

“Supplementary Information”

Naloxone-initiated mechanochemical synthesis of poly(lactic acid)

Mohammad A. Ebqa'ai,^{b,c} Sandun Bogahawaththa Kasthuri Dias,^a Andrew J. Kassick,^d Saadyah Averick,^d Toby L. Nelson^{*a,c}

^a Oak Ridge Innovation Institute, The University of Tennessee, Knoxville, TN 37996, USA. Email: tnelso31@utk.edu

^b Department of Chemistry, Physics, and Engineering, Cameron University, Lawton, Oklahoma 73505, USA.

^c Department of Chemistry, Oklahoma State University, Stillwater, Oklahoma 74078, USA.

^d Neuroscience Institute, Allegheny Health Network, Allegheny General Hospital, Pittsburgh, PA 15212, USA.

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1. Materials

Naloxone hydrochloride dihydrate was obtained from Sigma-Aldrich (St. Louis, MO) and then converted to its corresponding free base using acid–base extraction. (3*S*)-*cis*-3,6-Dimethyl-1,4-dioxane-2,5-dione (*L*-Lactide) and methanol were purchased from Sigma-Aldrich. 1-[3,5-Bis(trifluoromethyl)phenyl]-3-[(1*R*,2*R*)-(-)-2-(dimethylamino)cyclohexyl] thiourea catalyst (C-T) was obtained from Strem Chemicals. 4-Iodophenol and *p*-methoxyphenol were purchased from Alfa Aesar. Chloroform was obtained from an Innovative Technologies solvent purification system under ultra-pure argon. Ultra-pure water was purchased from Life Technologies, Grand Island, NY. All reagents were used as received unless otherwise noted.

2. Instrumentation

The speed-mixer experiments were carried out with a FlackTek Speed Mixer (DAC 330-100 PRO) instrument with SmartSnap stainless steel grinding jars (5 mL and 15 mL) and stainless-steel balls (5 mm and 7 mm). ¹H NMR spectra were measured on a Bruker Avance 400 MHz instrument. The number average molecular weight (M_n), weight average molecular weight (M_w) and dispersity (\mathcal{D}) were determined by gel permeation chromatography (GPC) using a Waters pump with a Waters 2410 refractive index detector. The temperature inside the grinding jars was measured immediately after milling via an infrared thermometer, Etekcity Laser grip 774. THF was used as an eluent at 35 °C with a flow rate of 1.0 mL/min. The instrument was calibrated with polystyrene standards and data were analyzed using Breeze software. Liquid chromatography–mass spectrometry (LC-MS) analysis was performed on a Dionex Ultimate 3000 uHPLC system

coupled to a Thermo Scientific TSQ Quantum Access MAX triple quadrupole mass spectrometer.

3. Synthesis, and Characterizations

Synthesis of naloxone-PLA polymers

In an argon-filled glovebox, L-Lactide (44 mg, 0.310 mmol, 10 equiv.), naloxone (10 mg, 0.031 mmol, 1 equiv.), thiourea catalyst (6.4 mg, 0.015 mmol, 5 mol %), and CHCl_3 (20 μL) were added to a stainless-steel grinding jar (5 mL), followed by stainless-steel balls (5 mm, 5 balls) (**Table 2**, entry 2). The grinding jar was sealed, removed from the glovebox, and placed in the vibrational speed mixer and subjected to milling for 60 minutes at 2100 rpm. The product was dissolved in CHCl_3 (1 mL) and filtered with a 0.45 μm syringe filter to remove any residue. Then, the polymer was purified by precipitation into 50 ml of cold methanol. The solid was allowed to settle for 60 min. Using a 50 mL centrifuge tube, the precipitate was collected by centrifugation at 3800 rpm for 30 min to obtain a pale yellow to off-white solid. After decantation of the supernatant liquid, the solid was then washed with 50 mL of methanol at 3800 rpm for 30 min (3 times). After each washing, the supernatant liquid was decanted. The resulting solid was dried under high vacuum overnight to afford an off-white solid (23 mg, 43%). ^1H NMR (400 MHz, CDCl_3) δ 6.85 (d, 1H), 6.68 (d, 1H), 5.83 (broad, 1H), 5.41-5.44 (m, 1H), 5.22-5.13 (q, 54 H), 4.69 (s, 1 H), 4.37-4.32 (m, 1 H), 3.27-3.02 (m, 5H), 2.70 (s,1H), 2.61 (broad, 1H), 2.41 (broad, 1H), 2.27-2.30 (m, 1H), 2.14 (broad, 1H), 1.87 (m, 1H), 1.56 (d, 166H) (See **Figure S2**). GPC: $M_n = 5.0$ kDa, $M_w/M_n = 1.16$. The ^1H NMR result was in excellent agreement with the result obtained by our previous work.¹

4. Nanoparticle characterization.

Dynamic light scattering (DLS)

The Z-average hydrodynamic size, polydispersity index (PDI), and zeta potential (ζ) of Naloxone-Poly(lactic acid) nanoparticles (NLX-PLA_{5,0} NPs) were measured using a Zetasizer Nano- ZS90 instrument (Malvern, Germany). Data were collected and analyzed using the Malvern Zetasizer software version 7.11 (Malvern Instruments, United States). Before DLS measurements, solutions of purified NLX-PLA_{5,0} NPs (0.1 mg/mL) were prepared in methanol and sonicated for 3 minutes. For sizing measurements, the solutions were poured in polystyrene disposable cuvettes, DTS0012. For zeta potential measurements, the solutions were poured into disposable folded capillary cells, DTS1070. DLS data was collected from four measurements.

Transmission electron microscopy (TEM)

Naloxone-PLA_{5,0} nanoparticles were also characterized via transmission electron microscopy. 1.0 mg of NLX-PLA_{5,0} polymer was suspended in 1.0 mL of methanol before being sonicated for 3 min at room temperature via a sonicator (BRANSONIC 220). Then, a 10 μ L drop of this solution was placed onto an ultrathin carbon film coated with 150 mesh copper TEM grid (Electron Microscopy Sciences, Hatfield, PA). The solution remained on the grid for 5 minutes to allow particle deposition. The solution was then dried by blotting with Whatman 3MM filter paper, and the sample was subsequently imaged on a JEOL JEM-2100 transmission electron microscope operating at 200 keV. The Image J program was used to analyze TEM data and determine the particle size.

5. Calculation Procedures and ¹H NMR spectra

Conversion (Conv.%) and initiator efficiency (IE%) were determined using the crude ¹H NMR spectra for polymers. However, degree of polymerization (DP) was determined the using the pure ¹H NMR spectra for polymers. The ¹H NMR spectra for the NLX-PLA_{5,0} polymer was used as a

representative example, displaying the necessary signals for performing the calculations (Figures S1-S2).

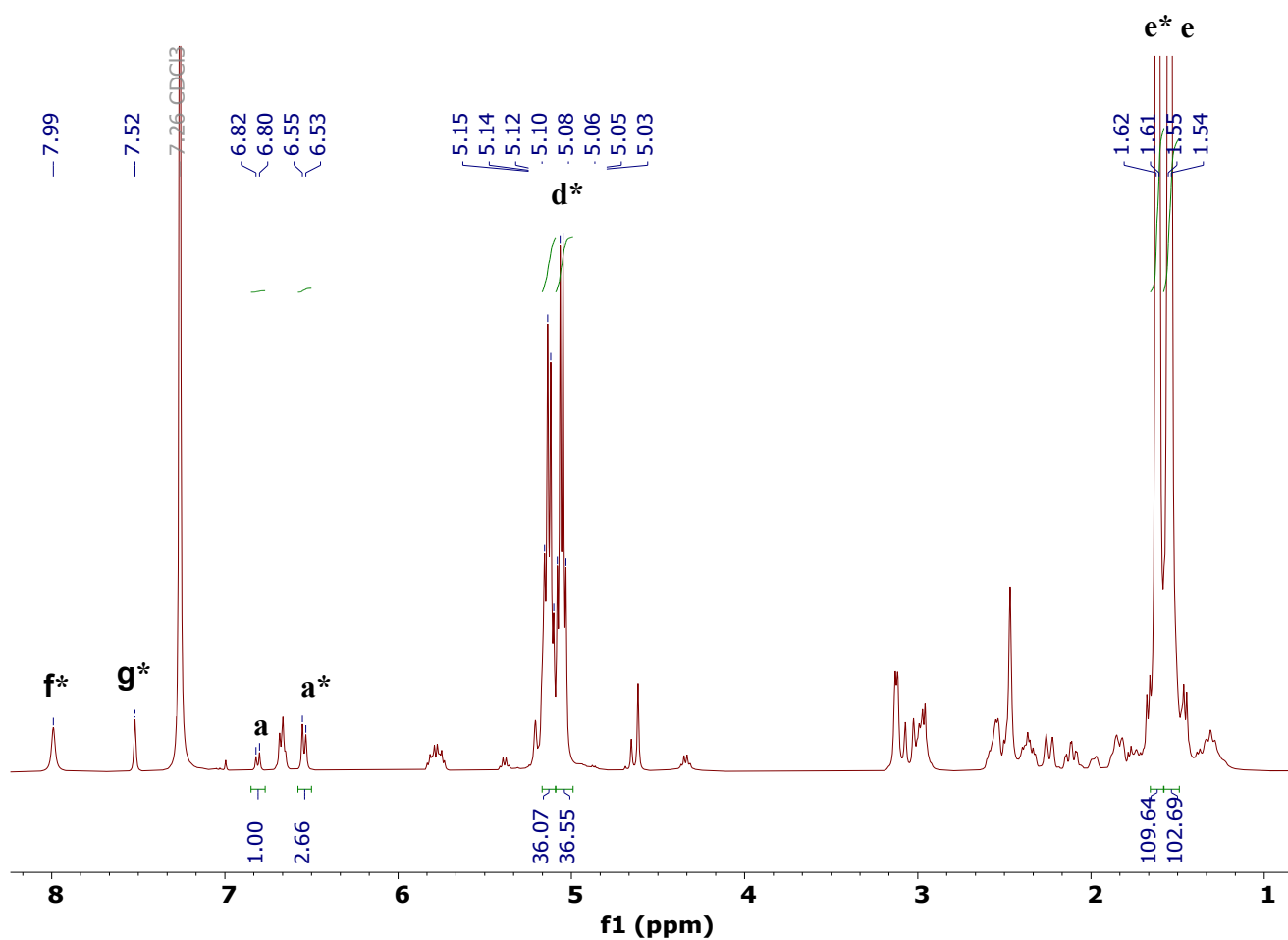
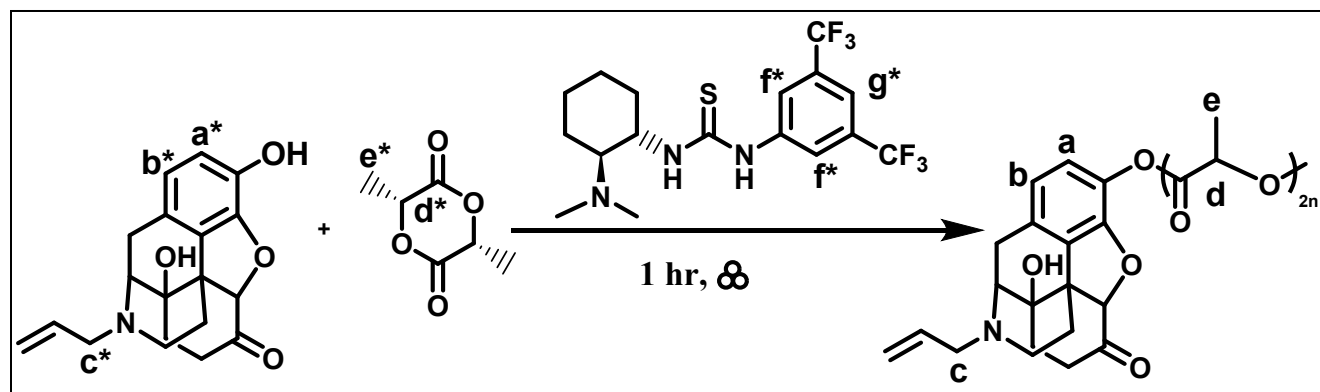


Figure S1. The crude ^1H NMR for NLX-PLA_{5.0} polymer.

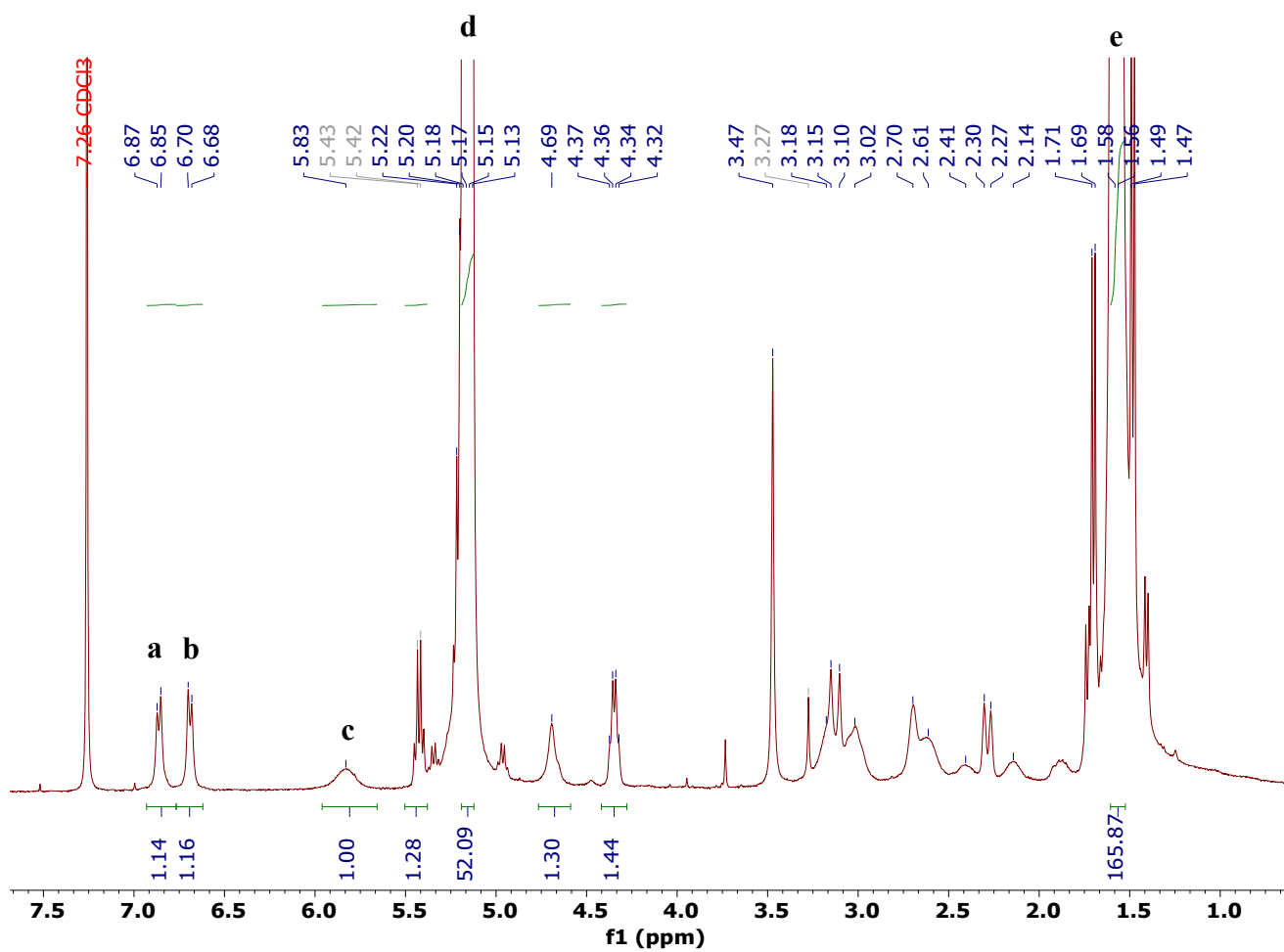
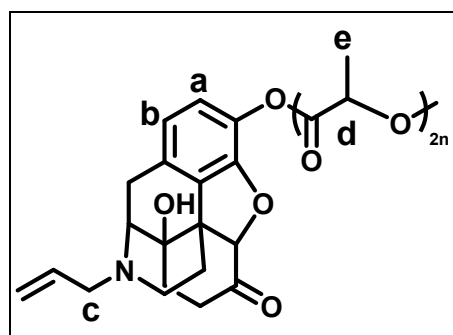


Figure S2. The pure ¹H NMR for NLX-PLA_{5.0} polymer.

Conversion % determination (Conv.%)

The conversion of ROP reactions was based on transformation of the monomer (L-lactide) into polymer. Conversions were calculated by the ratio of integrals obtained for the methine protons of poly(lactic acid) and unreacted L-lactide from the crude ^1H NMR spectra according to the following equation:

$$\text{Conversion\%} = \frac{\text{Integral methine (CH) proton (d) of polymer (mm)}}{\left[\text{Integral methine (CH) proton (d) of polymer (mm)} + \text{Integral methine (CH) proton (d*) of monomer (mm)} \right]} \times 100 \quad (\text{eq 1})$$

Initiator efficiency % determination (IE%)

The initiator efficiency (IE) of ROP reactions was based on the degree of incorporation of the initiators (naloxone) into the polymer chain. IE% values were calculated from the ratio of integrals obtained for the vinylic-CH protons of naloxone bound-polymer and unreacted naloxone from the

$$\text{IE\%} = \frac{\text{Integral of vinylic (CH) proton (a) of naloxone-bound polymer (mm)}}{\left[\text{Integral of vinylic (CH) proton (a) of naloxone-bound polymer (mm)} + \text{Integral of vinylic (CH) proton (a*) of unreacted naloxone (mm)} \right]} \times 100 \quad (\text{eq 2})$$

crude ^1H NMR spectra according to the following equation:

Degree of polymerization determination (DP)

DP = number of repeating units (n) that estimated from ratio of integrals of vinylic proton (**c**) from end group naloxone to methine proton (**d**) from lactic acid polymer chain.

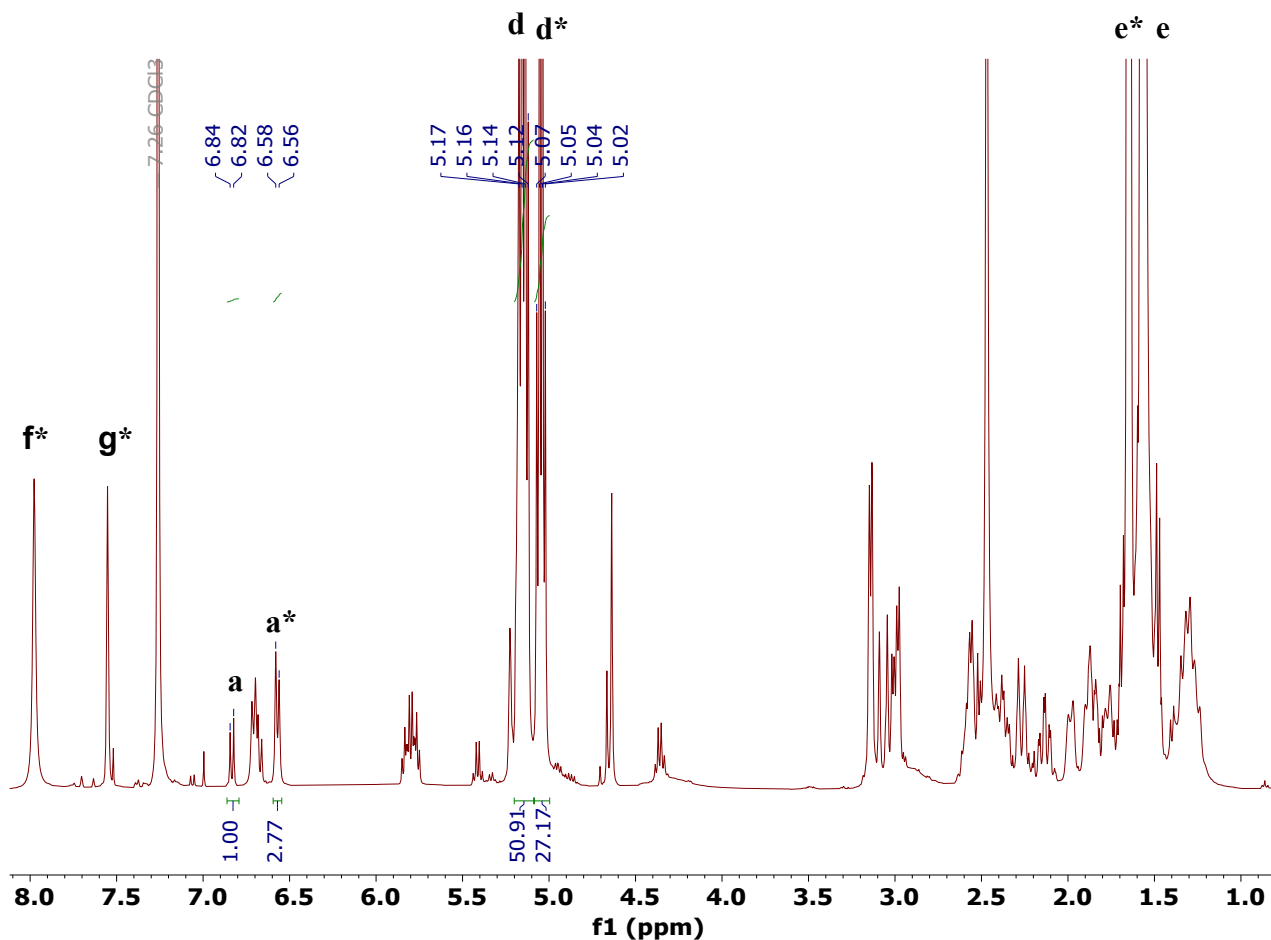
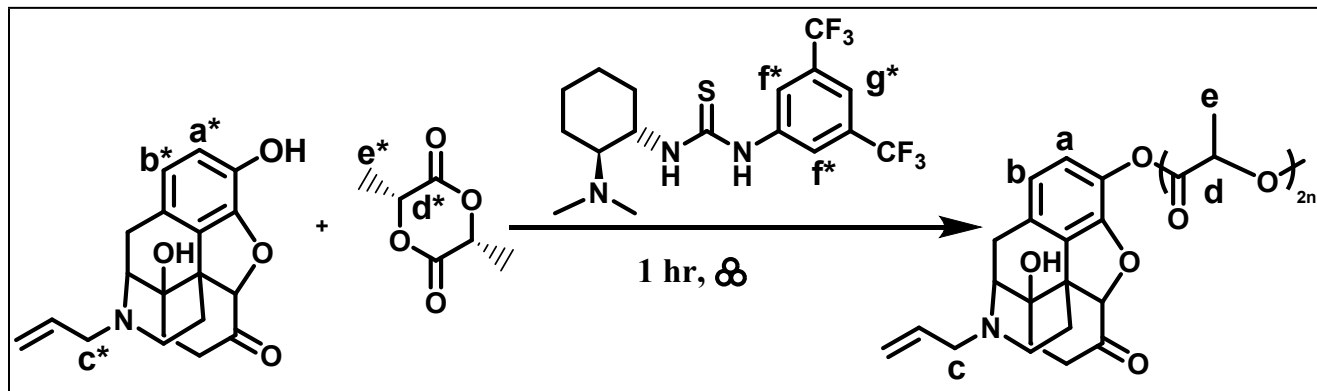


Figure S3. ¹H NMR for the crude NLX-PLA_{7.5} polymer.

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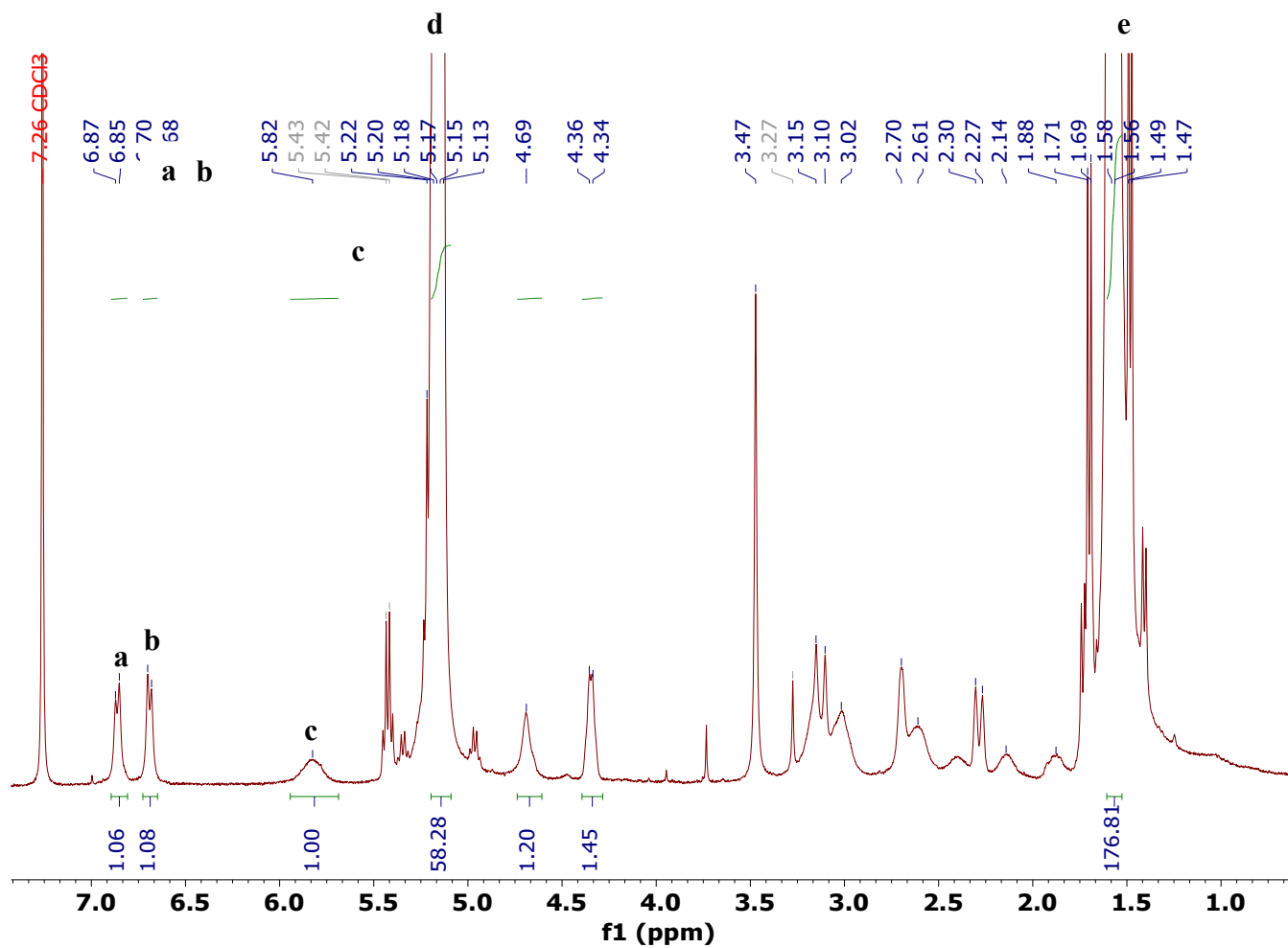
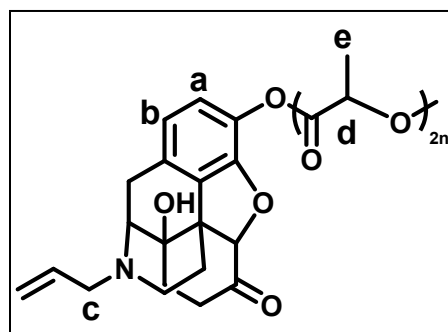


Figure S4. ^1H NMR for the pure (below) NLX-PLA_{7.5} polymer.

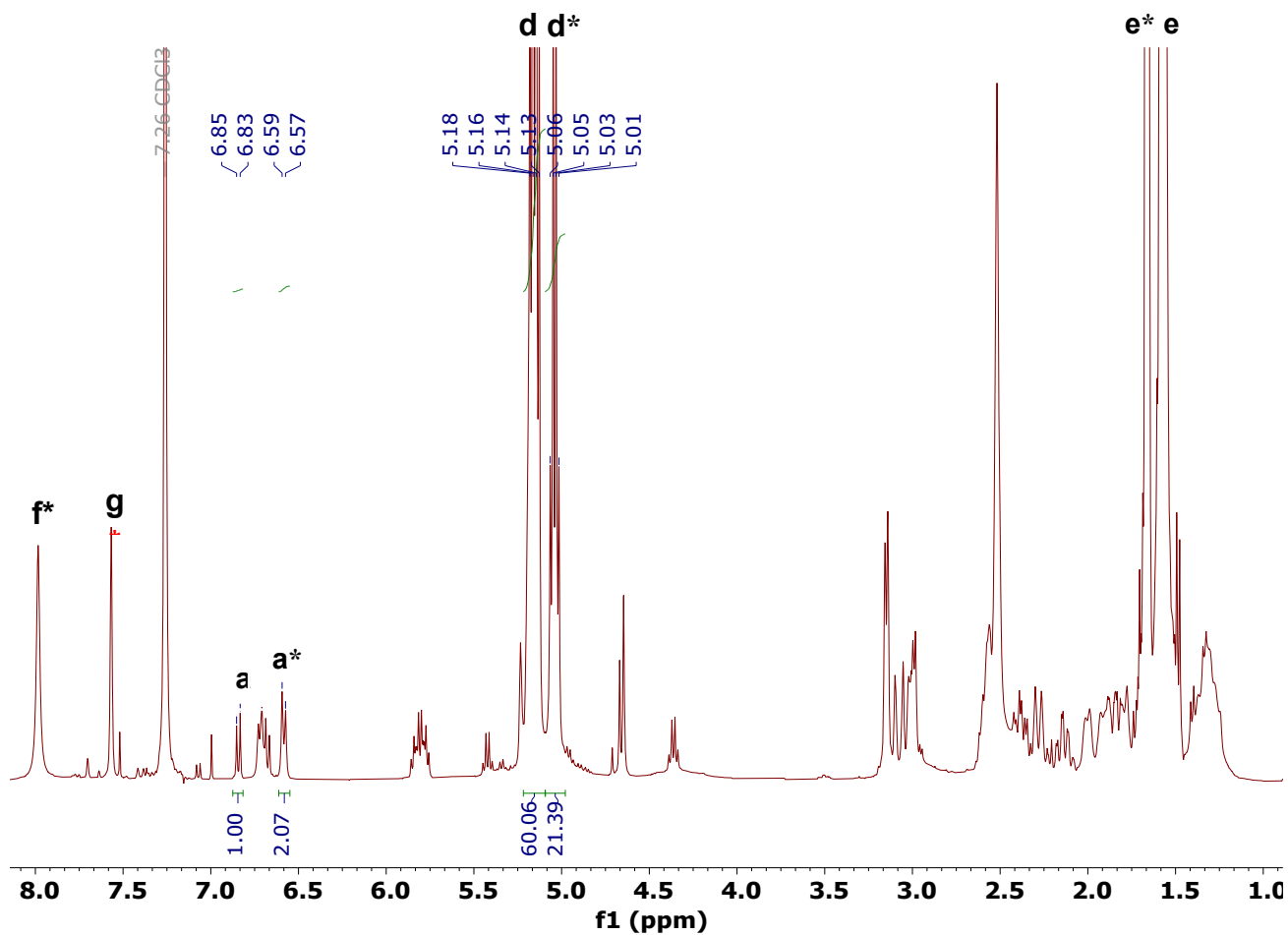
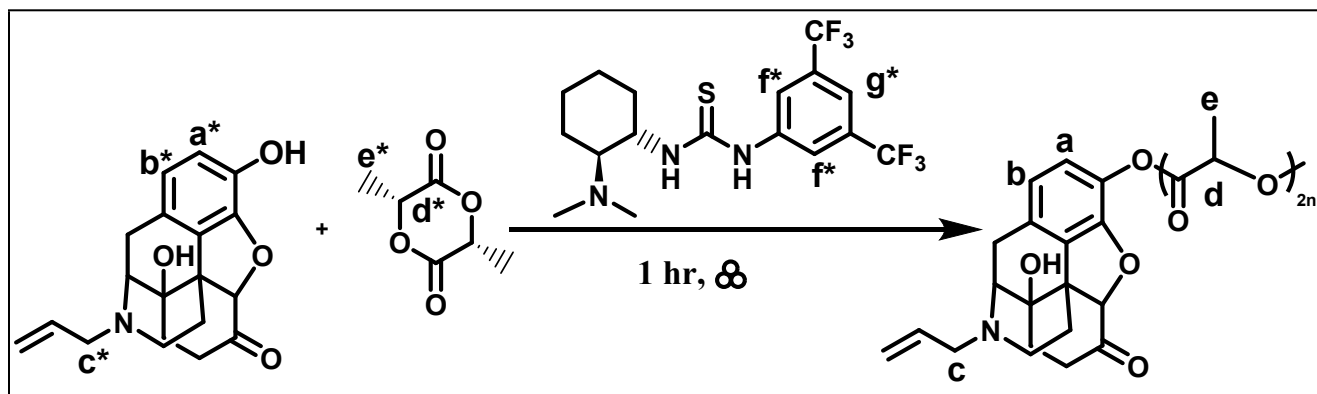


Figure S5. ^1H NMR for the crude NLX-PLA₁₀ polymer

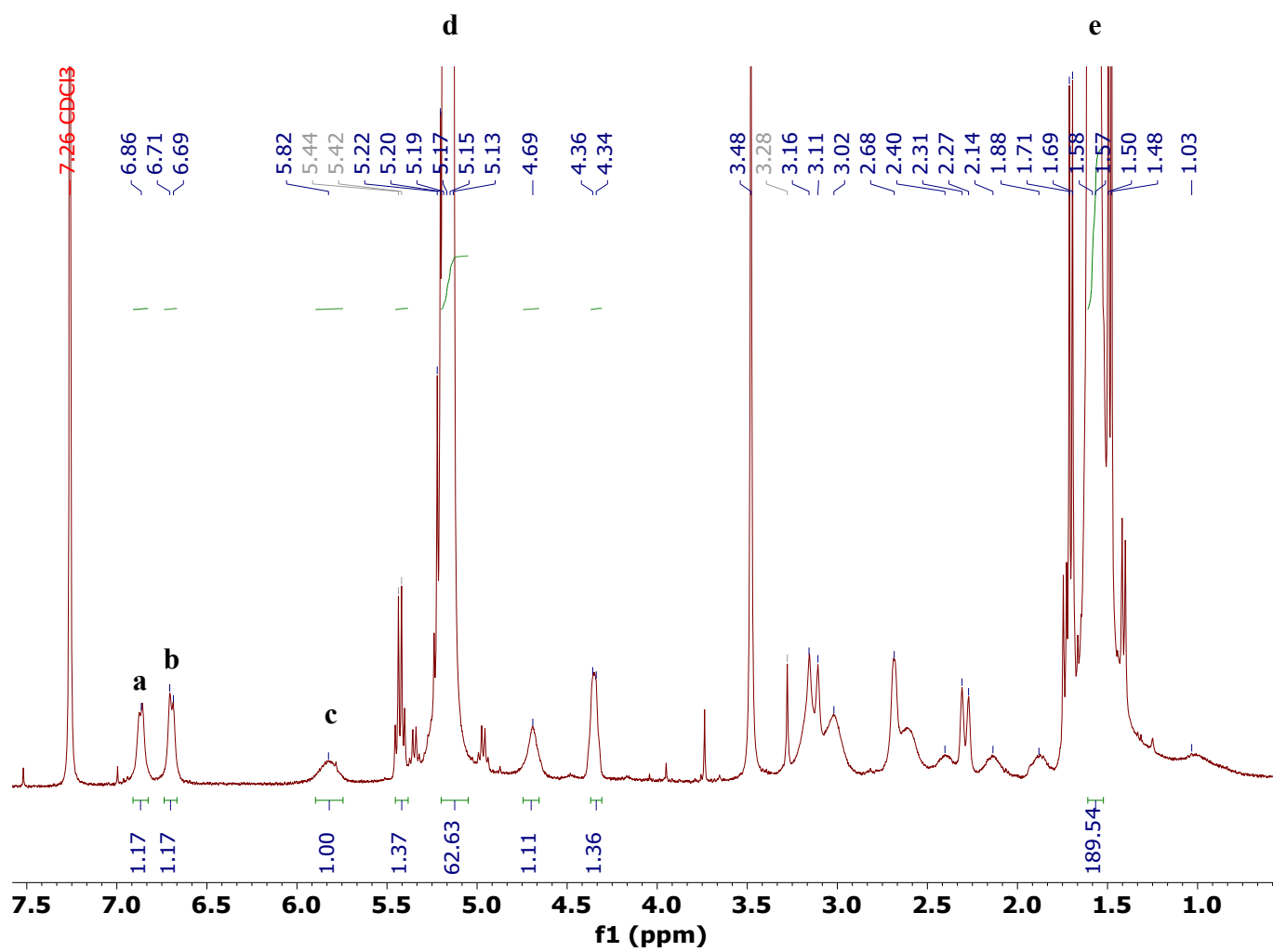
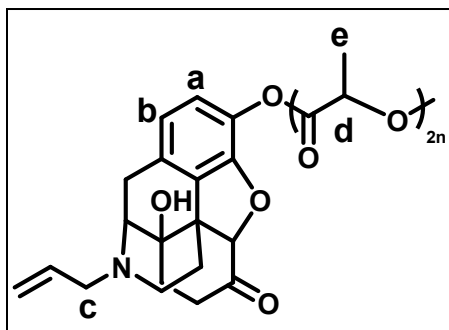


Figure S6. ^1H NMR for the pure (below) NLX-PLA₁₀ polymer

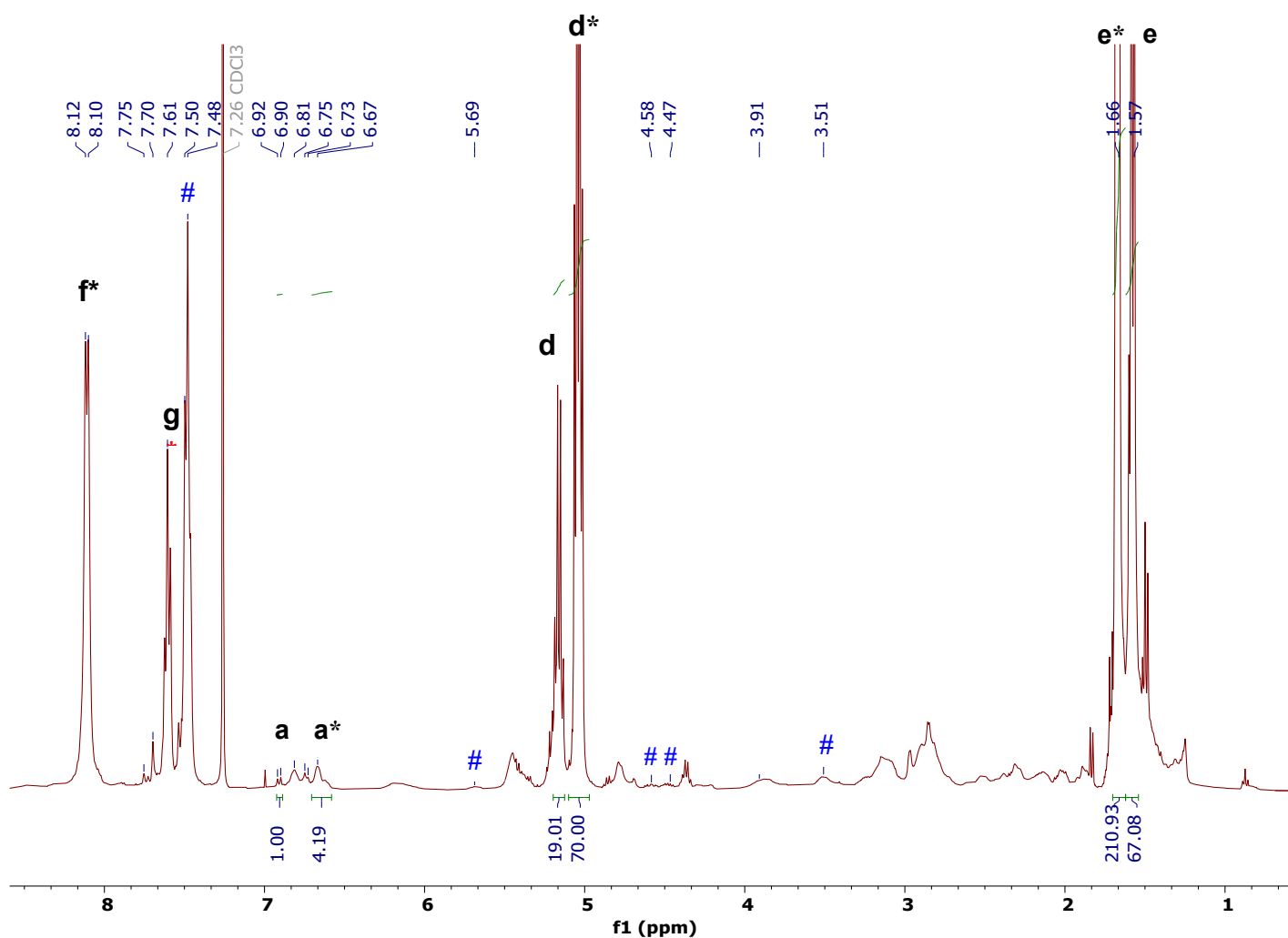
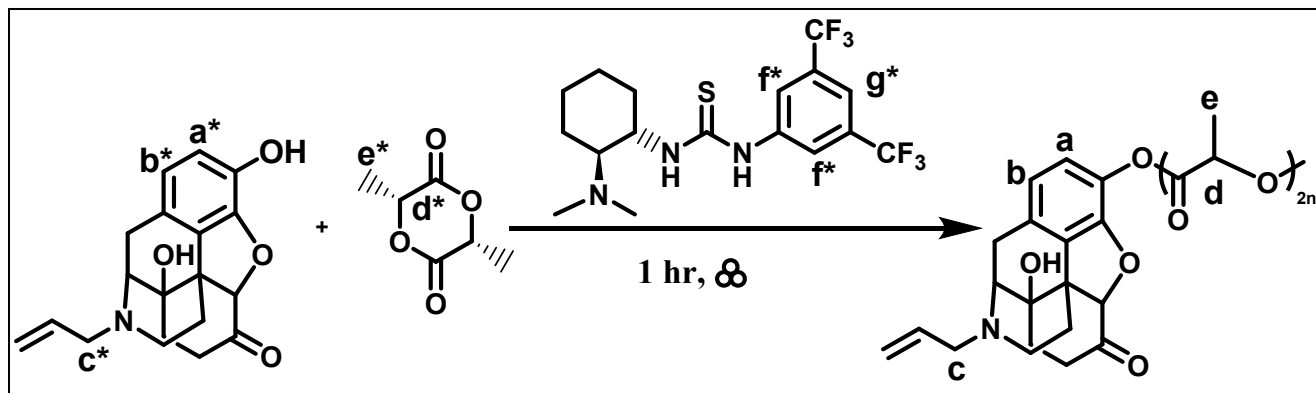


Figure S7. ^1H NMR for the crude NLX-PLA polymer at milling frequency of 2500 rpm.

Indicates to unknown impurities.

Drug loading (naloxone) determination:

A. Determine naloxone loading via ¹H NMR spectroscopy

For an initial estimation for naloxone loading, a standard ¹H NMR-based molecular weight analysis technique was used. After the calculation of the degree of polymerization (DP), naloxone loading can be determined based on equation (3) and (4) below:¹

$$M_n = nM_0 + M_e \quad (\text{eq 3})$$

Where; M_n = polymer MW, n = DP, M_0 = MW of one repeating unit, M_e = MW of end group (naloxone).

From the polymer MW (M_n), the naloxone loading can be determined as follows:

As an example, for NLX-PLA_{5.0} (DP = 52),

$$\% \text{ weight of naloxone} = \text{MW naloxone}/M_n \quad (\text{eq 4})$$

$$\% \text{ weight of naloxone} = \text{MW naloxone} / [(DP \times M_0) + \text{MW naloxone}]$$

$$= 327.38 / [(52 \times 72) + 327.38]$$

$$= 8.03 \% \text{ naloxone loading.}$$

B. Determine naloxone loading via UV-Vis spectroscopy

First: Preparation of a calibration curve

A direct evaluation method using UV-Vis spectrophotometry was applied to determine the naloxone loading % in NLX-PLA_{5.0} NPs. A standard curve was prepared from a 0.07 mg/mL stock solution of naloxone in DCM. This provided the essential equation for determining naloxone concentration from an unknown solution of NLX-PLA_{5.0} NPs (**Figure S5**).

Concentration (mg/ml)	Absorbance
0.07	0.39877
0.05	0.28196
0.03	0.17141
0.01	0.058969
0.005	0.029122

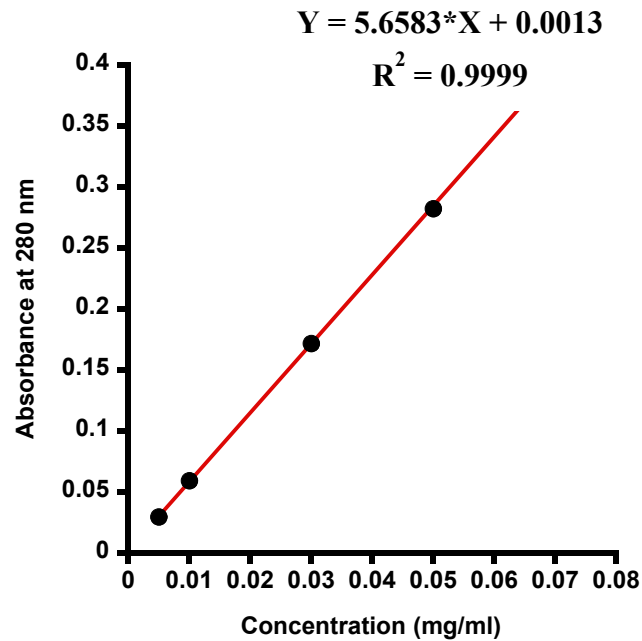


Figure S8. Calibration curve for 0.07 mg/mL naloxone in DCM.

Second: Determination of naloxone loading %

Naloxone concentration was determined by treating NLX-PLA (3.0 mg) with 10 mL of DCM (0.3 mg/mL), and then the absorbance at 280 nm was measured (**Figure S6**). As an example, the absorbance for **NLX-PLA_{5,0}** was measured to be 0.142366. Using the standard curve equation from the previous section, naloxone loading for the current NPs formulation was determined to be 8.31 w/w % as follows:

Absorbance at 280 nm for NLX in **NLX-PLA_{5,0}** (0.3 mg/ml) = 0.1423661

Y = 5.6583*X + 0.00132

0.142366 = 5.6583*X + 0.0013

X = NLX (in mg/ml) = 0.02493 mg/ml

NLX (in mg) = 0.02493 mg/ml × 10 mL = 0.2493 mg3

NLX loading % = Mass of NLX/ Mass of NLX-PLA x 100%4

NLX loading % = 0.2493 mg / 3.0 mg x 100 = **8.31 % w/w naloxone loading**

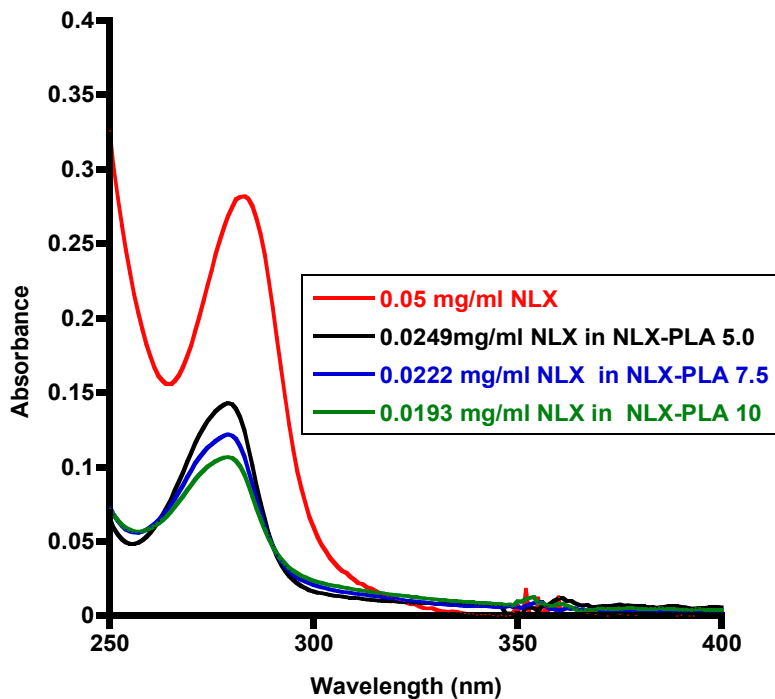


Figure S9. UV-Vis spectra of 0.05 mg/mL solution of naloxone and 0.3 mg/mL solution of NLX-PLA polymers.

C. Determine naloxone loading via LC-MS analysis

First: Preparation of a calibration curve via LC-MS

Naloxone loading for the NLX-PLA_{5,0} NPs (DP=52) sample was also determined via LC-MS by normalizing mass intensities taken from the extracted ion chromatogram (EIC) at each concentration for a stock solution. Preparation of a standard curve from a 0.25 mg/mL stock

Concentration (mg/mL)	EIC _{ave} × 20
0.250000	4.17 × 10 ⁸
0.125000	2.42 × 10 ⁸
0.062500	1.28 × 10 ⁸
0.031250	6.78 × 10 ⁷
0.015625	3.36 × 10 ⁷
0.0078125	1.91 × 10 ⁷

solution of naloxone in 1 M NaOH provided the requisite equation for determining naloxone concentration from an unknown solution of hydrolyzed NLX-PLA_{5,0} nanoparticles (**Figure S8**).

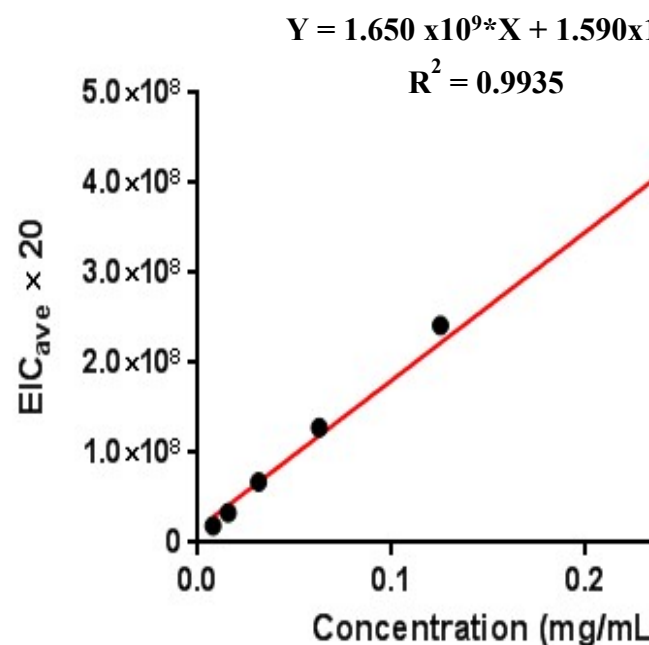


Figure S10. Calibration curve for 0.25 mg/mL naloxone in 1 M NaOH

Second: Determination of the naloxone loading %

Maximum naloxone *in vitro* release was determined by treating NLX-PLA_{5,0} NPs (15.3 mg) with 30.44 mL of 1 M NaOH (0.5 mg/mL), and then incubating the solution at 38 °C for 24 h. Given the fully degradable nature of PLA, complete hydrolysis and therefore complete naloxone release would be achieved under these conditions. Then, the maximum intensity released for naloxone was assessed via LC-MS to be 8.43×10^7 cps (count per second). Using the standard curve

equation, naloxone loading for the current NPs formulation was determined to be 8.2 w/w% as follows:

$$\text{Max release from NLX-NPs (1M NaOH, 38 }^\circ\text{C, 24 h)} = \text{EIC}_{\text{ave}} \times 20 = 8.43 \times 10^7 \dots\dots\dots 1$$

$$Y = 1.650 \times 10^9 * X + 1.590 \times 10^7 \dots\dots\dots 2$$

$$8.43 \times 10^7 = 1.650 \times 10^9 * X + 1.590 \times 10^7$$

$$X = \text{NLX (in mg/ml)} = 0.04145 \text{ mg/mL}$$

$$\text{NLX (in mg)} = 0.04145 \text{ mg/mL} \times 30.44 \text{ mL} = 1.262 \text{ mg} \dots\dots\dots 3$$

$$\text{NLX loading \%} = \text{Mass of NLX} / \text{Mass of NLX-PLA} \times 100\% \dots\dots\dots 4$$

$$\text{NLX loading \%} = 1.262 \text{ mg} / 15.3 \text{ mg} \times 100 = \mathbf{8.25\% \text{ w/w naloxone loading.}}$$

6. GPC Traces

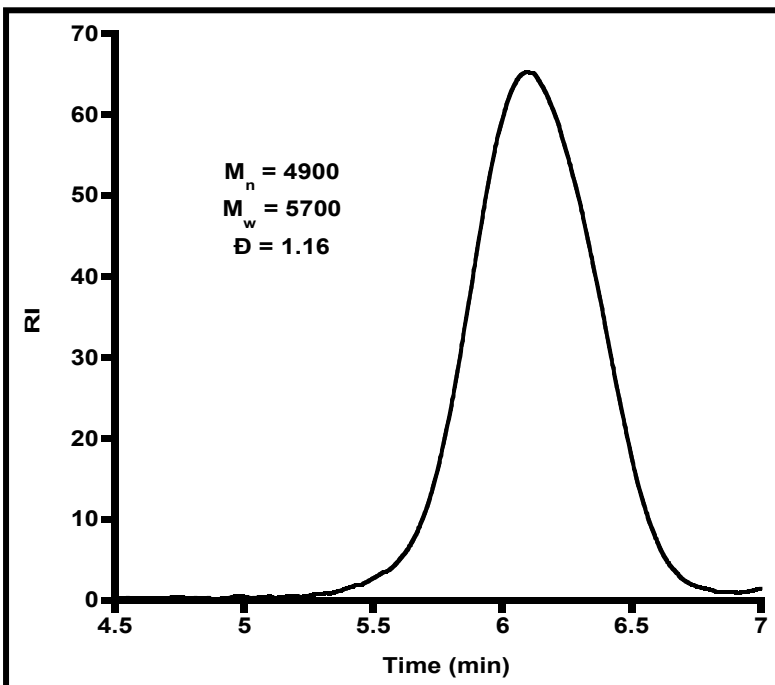


Figure S11. GPC trace of the pure NLX-PLA_{5.0}.

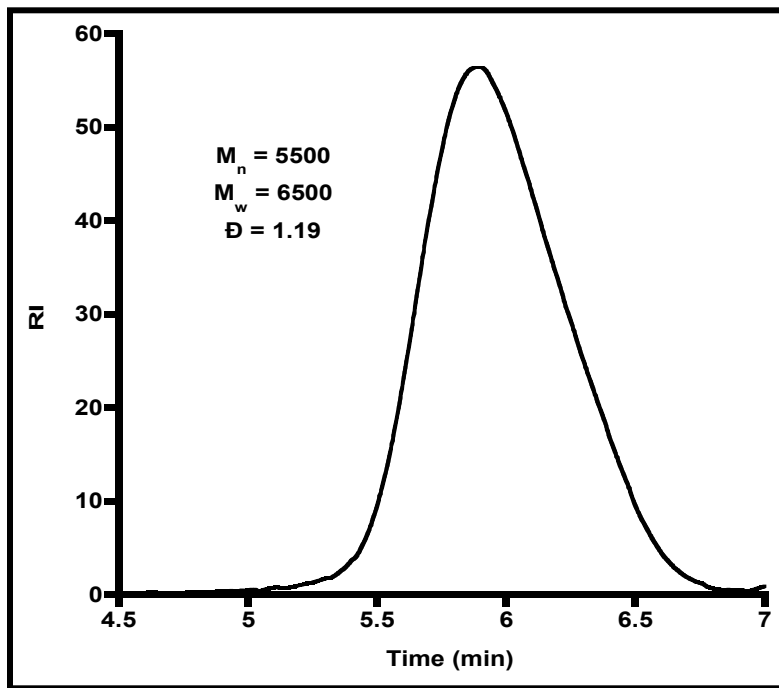


Figure S12. GPC trace of the pure NLX-PLA_{7.5}.

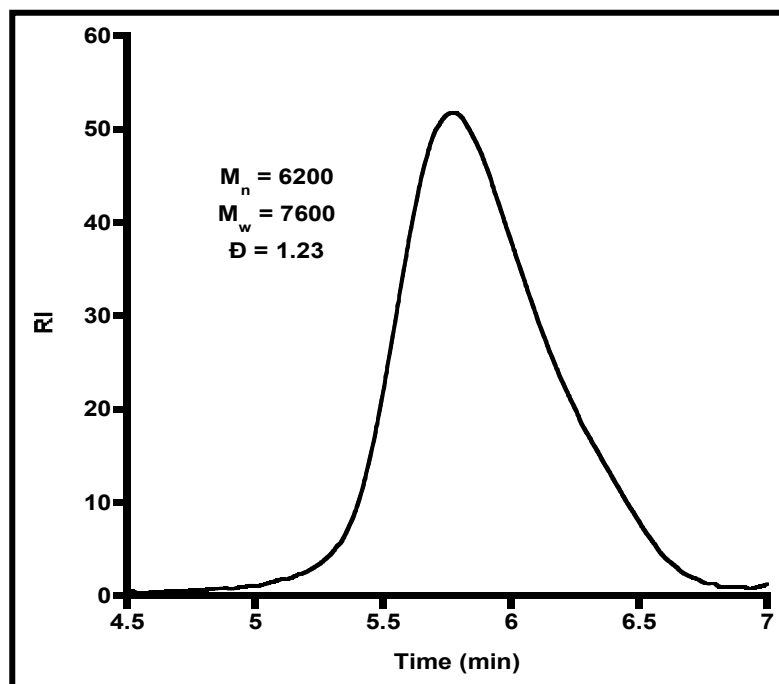


Figure S13. GPC trace of the pure **NLX-PLA₁₀**.

References

1. A.-J. Kassick, H.-N. Allen, S.-S. Yerneni, F. Pary, M. Kovaliov, C. Cheng, M. Pravetoni, N.-D. Tomycz, D.-M. Whiting, T.-L. Nelson, M. Feasel, P. Campbell, B. Kolber and S. Averick, *ACS Applied Biomaterials*, 2019, **2**, 3418.