Sigma-Aldrich. 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES, 99%) and chloroform-*d* ($CDCl_3$, 99%) were purchased from Acros Organics. Benzene (99%) and oleoyl chloride (80%) were purchased from TCI.

Unless stated otherwise, all reactions were performed in oven-dried glassware. Commercial reagents were used as received. Reactions were stirred magnetically and monitored by analytical thin-layer chromatography (TLC) using ALUGRAM® Xtra Sil G UV254 aluminum sheets. Reactions involving mPEG─OH were monitored by reverse-phase thin-layer chromatography using DC Kieselgel 60 RP-18 F254S aluminum sheets. Flash chromatography was performed employing silica gel (60 Å, 40–63 µm, 230–400 mesh, Macherey Nagel). Reverse-phase flash chromatography was performed employing reverse phase silica gel (90 Å, 40–63 µm, 230–400 mesh, Sigma-Aldrich).

Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a 400 MHz Bruker Avance II spectrometer at the NMR service of University of Minho in Braga, Portugal. Proton chemical shifts are expressed in parts per million (δ scale) and are calibrated using residual undeuterated solvent peak as an internal reference ($CDCl_3$: δ 7.26). Data for ^{1}H NMR spectra are reported as follows: chemical shift (δ ppm) (multiplicity, coupling constant (Hz), integration). Multiplicities are reported as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad, or combinations thereof. Carbon nuclear magnetic resonance (^{13}C NMR) spectra were recorded on a 101 MHz Bruker Avance II spectrometer. Carbon chemical shifts are expressed in parts per million (δ scale) and are referenced to the carbon resonances of the solvent ($CDCl_3$: δ 77.16). ^{1}H and ^{13}C resonances were assigned with the aid of additional information from 1D and 2D NMR spectra (H,H-COSY, DEPT 135, HSQC, and HMBC).

2. Synthesis of mPEG-C*n*-lipid

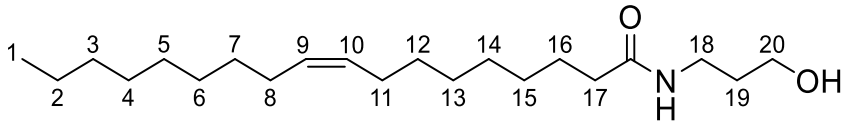
N-(2-Hydroxylpropyl)oleamide (4)



In a round-bottom flask under N_2, to a solution of Et_3N (516 mg, 5.10 mmol, 2 eq.) in dry CH_2Cl_2 (25.0 mL) was added 2-aminoethanol (**2**) (472 mg, 7.73 mmol, 3 eq.). Then, oleoyl chloride (**1**) (950 mg, 2.53 mmol, 1 eq.) was added dropwise and the mixture was stirred at RT overnight. A white precipitate formed, which was removed by filtration. The filtrate was concentrated to give a yellow oil, which solidified as it cooled down. Purification by flash chromatography (SiO_2; CH_2Cl_2/EtOAc 10:1→8:1→5:1→3:1) gave **4** (575 mg, 73% yield) as white solid.

^{1}H NMR (400 MHz, $CDCl_3$): 6.16 (s, H–N), 5.35 (m, 2 H, H–C(9, 10)), 3.73 (m, 2 H, H–C(19)), 3.43 (q, *J* = 5.2, 2 H, H–C(18)), 2.83 (s, H–O), 2.22 (t, *J* = 7.6 Hz, 2 H, H–C(17)), 2.01, (m, 4 H, H–C(8, 11)), 1.63 (m, 2 H, H–C(16)), 1.29 (m, 20 H, H–C(2, 3, 4, 5, 6, 7, 12, 13, 14, 15)), 0.88 (t, *J* = 6.9 Hz, 3 H, H–C(1)); ^{13}C NMR (101 MHz, $CDCl_3$): 174.6, 129.9, 129.6, 62.0, 42.3, 36.6, 31.8, 29.69, 29.65, 29.45, 29.24, 29.22, 29.09, 27.15, 27.10, 25.7, 22.6, 14.0.

N-(3-Hydroxypropyl)oleamide (5)

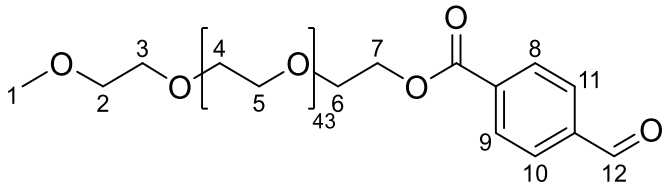


In a round-bottom flask under N_2, to a solution of Et_3N (494 mg, 4.88 mmol, 2 eq.) in dry CH_2Cl_2 (25.0 mL) was added 3-amino-1-propanol (**3**) (550 mg, 7.33 mmol, 3 eq.). Then, oleoyl chloride (**1**) (910 mg, 2.99 mmol, 1 eq.) was added dropwise, and the mixture was stirred at RT overnight. A white precipitate formed, which was removed by filtration. The filtrate was concentrated to give a yellow oil, which solidified as it cooled down. Purification by flash chromatography (SiO_2; CH_2Cl_2/EtOAc 1:0→8:1→5:1→3:1) gave **5** (790 mg, 88% yield) as white solid.

^{1}H NMR (400 MHz, $CDCl_3$): 6.27 (s, 1 H, H–N), 5.33 (m, 2H, H–C(9, 10)), 3.60 (t, *J* = 5.6 Hz, 2 H, H–C(20)), 3.45 (s, 1 H, H–O), 3.38 (q, *J* = 6.2 Hz, 2 H, H–C(18)), 2.17 (t, *J* = 7.6 Hz, 2 H, H–C(17)), 1.99 (m, 4 H, H–C(8, 11)), 1.66 (m, 2 H, H–C(19)), 1.61 (m, 2 H, H–C(16)), 1.27 (m, 20 H, H–C(2, 3, 4, 5, 6, 7, 12, 13, 14, 15)), 0.87 (t, *J* = 7.0 Hz, 3 H, H–C(1)); ^{13}C NMR (101 MHz, $CDCl_3$): 175.3, 130.6,

130.3, 59.8, 37.3, 36.8, 32.9, 32.5, 30.39, 39.34, 30.1, 29.94, 29.91, 29.90, 29.8, 27.84, 27.80, 26.5, 23.3, 14.7.

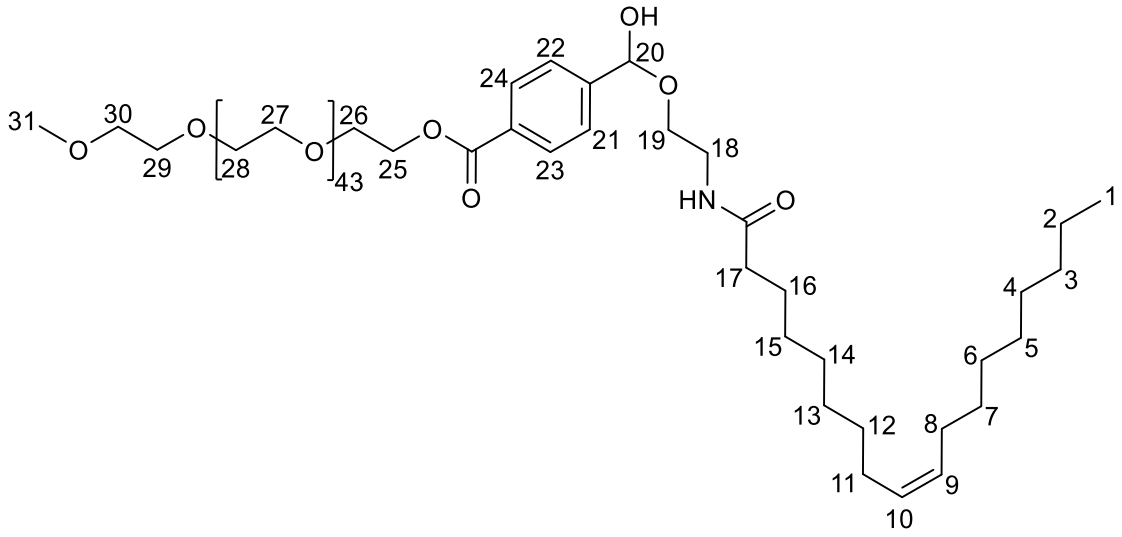
mPEG-benzaldehyde (6)



In a round-bottom flask under inert atmosphere, DCC (495 mg, 2.40 mmol, 8 eq.) was added to a solution containing 4-formylbenzoic acid (**8**) (360 mg, 2.40 mmol, 8 eq.) and DMAP (293 mg, 2.40 mmol, 8 eq.) in dry CH_2Cl_2 (15.0 mL). The reaction was stirred at RT for 15 minutes. Then, mPEG-OH (600 mg, 0.300 mmol, 1 eq.) was added to the reaction, and the mixture was stirred at RT overnight. A white precipitate formed, which was removed by filtration. The filtrate was concentrated by vacuum and precipitated 3 times from diethyl ether. Purification by flash chromatography (RP-SiO_2; MQ H_2O/MeCN 4:1→3:2) gave **6** (487 mg, 76% yield) as white solid.

^{1}H NMR (400 MHz, $CDCl_3$): 10.11 (s, H–C(12)), 8.22 (m, 2 H, H–C(10,11)), 7.96 (m, 2 H, H–C(8, 9)), 4.52 (m, 2 H, H–C(7)), 3.85 (m, 2 H, H–C(6)), 3.65 (m, 180 H, H–C(3, 4, 5)), 3.55 (m, 2 H, H–C(2)), 3.38 (s, 3 H, H–C(1)); ^{13}C NMR (101 MHz, $CDCl_3$): 191.5, 165.3, 139.0, 134.9, 130.1, 129.3, 71.7, 70.49, 70.44, 70.42, 70.37, 70.31, 68.9, 64.5, 58.9, 29.5.

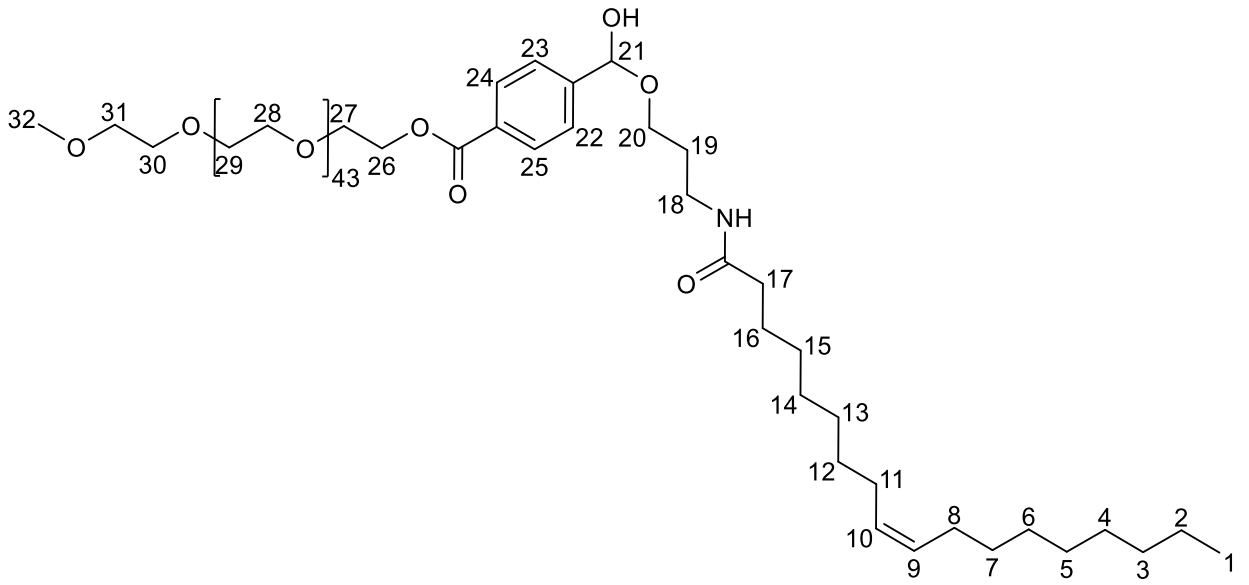
mPEG-C_2-lipid (9)



In a round-bottom flask, mPEG-benzaldehyde **8** (70 mg, 0.023 mmol, 1 eq.) and **3** (33 mg, 0.098 mmol, 5 eq.) were dissolved in benzene (3.0 mL). Then, *p*-TSOH (3.0 mg, 0.018 mmol, 1 eq.) was added. The reaction was left to stir for 2 h at 50 °C, after which the reaction mixture was heated to reflux for 18 h with Dean–Stark azeotropic removal of water. After cooling to RT, Et_3N (0.50 mL) was added to neutralize the acid. Then, the solution was concentrated under vacuum giving a yellow oil, which was dissolved in diethyl ether to precipitate product **9** (80 mg, 96% yield) as a brown solid.

^{1}H NMR (400 MHz, $CDCl_3$): 8.33 (s, 1 H, H–C(20)), 8.07 (m, 2 H, H–C(21, 22)), 7.78 (m, 2 H, H–C(24, 25)), 5.31 (m, 2 H, H–C(9, 10)), 4.47 (m, 2 H, H–C(19)), 4.38 (t, *J* = 5.6 Hz, 2 H, H–C(18)), 3.83 (m, 4 H, H–C(25, 26)), 3.63 (m, 172 H, H–C(27, 28)), 3.53 (m, 2 H, H–C(29)), 3.45 (m, 2 H, H–C(30)), 3.36 (s, 3 H, H–C(31)), 2.26 (t, *J* = 7.5 Hz, 2 H, H–C(17)), 1.97 (m, 4 H, H–C(8, 11)), 1.55 (quint, *J* = 7.6 Hz, 2 H, H–C(16)), 1.26 (d, *J* = 13.3 Hz, 20 H, H–C(2, 3, 4, 5, 6, 7, 12, 13, 14, 15)), 0.86 (t, *J* = 6.8 Hz, 3 H, H–C(1)); ^{13}C NMR (101 MHz, $CDCl_3$): 173.6, 165.9, 162.0, 139.7, 131.9, 129.9, 129.6, 127.9, 71.8, 70.56, 70.52, 70.50, 70.44, 70.39, 69.1, 64.2, 63.3, 59.8, 58.9, 34.1, 31.8, 29.6, 29.56, 29.54, 29.4, 29.2, 29.0, 28.98, 28.94, 28.93, 27.08, 27.04, 27.02, 24.8, 22.5, 14.0.

mPEG-C_3-lipid (10)



In a round-bottom flask, mPEG-benzaldehyde **8** (40 mg, 0.019 mmol, 1 eq.) and *N*-(3-hydroxylpropyl)oleamide **5** (33 mg, 0.098 mmol, 5 eq.) were dissolved in benzene (3.0 mL). Then, *p*-TSOH (3.0 mg, 0.018 mmol, 1 eq.) was added. The reaction was stirred at 50 °C for 2 h, after which the reaction mixture was heated to reflux for 18 h with Dean–Stark azeotropic removal of water. After cooling to RT, Et_3N (0.50 mL) was added to the mixture to neutralize the acid. Then, the solution was concentrated under vacuum to give a yellow oil, which was dissolved in diethyl ether to precipitate product **10** (56 mg, 97% yield) as brown solid.

^{1}H NMR (400 MHz, $CDCl_3$): 8.34 (s, 1 H, H–C(21)), 8.09 (m, 2 H, H–C(24, 25)), 7.78 (m, 2 H, H–C(22, 23)), 5.34 (m, 2 H, H–C(9, 10)), 4.49 (t, *J* = 5 Hz, 2 H, H–C(20)), 4.19 (t, *J* = 6.4 Hz, 2 H, H–C(18)), 3.83 (m, 4 H, H–C(26, 27)), 3.64 (m, 172 H, H–C(28, 29), 3.55 (m, 2 H, H–C(30)), 3.47 (m, 2 H, H–C(31)), 3.38 (s, 3 H, H–C(32)), 2.31 (t, *J* = 8 Hz, 2 H, H–C(17)), 2.04 (m, 6 H, H–C(8, 11, 19)), 1.62 (m, 2 H, H–C(16)), 1.28 (d, J = 16 Hz, 20 H, H–C(2, 3, 4, 5, 6, 7, 12, 13, 14, 15)), 0.88 (t, *J* = 8 Hz, 3 H, H–C(1)); ^{13}C NMR (101 MHz, $CDCl_3$): 173.6, 165.9, 160.4, 139.8, 131.6, 129.8, 129.5, 127.7, 71.7, 79.49, 70.44, 70.38, 70.32, 69.0, 68.97, 64.11, 64.05, 61.9, 58.8, 57.9, 34.1, 31.7, 29.7, 29.55, 29.49, 29.47, 29.43, 29.3, 29.2, 29.1, 28.96, 28.92, 28.9, 27.0, 26.97, 26.95, 24.8, 22.5, 13.99.

3. NMR Spectra

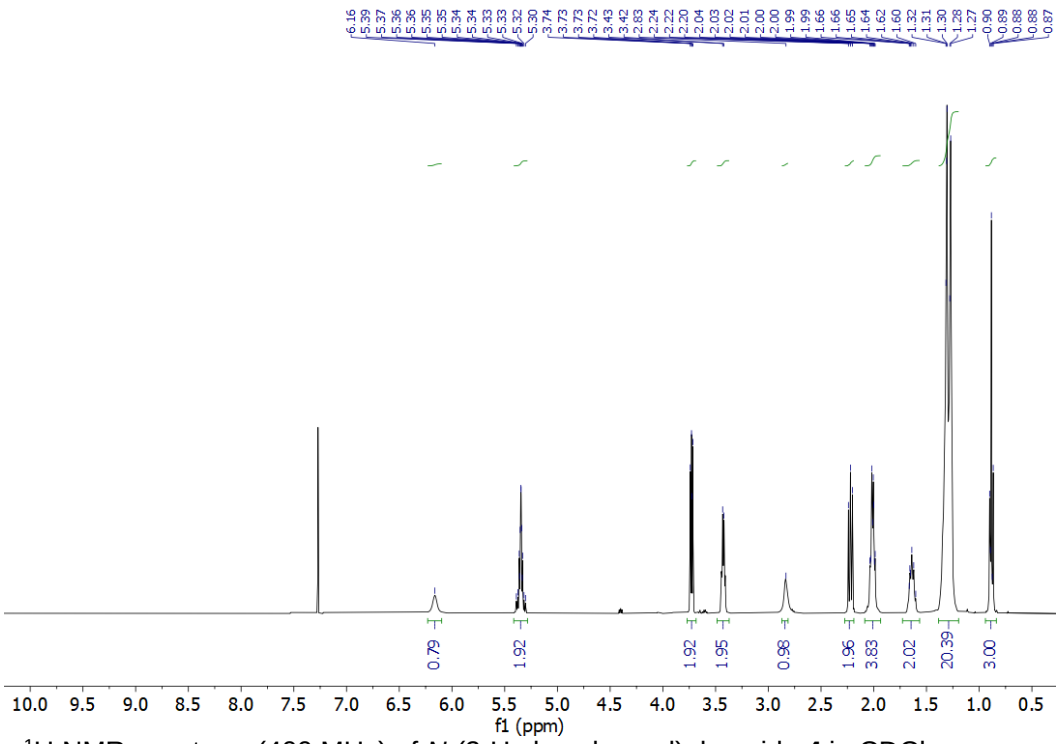


Figure 1S – ^{1}H NMR spectrum (400 MHz) of *N*-(2-Hydroxylpropyl)oleamide **4** in $CDCl_3$.

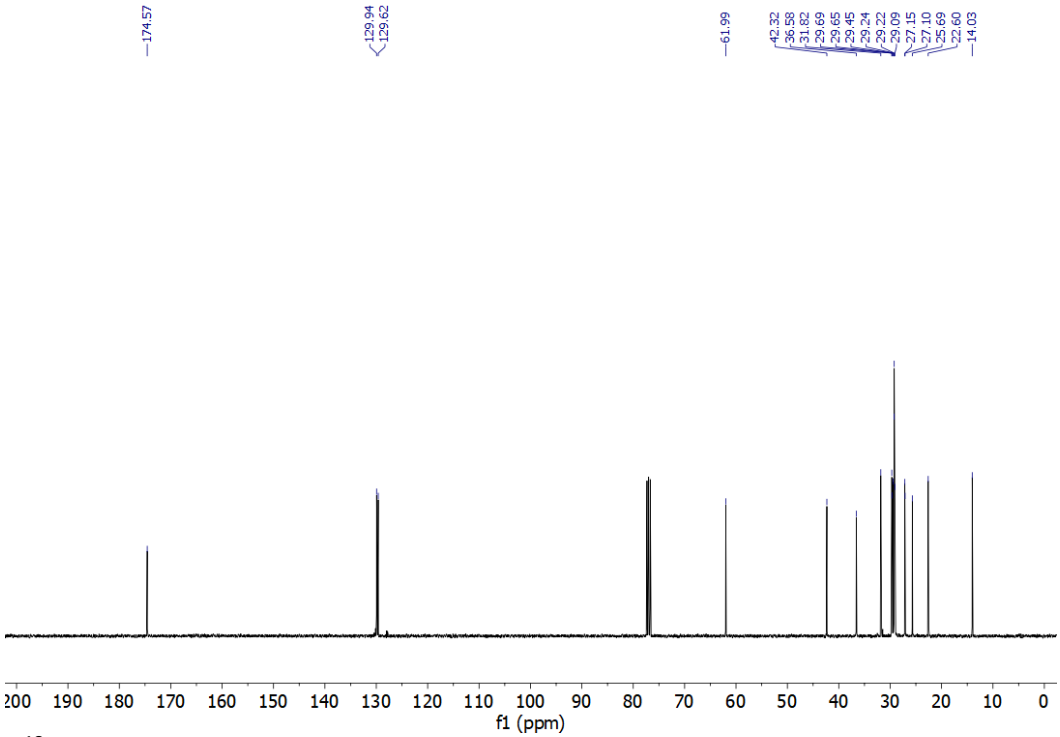


Figure 2S – ^{13}C NMR spectrum (101 MHz) of *N*-(2-Hydroxylpropyl)oleamide **4** in $CDCl_3$

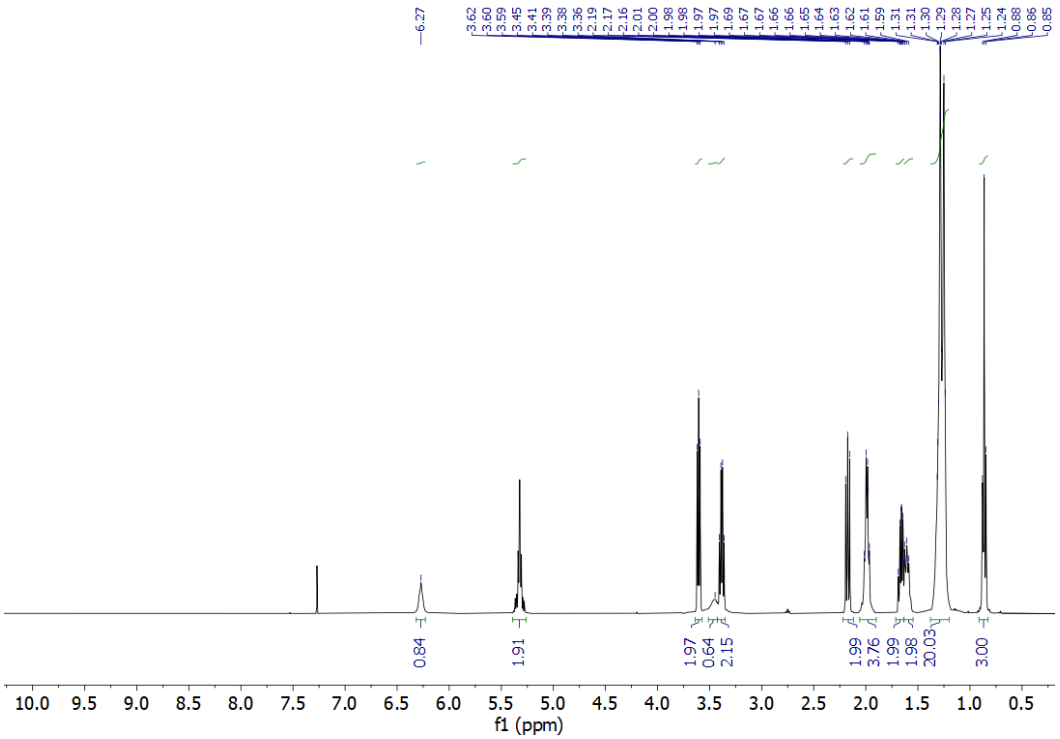


Figure 3S – ^{1}H NMR spectrum (400 MHz) of N-(3-Hydroxypropyl)oleamide **5** in $CDCl_3$.

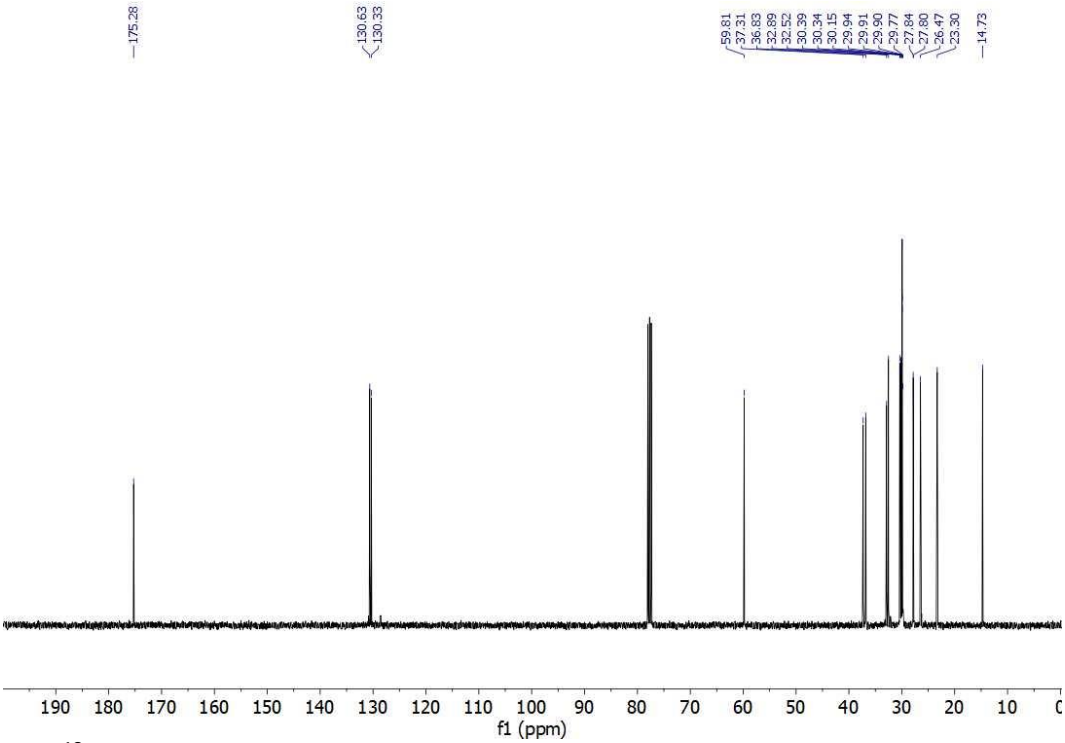


Figure 4S – ^{13}C NMR spectrum (101 MHz) of *N*-(3-Hydroxypropyl)oleamide **5** in $CDCl_3$

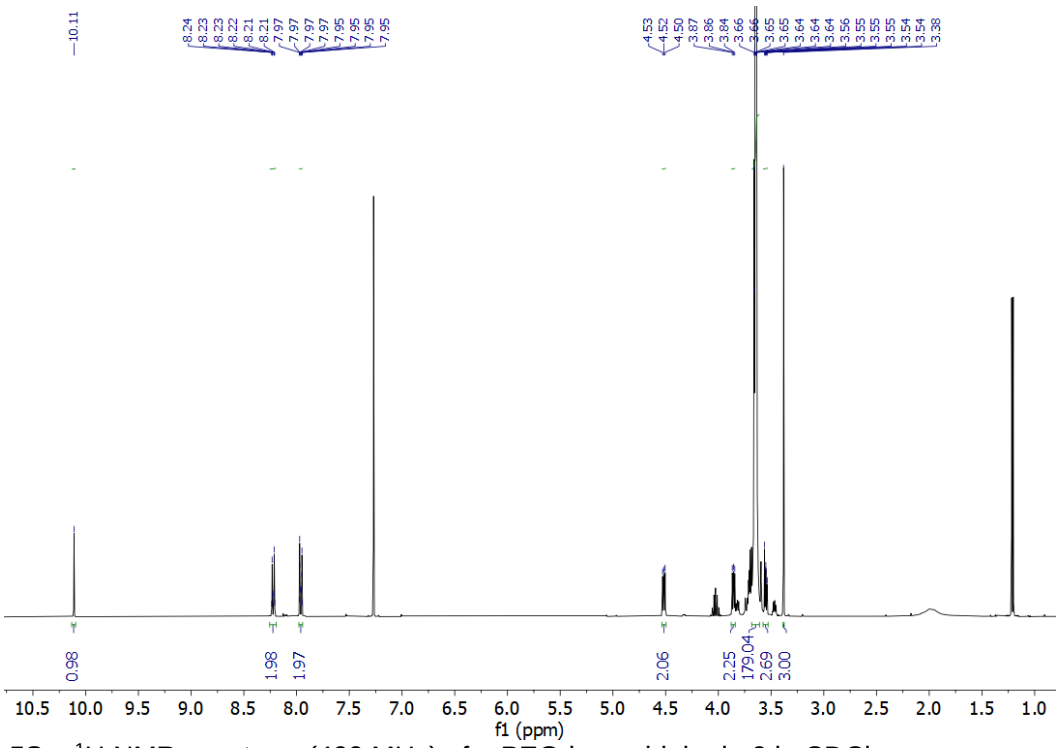


Figure 5S – ^{1}H NMR spectrum (400 MHz) of mPEG-benzaldehyde **6** in $CDCl_3$

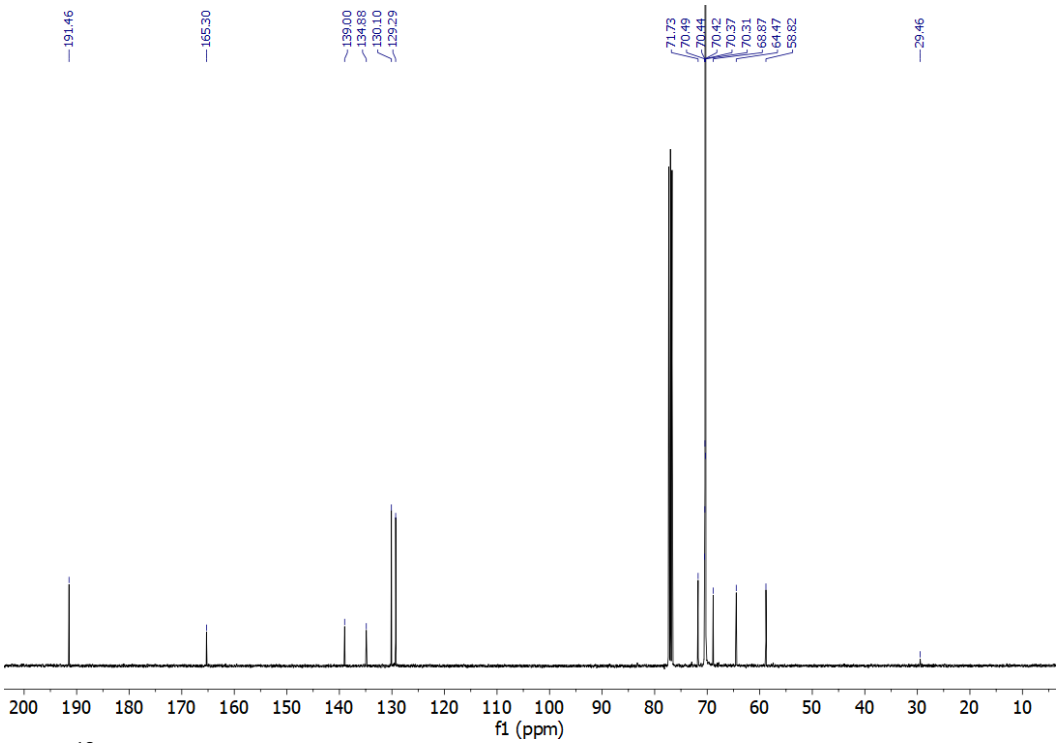


Figure 6S – ^{13}C NMR spectrum (101 MHz) of mPEG-benzaldehyde **6** in $CDCl_3$

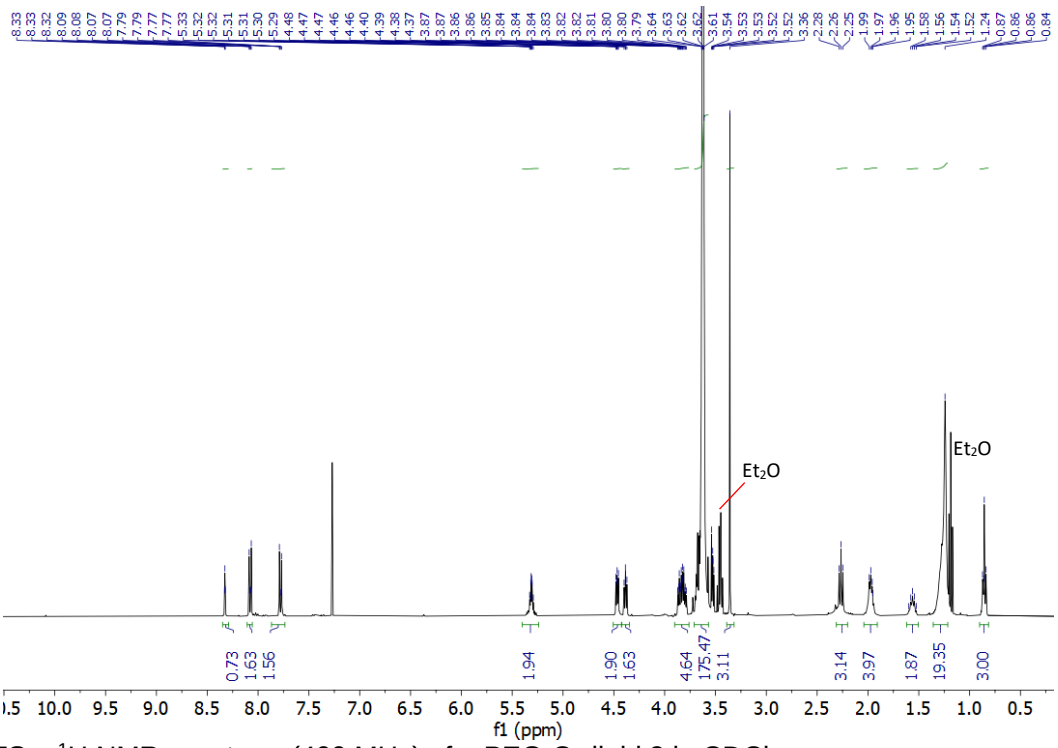


Figure 7S – ^{1}H NMR spectrum (400 MHz) of mPEG-C_2-lipid **9** in $CDCl_3$

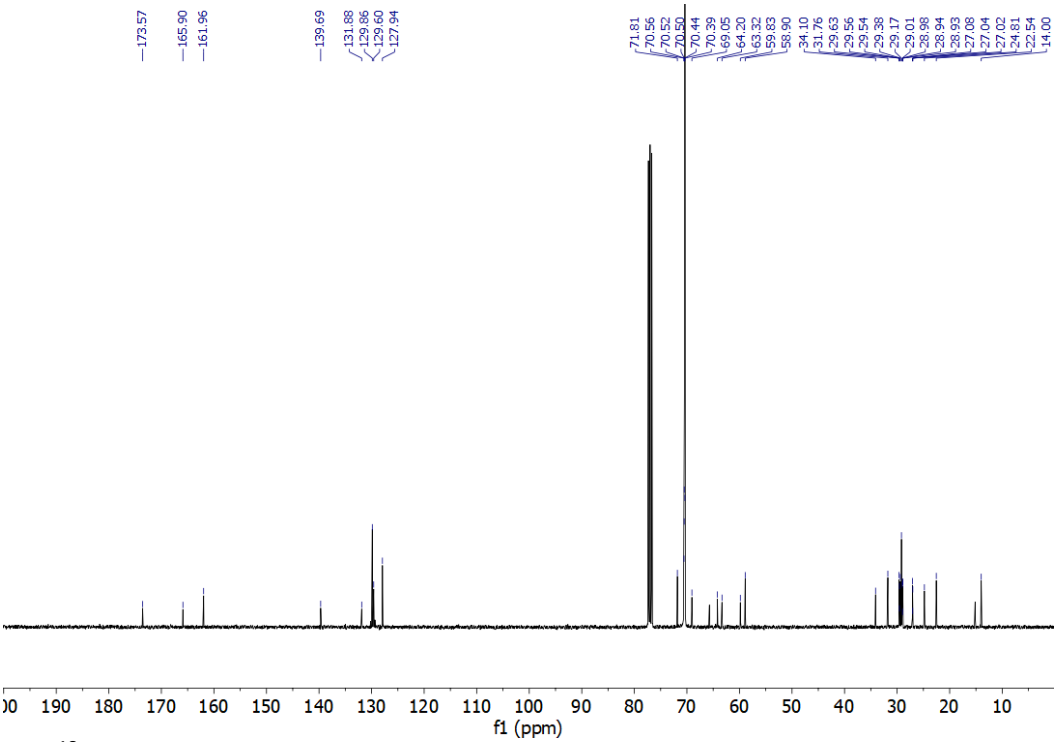


Figure 8S – ^{13}C NMR spectrum (101 MHz) of mPEG-C_2-lipid **9** in $CDCl_3$

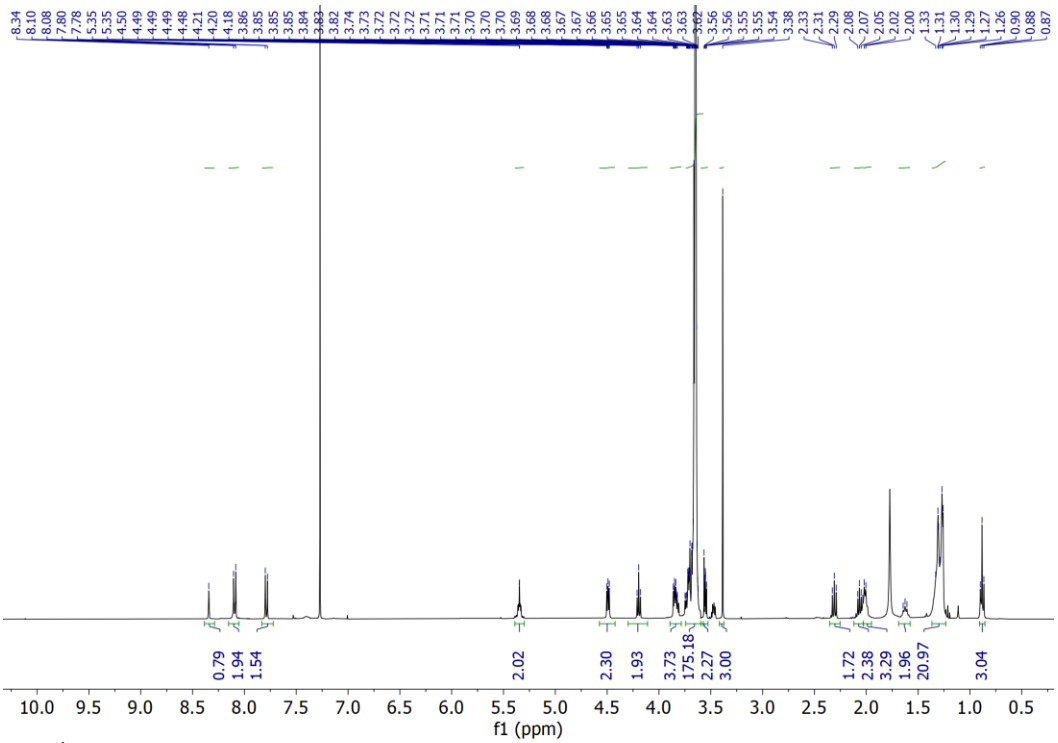


Figure 9S – ^{1}H NMR spectrum (400 MHz) of mPEG-C_3-lipid **10** in $CDCl_3$

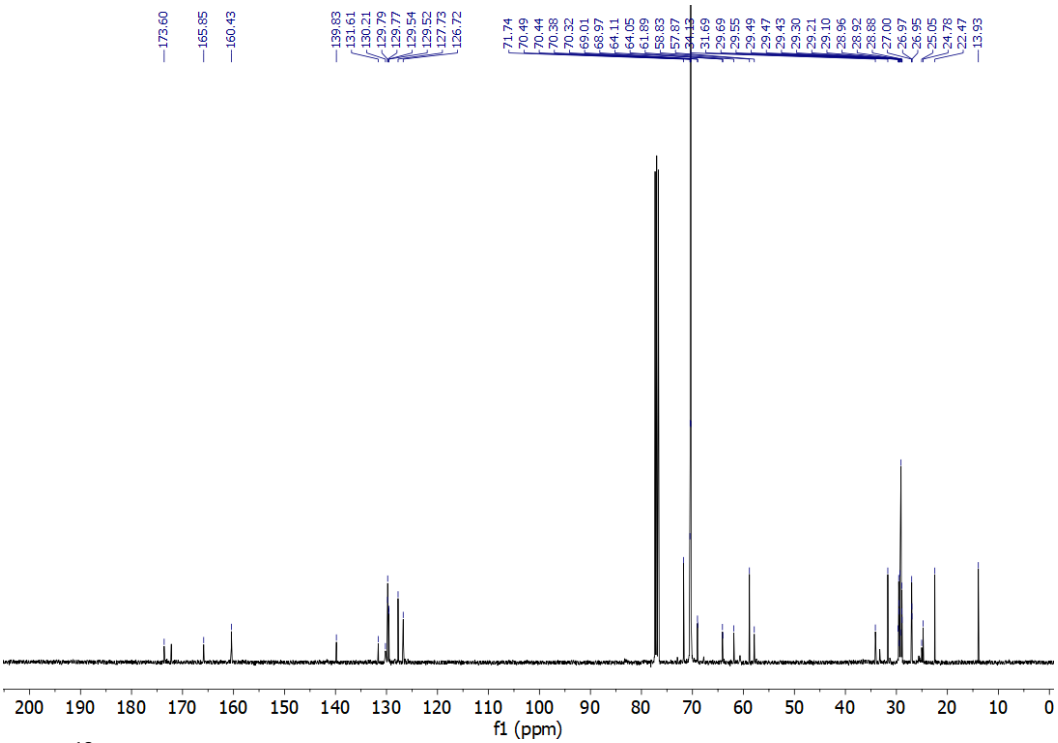


Figure 10S – ^{13}C NMR spectrum (101 MHz) of mPEG-C_3-lipid **10** in $CDCl_3$

4. Hydrolysis study of mPEG-C2-lipid

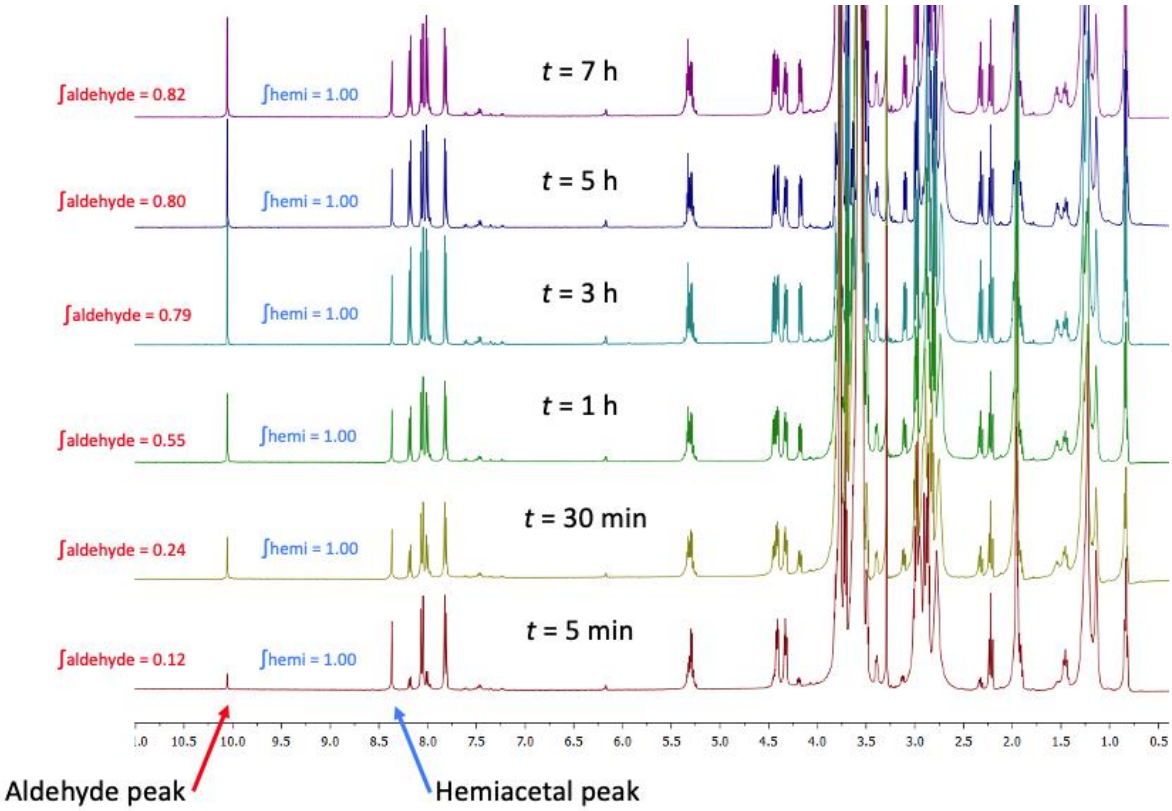


Figure 11S – Overlaid ^{1}H NMR spectra (400 MHz, HEPES buffer 0.2 M in D_2O) of mPEG-C_2-lipid at pH 7.4 over time. The hydrolysis percentage was set as the ratio of the aldehyde integral to the sum of aldehyde and hemiacetal integrals (equation 2).

5. Critical micellar concentration

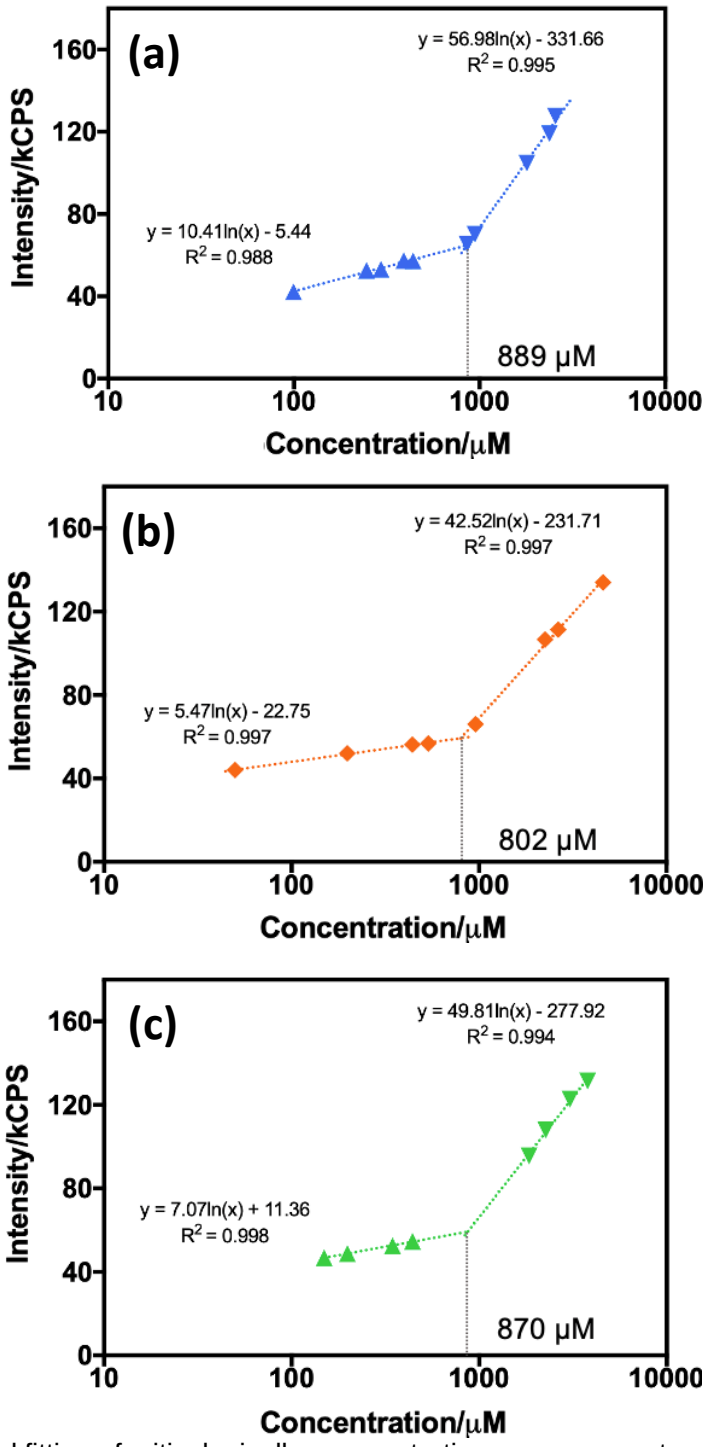


Figure 12S – Individual fitting of critical micellar concentration measurements represented as a plot of intensity of scattered light as a function of concentration of mPEG-C_2-lipid obtained by dynamic light scattering at 173° recorded as kilo counts per second (kCPS); (a) in ultrapure water, (b) second measurement in ultrapure water, and (c) at pH 8.0 in 25 mM TRIS buffer.