Synthesis and Characterization of Redox-Sensitive Polymer and Prodrug for Targeted Drug Delivery

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SYNTHESIS AND CHARACTERIZATION OF REDOX-SENSITIVE POLYMER AND PRODRUG FOR TARGETED DRUG DELIVERY

A Thesis
presented in partial fulfillment of requirements
for the degree of Master of Science
in the Department of Pharmaceutics and Drug Delivery
The University of Mississippi

by

CHANG-HEE WHANG

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Systemic administration of chemotherapeutics is associated with various side effects deriving from accumulation in off-target sites and acute toxicity of the drugs. In the past decade, engineering targeted drug delivery platforms arose as a novel paradigm to overcome such obstacles and ultimately achieve advanced forms of chemotherapy.

This thesis reports successful syntheses of l-RSP, a redox-sensitive self-immolative polymer, and DNS-SN38, thiol-sensitive SN-38 prodrug, as potential candidates for targeted drug delivery platforms.

By covalently conjugating a redox-trigger (p-nitrobenzyl alcohol) and self-immolative linker (p-hydroxybenzyl alcohol) to the cyclization spacer (n-2-(hydroxyethyl)ethylene diamine), a novel self-immolative monomer was obtained. Polymerization of the respective monomer yielded a linear redox-sensitive polymer (l-RSP) that is capable of systemic degradation via sequential 1,6-elimination and 1,5-cyclization reactions upon redox-stimulus. Ultimately, the polymer’s potential for biomedical application was simulated through in vitro redox-triggered release of paclitaxel from polymeric nanoparticles.

SN-38 (7-ethyl-10-hydroxy-camptothecin), a potent metabolite of irinotecan (CPT-11), has been extensively investigated in the past for direct usage in order to fully exploit its cytotoxic potency. Here, 2,4-dinitrobenzene sulfonyl (DNS) moiety was conjugated to SN-38 to furnish a thiol-sensitive prodrug, denoted as DNS-SN38, that can be activated in the intracellular regions with GSH abundance. Furthermore, due to strong electron-withdrawing potential of DNS, the inherent
fluorescence of SN-38 could be virtually quenched with intact conjugation. By investigating the prodrug’s activation property upon thiol-sensitive trigger cleavage via fluorescence activation and cytotoxicity against A2780 and mCherry+OCSC1-F2 cell lines, its vast potential as a viable theranostic agent was demonstrated.
LIST OF ABBREVIATIONS AND SYMBOLS

DCM: dichloromethane
TLC: thin-layer chromatography
EtOAc: ethyl acetate
NaOH: sodium hydroxide
NMR: nuclear magnetic resonance
THF: tetrahydrofuran
TEA: triethylamine
MeOH: methanol
PVA: poly(vinyl) alcohol
PBS: phosphate-buffered saline
DDI: deionized-distilled
PTX: paclitaxel
PDI: polydispersity index
ACN: acetonitrile
CDCl₃: deuterated chloroform
(CD₃)₂SO: dimethyl sulfoxide-d₆; deuterated dimethyl sulfoxide
D₂O: deuterium oxide
DMSO: dimethyl sulfoxide
DAPI: 4',6-diamidino-2-phenylindole
PFA: paraformaldehyde
# TABLE OF CONTENTS

ABSTRACT .......................................................................................................................... II

LIST OF ABBREVIATIONS AND SYMBOLS................................................................. IV

LIST OF FIGURES.......................................................................................................... VI

PART 1: OVERVIEW........................................................................................................ 1

  ORGANIZATION OF THE THESIS................................................................. 2

  TARGETED DRUG DELIVERY................................................................. 4

PART 2: L-RSP................................................................................................................ 6

  CHAPTER 1......................................................................................................... 7

  CHAPTER 2......................................................................................................... 10

  CHAPTER 3........................................................................................................ 23

  CHAPTER 4........................................................................................................ 33

LIST OF REFERENCES................................................................................................. 34

PART 3: DNS-SN38....................................................................................................... 39

  CHAPTER 1......................................................................................................... 40

  CHAPTER 2......................................................................................................... 43

  CHAPTER 3........................................................................................................ 47

  CHAPTER 4......................................................................................................... 59

LIST OF REFERENCES................................................................................................. 60

VITA................................................................................................................................ 63
LIST OF FIGURES

PART 2:

Scheme S1........................................................................................................... 16
Figure S1............................................................................................................. 16
Figure S2............................................................................................................. 17
Figure S3............................................................................................................. 18
Figure S4............................................................................................................. 19
Figure S5............................................................................................................. 20
Figure S6............................................................................................................. 21
Figure S7............................................................................................................. 22
Scheme 1............................................................................................................ 29
Figure 1 .............................................................................................................. 30
Figure 2.............................................................................................................. 31
Figure 3.............................................................................................................. 32
PART 3:
Scheme 1 ................................................................................................. 51
Scheme 2 ................................................................................................. 52
Figure 1 ..................................................................................................... 52
Figure 2 ..................................................................................................... 53
Figure 3 ..................................................................................................... 53
Figure 4 ..................................................................................................... 54
Figure 5 ..................................................................................................... 55
Figure 6 ..................................................................................................... 56
Figure 7 ..................................................................................................... 57
Figure 8 ..................................................................................................... 58
PART 1

OVERVIEW
ORGANIZATION OF THE THESIS

Herein, we propose synthesized l-RSP and DNS-SN38 as novel candidates for targeted drug delivery for advanced chemotherapy. This thesis consists of three parts:

PART 1 briefly describes the idea of targeted drug delivery and its potential impact in the advancement of conventional chemotherapy.

PART 2 describes linear redox-sensitive polymer (l-RSP). In the past decade, the self-immolative biodegradable polymer arose as a novel paradigm for its efficient degradation mechanism and vast potential for advanced biomedical applications. This study reports successful synthesis of a novel biodegradable polymer capable of self-immolative backbone cleavage. The monomer is designed by covalent conjugations of both pendant redox-trigger (p-nitrobenzyl alcohol) and self-immolative linker (p-hydroxybenzyl alcohol) to the cyclization spacer (n-2-(hydroxyethyl)ethylene diamine), which serves as the structural backbone. The polymerization of the monomer with hexamethylene diisocyanate yields a linear redox-sensitive polymer that can systemically degrade via sequential 1,6-elimination and 1,5-cyclization reactions within an effective timeframe. Ultimately, the polymer’s potential for biomedical application is simulated through in vitro redox-triggered release of paclitaxel from polymeric nanoparticles.

PART 3 describes thiol-sensitive SN-38 prodrug (DNS-SN38). SN-38 (7-ethyl-10-hydroxy-camptothecin), an active metabolite of irinotecan (CPT-11) and the most potent camptothecin analogue, is of significant interest for direct usage in order to fully exploit its potency. In this
study, 2,4-dinitrobenzene sulfonyl (DNS) was covalently conjugated as the redox-trigger to 10’ – OH of SN-38 to yield a thiol-sensitive prodrug, denoted as DNS-SN38, with virtually quenched fluorescence at 556 nm due to donor-excited photo-induced electron transfer (d-PeT). By investigating DNS-SN38’s activation property upon thiol-sensitive trigger-cleavage via fluorescence restoration and cytotoxicity against A2780 and mCherry+OCSC1-F2 cell lines, we have validated its vast potential as a viable chemotherapeutic agent simultaneously capable of real-time monitoring of SN-38.
TARGETED DRUG DELIVERY

According to an annual report of American Cancer Society, it is predicted that 1.6 million new cancer cases will arise and 600,920 deaths will occur in 2018 in the United States alone. Despite the tremendous efforts and a vast amount of financial resources that have been put into fighting cancer, the disease still remains as a major health problem that affects not only the United States, but the whole world. Although cancer may not be the most frequent cause of death, it is commonly recognized as the most fatal disease, to which no complete treatment is available once it steps into more advanced stages.

Currently, chemotherapy is considered to be one of the more effective clinical options against cancer in more advanced stages. However, the respective treatment is significantly hampered by adverse side effects like multidrug resistance (MDR) and systemic toxicities that affect not only the tumor cells, but also the normal cells. In order to effectively overcome these obstacles, the development of targeted drug delivery systems arose as a novel paradigm in the pharmaceutics industry.

Targeted drug delivery platform is a special type of platform that is designed to achieve selective delivery of drugs to either site of action or site of absorption. By preventing drug accumulation in non-targeted sites, the treatment efficacy can be significantly improved and associated side effects can be reduced. Ideally, these systems should be non-toxic, biocompatible, biodegradable or bioeliminable and are available in various forms such as dendrimer or polymer, prodrug, antibody drug conjugate, peptide drug conjugate, and liposome. The most significant property of
these systems is that they aim to exploit physiologic biomarkers that are associated with specific diseases for targeting: pH changes, redox status, reactive oxygen species (ROS), overexpressed enzymes, and proteins.
PART 2:

NOVEL BIODEGRADABLE POLYMER WITH REDOX-TRIGGERED BACKBONE CLEAVAGE THROUGH SEQUENTIAL 1,6-ELIMINATION AND 1,5-CYCLIZATION REACTIONS


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CHAPTER 1: INTRODUCTION

Over the past two decades, a significant progress has been made towards the development of synthetic biodegradable polymers for advanced biomedical applications such as tissue regeneration and controlled drug delivery.\(^1\) A number of biodegradable polymers could be engineered using various approaches to apply biocompatible materials into platform design. Although the respective polymers indeed displayed successful degradation upon hydrolysis, the lack of specificity in location as well as inconsistency in degradation rate have limited their translation into practical applications. In light of this, much research in recent years has focused on optimizing triggers that are responsive to different stimuli with the ambition to effectively control polymer degradation.\(^2\)

Among the corresponding functional groups, \(p\)-nitrobenzyl alcohol (\(p\)-NBA) has been widely utilized for its rapid electron cascade mechanism that is activatable by electronic reduction of its aryl nitro (NO\(_2\)) group. Upon activation, \(p\)-NBA trigger converts into a 1,6-elimination spacer called \(p\)-aminobenzyl alcohol (\(p\)-ABA).\(^3\) The redox-sensitive polymers are currently of significant interest in the controlled drug delivery research for targeting cancer cells that show notably altered redox environment compared to normal cells.\(^4\) As this difference in redox capacity also relates to positive correspondence towards tumor progression and elevated drug resistance, it is accepted to be one of the more viable targets for selective delivery of drug molecules.\(^5\)

With such stimuli-sensitivity in hand, furnishing efficient and predictable mechanism of polymer degradation became paramount for the advancement of novel polymer material
intended for drug delivery. In the past decade, the so-called ‘self-immolative’ system quickly arose as a novel paradigm in the research field for its extremely efficient domino-like disassembly mechanism. The respective system shows much resemblance to the biological system’s signal transduction and amplification processes, where a relatively small number of hormone molecules are capable of inducing significant cellular responses through a cascade of amplification steps. The analogous synthetic system mimics such processes as it is designed to degrade from head-to-tail upon cleavage at the focal point of a molecule.\textsuperscript{6}

The initial form of the self-immolative polymer was a dendrimer theoretically intended for significant functional amplification through highly-ordered dendritic chain.\textsuperscript{7} However, due to complexity in synthesis for such molecule to achieve dramatic amplification, current interest in achieving similar advantage resides in synthesizing linear polymeric system for its facile one-step polymerization with appropriately designed monomer.\textsuperscript{8} Several linear biodegradable polymers with self-immolative property have been synthesized by several groups for effective amplification of stimuli-sensitivity in mind. The first reported of such system was synthesized by Shabat’s group, in which the whole compound could be degraded entirely by 1,6-elimination decarboxylation reactions. Considering their polymer chain consisted of over 15 monomer units, its functional efficacy could be well displayed.\textsuperscript{9} Since then, tremendous progress has been made in order to translate such innovative approach into various applications including drug delivery and biosensors. For example, nanoparticle drug delivery application was demonstrated through preparation of amphiphilic block copolymer by conjugating hydrophilic end caps on a hydrophobic self-immolative polymer.\textsuperscript{10}
In this article, we report on the synthesis of linear redox-sensitive polymer (\(l\)-RSP) with self-immolative degradation mechanism. The design of our monomer comprises of three components that can be readily degraded by a cascade of irreversible electron-pushing mechanisms induced by focal point reduction. One of the significances of this work resides in the introduction of a new self-immolative polymer backbone, n-2-(hydroxyethyl)ethylene diamine (N-2-HED), which appends to the very limited number of currently available backbones. To this backbone, a redox trigger, p-NBA, and self-immolative linker, p-HBA, are covalently conjugated to its primary amine and secondary amine, respectively (Scheme 1). The incorporation of p-NBA as the redox-trigger highlights the polymer’s functional variation from other redox-sensitive systems that primarily utilize disulfide bridge reduction. This nitroaryl trigger allows potential enzymatic reduction, including nitroreductases (NTR), which are commonly expressed in pathological bacteria.\(^{11}\)

Through sequential 1,6-elimination decarboxylation and 1,5-intramolecular cyclization mechanisms, the respective polymer system is expected to degrade into biocompatible fragments for potential biomedical applications.
CHAPTER 2: EXPERIMENTAL MATERIALS.

4-nitrobenzyl alcohol (p-NBA) and 4-nitrophenyl chloroformate (4-NPC) were purchased from Sigma-Aldrich (St. Louis, MO); n-(2-hydroxyethyl)ethylene diamine (N-2-HED) and 4-hydroxybenzyl alcohol (p-HBA) was purchased from Alfa Aesar (Ward Hill, MA); polystyrene standards were purchased from Polysciences, Inc. (Warrington, PA); paclitaxel (PTX) was purchased from LC Laboratories® (Woburn, MA, USA); All other chemicals and solvents were purchased from Fisher Scientific (Pittsburgh, PA).

I. Synthesis of Linear Redox-Sensitive Polymer (l-RSP) (Schemes 1 and S1)

I.1. Synthesis and \(^1\)H NMR Characterization of (NBA-HED).

p-NBA (12 mmol) was dissolved in 10 ml of anhydrous DCM, to which excess amount of pyridine was added (50 mmol). Next, 4-NPC (10 mmol), dissolved in 10 ml of anhydrous DCM, was added to the reaction mixture in a drop-wise fashion over 30 minutes at 0°C. The mixture was then left to stir at room-temperature for 5 hours. After checking the reaction progress using TLC (EtOAc:Hexane = 8:2), one-pot synthesis was carried out. In a separate round-bottom flask, N-2-HED (50 mmol) was dissolved in 10 ml of anhydrous DCM, to which excess amount of pyridine was added again. The first reaction batch was then added drop-wise over 30 minutes at 0°C. After stirring for >16 hours at room-temperature, the crude product was obtained by extraction (0.1 M
NaOH and brine) and evaporation under reduced pressure. The solid product was further purified by recrystallization (EtOAc:Ethyl Ether = 5:5), yielding white solid powder.

% Yield = 60%; $^1$H NMR (400 MHz, CDCl$_3$) δ ppm: 8.21 (2H, d), 7.51 (2H, d), 5.52 (-OH, br.), 5.19 (2H, s), 3.69 (2H, t), 3.36 (2H, m), 2.83 (4H, m).

I.2. Synthesis and $^1$H NMR Characterization of (HBA-NPC).

p-HBA (10 mmol) was dissolved in 10 ml of anhydrous THF in a reaction flask to which excess TEA (30 mmol) was added. To the reaction mixture, 4-NPC (10 mmol), dissolved in 10 ml of anhydrous THF, was added in drop-wise fashion over 90 minutes at 0 °C. After the addition was complete, the reaction mixture was left to stir for 2 hours at room temperature. The resulting organic phase was then evaporated under reduced pressure, to which DCM was added for extraction with water and brine. The crude solid product was further purified by recrystallization (Toluene:DCM) to yield light-yellow solid powder.

% Yield = 76 %; $^1$H NMR (400 MHz, CDCl$_3$) δ ppm: 8.33 (2H, d), 7.50 (2H, d), 7.46 (2H, d), 7.29 (2H, d), 4.74 (2H, s).


NBA-HED (4.2 mmol) was dissolved in 10 ml of anhydrous THF, to which excess pyridine (20 mmol) was added. After 15 minutes of stirring at room temperature, HBA-NPC (5.0 mmol), dissolved in THF, was added to the reaction mixture drop-wise at 0°C. The solution was proceeded under reflux at 50°C with vigorous stirring for 24 hours. The reaction progress was checked using TLC and the resulting organic phase was concentrated under reduced pressure for purification through flash column chromatography (EtOAc:Hexane = 7:3). The crude product was then recrystallized (DCM) into white solid powder. The $^1$H NMR spectrum is shown in Figure S2 with appropriate proton assignments.
% Yield = 54%; \( ^1 \)H NMR (400 MHz, CDCl\(_3\)) \( \delta \) ppm: 8.15 (2H, d), 7.47 (2H, d), 7.31 (2H, t), 7.05 (2H, d), 5.48 (NH, s), 5.20 (2H, d), 4.68 (2H, s), 3.88 (2H, s), 3.66-3.55 (6H, m).


The monomer was dissolved in 10 ml of anhydrous THF, to which diluted HMDI (in 5 ml of anhydrous THF) was added in drop-wise fashion over 15 minutes at 0°C. After stirring for 15 minutes, a drop of dibutyltin diaurate was added into the mixture at the same temperature. The reaction mixture was then left to stir overnight at room temperature. The polymer was extracted through precipitation in excess of cold hexane as white gooey solid, which hardened into crystalline-like solid after overnight vacuum evaporation.

II. Polymer Characterization via \( ^1 \)H NMR and GPC.

The % yield of polymerization reaction was determined to be 73%. The chemical structure of the synthesized polymer was analyzed and confirmed through \( ^1 \)H-NMR spectrum with Bruker Avance 400 MHz spectrometer, in which (CD\(_3\))\(_2\)SO:D\(_2\)O (v/v 6:1) was used as the reference solvent. The corresponding spectrum is shown in Figure S3 (with corresponding proton assignments), where peak-broadening – compared to that of the monomer – as well as HMDI proton peaks were observed. The molecular weight of the polymer, in weight-average (M\(_w\)), was determined through gel permeation chromatography (GPC) system purchased from (Waters, Milford, MA), which was equipped with Waters 1525 binary pump, Waters 717plus auto sampler, Waters 2414 refractive index detector, and Phenogel 10\( \mu \) 10E3A column (300x7.8 mm, 10 micron). The mobile phase was composed of 100% HPLC-grade THF, eluting at a flow rate of 1.0 mL/min. at 25 °C. Using Breeze 3.3 software, a calibration curve (R\(^2\) = 0.999) was constructed from the polystyrene standards ranging from 1,200 to 50,000 g.mol\(^{-1}\).

III. Preparation of Polymeric Nanoparticles and Encapsulation of PTX.
The preparation of redox-sensitive polymeric nanoparticles was carried out by single oil-in-water nanoemulsion method. First, 25 mg of the polymer was completely dissolved in 2 ml of CH$_2$Cl$_2$:MeOH (19:1) solution, which was slowly added into 20 mL of PVA (1% w/v)-PBS buffer solution (pH = 7.4) with constant stirring at room-temperature. Next, the mixture was emulsified by probe sonication for one minute using Sonics Vibra Cell, CV18. The solution was then left to stir overnight at room-temperature for evaporation of the organic phase. After filtering out large particles using 0.45 µm filter, the nanoparticles were washed with DDI water (deionized-distilled) twice and re-dispersed in mannitol-water solution (10 mg). The solution was then lyophilized to obtain white solid particles. The PTX-encapsulation process was carried out in the same manner, in which 2.5 mg of PTX was dissolved along with polymer in the organic solution before the emulsion step.

IV. Characterization of Polymeric Nanoparticles via Dynamic Light Scattering (DLS), Transmission Electron Microscopy (TEM) and High-Performance Liquid Chromatography (HPLC).

Using Zetasizer Nano ZS purchased from Malvern Instruments (Malvern, UK), the size distribution (by intensity) of the polymeric nanoparticles was determined via dynamic light scattering (DLS) for both blank (Z-average diameter = 210.3 d.nm; PDI = 0.158) and PTX-loaded (Z-average diameter = 232.3 d.nm; PDI = 0.296) samples (Figure S1). The respective particles were also characterized using TEM using Tecnai T12 microscope (FEI, Hillsboro, OR) operating at 80.0 kV. All samples were sonicated briefly before being mounted on a carbon-coated Formvar cooper grid (400 mesh). After appropriate drying process, the samples were negatively-stained with uranyl acetate (2% w/v) prior to imaging. The PTX-loading content (w/w) of 2.3% was determined by completely dissolving the nanoparticles in THF and quantitating the drug
concentration in the solution using the PTX-calibration curve ($R^2=0.9998$) constructed using Waters HPLC system ($\lambda_{\text{Detection}} = 230 \text{ nm}$) with Waters 1525 binary pump, Waters 717plus autosampler, Waters 2487 UV detector, and Phenomenex Luna C18 column (150x4 60 mm, 5 micron). The mobile phase was ACN:H$_2$O mixture (55:45, v/v) at a flow rate of 1.0 ml/min.

V. *In Vitro* Redox-Triggered Release Study

V.1. **PTX Release Study.**

The PTX release from polymeric nanoparticles was studied under 200-molar excess of sodium dithionite (to estimated number of p-nitrobenzyl trigger). Drug-loaded nanoparticles (PTX = 34.5 $\mu$g) were suspended in 2.0 mL of PBS buffer (pH 7.4) – 0.8 M sodium salicylate solution. The reduced sample contained sodium dithionite, while the control sample did not. Both samples were incubated at 37$^\circ$C for 24 hours, during which sampling (centrifugation 13.0 K RPM for 15 minutes and aliquot collection) was carried out at pre-determined time-points (hrs.): 0, 1, 3, 6, 12, 24. The step-wise release trend was also studied using the respective nanoparticles. However, the particles were introduced to excess sodium dithionite condition after 12 hours of incubation in the same release medium (and temperature). The samplings were carried out in the same manner at following time-points (hrs.): 0, 1, 3, 6, 12, 16, 24. The volume of the release medium was initialized after every sampling. The HPLC system flow rate (ACN:H$_2$O = 55:45; v/v) was set at 1.0 mL/min. and $\lambda_{\text{Detection}}$ at 230 nm.

V.2. **p-HBA Release Study.**

The release of p-HBA from reduced polymer sample was examined via HPLC analysis. 5 mg of the polymer was dissolved in 1.4 mL of DMSO:H$_2$O (6:1; v/v) solution, to which 200-molar excess of sodium dithionite was added. The sample was vigorously vortexed and then incubated at 37 $^\circ$C for 24 hours until sampling (centrifugation and aliquot collection) with the mobile phase –
MeOH:H₂O:2-Propanol (35:55:10). The HPLC system flow rate was set at 0.5 mL/min. and λ_{Detection} at 270 nm. The effect of sodium dithionite on 4-HBA stock sample was also tested (Figure S4).

VI. Polymer Biocompatibility Study

Polymer biocompatibility was investigated using live and dead (viability/cytotoxicity) kit and CyQuant cell proliferation assay (Invitrogen, Carlsbad, USA). Specifically, bone marrow mesenchymal stem cell (hMSCs) were purchased from Lonza, Inc. (Walkersville, MD). hMSCs within passage numbers 4 were used for the experiments and grown with MSCGM™ Mesenchymal Stem Cell Growth medium kit from Lonza, Inc. The hMSCs were seeded onto 48 well plate at a cell seeding density of 10,526 cells/cm² (1x10⁴ cells) per well. After the cells were attached to the wells, different concentrations of polymer were applied. The polymer concentrations tested were: (1) 0 mg/mL (control), (2) 0.1 mg/mL, (2) 0.01 mg/mL, (3) 0.0001 mg/mL. Each condition was conducted in 6 replicates including 3 replicates for CyQuant cell proliferation assay and 3 replicates for live and dead cell (viability/cytotoxicity) imaging. Cell cultures in the plate were maintained under standard culture conditions (37 °C, 95% relative humidity, and 5% CO₂). The live and dead viability/cytotoxicity kit was performed on day 1 and day 4 of polymer treatments. The live and dead viability/cytotoxicity kit can provide two color fluorescence to indicate viable cells (green fluorescence) and dead cells (red fluorescence). The live and dead cell imaging was performed using fluorescent microscope (Nikon, USA). After live and dead assay imaging, the samples were collected and stored at -80 °C for cyquant proliferation assay based on the manufacturer’s protocol. In the process of CyQuant cell proliferation assay, the fluorescence intensity of samples was measured using a microplate reader (EL×800, BIO-TEK Instrument, VT) at 480 nm (excitation) and 520 nm (emission). Finally, the
fluorescence intensity was converted to the cell number by using the cell number standard curve. The results (Figures S6 and S7) proved that the polymer treatment did not induce cytotoxicity to hMSCs at indicated polymer concentration range (0 to 0.1 mg/mL) as cell proliferation was not negatively impacted by the polymer presence.

VII. Statistical Analysis

The triplicated measurements were presented as mean ± standard deviation (SD). The student’s t-test analysis was applied for the experimental data, where statistical significance was considered for $p < 0.05$.

Scheme S1 Synthesis scheme for intermediates and monomer.
Figure S1 Polymeric nanoparticle size determination via DLS.

- Blank
- PTX-Loaded

Figure S2 $^1$H NMR (400 MHz, CDCl$_3$) spectrum of the monomer with proton assignments.
Figure S3 $^1$H NMR (400 MHz, (CD$_3$)$_2$SO:D$_2$O = 6:1) spectrum of the polymer with proton assignments.
Figure S4 HPLC detection ($\lambda_{\text{Detection}} = 270$ nm) of p-HBA release from redox-triggered polymer degradation. (A) stock p-HBA sample; (B) stock p-HBA sample with sodium dithionite; (C) polymer sample after 24 hours of degradation with sodium dithionite.
Figure S5 Overlay of DLS plots showing decreasing particle size over time.
Figure S6 Cell number (%) for each well was determined in comparison to the control (without polymer treatment) group. (a) Day 1 of treatment; (b) Day 4 of treatment. Error bars represent ± standard deviation ($n = 3$).
Figure S7 Live and dead assay images of (a) control at day 1; (b) hMSCs treated with 0.1 mg/mL polymer at day 1; (c) hMSCs treated with 0.01 mg/mL polymer at day 1; (d) hMSCs treated with 0.0001 mg/mL polymer at day 1; (e) control at day 4; (f) hMSCs treated with 0.1 mg/mL polymer at day 4; (g) hMSCs treated with 0.01 mg/mL polymer at day 4; (h) hMSCs treated with 0.0001 mg/mL polymer at day 4. Scale bar: 200 µm.
CHAPTER 3: RESULTS AND DISCUSSION

Polymer Design and Characterization

A redox-sensitive polymer, synthesized primarily by carbamate bonds, was successfully obtained from a monomer consisting of p-NBA trigger. Initially, NBA-HED was synthesized by conjugating activated p-NBA (with 4-nitrophenyl chloroformate) to N-2-HED. Next, similarly activated p-HBA was conjugated to NBA-HED to yield a redox-sensitive monomer (Scheme S1, Supporting Information). This monomeric compound was then polymerized into polyurethane with pendant p-NBA using hexamethylene diisocyanate (Scheme 1 (a)). The synthesis yield of the polymer was calculated to be 73% after precipitation in hexane. Using gel permeation chromatography (GPC) system (Waters, Milford, MA), the weight-average (Mw) molecular weight and PDI of the polymer were determined to be around 9,815 Da and 2.05, respectively.

The triggered-fragmentation of the polymer is expected to start once the end aryl nitro group of pendant p-NBA reduces into amine, which can spontaneously initiate a cascade of self-immolative processes throughout all the repeating units (Scheme 1 (b)). First, the trigger moiety detaches from the backbone’s primary amine through 1,6-elimination decarboxylation, resulting in p-aminobenzyl alcohol (p-ABA) and CO₂ fragments. Next, the unmasked primary amine readily causes a 1,5-intramolecular cyclization of the backbone, leading to its cleavage from the compound. The electron-pushing cascade then
continues on through p-HBA in a similar manner, ultimately resulting in systemic degradation of the polymer within an effective timeframe.

Redox-Triggered Degradation Monitored via $^1$H NMR

In order to confirm our hypothesis, the electronic reduction-induced structural changes of the synthesized polymer were observed and analyzed through $^1$H NMR (400 MHz, 

$\text{(CD}_3\text{)}_2\text{SO:D}_2\text{O} = 6:1 \text{ v/v}$) measurements, where 200-fold molar excess of sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$) over aryl nitro groups was used as the reductant. In Figure 1, observed proton shifts clearly suggest the polymer degradation sequence in accordance with our prediction.

The figure shows that the initial shifts occurred for peaks (a) and (b), which represent benzylic protons of the nitrobenzyl trigger – assigned in Figure S3, Supporting Information. As observed in spectrum B of the figure, the corresponding peaks shifted up-field compared to their initial chemical shifts in spectrum A. This is most-likely in response to the electronic re-shielding effect brought upon by the reduction of electron-withdrawing nitro group into an electron-donating amine group.$^{12}$ The consequent electron cascade resulting from such reduction then led to the formation of p-ABA and p-HBA, which was represented by the merging of their methylene proton peaks (c) and (d) – assigned on spectrum C. Such merging is possible, as the electronic environments for the corresponding methylene protons of each group become virtually equal when both moieties are cleaved off to be independent from the polymer backbone. This claim is supported by several studies, in which the corresponding protons were assigned to be around 4.50 ppm ($^1$H NMR) for both p-HBA$^{13}(a)$ and p-ABA$^{13}(b)$.

Furthermore, the rearrangement of peak (e) – which represents 4 protons of 3’ and 4’ methylene groups of N-2-HED backbone (shifts assigned in Figure S3, Supporting
Information) – into a single peak seen on spectrum C suggests that the 1,5-cyclization fragment was indeed created during the process. The formation of 2-imidazolidinone ring (Scheme 1 (b)) could provide similar electronic environment to those protons.\textsuperscript{14} This is analogous to the previous explanation for merging of (c) and (d) peaks.

The probable reason for relatively slow formation of the cyclization fragment compared to the initial 1,6-elimination reaction may be the distance that the exposed amine group must cyclize in order to reach the carbamate linkage connecting p-HBA and the backbone. Here, poor electrophilicity of the carbamate bond or slight protonation of amine in aqueous environment can further slow the reaction down, as suggested previously.\textsuperscript{15} This issue is a prevalent theme among the molecules aiming degradation through intramolecular cyclization based on n,n’-dimethylethylenediamine spacer, as some are reported to require days to reach complete disassembly.\textsuperscript{15,16} Therefore, it is advisable to avoid carbamate linkage when incorporating cyclization reactions as the sole routine for targeting rapid degradation. As successfully demonstrated previously, converting such linkage into either carbonate or thiocarbonate could effectively address the issues mentioned above and thereby induce significantly faster degradation.\textsuperscript{15} In regards to our polymer, however, the alternating mechanisms of rapid 1,6-elimination reaction and 1,5-intramolecular cyclization based on N-2-HED prove to be effective in tuning the overall degradation kinetics, as the process completes within 24 hours according to our \textsuperscript{1}H NMR monitoring.

Polymeric Nanoparticle Characterization via DLS and TEM

After observing redox-triggered structural changes of the polymer through \textsuperscript{1}H NMR, polymeric nanoparticles were prepared through single oil-in-water emulsion method using poly(vinyl alcohol) (PVA) as the emulsion stabilizer to test chemical release in a redox-
sensitive manner. With Zetasizer Nano ZS (Malvern, UK), the particle size distribution was determined by dynamic light scattering (DLS) method, where the Z-Average diameter was determined to be 210.3 d.nm. (PDI = 0.158) for blank particles and 232.3 d.nm (PDI = 0.296) for PTX-loaded particles (Figure S1, Supporting Information).

The redox-sensitivity of the polymer was then further characterized using the respective nanoparticles by suspending them in reductive aqueous environment (dissolved excess amount of Na₂S₂O₄). As shown in Figure 2 (a), the addition of reducing agent led to rapid increase in particle size, up to maximum of 623.2 d.nm. within 80 minutes of reduction. This extreme behavior was in parallel with previous reports on redox-sensitive micelles/nanoparticles, in which the experiments were conducted in similar manners. As explained in those studies, the trend may be explained by the solubility reversal of the polymer core’s hydrophobic nature into hydrophilic nature, due to successful unmasking of amine group in its backbone.¹(a),¹⁷ The consequent hydration of the particles induced drastic swelling, which can be correlated with observed particle size increase.

Also, in previous studies on polymer particulate encapsulation of paclitaxel (PTX), it has been suggested that the particle size may increase more than two-folds due to hydrophobic interactions between drug molecules and polymer core.¹⁸ However, in case of our polymeric nanoparticle encapsulation of PTX, the respective size increase was insignificant as Z-average diameters of PTX-encapsulated and blank particles displayed near-identical results (Figure S1, Supporting Information). This negligible difference may be explained by our nanoparticles’ relatively low drug-loading content of 2.3% (w/w) compared to as high as 18% (w/w) for amphiphilic polymer micellar-encapsulation of PTX using a redox-responsive polymer based on quinone propionic acid.¹(a) As enhanced
hydrophobicity of particle’s core is positively correlated to the increased drug loading efficiency and low drug release, this suggested that our polymer core exhibited relatively low hydrophobicity.\textsuperscript{19}

The transmission electron microscopy (TEM) images of our nanoparticles indeed showed morphologies that qualitatively supported such negligible size difference between blank and drug-loaded particle samples, in which both displayed stable spherical shape (Figure 2 (b)). The slight size estimation difference between the two methods (TEM and DLS) may simply be explained by the variation in physical conditions provided for the particles. The hydrodynamic particle size determined by DLS is accepted to be always greater than the estimation of TEM, as aqueous suspension in DLS provides solvation-layer coating around the particles.\textsuperscript{20}

It should be noted that the hydrodynamic estimation may be considered more relevant to potential biomedical applications, as it mimics the biological conditions that particles must be optimized to. For example, in designing polymeric particles for cancer-selective drug delivery, procurement of nanoscopic size range is imperative as it enhances the particles’ tendency to selectively accumulate in the tumor tissues via enhanced permeability and retention (EPR) effect.\textsuperscript{21} Although there are slight variations in accepted ranges for the particles to capitalize EPR effect, 10 nm to 100 nm range is generally known to be most ideal for avoiding both kidney and reticuloendothelial clearances.\textsuperscript{22} In this sense, our nanoparticles have room to improve in further minimizing its size distribution. One approach towards achieving such optimization may be through PEGylation at the polymer ends to yield an amphipathic compound which can self-assemble into micelles without the aid of surfactant(s). The corresponding modification may not only significantly reduce the
overall hydrodynamic size distribution, but also prevent inevitable hydrophobic aggregation of the particles, as PEG outer-layer minimizes electrostatic attractions.23

Redox-Triggered PTX-Release Study via HPLC

In order to further confirm the polymer’s redox-sensitivity, *in vitro* PTX release from the drug-loaded nanoparticles in the presence of sodium dithionite was quantified using high-performance liquid chromatography system (Waters, Milford, MA) (Figure 3). The release trends for both control (without sodium dithionite) and reduced samples were monitored over the course of 24 hours at following predetermined time points: 1, 3, 6, 12, and 24 hours. (Figure 3 (A)). As depicted in the figure, the reduction clearly induced significantly faster release of the drug from the particles. By the 24-hour mark, the reduced polymer nanoparticles could release around 84% of the encapsulated drug content, while the control sample release remained around 37%. Here, our control sample’s relatively high drug release can be attributed to aforementioned low hydrophobicity of our polymer core, which could enhance drug diffusion to PTX-soluble release medium containing 0.8 M sodium salicylate.24 The rapid release induced by reduction was also displayed in a step-wise fashion (Figure 3 (B)), where the reducing agent was applied after 12 hours of particle incubation in the same release medium. As this plot shows, a dramatic increase in release rate was observed once the reduction occurred. The reduction-mediated release trends shown through these *in vitro* studies were indeed comparable to the results of the past studies.1(a),17

Furthermore, the release of p-HBA could be also detected from the reduced polymer sample via HPLC (Figure S4, Supporting Information). This detection suggested that our polymer backbone was indeed cleaved by the alternating mechanism involving 1,6-
elimination of p-HBA as originally hypothesized. Taken together, it was clear that the reducing agent was able to cause physicochemical changes on our polymer for ultimately desired degradation. Thus, this approach may be further applied for the nanoparticle preparation of other biodegradable polymers suitable for biomedical applications.

Scheme 1 (a) Polymerization scheme to synthesize l-RSP (b) Focal point reduction-induced disassembly of the l-RSP.
Figure 1 $^1$H NMR (400 MHz, (CD$_3$)$_2$SO:D$_2$O = 6:1 v/v) monitoring of structural changes the polymer experiences upon reduction. (A) control, without sodium dithionite; (B) 1 hour of reduction; (C) 24 hours of reduction.
Figure 2 (a) Redox-induced size increase of polymeric nanoparticles monitored via DLS; (b) TEM images of nanoparticles (Right) PTX-Encapsulated (Left) Drug-free particles. Error bars represent ± standard deviation (n = 3).
Figure 3 Redox-triggered PTX-release profiles of nanoparticles monitored via HPLC ($\lambda_{\text{Detection}} = 230$ nm). (a) reduced group compared to the control (non-reduced) group; (b) reduction (sodium dithionite) applied at 12-hour mark. Error bars represent ± standard deviation ($n = 3$).
CHAPTER 4: CONCLUSIONS

In conclusion, we reported successful synthesis and characterization of a novel redox-sensitive polymer with spontaneous backbone cleavage through alternating 1,6-elimination and 1,5-intramolecular cyclization reactions. Simple covalent conjugations of redox-trigger (p-NBA), 1,5-cyclization spacer (N-2-HED), and self-immolative linker (4-HBA) yielded a redox-sensitive monomer that was polymerized into a biodegradable polyurethane. In both qualitative and quantitative manners, polymer characterizations were carried out to confirm its redox-triggered self-immolative disassembly.
LIST OF REFERENCES
*PART 2 was directly extracted from accepted manuscript version of

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PART 3:
SYNTHESIS OF DNS-SN38, A NOVEL THIOL-SENSITIVE SN-38 PRODRUG FOR BIMODAL THERAPY.
SN-38 (7-ethyl-10-hydroxy-camptothecin), an active metabolite of irinotecan (CPT-11), is considered to be the most potent antineoplastic agent among the camptothecin (CPT) derivatives. The CPT analogues are known to act as inhibitors of topoisomerase 1 (Top-1), which plays a crucial role in relieving the torsional strain in DNA. In clinical applications, CPT-11 has been found effective against various types of cancer, including colorectal, lung, and ovarian cancers. The therapeutic efficacy of CPT-11 is considered to be directly related to its metabolic conversion into SN-38 by carboxylesterases 1 and 2, as SN-38 exhibits potency of up to 1000-fold relative to that of CPT-11. Due to relatively low conversion rate (<10%) and significant interpatient discrepancies in carboxylesterase efficacy, however, direct usage of SN-38 is accepted as an ideal path to fully exploit its cytotoxic potency. Despite this, the translation of SN-38 into an appropriate chemotherapeutic option has been hampered by a number of limitations deriving from its low solubility and acute toxicity.

In the past decade, targeted prodrug strategy has gained a considerable attention in the biomedical field for its advantageous traits over conventional drugs used in the clinic. Through covalent conjugation of functional moieties that are sensitive to biological signals found in the disease sites, such as pH changes, reactive oxygen species (ROS),
enzyme overexpression\(^7\), and elevated biothiol levels\(^8\), various innovative prodrug systems could be engineered to display enhanced activation efficiency as well as site specificity in drug release. In particular, such strategy has been extensively implemented on anticancer therapeutics that are prone to causing systemic toxicity to effectively prevent dose-limiting side effects and improve therapeutic efficiency of the drugs.

Accordingly, various SN-38 prodrug platforms have been reported. For example, polyethylene glycol (PEG)-conjugate of SN-38 (EZN-2208) reached clinical trials for triple negative breast cancer for its increased solubility and prolonged biological half-life.\(^9\) Similarly, PEG-poly(glutamate)-SN38 conjugate (NK012) demonstrated self-assembly capability into micelles with enhanced solubility, half-life, and drug accumulation in tumors via the presumed enhanced permeation and residence (EPR) effect.\(^10\) Furthermore, quinone propionic acid (QPA) trigger was conjugated to SN-38 for redox-stimulus activation of the drug at tumor sites with upregulated level(s) of a detoxifying redox enzyme called NAD(P)H:quionone oxidoreductase 1 (NQO1 or DT-diaphorase).\(^11\) Here, the respective enzyme could mediate a two-electron reduction of the trigger and intact SN-38 was released upon its cleavage. Likewise, arylnitro groups such as \(p\)-nitrobenzyl and 2-nitroimidazole were also incorporated as redox-triggers in the syntheses of hypoxia-sensitive bioreductive SN-38 prodrugs.\(^12\) Upon successful reduction, the arylnitro moieties could transform into arylamines that spontaneously initiated intramolecular electron pushing processes to release intact SN-38.

Herein, we report synthesis of DNS-SN38, a novel bimodal SN-38 prodrug that is activatable in the presence of glutathione (GSH). GSH is an endogenous tripeptide, found predominantly in the intracellular regions, that plays crucial roles in important cellular
It is widely recognized that GSH concentrations in hypoxic tumor microenvironments are notably higher than those in normal cells and thus, furnishing GSH-sensitivity in anticancer prodrug design is a trending paradigm in the industry. In this prodrug, 2,4-dinitrobenzene sulfonyl (DNS) moiety is conjugated as the cleavable trigger via nucleophilic addition of the sulfhydryl (R-SH) group in the biothiols, such as glutathione (GSH) and cysteine (Cys). Recently, the DNS moiety has emerged as a useful trigger for the design of thiol-sensitive prodrugs and fluorescent imaging probes. This trigger group was found to be particularly noteworthy in our study, where it was conjugated to the hydroxyl (–OH) group at C10 site of SN-38, as it virtually quenched the drug’s inherent fluorescence. According to previously reported systems that utilized DNS, such fluorescence quenching mechanism was attributed to either donor-excited photo-induced electron transfer (d-PeT) or intramolecular charge transfer (ICT), in which the strong electron withdrawing potential of DNS allowed transfer-acceptance of excited electron from the conjugated fluorophore. Thus, the thiols-mediated cleavage of DNS was expected to not only release intact SN-38 in the intracellular compartment, but also simultaneously restore the fluorescence of SN-38 to achieve both anticancer therapy as well as diagnostic molecular imaging of the drug. As such, we have investigated DNS-SN38’s potential as a bimodal chemotherapeutic agent by studying its thiol-triggered activation through fluorescence restoration and cytotoxicity in comparison to that of SN-38.
CHAPTER 2: EXPERIMENTAL

Materials

SN-38 (7-ethyl-10-hydroxy-camptothecin) was purchased from Carbosynth, LLC (San Diego, CA). 2,4-dinitrobenzene sulfonyl chloride and triethylamine (TEA) was purchased from Alfa Aesar (Ward Hill, MA). All other chemicals and solvents were purchased from Fisher Scientific (Pittsburgh, PA).

1. Synthesis of DNS-SN38

SN-38 (0.255 mmol, 1 eq.) was first dissolved in 10 mL of anhydrous DCM and excess TEA was added (0.765 mmol, 3.0 eq.). The mixture was then lowered to 0°C and stirred for 15 minutes. Next, 2,4-dinitrobenzene sulfonyl chloride (0.3 mmol, 1.2 eq.) dissolved in 5 mL of anhydrous DCM was added to the mixture slowly in drop-wise fashion over 30 minutes. Once addition was finished, the reaction mixture was left to stir for additional 1.5 hours at 0°C. After monitoring the reaction progress via TLC, the organic phase was washed with saturated NaHCO₃ solution and brine. The organic phase was then concentrated under vacuum and crude product was recrystallized with acetone to yield slightly-yellow solid powder as the final product. (%Yield = 65%)

2. ¹H NMR and HPLC Characterization of DNS-SN38

¹H NMR (Bruker Avance, 400 MHz, DMSO-d₆; ppm): 9.14 (d, 1H), 8.59 (dd, 1H), 8.35 (d, 1H), 8.23 (d, 1H), 8.05 (d, 1H), 7.66 (dd, 1H), 7.34 (s, 1H), 5.44 (s, 2H), 5.34 (s, 2H), 3.13 (q, 2H), 1.87 (m, 2H), 1.17 (tr, 3H), 0.87 (tr, 3H).
High performance liquid chromatography (HPLC) was utilized to characterize DNS-SN38 in comparison to SN-38. Waters (Milford, MA) HPLC system ($\lambda_{\text{Detection}} = 365$ nm) with Waters 1525 binary pump, Waters 717plus auto sampler, Waters 2487 UV detector, and Phenomenex Luna C18 column (150×4 60 mm, 5 micron). The mobile phase was 25 mM NaH$_2$PO$_4$ (pH 3.1):ACN = 50:50 ($v/v$) eluting at a flow rate of 1.0 ml/min. All samples were filtered with 0.45 µM filters before injection into HPLC system.

3. UV-Vis Spectroscopy

The UV-Vis spectroscopy was performed for both DNS-SN38 and SN-38 using Genesys 10S UV-Vis Spectrophotometer from Thermo Fisher Scientific (Waltham, MA) in DMSO:PBS (pH 7.4) = 1:2 ($v/v$) solution.

4. GSH-Activation of DNS-SN38 via Fluorescence Restoration

For this study, LC500 Fluorescence Spectrophotometer from Perkin-Elmer (Waltham, MA) was used for fluorescence measurements. Based on the result of UV-scan of both SN-38 and DNS-SN38, the excitation wavelength was set at 365 nm. The solution used to prepare SN-38 and DNS-SN38 stock samples was DMSO:PBS (pH 7.4) = 1:2 ($v/v$) solution. Appropriate dilutions of these stock samples were carried out with the same solution. For time-dependent studies, FL WinLab software’s kinetic study mode was utilized with consistent delay of 60 seconds between the scans. The first scan was made 30 seconds after GSH injection into sample solution.

5. Fluorescence Imaging of DNS-SN38 in B16-F10 cells

For tracking of internalization of SN-38 in B16F10 cells, confocal images were obtained on a Nikon TiE confocal microscope using the NIS-Elements software. B16F10 at a density of 1 X $10^5$ cells per well were seeded on the coverslips in 24 well plate under the standard conditions
at 37 °C with 5 % CO₂ and 100 % humidity. When the cell confluency was around 90 %, 10 μM DNS SN-38 was treated four wells and another well without DNS SN-38 was used for control. Then, cells were incubated for 1, 5, 15, and 30 mins. For cell fixation, all media were aspirated at different time points and washed with 1 mL of PBS for 5 min. Then, 500 μL of 4 % PFA was treated at room temperature for 10 minutes. All wells were rinsed with 1 mL of PBS for three times. Then, 500 μL of DAPI was added to all wells and incubated at room temperature for 10 min. All DAPI solution was removed and washed with 1 mL of PBS for 5 min. Then, the coverslip was mounted on the slide glass for confocal microscopy.

The reported percentage values were normalized DNS SN-38 fluorescence intensity compared to the DAPI intensity. ImageJ software was used to measure the mean gray values of DAPI and SN-38 from four different regions. The normalized DNS SN-38 fluorescence intensity is given by the following equation:

\[
\text{Normalized DNS SN 38 (\%)} = \left( \frac{\text{DNS SN 38 fluorescence intensity}}{\text{DAPI intensity}} \right) \times 100
\]

6. Cytotoxicity Test On Ovarian Cancer Cell Lines: A2780 & mCherry + OCSC1-F2

The A2780 ovarian cancer cells were seeded into 96-well plates at 1 x 10⁴ cells/well and incubated at 37 °C overnight before experiments. After cells were attached to the plates, different concentrations (0.01, 0.1, 1, 10, 100, 1,000, and 10,000 nM) of DNS-SN38 and SN-38 were incubated with cells for 48 hours, followed by the MTT assay. The absorbance was determined at 570 nm with a reference wavelength of 630 nm by a microplate reader.
Similar process was applied to the mCherry-labeled ovarian cancer cell line OCSC1-F2, which was generated and propagated as previously described. The excitation for mCherry was carried out at 550 nm with emission at 635 nm.
CHAPTER 3: RESULTS AND DISCUSSION

The synthetic route for the prodrug is depicted in Scheme 1. By precisely controlling the reagent ratio, temperature, and addition rate, selective conjugation of DNS to the phenolic hydroxyl group (10’ –OH) was accomplished. In addition to 1H NMR validation, we utilized HPLC to confirm the success of respective modification. Based on the detection conditions listed in details in the experimental section, the elution times for SN-38 and DNS-SN38 were determined to be 2.1 minutes and 8.6 minutes, respectively. Considering chromatography was performed in reversed-phase, this result indicated that DNS-SN38 was more hydrophobic than SN-38. Such alteration may be explained by the masking of polar hydroxyl group with relatively non-polar DNS moiety. Previously, SN-38 has been modified with various hydrophobic groups, such as oleic acid, valine, and α-tocopherol to increase lipid solubility of the drug. The potential benefits of such modifications were: enhanced drug loading efficiency in lipid-based formulations and improved permeability through the cell membranes. Although DNS moiety’s hydrophobicity may not be on par with the aforementioned groups, its presence may still be sufficient enough to extend out to lipid-based delivery systems in future studies.

The large variation in cytotoxic potency between the CPT analogues is known to be caused by their differences in structural arrangements and/or substitutions. For example, the presence of a chiral center at C20 creates 20S- and 20R-isomers, where S-form exhibits up to 100-fold potency relative to its counterpart. In the case of SN-38, the hydroxyl
group substituted at C10 – along with ethyl group at C7 – enhances drug stability in physiological conditions, which largely contributes to SN-38’s unparalleled cytotoxicity. For that, masking of this hydroxyl group via covalent conjugation has proven to significantly lower toxicity of the drug as exemplified by several cases such as CPT-11 and SN38-glucuronide (SN38G). Based on this, DNS-SN38 is expected to sufficiently follow the most fundamental principle of prodrugs – pharmacological inertness prior to activation.

In order to test our hypothesis on thiol-activation of the synthesized prodrug, 2 µM of DNS-SN38 was treated with 1 mM GSH in DMSO:PBS (10 mM, pH 7.4) solution at 37 °C. After 10 minutes of incubation, the sample was immediately analyzed via HPLC. In the resulting chromatogram, Figure 2, the characteristic peak at 8.6-minute representing DNS-SN38 was absent, but a new peak appeared at 2.1-minute which was previously characterized to represent SN-38 standard. As the control group (without GSH-treatment) remained intact in the same incubation conditions, such result indicated that GSH-treatment could indeed cleave the DNS-trigger from DNS-SN38 via nucleophilic substitution of sulphydryl group present in GSH and thereby release intact SN-38.

Encouraged by this preliminary study, DNS-SN38’s activation was further studied in terms of fluorescence quenching and restoration. As mentioned previously, we predicted that the covalent conjugation of DNS-trigger to the hydroxyl group at C10 site of SN-38 would quench the inherent fluorescence of the drug at 556 nm due to d-PeT/ICT process. Therefore, we first examined the optical properties of DNS-SN38 in comparison to those of SN-38. The UV-absorption spectroscopy of DNS-SN38 and SN-38 revealed that the
absorption maxima for both compounds overlapped at 365 nm and 380 nm (Figure 1). As such, the excitation wavelength ($\lambda_{\text{excitation}}$) was set at 365 nm for the fluorescence study.

In accordance to our prediction, the fluorescence emission at 556 nm of 20 $\mu$M SN-38 was measured to be 41-times higher than that of 20 $\mu$M DNS-SN38 in DMSO:PBS (10 mM, pH 7.4) medium as depicted in Figure 3. Next, in order to verify thiol-sensitive prodrug activation, 20 $\mu$M DNS-SN38 sample was treated with 100 $\mu$M GSH and fluorescence restoration was monitored in a time-dependent manner Figure 4. Consistent with the preliminary study carried out using HPLC, the activation of DNS-SN38 completed within 10 minutes, as the plateau fluorescence intensity was reached by the 9.5-minute mark. Considering [GSH:DNS-SN38] molar ratio was much lower in our fluorescence activation study [10:2] than in preliminary HPLC study [1000:2], such rapid activation rate suggested that thiol-sensitive DNS-cleavage do not require significant excess of biothiols.

Motivated by such indication, we next assessed fluorescence activation/restoration of DNS-SN38 in a GSH concentration-dependent manner in similarly prepared conditions. As shown in Figure 5, an array of GSH concentrations (20, 40, 60, and 100 $\mu$M) was tested against 20 $\mu$M of DNS-SN38, where the fluorescence activation rate indeed increased along with increase in GSH concentrations. Furthermore, the rapid activation trend observed in previous studies remained unchanged, as the plateau fluorescence intensity was reached within 10 minutes for all tested samples. However, it was notable that the maximum/plateau fluorescence intensity of 20 $\mu$M GSH sample [1:1] was around 82% of those of remaining samples with higher GSH content, which indicated that slight molar excess of GSH over DNS-SN38 is still required in order to induce complete
activation of the prodrug. Lastly, the control group (without GSH-treatment) remained quenched in fluorescence intensity in same incubation conditions, clearly confirming that fluorescence restoration was induced by GSH.

These results collectively showed that GSH-dependent activation of DNS-SN38 is highly efficient and rapid. Although the results obtained from such simulated *in vitro* conditions cannot be used as the sole reference to conclude about *in vivo* conditions, they were sufficient to make a rational prediction that DNS-SN38 activation at tumor sites – with GSH in millimolar ($10^{-3}$ M) range – is extremely probable. Such activation trend was also in conjunction to previous reports on DNS-based molecules intended for biothiol-sensitivity.

Next, we examined real-time drug monitoring capability of DNS-SN38 by demonstrating the prodrug’s fluorescence response in B16F10 cell line, stained with DAPI, via confocal microscopy (Figure 6). Here, 10 µM of DNS-SN38 was incubated with $10^4$ cells. As Figure 6 shows, rapid fluorescence activation was observed from the cells over 30 minutes. Furthermore, merging of DAPI fluorescence and activated DNS-SN38 fluorescence images revealed that DNS-SN38 was indeed internalized and activated in the cytosol as the green fluorescence of SN-38 surrounded the blue fluorescence of DAPI. Considering DAPI was used to stain the cell nuclei, it was evident that the prodrug was activated once it was internalized into the cell cytosol. Taken together, it was clear that DNS-SN38 activation could be achieved at tumor sites at a very rapid rate. However, considering normal cells also consist of adequate levels of GSH, targeted chemotherapy using DNS-SN38 would be most ideal when supplemental
delivery vehicle(s) can be implemented to exploit passive-targeting via the enhanced permeation and retention (EPR) effect.

To further evaluate anticancer activity of DNS-SN38, we then carried out 48-hour cell viability tests in comparison to SN-38 on A2780 and mCherry+OCSC1-F2 (mCherry-labeled ovarian cancer stem cell) lines in MTT assay (Figure 8). The results displayed extremely potent anticancer profiles of DNS-SN38 on both cell lines, which were nearly identical to the profiles of SN-38. The corresponding IC\textsubscript{50} (50\% inhibitory concentration) values were determined to be within 10 nM against both cell lines. Considering SN-38’s parent drug, CPT, typically displays IC\textsubscript{50} in micro-molar range, resulting cytotoxicity of DNS-SN38 proved to be superior and comparable to that of intact SN-38. Moreover, such result further validated the activation efficiency of DNS-SN38 in agreement to the results of our previous experiments.

Scheme 1. Synthetic scheme for DNS-SN38
Scheme 2. Schematic illustration of DNS-SN38 fluorescence activation in the presence of biothiols. The quenched fluorescence of SN-38 is resumed upon DNS-cleavage by thiol substitution.

Figure 1 UV-absorbance spectra of DNS-SN38 and SN-38 in DMSO:PBS (pH 7.4) = 1:2 (v/v).
Figure 2. HPLC chromatograms of DNS-SN38, GSH-treated (10 minute) DNS-SN38, and SN-38 standard.

Figure 3. Fluorescence emission at 556 nm ($\lambda_{\text{excitation}} = 365$ nm) of SN-38 standard and quenched emission of DNS-SN38.
Figure 4. Fluorescence activation of DNS-SN38 with 100 µM GSH over 9.5 minutes with consistent 60-second measurement intervals.
Figure 5. GSH concentration (control or 0, 20, 40, 60, 100 µM)-dependent fluorescence activation rate of DNS-SN38 (20 µM). The error bars represent standard deviation (n=3).
Figure 6. Confocal microscopy images with the different time points of B16F10 cells incubated with 10μM of DNS SN-38 and DNS SN-38 fluorescence intensity analysis. B16F10 cells exposed to 10μM of DNS SN-38 for 1, 5, 15, and 30 min were stained with DAPI and imaged by a Nikon TiE confocal microscope using the NIS-Elements software. Green fluorescence shows the location of DNS SN-38. DAPI staining was used for the nucleus (blue). Expanded images represent the region of interest (ROI) which is the yellow box of the merged images. Scale bar of DAPI, DNS SN38, and merged images corresponds to 100 μm. Scale bar of the expanded image corresponds to 20 μm.
Figure 7. Normalized DNS SN-38 fluorescence intensities were calculated by ImageJ software from different regions (n=4).
Figure 8. (A) 48-hour cell viability profiles for A2780 cell line treated with either DNS-SN38 or SN-38 at varying concentrations (nM); (B) 48-hour cell viability profiles for mCherry+OCSC1-F2 cell line treated with either DNS-SN38 or SN-38 at varying concentrations (nM). The error bars represent standard deviation ($n \leq 3$).
CHAPTER 4: CONCLUSIONS

In summary, we report successful synthesis and preliminary characterization of a novel SN-38 prodrug called DNS-SN38. By covalently masking 10’-OH site of SN-38 using 2,4-dinitrobenzensulfonyl (DNS) group, a highly thiol-sensitive anticancer prodrug with quenched fluorescence via d-PeT was yielded. Upon nucleophilic substitution of sulfhydryl group in biothiols, the DNS-trigger could be rapidly cleaved off and thereby release intact SN-38 with restored fluorescence. Through various studies, we have demonstrated the prodrug’s capability for real-time monitoring of drug distribution and its extremely potent cytotoxicity upon activation. Thus, we propose DNS-SN38 as a viable agent that can potentially translate into a useful chemotherapeutic option against various types of cancer.
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PUBLICATIONS


PRESENTATIONS


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64