Covalent Poly(lactic acid) Nanoparticles for the Sustained Delivery of Naloxone

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*Supporting Information

ABSTRACT: The opioid epidemic currently plaguing the United States has been exacerbated by an alarming rise in fatal overdoses as a result of the proliferated abuse of synthetic mu opioid receptor (MOR) agonists, such as fentanyl and its related analogues. Attempts to manage this crisis have focused primarily on widespread distribution of the clinically approved opioid reversal agent naloxone (Narcan); however, due to the intrinsic metabolic lability of naloxone, these measures have demonstrated limited effectiveness against synthetic opioid toxicity. This work reports a novel polymer-based strategy to create a long-acting formulation of naloxone with the potential to address this critical issue by utilizing covalent nanoparticle (cNP) drug delivery technology. Covalently loaded naloxone nanoparticles (Nal-cNPs) were prepared via the naloxone-initiated, ring-opening polymerization (ROP) of L-lactide in the presence of a bifunctional thiourea organocatalyst with subsequent precipitation of the resulting naloxone-poly(L-lactic acid) polymer. This protocol afforded well-defined nanoparticles possessing a drug loading of approximately 7% w/w. The resulting Nal-cNPs demonstrated excellent biocompatibility, while exhibiting sustained linear release kinetics in vitro and blocking the effects of high dose (10 mg/kg) acute morphine for up to 98 h in an in vivo rodent model of neuropathic pain.

KEYWORDS: naloxone, drug delivery, covalent nanoparticles, controlled release, ring-opening polymerization

INTRODUCTION

For centuries, mu opioid receptor (MOR) agonists have been routinely employed by the medical community as an effective and reliable treatment for both moderate and severe pain. Despite their excellent therapeutic profiles, the heightened potential for addiction and abuse displayed by MOR agonists presents a serious liability.1−5 This increased abuse potential has recently manifested in an unparalleled epidemic of opioid overdoses and deaths in the United States (U.S.) fueled by highly potent, synthetic MOR agonists, such as fentanyl (1) and its derivatives (Figure 1). According to a recent report by the Centers for Disease Control (CDC), the pervasive abuse of both prescription and illicit forms of synthetic opioids has been implicated in the exponential rise in opioid-related deaths observed in the U.S. since 2012.6 Moreover, it has been estimated that, of the nearly 48 000 opioid-related mortalities reported in 2017, more than half have been attributed to synthetic opioid overdose.6 Given this dramatic increase in

Received: May 3, 2019
Accepted: July 25, 2019
Published: July 25, 2019

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synthetic opioid-related deaths and the growing concern regarding the potential threat posed by these compounds to the general public, the opioid epidemic in the U.S. has recently been declared a national public health emergency.

While prompt administration of the MOR antagonist naloxone (2) has long been the primary means of treating an opioid overdose, this historical gold standard for opioid reversal has proven to be limited in its ability to effectively combat the deleterious effects of 1 and other highly potent synthetic opioids. This diminished efficacy can be explained by the rapid metabolism and clearance of naloxone via UGT2B7-mediated glucuronidation of the C3 phenol moiety coupled with the high potency and hydrophobicity possessed by synthetic MOR agonists. The hydrophobic nature of fentanyl-related analogues enables the facile absorption of these compounds into adipose tissue, thereby attenuating metabolism and effectively increasing their circulatory half-life.7 Synthetic opioids can remain sequestered in tissues well after a therapeutic dose of naloxone has been eliminated from the body, resulting in a dangerous and potentially fatal condition known as renarcotization, in which a patient, despite being treated with naloxone, can experience the recurrence of toxicity from the slow permeation of residual synthetic opioids from adipose tissue.8−11 As a result, the effective reversal of synthetic opioid overdose and prevention of renarcotization can require the administration of larger or multiple doses of naloxone, greatly complicating antidote delivery logistics. This phenomenon has been recently documented in several reports in patients treated with intravenous (IV), intramuscular (IM), or intranasal (IN) forms of naloxone.9,11−14 Therefore, a critical need exists to develop new formulations of opioid reversal agents with improved pharmacokinetic profiles that are capable of providing longer-lasting antagonistic effects.

Given the challenges associated with identifying and optimizing entirely new chemical entities, we elected to incorporate the existing MOR antagonist, naloxone, into a delivery system capable of providing a sustained, therapeutic dose of drug in an attempt to more effectively treat the toxic effects of synthetic opioids. A promising strategy to achieve prolonged antidote infusion was anticipated through the application of biodegradable covalent nanoparticle (cNP) technology. We have previously demonstrated the feasibility of utilizing poly(lactic acid) (PLA)- and poly(lactide-co-glycolide) (PLGA)-derived cNPs as drug delivery systems in our exploration of extended release fentanyl-based analogues for improved pain management.15 Our current approach to achieve sustained MOR antagonist is predicated on the analogous two-step covalent nanoparticle formulation strategy illustrated in Scheme 1. We speculated that the formation of covalently loaded naloxone nanoparticles (Nal-cNPs) would occur by grafting commercially available L-lactide from the phenolic hydroxyl moiety of naloxone, resulting in a low degree of polymerization (DP) naloxone−poly(lactic acid) polymer of type 4. Subsequent precipitation of polymer 4 from an appropriate solvent system would then furnish well-defined polymeric nanoparticles with a high percent loading of naloxone, which, under physiological conditions, could degrade and release the desired opioid reversal agent. This method of covalent conjugation of drug to nanoparticle possesses several advantages over traditional, noncovalent nanoparticle delivery systems as it permits higher drug loadings and promotes batch-to-batch consistency in the preparation of NPs, while also avoiding the unwanted phenomenon of burst release,16−19 which may precipitate withdrawal symptoms in opioid dependent individuals.20 These advantages have been documented in several recent investigations comparing covalently conjugated NP formulations of fentanyl derivatives15 and fluorescent Cy5 cyanine dyes21 to their

Scheme 1. Schematic Representation of Covalent Naloxone Nanoparticle (Nal-cNP) Formation

(a) Naloxone-initiated ring-opening polymerization of lactide affords naloxone−poly(lactic acid) polymer 4. (b) Polymer 4 is converted to the corresponding nanoparticles (Nal-cNPs). (c) Ester hydrolysis releases naloxone in a controlled manner.

Figure 1. Fentanyl and naloxone (Narcan).
corresponding noncovalently encapsulated variants. Herein we report our results for the preparation and characterization of covalently loaded naloxone/PLA nanoparticles (Nal-cNPs) that demonstrate linear release kinetics in vitro and maintain an extended MOR blockade against the effects of high dose morphine vs free naloxone in a rodent model of neuropathic pain.

**EXPERIMENTAL SECTION**

**Materials and Methods.** Naloxone hydrochloride dihydrate was purchased from Sigma-Aldrich (St. Louis, MO) and subsequently converted to the corresponding free base (2) via acid–base extraction with saturated aqueous sodium bicarbonate (NaHCO₃). 1-[3,5-Bis(trifluoromethyl)phenyl]-3-{[1(R,2R)-(−)-2-(dimethylamino)cyclohexyl]thiourea was obtained from Strem Chemicals, Inc. (Newburyport, MA). (3S)-cis-3,6-Dimethyl-1,4-dioxane-2,5-dione (lactide), anhydrous dichloromethane (CH₂Cl₂), dichloroethane (DCE), and toluene (PhCH₃) were purchased from Sigma-Aldrich (St. Louis, MO). Naloxone was dissolved in 0.9% saline at a dose of 10 mg/kg. Morphine sulfate, purchased from Sigma-Aldrich (St. Louis, MO), was dissolved in 0.9% saline at a dose of 10 mg/kg. Doses were determined based on previous publications.¹² Water was purified via a Millipore Synergy water purification system. All reagents and solvents were used as received unless otherwise noted.¹¹ H NMR spectra were measured in deuteriochloroform (CDCl₃) or DMSO-d₆ on a Bruker Avance 500 MHz spectrometer. Chemical shifts are reported in ppm employing the residual solvent resonance as the internal standard (CHCl₃ δ 7.26 ppm, DMSO δ 2.50 ppm). UV–vis spectra were measured on a DeNovix DS-11 spectrophotometer using a 10 mm quartz cuvette. Gel permeation chromatography (GPC) analysis was performed on a Dionex Ultimate 3000 uHPLC system coupled to a Thermo Scientific TSQ Quantum Access MAX triple quadrupole mass spectrometer. Reverse-phase chromatographic separation was accomplished on an Agilent ZORBAX Eclipse Plus C18 column (3.5 µm, 100 mm × 4.6 mm) with acetonitrile (CH₃CN) and water (H₂O), modified with 0.1% formic acid, as the mobile phase solvents. The standard HPLC method consisted of a linear gradient from 1 to 95% CH₃CN over 5 min followed by a hold at 95% CH₃CN for 1 min and then a re-equilibration at 1% CH₃CN for 2.5 min. (total run time = 10 min, flow rate = 0.400 mL/min, injection volume = 10 μL; T < naloxone = ~5.2 min).

**Solvent-Free Synthesis of Naloxone–PLA Polymers.** (3S)-cis-3,6-Dimethyl-1,4-dioxane-2,5-dione (3, 1.0 g, 6.94 mmol, 1 equiv) was added to an oven-dried 20 mL scintillation vial equipped with a magnetic stir bar under N₂. Lactide 3 was melted at 130 °C and then treated with a mixture of naloxone (0.227 g, 0.694 mmol, 10 mol %) and thiourea catalyst 5 (0.144 g, 0.347 mmol, 5 mol %). The reaction mixture became a clear solution and was maintained at 100 °C to dissolve and was then transferred to the solution of naloxone and catalyst. The reaction mixture became a clear solution and was maintained at 100 °C for 24 h. The reaction was cooled to ambient temperature and then added to 75 mL of cold MeOH slowly dropwise via syringe. The resulting white suspension was centrifuged at 4000 rpm for 25 min (2 × 50 mL centrifuge tubes). The supernatant liquid was decanted, and the precipitate was resuspended in MeOH (25 mL each tube) and then centrifuged at 4000 rpm (repeated 3×). The resulting product was dried under a vacuum to obtain 286 mg (47%) of a white solid. GPC: Mₙ = 2700, Mₘ/Mₙ = 1.10.

**Preparation of Covalently Linked Naloxone–PLA Nanoparticles.** A solution of the Nal-PLA polymer hybrid (40 mg) in 4 mL of CH₂CN was added slowly dropwise via syringe pump to a solution of 0.3% PVA in H₂O (30 mL) at a flow rate of 20 μL/min (0.02 mL/min) with rapid stirring. Upon completion of the addition, the resulting white turbid mixture was maintained overnight at ambient temperature with rapid stirring. Nanoparticles were initially collected by centrifugation at 4500 rpm and then subsequently washed with H₂O (3 × 10 mL) with centrifugation at 4500 rpm for 30 min. The supernatant liquid was decanted, and the resultant precipitate was resuspended in about 10 mL of H₂O and lyophilized to yield 25 mg of a fluffy, white solid.

**Covalent Nanoparticle Characterization.** Size and ζ potential of the covalent nanoparticles were measured using a Particulate Systems NanoPlus3 dynamic light scattering (DLS) instrument and ζ potential analyzer equipped with a pH autotitrator. Solutions of covalent nanoparticles were prepared in ultrapure water at ~0.1 mg/mL.

**Transmission Electron Microscopy.** A small drop of solution containing the sample was placed on a Formvar coated 300 mesh copper grid (Electron Microscopy Services, Hatfield, PA). After 30 s, the drop was removed by blotting with filter paper. The sample solution that remained on the grid was allowed to dry before inserting the grid into the microscope. The grids were viewed on a Hitan H-7100 transmission electron microscope operating at 75 kV. Digital images were obtained using an AMT Advantage 10 CCD Camera System.

**Naloxone Release Rate Determination.** Nal-cNP (4.2 mg) was suspended in 4.2 mL of pH 7.4 phosphate buffered saline (1X PBS) with subsequent ultrasonication. The reaction vial was sealed then horizontally shaken in a GeneMate incubated shaker at 37 °C and 110

1. H NMR (500 MHz, CDCl₃): δ 6.85 (d, J = 8.3 Hz, 1H), 5.87–5.77 (m, 1H), 5.19–5.13 (q, J = 7.1 Hz, 62 H), 4.67 (s, 1 H), 4.38–4.32 (m, 2 H), 3.21–3.09 (m, 3H), 3.06–2.96 (m, 2H), 2.65–2.56 (m, 2H), 2.40 (ddd, J = 5.1, 12.7, 12.7 Hz, 1H), 2.28 (ddd, J = 3.2, 3.2, 14.6 Hz, 1H), 2.13 (ddd, J = 3.8, 12.4, 12.4 Hz, 1H), 1.87 (ddd, J = 3.2, 5.0, 13.5 Hz, 1H), 1.58 (d, J = 7.1 Hz, 194H), GPC: Mₙ = 3000, Mₘ/Mₙ = 1.13.

**Solution-Based Synthesis of Naloxone–PLA Polymers.** A solution of the Nal-PLA polymer hybrid (40 mg) in 4 mL of CH₂CN was added slowly dropwise via syringe pump to a solution of 0.3% PVA in H₂O (30 mL) at a flow rate of 20 μL/min (0.02 mL/min) with rapid stirring. Upon completion of the addition, the resulting white turbid mixture was maintained overnight at ambient temperature with rapid stirring. Nanoparticles were initially collected by centrifugation at 4500 rpm and then subsequently washed with H₂O (3 × 10 mL) with centrifugation at 4500 rpm for 30 min. The supernatant liquid was decanted, and the resultant precipitate was resuspended in about 10 mL of H₂O and lyophilized to yield 25 mg of a fluffy, white solid.

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**Transmission Electron Microscopy.** A small drop of solution containing the sample was placed on a Formvar coated 300 mesh copper grid (Electron Microscopy Services, Hatfield, PA). After 30 s, the drop was removed by blotting with filter paper. The sample solution that remained on the grid was allowed to dry before inserting the grid into the microscope. The grids were viewed on a Hitan H-7100 transmission electron microscope operating at 75 kV. Digital images were obtained using an AMT Advantage 10 CCD Camera System.

**Naloxone Release Rate Determination.** Nal-cNP (4.2 mg) was suspended in 4.2 mL of pH 7.4 phosphate buffered saline (1X PBS) with subsequent ultrasonication. The reaction vial was sealed then horizontally shaken in a GeneMate incubated shaker at 37 °C and 110
The experimenter was blinded to treatment until after all data was analyzed. Due to the large sample size (n = 32), statistical analysis of all data was accomplished using GraphPad Prism 7 software. All data is shown as mean ± SEM. Von Frey behavioral data was analyzed using two-way Analysis of Variance (ANOVA) followed by Bonferroni post hoc tests. Data from the cytotoxicity assay was subjected to Analysis of Variance (ANOVA) followed by Tukey’s post hoc test for multiple comparisons between treatment groups and the untreated control group. Statistical significance was defined as p < 0.05.

Spared Nerve Injury Surgery. Spared nerve injury was completed as previously described. Briefly, mice were anesthetized with 3% isoflurane, and the fur over the left hindlimb was shaved with electric clippers. A small 1 cm incision was made in the skin parallel to the sciatic nerve, the biceps femoris muscle was moved aside, and the nerve was exposed. The tibial and common peroneal branches of the sciatic nerve were ligated with silk sutures and cut 2 mm distal to the ligatures. The sural nerve was left unmanipulated and intact. The skin was then closed with sutures over the surgical site, and mice recovered on a heating pad.

Behavioral Assay. Mechanical sensitivity was assayed with von Frey filaments to determine 50% withdrawal thresholds using the up/down method. Animals were placed on wire mesh in individual Plexiglas boxes and allowed to habituate for 2 h prior to testing. Filaments ranging from 0.02 to 2.56 g were used to assay 50% withdrawal thresholds prior to SNI surgery (baseline). Seven days after surgery, mice were assessed for mechanical sensitivity again to confirm surgical success. An a priori threshold effect of SNI was set at 50%. In other words, animals exhibiting a less than 50% decrease in withdrawal threshold from the baseline were not included in the remainder of the study. Only 2 of 38 animals were excluded from the study based on this threshold. The remaining mice were divided into morphine or saline groups. On day 8 after SNI, intraperitoneal (IP) injections (100 µL) of either morphine (10 mg/kg) or saline vehicle were administered in a blinded fashion and mechanical sensitivity was assessed again at 2, 4, and 24 h post injection to demonstrate the time course of morphine efficacy in rodents with neuropathic pain. On day 10, 48 h following the first morphine/saline injection, animals again received IP injections (100 µL) of either morphine (10 mg/kg) or saline vehicle combined with a subcutaneous injection (10 µL/g) of free naloxone (10 mg/kg), Nal-cNP (1 mg/kg @ 7% w/w loading), or empty NP (NP-empty), and mechanical sensitivity was assayed at 2 and 4 h post injection. Finally, morphine (10 mg/kg) or saline vehicle was injected again (100 µL) 1 day (day 11 post-SNI) and 3 days (day 14 post-SNI) after nanoparticle administration and mechanical sensitivity was assayed at 2 and 4 h time points following injection (corresponding to 26/28 and 98/100 h time points).

Statistical Analysis. Statistical analysis of all data was accomplished using GraphPad Prism 7 software. All data is shown as mean ± SEM. Von Frey behavioral data was analyzed using two-way Analysis of Variance (ANOVA) followed by Bonferroni post hoc tests. Data from the cytotoxicity assay was subjected to Analysis of Variance (ANOVA) followed by Tukey’s post hoc test for multiple comparisons between treatment groups and the untreated control group. Statistical significance was defined as p < 0.05.
RESULTS AND DISCUSSION

Synthesis of Naloxone–Polyactic Acid Polymer Hybrids. Aliphatic polyesters have traditionally been prepared via the catalytic ring-opening polymerization (ROP) of various lactide or lactone monomers using organometallic reagents. Among the most widely employed metal-derived polymerization catalysts are tin-based species including the commercially available tin(II) 2-ethylhexanoate. While these compounds have been successfully utilized for the synthesis of polylactide polymers, their associated potential for toxicity remains a concern, especially when generating materials to be employed in biomedical applications. As a result, we sought to identify a novel means of arriving at the requisite naloxone-initiated polymer 4 without relying on tin-based ROP catalysis.

Figure 2. Characterization of naloxone functionalized PLA 4 prepared via organocatalyzed, solvent-free ROP of L-lactide: (a) $^1$H NMR of Nal-PLA 4 in CDCl$_3$. Integration of vinylic CH vs lactic acid CH reveals DP of 64 (∼7 wt % drug loading). (b) $^1$H NMR of naloxone in CDCl$_3$. (c) GPC trace of Nal-PLA 4.
Recent advances in the organocatalyzed ROP of cyclic esters have offered attractive alternatives to the standard Sn(II)-catalyzed polymerization conditions typically employed to arrive at the PLA-derived biopolymers of type 4 thus avoiding the use of potentially toxic heavy metals. Hydrogen-bond-mediated catalyst systems derived from amidine or guanidine bases such as 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) and 1,5,7-triazabicyclo[4.4.0]dec-5-ene (TBD) or various thiourea/tertiary amine base cocatalysts have been shown to grant unprecedented control over the molecular weight and polydispersity obtained in ROPs of lactone monomers with alcohol initiators. With regard to L-lactide, literature examples have suggested that thiourea/amine cocatalyst systems are more effective than TBD for the ROP of lactide, allowing for the best control over the desired PLA polymer properties. However, these organocatalytic polymerization reactions typically employ benzyl and primary alkyl alcohols as initiators and, to the best of our knowledge, no examples of organocatalyzed, phenol-initiated ROP reactions have been described. We therefore pursued a novel synthetic approach to the desired PLA polymer hybrid 4 via an organocatalyzed ROP of L-lactide from naloxone using a thiourea/tertiary amine catalyst system (Scheme 2).

Our studies to determine optimal reaction conditions for the thiourea/tertiary amine-catalyzed ROP of lactide are summarized in Table 1. In order to achieve a higher drug loading of naloxone, a low DP polymer (DP ∼ 20) was targeted by selecting a 10:1 molar ratio of lactide monomer to initiator in the ROP reactions. Bifunctional thiourea catalyst 5, originally developed by Takemoto and co-workers, was selected for these screening experiments as it offered the convenience of containing both thiourea and tertiary amine functional groups required for monomer and nucleophile activation, respectively (Scheme 2). Our catalyst loading (5 mol %) reflected standard loadings employed for ROP reactions with thiourea reported in the literature. We initially examined the effectiveness of 5 under solvent-free conditions but also explored the possibility of carrying out the polymerizations under milder solvent-based reaction conditions. Under solvent-free conditions, a mixture of solid thiourea 5 (5 mol %) and naloxone (10 mol %) was added in one portion to premelted L-lactide at 130 °C. For the solvent-based reactions, L-lactide 3 was dissolved in solvent (∼0.7 M in lactide) and a solution of naloxone and catalyst 5 was added to start the polymerization; however, a reverse

**Table 2. Preparation and Characterization Summary for Nal-cNPs**

<table>
<thead>
<tr>
<th>entry</th>
<th>flow rate (μL/Min)</th>
<th>concentration a (mg/mL)</th>
<th>diameter b (nm)</th>
<th>PDI b</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>60</td>
<td>10</td>
<td>405</td>
<td>0.24</td>
</tr>
<tr>
<td>b</td>
<td>60</td>
<td>20</td>
<td>1314</td>
<td>0.64</td>
</tr>
<tr>
<td>c</td>
<td>20</td>
<td>10</td>
<td>243</td>
<td>0.15</td>
</tr>
</tbody>
</table>

aConcentration of Nal-PLA in CH₃CN prior to slow addition into 0.3% PVA (aq)
bAverage of 4 measurements

**Figure 3.** Characterization of covalently loaded Nalo-PLA NPs: (a) size distribution of Nal-cNP via DLS analysis and (b) transmission electron micrograph of Nal-cNP.

**Figure 4.** Cumulative release of naloxone from Nal-cNP in PBS buffer (pH 7.4). As indicated by the line of best fit ($R^2 = 0.9730$), there is a substantive linear release of naloxone from the Nal-cNPs.

**Figure 5.** Biocompatibility assays illustrating the effect of 72 h treatment of cell lines with Nal-cNP versus the negative control (untreated) and positive cytotoxic control (Saponin). Data presented is the average of three replicates. NS = not significant compared to untreated, ***p ≤ 0.001 compared to untreated.
addition of lactide to naloxone and thiourea at 100 °C was required in the case of toluene solvent due to the poor solubility of the initiator and catalyst. Reactions were conducted for the prescribed times (Table 1), and the resulting polymers were purified via precipitation into cold MeOH. HPLC traces of the purified polymer demonstrated no free catalyst or monomer was present after purification (Figure S1).

As documented in Table 1, solvent-free conditions (entry a) resulted in the rapid and complete conversion of L-lactide to the desired polymer 4 within 15 min and afforded excellent control over the molecular weight and polydispersity ($M_n = 3000, M_w/M_n = 1.13$) as determined by GPC analysis (Figure 2c). Comparable results were obtained for the solvent-based reaction employing toluene (entry d), wherein complete conversion to the desired polymer 4 was observed after 24 h at 100 °C ($M_n = 2700, M_w/M_n = 1.10$). Attempts at polymerization under milder temperatures with solvents like CH$_2$Cl$_2$ and DCE (entries b and c) resulted in lower conversions, indicating the need for elevated temperatures to achieve the desired reactivity. Given its operationally simple reaction setup, faster reaction times, and avoidance of potentially toxic solvents, we opted to utilize the solvent-free route for the production of larger batches of Nal-PLA 4 for further studies.

The polymer structure and degree of polymerization (DP) for Nal-PLA prepared via both solvent-free and solution-based methods were confirmed by $^1$H NMR analysis (Figure 2 and Figures S2–S4), and the corresponding drug loading of naloxone was determined using standard polymer molecular weight analysis techniques (eqs S3–S5). $^1$H NMR spectra recorded in both CDCl$_3$ (Figures 2a,b) and DMSO-$d_6$ (Figures S2a,b) revealed a significant downfield shift in the aryl ring proton resonances of naloxone (a and b) following ROP, suggesting that the polymerization of lactide occurred from the C3 phenol, a result consistent with the characteristic

Figure 6. In vivo efficacy of Nal-cNP and free naloxone in a spared nerve injury (SNI) mouse model treated with morphine (a) or saline (b). Bonferroni post hoc tests (a) represent significance ($p < 0.001$) between cNP-empty and Nal-cNP, (b) represent significance ($p < 0.001$) between cNP-empty and naloxone, (c) represent significance ($p < 0.001$) between naloxone and Nal-cNP, (d) represent significance ($p < 0.05$) between naloxone and Nal-cNP, and e represent significance ($p < 0.01$) between cNP-empty and Nal-cNP; $n = 6$ per group.
deshielding effects of an adjacent ester functionality. Further support for this assignment was obtained upon inspection of the $^1$H NMR spectra of naloxone and Nal-PLA 4 in DMSO-$d_6$, where the disappearance of the phenolic proton resonance $i$ from the Nal-PLA spectrum can be clearly noted (Figures S2a,b). $^1$H NMR analysis in DMSO-$d_6$ is necessary in this instance due to the rapid exchangeability of the phenolic proton in CDCl$_3$. Selective incorporation of lactide from the phenolic hydroxyl is also demonstrated through the lack of appreciable changes in chemical shift exhibited by the protons adjacent to the C14 hydroxyl moiety ($m$ and $t$), as well as the presence of the tertiary alcohol proton ($s$) (Figures S3a,b and S4a,b). This chemoselective acylation of the phenolic hydroxyl moiety of naloxone in the presence of its free C14 tertiary alcohol has also been well documented in the literature, thus permitting the present polymer structure to be assigned by analogy. $^{15,47}$ Polymer DPs, presented in Table 1, were based on the ratio of integrals between the methine protons of the polymer chain of Nal-PLA ($d$), including carboxylic and hydroxyl end groups ($d_{\text{carboxyl}}$ and $d_{\text{hydroxyl}}$), and the vinylic proton resonance of the naloxone chain end ($e$) (Figure 2a). From these values, it could be determined that drug loadings of approximately 7 wt % were achieved for both solvent-free and PhCH$_3$-based preparation methods (eqs S4 and S5, respectively). Further confirmation of the naloxone content of our Nal-cNPs was obtained via UV−vis spectrophotometric analysis of a 0.3 mg/mL solution of Nal-PLA 4 (DP = 64, Figure S5). The naloxone concentration of the sample was determined to be 0.0197 mg/mL or a 6.6% w/w naloxone loading, which was in excellent agreement with our initial $^1$H NMR estimation. It should be noted that, while high conversions of lactide to Nal-PLA were generally observed at elevated temperatures, $^1$H NMR analysis of the crude product mixtures showed that reactions proceeded with incomplete initiation of polymer growth from naloxone. The resulting initiator efficiencies (IE) for the reaction conditions investigated ranged from 16 to 38% (Table 1 and eq S2). Further optimization of IE could be used to arrive at enhanced DPs and, as a result, polymers bearing higher naloxone loadings.

**Nanoparticle Preparation and Characterization.** Having arrived at a method to synthesize naloxone terminal PLA polymers possessing suitable drug loadings, attention was focused on the preparation of the desired covalently functionalized NPs. Nal-PLA polymer 4 was converted to the corresponding cNPs using a previously described nanoprecipitation method. $^{15,48}$ Nal-PLA polymer 4 in acetonitrile (10 mg/mL) was slowly added via syringe pump (20 μL/min) to a stirred 0.3% aqueous solution of poly(vinyl alcohol) (PVA). The resulting cNPs were collected by centrifugation, then purified, and lyophilized to afford the desired Nal-cNPs. Subsequent characterization of Nal-cNP was achieved via dynamic light scattering (DLS), ζ potential, and transmission electron microscopy (TEM). DLS measurements revealed the formation of well-defined particles that possessed an average diameter of 243 nm and a narrow, monomodal size distribution with polydispersity of 0.15 (Figure 3a). Nal-cNP also exhibited a strong, negative ζ potential value ($\zeta = -29$ mV) consistent with the presence of lactic acid chain ends and their observed stability and lack of particle aggregation upon dispersion in water. $^{49-51}$ These results were confirmed by TEM morphological analysis, which provided images of well-dispersed, spherical Nal-cNPs lacking any visible surface cracks or voids (Figure 3b).

We attempted to prepare additional Nal-cNPs possessing different sizes by varying the parameters of polymer addition rate and concentration as depicted in Table 2. An increase in hydrodynamic diameter was observed when the infusion rate of polymer was increased (entry a), while maintaining the original polymer concentration (10 mg/mL). Larger particles were obtained by adding a more concentrated polymer mixture (entry b) for precipitation into 0.3% aqueous PVA. Although DLS analysis revealed the ability to form larger diameter Nal-cNPs, these particles were not selected for further evaluation due to their broader molecular weight distributions as characterized by their higher PDIs (0.235 and 0.635, respectively). It has been recently demonstrated in the literature that the sustained release properties of NPs are impacted by their corresponding molecular weight distributions. $^{52,53}$ Given that more disperse particles were typically found to produce a greater variability in release kinetics, we elected to pursue our low PDI cNPs (entry c) due to their more homogeneous particle population size characteristics to ensure a more uniform release of drug.

**In Vitro Naloxone Release Studies.** Controlled release studies were performed to evaluate the ability of Nal-cNP to provide a linear, sustained dose of naloxone. In vitro naloxone release rates were determined by incubation of Nal-cNP at 37 °C in 1X phosphate buffered saline (pH 7.4) over the course of 7 weeks with concomitant monitoring of the appearance of naloxone via LC−MS. Data points were normalized to maximum naloxone release obtained via base-mediated cNP hydrolysis in the presence of 1 M NaOH at 37 °C for 24 h. As shown in Figure 4, our preliminary in vitro release studies illustrate a prolonged, linear release of naloxone demonstrating that Nal-cNP is a suitable vehicle for the sustained delivery of naloxone as it avoids undesired burst release kinetics. While the observed drug release was very slow, requiring approximately 7 weeks to achieve the cumulative release of 65% naloxone, it was anticipated that Nal-cNP would exhibit faster rates of drug release in vivo due to exposure to endogenous hydrolytic enzymes not present in the in vitro experiment, a known phenomenon for drug-eluting nanoparticles. $^{54}$

**In Vitro Biocompatibility Assay.** Our assessment of Nal-cNP for potential cytotoxic activity against multiple cell lines is presented in Figure 5. Human embryonic kidney cells (HEK293), murine embryonic fibroblasts (NIH3T3), and human keratinocytes (HaCaT) were evaluated as they represent tissues that could be exposed to cNPs following a subcutaneous or intramuscular injection, the preferred routes of cNP administration. To determine biocompatibility, cell treatment groups were incubated for 72 h in the presence of various concentrations of Nal-cNP and then compared to both untreated cells and an additional grouping incubated with 5 μg/mL of the cytotoxic agent, saponin, as a positive control. Gratifyingly, Nal-cNP showed no significant influence on cell viability across the tested concentration range for all three cell lines (250 μg/mL to 3 mg/mL), clearly demonstrating its excellent biocompatibility.

**In Vivo Efficacy.** We measured the in vivo efficacy of Nal-cNP by blocking the analgesic effects of free morphine in a mouse model of neuropathic injury. In this experiment, animals exhibited mechanical hypersensitivity associated with a spared nerve injury (SNI). We sought to (1) block this hypersensitivity acutely with morphine and (2) evaluate whether Nal-cNP (7% w/w; 1 mg/kg) could in turn block the analgesic effects of morphine compared to free naloxone.
organocatalyst. The resultant polymers were characterized by GPC analysis and 1H NMR spectroscopy demonstrating good control over molecular weight and a high drug loading of naloxone (7% w/w). Subsequent conversion to the corresponding cNPs was accomplished by employing a previously reported nanoprecipitation protocol that yielded well-defined, spherical particles as confirmed by DLS and TEM analysis. The Nal-cNP particles showed no signs of in vitro cytotoxicity against multiple cell lines (up to 3 mg/mL), demonstrating their excellent biocompatibility. Controlled release studies demonstrated a linear, sustained release of naloxone without the undesired phenomenon of burst release. In vivo studies in SNI mice revealed the enhanced capability of Nal-cNP to promote superior levels of extended MOR receptor blockade against high dose morphine relative to free naloxone (up to 98 h), suggesting that our functionalized cNPs have the potential to dramatically improve the ability of naloxone to combat the toxic effects of fentanyl and other synthetic opioids. Additional in vitro and in vivo studies toward the further optimization of Nal-cNP for synthetic opioid reversal are currently underway and will be reported in due course.

**ASSOCIATED CONTENT**

* Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsabm.9b00380.

Experimental calculations, characterization data, NMR spectra, HPLC traces, and UV–vis spectra (PDF)

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**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

We gratefully acknowledge The Army Research Office (award no. 68271-CH) Young Investigator Program (W911NF-17-1-0015) and the Neuroscience Institute at AHN for funding. NMR measurements and instrumentation at CMU, which was partially supported by NSF (CHE-0130903 and CHE-1039870), the National Institutes of Health (UL1TR001857) and will be reported in due course.

**REFERENCES**


