2015

Synthetic Materials with Redox-Triggers for Imaging and Drug Delivery

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SYNTHETIC MATERIALS WITH REDOX-TRIGGERS FOR IMAGING AND DRUG DELIVERY

A Dissertation
presented in partial fulfillment of requirements
for the degree of Doctor of Philosophy
in the Department of Pharmaceutics and Drug Delivery
The University of Mississippi

by
JUNGEUN BAE
December 2015
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ABSTRACT

Synthetic materials embedded with stimuli-responsive cleavable bonds have been extensively utilized for the development of site-targeted drug delivery system and selective imaging reporters. We developed stimuli-sensitive materials with two different redox switches including trimethyl-locked quinone propionic acid and nitrobenzyl moieties. Novel redox-sensitive polymeric nanoparticles and micelles were prepared from a synthesized monomer containing trimethyl-locked quinone propionic acid as a redox trigger. A hydrophobic cancer drug, paclitaxel, was incorporated into the redox-responsive polymeric nanoparticles and micelles prepared from the synthesize polymers. The effect of redox stress on the nanoparticles and micelles was evaluated with a chemical reductant, sodium dithionite demonstrating that the significant amount of paclitaxel was released at a simulated redox-state compared to the control. Cell viability studies revealed that the polymer was non-toxic and the nanoparticles and micelles could release paclitaxel to suppress breast cancer cell growth. In addition to the drug delivery system, a p-nitrobenzyl moiety redox trigger was utilized to develop a novel NIR fluorescent probe, p-nitrobenzyl 3,7-bis(dimethylamino)-10H-phenothiazine-10-carboxylate for the selective imaging and therapy. Conjugating methylene blue with a p-nitrobenzyl moiety enables it to quench electron transfer within the molecules. Moreover
the redox trigger caged probe released active methylene blue by nitroreductase-mediated 1,6-elimination resulting exhibition of strong fluorescence ($\lambda_{\text{max}} = 680$ nm) with the excitation at 580 nm. The fluorescence emission was also induced by incubation with live *Escherichia coli* bacteria. It was demonstrated that the NIR fluorescent probe generated singlet oxygen after nitroreductase mediated reduction with a laser irradiation at a wavelength of 634 nm. The redox trigger caged NIR fluorescent probe would be a suitable imaging sensor to detect bacteria expressing NTR. In addition, the probe also possesses potential as an MB prodrug which can be applied for the treatment of various diseases.
DEDICATION

This research work is dedicated to my loving family.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADEPT</td>
<td>Antibody-directed enzyme prodrug therapy</td>
</tr>
<tr>
<td>CB1954</td>
<td>2,4-dinitrobenzamide</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>CMC</td>
<td>Critical micelle concentration</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DFMO</td>
<td>$\alpha$-difluoromethylornithine</td>
</tr>
<tr>
<td>DI</td>
<td>Deionized</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DOPE</td>
<td>1,2-dioleoyl-sn-glycero-3-phosphoethanolamine</td>
</tr>
<tr>
<td>DPH</td>
<td>1,6-diphenyl-1,3,5-hexatriene</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EPR</td>
<td>Enhanced permeability and retention</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FMN</td>
<td>Flavin mononucleotide</td>
</tr>
<tr>
<td>GDEPT</td>
<td>Gene-directed enzyme prodrug therapy</td>
</tr>
<tr>
<td>GPC</td>
<td>Gel permeation chromatography</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Half maximum inhibitory concentration</td>
</tr>
<tr>
<td>MB</td>
<td>Methylene blue</td>
</tr>
<tr>
<td>mPEG-PDLLA</td>
<td>Monomethoxy poly(ethylene glycol)-block-poly(D,L-lactide)</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular weight cut-off</td>
</tr>
<tr>
<td>NAD(P)H</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NHS-QPA</td>
<td>n-hydroxysuccinimidyl QPA</td>
</tr>
<tr>
<td>NIR</td>
<td>Near-infrared</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NPs</td>
<td>Nanoparticles</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non-small-cell-lung cancer</td>
</tr>
<tr>
<td>NTR</td>
<td>Nitroreductase</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PDI</td>
<td>Polydispersity index</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PET</td>
<td>Photoinduced electron transfer</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly(D,L-lactide-co-glycolid)</td>
</tr>
<tr>
<td>Pluronic®</td>
<td>Poly(ethylene glycol)-poly(propylene glycol)-poly(ethylene glycol)</td>
</tr>
<tr>
<td>p-NBMB</td>
<td>p-nitrobenzyl 3,7-bis(dimethylamino)-10H-phenothiazine-10-carboxylate</td>
</tr>
<tr>
<td>PTX</td>
<td>Paclitaxel</td>
</tr>
<tr>
<td>PVA</td>
<td>Polyvinylalcohol</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>QPA</td>
<td>Timethyl-locked quinone propionic acid</td>
</tr>
<tr>
<td>RES</td>
<td>Reticuloendothelial system</td>
</tr>
<tr>
<td>RNO</td>
<td>( p )-nitrosodimethylaniline</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscope</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SRB</td>
<td>Sulforhodamine B</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
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CHAPTER 1

INTRODUCTION
The aim of this chapter is to provide an insight into the principles behind the experimental work reported in this dissertation work. First, an introduction to redox microenvironment and its associated therapeutic opportunities are given, followed by an overview of redox triggers applied in the design of redox-responsive delivery system. The chapter concludes by discussing the advantages redox triggers may offer for targeted drug delivery systems and selective imaging.

Numerous modern drug delivery strategies seek to maximize the therapeutic potential of drug molecules at the targeted site and to minimize their side effects in healthy tissues. Potent anticancer therapeutic agents are generally non-selective to normal cells, leading to severe cytotoxic complication. Targeted drug delivery systems have been considered as a promising tool to alleviate such toxicity by distributing the cytotoxic drugs favorably to tumor sites in the body. For selective drug release in the desired site, stimuli-responsive polymer-based drug delivery systems have been extensively explored. The bioresponsive delivery systems can undergo conformational and/or physiochemical property changes and release incorporated drugs in response to the signals stemming from tumor microenvironments such as low pH, altered oxidation-reduction (redox), over-expressed enzymes and hyperthermia. In addition to the targeted drug delivery system approach for the improvement of therapeutic effect, selective imaging is also pivotal in achieving accurate disease diagnostics and monitoring
therapeutic efficacy and processes. The key to an effective imaging strategy is the use of reporters that can be detected by various imaging devices. Radiotracers, magnetic probes and fluorescent agents are widely utilized reporters in research settings. Among these, the use of fluorophores is a promising tool for imaging due to advantages including high resolution, sensitivity and non-invasiveness. However, fluorescent probes with emission at short wavelengths have the limitation of poor tissue penetration owing to interference with the background auto-fluorescence from biomolecules. To overcome such limitations, fluorophores with emission in the far red and near-infrared (NIR), in the range of 600-1000 nm, have been investigated because absorption and autofluorescence are minimal in those wavelength ranges. Various approaches have been made to achieve selective imaging sensitivity of NIR fluorophores. Latent fluorescent probes, by virtue of being switchable/activatable, can be turned on by undergoing a user-designated chemical reaction triggered by specific stimuli. These activatable molecular probes improve the achievable target-to-background ratios. Such activatable probes are generally dependent on targeting groups such as a small molecules, peptides, and proteins. Recently the altered redox status between intracellular and extracellular compartment has gained attention as an emerging strategy to achieve the targeted drug release and selective imaging.

1.1. **Cellular redox microenvironments and therapeutic opportunities**

Cellular metabolism and integrity are maintained by balancing the redox state of all the cellular components to maintain optimal overall functions. Redox homeostasis is
therefore vital to all organisms and is closely involved with redox reactions in which electrons and/or hydrogen atoms are transferred between donor and acceptor molecules. In response to redox imbalance, however, the redox gradient between intracellular and extracellular compartments becomes notably greater, which induces new metabolic pathways to restore optimal overall function. During these intracellular redox-regulatory processes, various redox agents are expressed\textsuperscript{12} and can serve as an important indicator of the redox status.\textsuperscript{13} In conjunction with the disease-related cellular redox changes, the redox gradient between intracellular and extracellular spaces has inspired the paradigm of redox-responsive biomaterials intended for intracellular drug delivery.\textsuperscript{14}

1.2. Redox triggers

Synthetic and macromolecular chemistry provides access to a wide range of redox-susceptible materials. Considering a number of redox agents expressed during the intracellular redox-regulatory pathways, it is crucial to select an appropriate redox trigger which responds specifically and selectively at the desired site of action for the responsive material architecture. Selected redox-triggers including disulfide, quinone and nitro compounds are discussed with their applications in biomedical materials as follows.

1.2.1. Disulfide bond and glutathione

A disulfide bond, formed through the oxidation of two thiols is a valuable functional group in a variety of chemical and biological agents exhibiting potent
reactivity or biological activities. This bond has already been found in proteins, oxidized glutathione, and even in numerous natural products including some drugs (e.g., mitomycin disulfides, leinamycin, etc.). In mildly oxidizing environments, the disulfide bond is relatively stable. However, disulfide bonds are rarely found inside cells, since disulfide bond can be easily cleaved by glutathione (GSH) which is the most abundant thiol-containing small molecule. In the human body, intracellular GSH concentration reaches 10 mM in cellular fluids while plasma and other body fluids drops the GSH concentration to 20 µM. In addition, tumor cells have also shown an elevated intracellular reduced glutathione concentration, a ~7-fold increase in vitro, in comparison with that of normal cells. Therefore, intracellular drug delivery has heavily taken

**Figure 1.1.** The reduction of a disulfide substrate to free thiols. (i) Formation of a glutathione-substrate mixed disulfide and substrate free thiol; (ii) release of the second substrate free thiol and formation of GSSG; (iii) reduction of GSSG to GSH by GSSG reductase.
advantage of GSH-mediated thiol exchange. GSH can react with disulfide bonds through its cysteine-based thiol, which reduces the disulfide substrates to their corresponding free thiols and oxidizing glutathione to glutathione disulfide (Figure 1.1.). Because of this unique property of disulfide bond, it has been extensively applied for cancer research as an efficient redox trigger.

The disulfide chemistry applied for a variety of materials including prodrugs\(^{18}\), dendrimer\(^{19}\), liposome\(^{20}\), and polyplex for siRNA delivery\(^{21}\) could indeed release drug under elevated redox potential in tumor. Long et al. synthesized the disulfide-linked polyethylene glycol (PEG)–GSH conjugates 1 and 2 for GSH

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**Figure 1.2.** GSH-transporter-targeted prodrug conjugates 1 and 2.

---

**Figure 1.3.** Chemical structure of disulfide linked-amphiphilic polymer.
delivery (Figure 1.2). PEG is employed to protect the GSH moiety from oxidation until it is delivered into the cells. GSH is oxidized to GSSG during oxidative stress in cells, thereby restricting the oxidation of other molecules. Therefore, it plays an antioxidant role to protect cells from oxidative stress. When administered to the SH-SY5Y human neuroblastoma cell line under oxidative stress induced by \( \text{H}_2\text{O}_2 \), 1 did not display protecting activity against oxidative stress (Figure 1.2.). However, 2 was 100% effective on inhibition of oxidative stress since 2 enabled to release GSH via the reduction of its disulfide bond (Figure 1.2.). Further improvements in utilizing micelles that contain the S–S bond were investigated by Thayumanavan et al.\(^{23}\) In this study, a disulfide bond was exploited to conjugate the backbone chain to a hydrophobic alkyl chain resulting in the formation of micelle-like nanoassembly in aqueous media. In the presence of GSH, the doxorubicin loaded micelles were disassembled and thereby released the incorporated drug upon the solubility change in polymer after disulfide cleavage. It was demonstrated that the drug release from the micelles was dependent on the concentration of a reducing agent, GSH. However, these micelle-based approaches were not feasible to release drug release under tumor cell-relevant GSH levels. In addition, there have been many attempts to obtain dual-sensitivity by combining pH-sensitivity with disulfide-based redox-sensitivity for selective tumor targeting.\(^{24}\) A disulfide group was also utilized for a redox-responsive cross-linker to stabilize micellar structure and control the release of the encapsulated cargo in response to D,L-dithiothreitol.\(^{25}\)
1.2.2. Trimethyl-locked quinone propionic acid and DT-diaphorase

Trimethyl-locked quinone propionic acid (QPA) group can undergo two-electron reduction by reducing agents to yield the respective hydroquinone propionic acid. The hydroquinone is immediately transformed into its corresponding lactone via intramolecular cyclization which eventually leads to the cleavage of any group linked through the carboxyl functionality as shown in Scheme 1.1. QPA can be reduced not only by chemical agents such as sodium dithionite and sodium borohydride but also by reductive enzyme, DT-diaphorase.

Scheme 1.1. Chemical reduction of the trimethyl-locked QPA based redox-sensitive material.

DT-diaphorase (NAD(P)H:quinone oxidoreductase (NQO) (EC 1.6.99.2) is a cytosolic flavoprotein and was first isolated in 1958 by Lars Ernster.\textsuperscript{26} DT-diaphorase catalyzes the two-electron reduction of diverse substrates including quinones, quinone epoxides, aromatic nitro and nitroso compounds, azo dyes and Cr(VI) compounds using NADH or NADPH as electron donors. DT-diaphorase has been regarded as a chemoprotective enzyme capable of catalyzing the reduction and detoxification of
exogenous quinones. The enzyme is also known to be involved in the bioactivation of molecules such as MMC into toxic DNA-damaging agents. This enzyme is interestingly over-expressed in a number of tumor types, including non-small cell lung carcinoma, colorectal carcinoma, liver cancers, pancreatic cancers and breast carcinomas, when compared to normal tissues.\textsuperscript{27} In the case of non-small-cell-lung (NSCLC) tumors, DT-diaphorase activity determined using 2,6-dichlorophenolindophenol was found to be 123 nmol min\(^{-1}\) mg\(^{-1}\) compared to 6.4 nmol min\(^{-1}\) mg\(^{-1}\) in normal cells, a 20-fold increase\textsuperscript{28}. Since DT-diaphorase detoxifies and protects the cell from toxins and mutagens, it tends to be expressed at higher levels in the early carcinogenic process. This upregulation may involve in epigenetic factors such as hypomethylation of DT-diaphorase gene, elevation of c-fos proto-oncogenes, altered promoter and/or repressor function.\textsuperscript{29} The previous study reported that it might play a role in the stabilization of tumor suppressor protein p53. Hence the last decade has seen increased focus on DT-diaphorase as an important target in oncology and a QPA group has potential to develop into an effective redox trigger to design redox-sensitive material in cancer research communities.
Redox-triggered bioimaging probes composed of QPA have been synthesized to release fluorescent indicators after the DT-diaphorase-mediated activation. McCarley and coworkers have developed a QPA caged fluorophore using rhodamine (Figure 1.4). It demonstrated that the QPA-based imaging probe generated fluorescence in the presence of DT-diaphorase.

**Figure 1.4.** Activation of QPA caged fluorophore by human DT-diaphorase to yield the highly fluorescent dye, rhodamine.
of DT-diaphorase utilizing DT-diaphorase-positive human cancer cells. Moreover, redox-sensitive self-immolative fluorophore was synthesized with a QPA trigger group, an N-methyl-p-aminobenzyl alcohol self-immolative linker and naphothalimide fluorophore to detect DT-diaphorase levels for cancer diagnosis. These studies investigated kinetics of DT-diaphorase with the redox-sensitive fluorophores and verified the selectivity of the QPA caged fluorophore to DT-diaphorase. However, monitoring fluorescence emission from these QPA caged fluorophores has not been accomplished. Recently Liu et al. synthesized DT-diaphorase-activatable theranostic prodrug which capable of assessing fluorescent emission to detect DT-diaphorase level. Camptothecin, an inhibitor of topoisomerase I, was selected as a fluorophore and its fluorescence was quenched by the covalently attached QPA trigger via photoinduced electron transfer (PET). The camptothecin released from the QPA caged fluorophore selectively destroyed the cancer which overexpresses DT-diaphorase with high efficacy.

![Diagram](image)

**Figure 1.5.** Activation of QPA caged self-immolative fluorophore by human DT-diaphorase to yield the highly fluorescent dye naphothalimide.
In addition to the QPA caged imaging probes, the QPA trigger was applied to the synthesis of prodrug for DT-diaphorase targeted release. A QPA-based aniline mustard prodrug synthesized by Volpato et al. had showed that cyclized product and the active drug were generated a time- and DT-diaphorase-dependent manner. The active drug exerted cytotoxicity on T47D cells upon the reductive activation by oxidoreductases at low oxygen concentration\(^{33}\). The QPA trigger was also exploited to design redox-triggered liposomes which electrochemically release chemicals. The liposomes were composed of 1,2-dioleoyl-\textit{sn}-glycero-3-phosphoethanolamine (DOPE) and a QPA trigger attached to the head group of the lipid moiety.\(^{34}\) Chemical reduction-induced cyclization of QPA head group on the liposomes led to conformational change in the supramolecular assemblies, resulting in the release of encapsulated hydrophilic dye, calcein. Even though the QPA trigger has been extensively investigated in the development of redox-sensitive materials, it has never been introduced to a polymer network for drug delivery until our

\textbf{Figure 1.6.} Activation of QPA caged self-immolative fluorophore by human DT-diaphorase to yield the highly fluorescent dye naphothalimide.
group started to actively pursue the formulation of QPA based redox-sensitive polymer in 2012. The approach with polymeric nanocarriers to deliver drug is advantageous over prodrugs and liposomes. Polymeric materials have a wide variety of available drug or dye selection to be incorporated inside them in spite of its hydrophobicity. Furthermore, the modification of polymers for further application in targeted delivery might be more easily achieved than prodrug or liposomes.

1.2.3. Nitrocompounds and Nitroreductase

Nitroreductases (NTR) play an important role in catalyzing the reduction of nitroaromatic compounds, which are wildly distributed in the environment. Nitroreductase is a flavoenzyme bound with flavin mononucleotide (FMN) cofactor and a homodimer of 24 kDa subunits expressed in Escherichia coli. It acts via a substituted enzyme mechanism, whereby the bound FMN is first reduced by nicotinamide adenine dinucleotide phosphate (NAD(P)H) and the NAD(P)⁺ produced dissociates. In the second step, various substrates can bind to the enzyme and be reduced, in turn, by FMN. In the case of nitroaromatic compounds, nitroreductase catalyzes the reduction of a nitro group to hydroxylamine, which is subsequently converted to an amine in the presence of NAD(P)H. The large electronic change resulting from the conversion of the electron-withdrawing nitro group to the electron-donating hydroxylamino group provides an effective ‘switch’ mechanism for the activation of an inert compound and the subsequent release of the active form of the agent. Based on this switch mechanism, various prodrugs
or switchable imaging probes have been designed with nitro groups as substrates to be triggered upon reduction by NTR.

According to previously reported NTR-catalyzed reactions, aromatic substrates show relatively high reactivity with NTR caused by hydrogen bonding, $\pi-\pi$ stacking interaction, hydrophobic effect, and etc. The nitro groups in substrates can be reduced first to a nitroso group, then a hydroxylamine group, and ultimately to an amino group. In addition, the electron withdrawing group induces an electron-transfer process, quenching the fluorescence emission of a substrate, and the reduction of nitro groups by NTR leads to a change in fluorescence intensity. These fundamental concepts underpin an important strategy in the design of fluorescent probes for NTR detection and NTR activatable prodrugs.

2.3.1. Nitroreductase activatable prodrugs and sensors

The first identified NTR prodrug was 2,4-dinitrobenzamide, CB1954 that is also known as tretazicar. It consists of a 2,4-dinitrobenzylamide ring linked at the 5-position to an aziridinyl substituent. Both nitro groups were reduced by bacterial type I NTR generating 2- and 4-hydroxylamine metabolites which have cytotoxic activities in mammalian systems. In vitro analysis has shown not only that CB1954 functions as a substrate for both T. brucei and T. cruzi NTR and but also that it blocks replication of the mammalian forms of the parasites. Currently CB1954 proceeded to clinical trials in combination with E. coli NTR as a potential gene-directed enzyme prodrug therapy (GDEPT). A dinitrobenzamide mustard prodrug (PR-104) was also developed as an
NTR activatable prodrug. PR-104 is the first developed tumor hypoxia-activated (GDEPT independent) therapeutic agent and has advanced to multinational Phase II clinical trials. The previous study has shown that PR-104 was tolerated by humans at up to twenty times the maximum dose of CB1954 on a molar basis. Moreover, the activated metabolites of dinitrobenzamide mustard prodrugs possess greater potency and bystander effect than CB1954.

2.3.2. \( p\)-nitrobenzyl-based prodrugs and sensors

Thanks to its high selectivity and sensitivity, application of NTR-catalyzed reaction in the design of prodrug and imaging probes have attracted considerable attention in the drug targeting field. Among various nitroaromatic compounds, however, only a limited number of studies on a nitrobenzyl trigger had been carried out in the past. Hu et al. first synthesized an NTR activatable phosphoramide mustard prodrug using a 4-nitrobenzyl moiety as a redox-trigger. While the prodrug has low cytotoxicity in the absence of NTR, it showed 167,500\( \times \) selective cytotoxicity toward nitroreductase-expressing V79 cells with an IC\( _{50} \) of 0.4 nM. It suggested that 4-nitrobenzyl phosphoramide mustard is about 100\( \times \) more active and 27\( \times \) more selective than CB1954, indicating that a nitrobenzyl functionality is a highly selective trigger to NTR. To explore nitrocompound structure–activity relationships as substrates of NTR, Hu et al. have also synthesized a series of nitrobenzyl phosphoramide mustards. The results implied that a nitrobenzyl based prodrug offers the best enzyme activity and antiproliferative effect against both mammalian and trypanosomatid cells. Hu’s group has also applied a
nitrobenzyl group in the design of α-difluoromethylornithine (DFMO or Efornithine) prodrug. Moreover, Charkrapani’s group has synthesized a nitrobenzyl based diazeniumdiolate prodrug. Cell viability assays for DFMO prodrug and diazeniumdiolate prodrug have revealed that cytotoxic effects towards cancer cell lines is significantly enhanced in the presence of NTR.

**Scheme 1.2** Chemical reduction of nitrobenzyl trigger based redox-sensitive prodrug.

In addition to the aforementioned nitrobenzyl trigger based prodrugs, various selective imaging probes possessing a nitrobenzyl moiety have also been developed during the past several years. Cui et al. have synthesized an NTR-activatable fluorescent probe using a nitrobenzyl trigger and naphothalimide in 2011. The spectroscopic evaluation using reducing agents including dithiothreitol, glutathione, cysteine, homocysteine, NADPH and NTR verified that the probe was feasible to be reduced to release fluorescence at 550 nm. Even though a nitrobenzyl trigger weakens an intramolecular charre transfer of fluorophore, the nitrobenzyl trigger conjugated probe emitted fluorescence at 475 nm and at 550 nm, before and after reduction, which works in the relatively high energy region (400-650 nm) of the electromagnetic spectrum. To minimize biological auto-fluorescence, a NTR-activatable sensor with a fluorescence emission wavelength in the NIR region was recently developed by Shi et al. Cyanine
dyes were selected as a fluorophore with signals observed in the NIR region (>700 nm) and the introduction of sulfonate groups to Shi’s dyes enable the sensor to improve water solubility and prevent the aggregation of cyanate dye. It was demonstrated that the nitrobenzyl trigger caged sensor did not emit significant fluorescence but strong NIR emission at 708 nm in the presence of NTR. However, the antibacterial activity of this sensor has not been tested. Despite the previous extensive investigation about the use of a nitrobenzyl redox trigger for NTR activatable materials, such triggers have not been applied for the development of bifunctional compounds, which can serve as a tool for selective imaging and therapy.

1.3. Organization of the dissertation

Chapter 2 discusses a QPA trigger based redox-responsive polymer for potential application in a tumor targeted drug delivery system. The synthesized polymers were utilized to prepare hydrophobic drug loaded nanoparticles. Redox-triggered drug release of nanoparticles was evaluated using sodium dithionite and their potential application in the development of tumor targeted drug delivery system was delineated as well.

Chapter 3 includes a redox-responsive amphiphilic polymer comprising a QPA redox trigger. Coupling of mPEG to a hydrophobic redox-responsive polymer provided the amphiphilic polymer with its self-assembling capability to incorporate hydrophobic drug into the core of micelles. The dimensional and chemical structural changes
occurring to the micelles in the presence of a redox chemical were characterized to verify if the drug releases in a redox-triggered manner. *In vitro* drug release kinetics and cytotoxicity of micelles were also discussed.

**Chapter 4** elaborates the synthesis and characterization of a nitrobenzyl redox trigger caged NTR imaging probe. Selective uncaging of the probe via nitro reduction was evaluated with live *E. coli* for imaging. A potential therapeutic application of the compound in photodynamic therapy was also addressed.

Last, in **Chapter 5**, we summarize the dissertation research work consisting of those two materials prepared from redox triggers and discuss their challenges and future research directions.
CHAPTER 2

REDOX-RESPONSIVE POLYMERIC NANO行李ORES FOR DRUG DELIVERY
Bioresponsive polymeric nanoparticles have been extensively pursued for the development of tumor-targeted drug delivery. Novel redox-sensitive polymeric nanoparticles were prepared from a synthesized monomer containing trimethyl-locked quinone propionic acid as a redox trigger. A hydrophobic cancer drug, paclitaxel, was incorporated into the redox-responsive polymeric nanoparticles prepared from the synthesize polymers. The particle size of nanoparticles was determined to be 180 nm (PD = 0.138) by dynamic light scattering. The effect of redox stress on the nanoparticles was evaluated with a chemical reductant, sodium dithionite demonstrating that the nanoparticles released >80% of paclitaxel over 24 h at a simulated redox-state compared to 26.5% to 41.2% release from the control. Cell viability studies revealed that the polymer was non-toxic and the nanoparticles could release paclitaxel to suppress breast cancer cell growth.
2.1. INTRODUCTION

Cancer has been a major cause of mortality across the world.\textsuperscript{46} Tremendous efforts have already been made to improve cancer therapy, including chemotherapy, in conjunction with nanotechnology as well as bioimaging.\textsuperscript{47} Potent anticancer therapeutic agents are generally non-selective and thus affect normal cells, leading to severe side effects. Targeted drug delivery systems have been considered as a promising tool to minimize the toxicity by distributing the cytotoxic drugs favorably to tumor sites in the body. In particular, abnormal leaky tumor vasculatures and deficiency of lymphatic drainage in tumor enable nanotechnology-based targeted drug delivery to favorably accumulate cancer therapeutics in solid tumor while lessening their toxicity to normal tissues.\textsuperscript{48} For selective drug release in tumor sites, stimuli-responsive polymer-based drug delivery systems have been extensively explored.\textsuperscript{1} The bioresponsive delivery systems can undergo conformational or physiochemical property changes and release incorporated drugs in response to the signals stemming from tumor microenvironments such as acidic pH\textsuperscript{2}, altered oxidation-reduction (redox)\textsuperscript{3}, over-expressed enzymes\textsuperscript{4} and hyperthermia\textsuperscript{5}.

Since redox changes associated with tumor hypoxia have been identified as a viable biomarker for tumor progression and cancer drug resistance, reductive enzymes overexpressed in tumor microenvironments, such as DT-Diaphorase (EC 1.6.99.2),
provide an important methodology for selective tumor targeting. Various bioreductive prodrugs that can be activated by the reductive enzymes specifically in hypoxic tumor microenvironments have been reported and some of them are currently under clinical trials. As demonstrated from aniline mustard protected with a bioreductive protecting group, drug activation under hypoxia, tested on T47D cells, resulted in cytotoxicity. However, the prodrug approach can be complicated with ubiquitous expression of various reductive enzymes in normal cells. Even though redox-triggered liposomes that release the incorporated molecule upon chemical reduction have been studied already, the delivery platform based on the redox-sensitive polymeric nanoparticles have not been conceived yet for targeted drug delivery applications. The approach with polymeric nanoparticles to target hypoxic solid tumors would have advantages over the bioreductive prodrugs and liposomes. They can be easily modified with the ligands that selectively interact with the molecules expressed on cancer cells to achieve active targeting to cancer cells. In addition, polymeric nanoparticles cannot be limited by the physicochemical properties of the drugs to be loaded. Unlike to liposomes, polymeric nanoparticles can easily adopt various cytotoxic drugs, either hydrophobic or hydrophilic for drug targeting. Furthermore, the polymeric nanoparticles can be modulated for increased circulation time and favorable accumulation in tumor.

DT-diaphorase (NAD(P)H:quinone oxidoreductase (NQO1), EC 1.6.99.2) is an obligate two-electron cytosolic reductase which reduces and detoxifies quinones and their derivatives as a part of an electrophilic and/or oxidative stress-induced cellular defense mechanism. DT-diaphorase is overexpressed in many cancerous tissues compared to
surrounding normal tissues. In the case of non-small-cell-lung (NSCLC) tumors, a DT-diaphorase activity determined using 2,6-dichlorophenolindophenol was found to be 123 nmol min⁻¹ mg⁻¹ compared to 6.4 nmol min⁻¹ mg⁻¹ in normal cells, a 20-fold increase. Since DT-diaphorase was known to activate quinone-bearing anticancer drugs, various quinone derivatives have been studied as a viable component of bioreductive cancer chemotherapeutics. In addition, quinone-based bioreductive prodrugs which release active cytotoxic drugs have been designed to selectively target cancer cells by overexpressed DT-diaphorase. Interestingly, trimethyl-locked quinone propionic acid (QPA) has been known to readily undergo intramolecular cyclization by DT-diaphorase-mediated two-electron reduction and release a lactone. Because of this unique property of the QPA group, it has been extensively applied for cancer research as an efficient redox trigger.

Redox-triggered bioimaging probes consisting of QPA have been synthesized to liberate fluorescent indicators after DT-diaphorase-mediated activation. Furthermore, QPA has been applied for the synthesis of bioreductive prodrugs based on aniline mustard and oxindoles for selective tumor targeting. Even redox-triggered liposomes that electrochemically release chemicals have been designed with the QPA chemistry. Chemical reduction-induced shedding of QPA from the liposome surface resulted in the structural change of the supramolecular assemblies, which later destabilized the redox-sensitive liposomes to electrochemically release a hydrophilic fluorescent dye, calcein. However, the QPA-based redox chemistry has never been applied for a polymeric drug delivery system thus far. The approach to use QPA-based redox-sensitive polymeric
nanoparticles is advantageous over the QPA-based prodrugs or liposomes to target the redox stress in solid tumors. Polymeric nanoparticles can be easily modified with the ligands that selectively interact with the molecules expressed on cancer cells to achieve active targeting. In addition, the nanoparticles may be more flexible than the above-mentioned liposome for the formulation of hydrophobic cancer drugs since they may disrupt exquisitely balanced non-covalent interactions of QPA at the liposome surface. Furthermore, the nanoparticles can easily achieve prolonged circulation in blood and preferred accumulation in tumor by poly(ethylene glycol) (PEG) coupling.

Scheme 2.1. Chemical reduction of the redox-sensitive polymers based on trimethyl-locked benzoquinone.

In this chapter, we describe a novel redox-sensitive polymer containing amino groups protected with a QPA redox trigger, which is able to respond to the redox variation with polymer property changes. The polymer is designed to release a lactone from QPA and unmask free amino groups via the two-electron reduction mediated by chemical agents, such as sodium dithionite (Na₂S₂O₄). As a result, the reduced polymer would have enhanced water solubility at neutral pH upon protonation of free amino groups. Thus, nanoparticles prepared with the redox-sensitive polymer would be able to
release incorporated drugs upon polymer hydration in response to redox changes (Scheme 2.1). We first synthesize the monomers with QPA trigger to obtain redox-sensitive polymer. The polymers were utilized for the preparation of PTX-loaded nanoparticles. The nanoparticles were evaluated for redox-triggered drug release under a simulated redox state. *In vitro* cytotoxicity of nanoparticles was determined in human breast tumor T47D and MDA-MB-231 cells to determine cancer cell-mediated drug release.

2.2. EXPERIMENTAL

2.2.1. Materials

Methanesulfonic acid, 2,3,5-trimethyl hydroquinone, methyl β,β-dimethylacrylate, 2-amino-1,3-propanediol, adipoyl chloride and glutaryl chloride were obtained from Alfa Aesar (Ward Hill, MA). Adipoyl chloride and glutaryl chloride were distilled under reduced pressure before the reaction. Paclitaxel (PTX) was purchased from LC Laboratories® (Woburn, MA). Polyvinyl alcohol (PVA; $M_w$ 30,000 - 70,000) was obtained from Sigma-Aldrich (St. Louis, MO). All other chemicals purchased from Fisher Scientific (Pittsburg, PA) were used as received.

2.2.2. Computational analysis

Polymer Construction
QPA-based redox-sensitive polyester structures were constructed by combinations between N-(1,3-dihydroxypropan-2-y1)-3-methyl-3-(2,4,5-trimethyl-3,6-dioxocyclohexa-1,4-dien-1-yl) (monomer 1 in scheme 2.2, QPAMN) and two dicarboxylic acids, glutaric acid and adipic acid. According to the nature of the repeating units, the polymers were constructed head-to-tail in 3D using the polymer builder tool of Molecular Operating Environment (MOE) software package (ver. 2011.10, Chemical Computing Group, Montreal, Canada). We considered the three possible degrees of reduction—non-reduced, partially reduced and reduced—of QPA groups because of their potential effects on polymer solubility and drug release. Polymer chains of two molecular weights ~3000 and ~5000 Daltons were sketched.

**Calculation of logP**

The logP calculations used the weighted-method of ChemAxon suite (MarvinSketch 5.6.0.1, 2011, www.chemaxon.com), which is a combination of three algorithms, VG method (logP calculation according to various atom types)\(^55\), KLOP method (group contribution approach)\(^56\) and PHYSPROP (based on a logP database).\(^{57}\) The logP calculated in this way is the arithmetic average of the three methods.

**2.2.3. Synthesis of monomer**

NHS-activated benzoquinone compound 4, NHS-QPA (scheme 2.2) was synthesized as reported previously.\(^9^a\) To a mixture of 2-amino-1,3-propanediol (1.639 g, 18 mmol) and triethyl amine (5 mL) in isopropanol (100 mL), the synthesized compound
4 (4.168 g, 12 mmol) in THF (50 mL) was added dropwise at room temperature. After being stirred overnight at room temperature, the mixture was collected and concentrated under reduced pressure. The crude compound was washed with NaHCO₃ three times and extracted by ethyl acetate (50 mL × 3), then dried over by sodium sulfate. The reductive monomer 1 (yellow crystalline) was purified by recrystallization in ethyl acetate with an isolated yields of 61.3% (2.379 g). NMR spectra were recorded on a Bruker Ultrashield™ 400 PLUS at 400 and 100 MHz for ¹H and ¹³C, respectively. The residual solvent peak (δ = 2.50 or 7.24 for DMSO-d₆ or CDCl₃, respectively) were used as the solvent residual references for ¹H NMR spectra, and chemical shifts of the solvent peaks (δ = 39.52 or 77.00 for DMSO-d₆ or CDCl₃, respectively) were used as the reference for ¹³C NMR spectra.

Monomer 1, yellow crystal; mp 161–162 °C (from EtOAc), ¹H NMR (400 MHz, DMSO-d₆) δ = 7.54 (d, J = 8.3 Hz, 1H), 4.56 (t, J = 5.5 Hz, 2H), 3.60 (dt, J = 8.2, 5.6 Hz, 1H), 3.32 (m, 4H), 2.71 (s, 2H), 2.01 (s, 3H), 1.90 (s, 3H), 1.87 (s, 3H), 1.32 (s, 6H); ¹³C NMR (101 MHz, DMSO-d₆) δ = 190.20, 186.85, 171.31, 154.94, 144.03, 136.07, 135.37, 60.13, 52.70, 47.58, 37.69, 28.06, 13.69, 12.77, 11.70; IR (neat, cm⁻¹) 1528, 1600, 1638, 3333; Found: C, 63.20; H, 7.71. Calcd for C₁₇H₂₅O₅: C, 63.14; H, 7.79.
Scheme 2.2. Synthetic reactions for a trimethyl-lock quinone-based redox-sensitive monomer.
Figure 2.1. $^1$H and $^{13}$C NMR spectra of monomer 1 in DMSO-$d_6$. 
Scheme 2.3. Schematic synthetic reactions for redox-sensitive polymer with QPA and mechanism under reduction.

2.2.4. Synthesis of polymer

Freshly distilled glutaryl chloride (242.7 mg, 1.44 mmol) was diluted ten times with dry THF and slowly added to the solution of monomer, QPAMN (464.3 mg, 1.44 mmol) in pyridine at room temperature (Scheme 2.3). The reaction was further carried on at room temperature overnight. After being poured into an excess amount of ethanol and mixed for 6 hours, it was extracted with deionized (DI) water and later with CH$_2$Cl$_2$. The collected organic phase was concentrated by a rotary evaporator and precipitated into cold diethyl ether to obtain the crude product, (3a, 470.8 mg). The adipic acid based polymer (3b, 571.4 mg) was synthesized with the same procedure shown above using 1.3 mL of pyridine monomer 1 (517.5 mg, 1.60 mmol) and dried adipoyl chloride (292.6 mg,
1.60 mmol). The structures of the synthesized polymers, 3a and 3b, were elucidated by ¹H-NMR spectra using a Bruker Avance 400 MHz spectrometer. The molecular weight and polydispersity index (PDI) of the synthesized polymers were determined with a Waters gel permeation chromatography (GPC) system (Waters, Milford, MA). GPC instrument is equipped with a binary pump (Waters 1525), a refractive index detector (Waters 2414), and a Styragel HR4E column (300 × 7.8 mm ID, 5 µm particle size). The measurement of the molecular weight was performed using HPLC grade THF as a mobile phase at a flow rate of 1.0 mL min⁻¹ at 25 °C. The molecular weight of the synthesized polymers was determined with the calibration curve obtained using polystyrene standards (600-50,000 Da). Under these conditions, retention times of 3a and 3b were 8.559 min and 8.609 min, respectively.

Polymer 3a; ¹H-NMR (400 MHz, CDCl₃): δ 6.32 (1H, s), 4.38 (1H, s), 4.12 (2H, s), 4.06 (2H, s), 2.85 (2H, s), 2.38 (4H, t), 2.13 (3H, s), 1.95 (8H, m), 1.39 (6H, s).

Polymer 3b; ¹H-NMR (400 MHz, CDCl₃): δ 6.30 (1H, s), 4.37 (1H, s), 4.12 (2H, s), 4.06 (2H, s), 2.83 (2H, s), 2.36 (4H, t), 2.11 (3H, s), 1.95 (10H, m), 1.39 (6H, s).

2.2.5. Preparation of blank and PTX-loaded nanoparticles

The blank and PTX-loaded nanoparticles (NPs) were prepared by an single emulsion method.⁵⁸ NPs-3a from polymer 3a and NPs-3b from polymer 3b were obtained through the same procedure. For preparation of PTX-loaded NPs, 40 mg of the synthesized redox-responsive polymer and 4 mg of PTX were dissolved in 2 mL of
CH₂Cl₂. This organic phase was slowly added to 18 mL of phosphate-buffered saline (PBS, pH 7.4) containing 0.7% of PVA mixing under magnetic stirring. The mixture was emulsified for 1 min in an ice bath by a probe sonicator (Qsonica, LCC. XL-2000, Newtown, CT). The emulsion was stirred overnight at room temperature to evaporate CH₂Cl₂. Aggregated NPs were filtered off with a 0.45 µm filter. The NPs were collected from the filtered solution by centrifugation (VWR TM Galaxy 14D, 6,000 rpm, 15 min). The NPs were washed twice with PBS, 20 mL each time, and later with 20 mL of DI water to remove emulsifier. The produced suspension was lyophilized with the addition of 20 mg of mannitol to obtain fine powder of NPs. To prepare blank NPs, the above-mentioned procedures were employed except for leaving out PTX.

2.2.6. Characterization of blank and PTX-loaded NPs

The amount of PTX loaded in NPs was determined by dissolving a known weight (1.05 mg) of freeze-dried drug loaded NPs into 1 mL of acetonitrile and was analyzed via HPLC. The HPLC system is equipped with a Luna C18(2) chromatographic column (Phenomenex, 150 x 4.6 mm, 5 µm), a binary pump (Waters 1525), and an auto-sampler (Water 717). The mobile phase, a mixture of acetonitrile and water at 55/45% v/v, was used at a flow rate of 1 mL min⁻¹ after injecting a 20 µL sample.⁵⁹ PTX was detected at a retention time of 4.9 min using a UV detector (Water 2487) at 227 nm. The concentration of released PTX was calculated using the calibration curve obtained from different concentrations of PTX standard solutions in acetonitrile. The percentage of PTX
incorporated into NPs was calculated by dividing the amount of incorporated PTX with the initial PTX amount and multiplied by 100.

The size distribution and Z-average diameter of the NPs were determined by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). NP suspensions were diluted to a concentration of 1.25 g NP in 1 mL of DI water. NP sizes were measured before freeze-drying and after reconstituting the NPs freeze-dried in the presence of mannitol in order to test the protective effect of mannitol during drying NPs.

For scanning electron microscope (SEM) analysis, NPs were reconstituted at a concentration of 1 mg mL\(^{-1}\) in DI water and homogeneously suspended by a 5 min sonication. A 50 µL aliquot of the reconstituted sample was mounted on an aluminum plate and dried. Then, the sample was sputter coated with gold and palladium. Quanta FEG 650 (FEI) at an accelerating voltage of 10 or 15 kV was used to image NPs.\(^6\) The polymeric nanoparticles were also characterized by transmission electron microscopy (TEM) using a Tecnai T12 microscope (FEI, Hillsboro, OR) operated at 80 kV. Each sample was sonicated for 5 minutes before being mounted on a carbon coated Formvar cooper grid (400 mesh) and dried for 3 minutes. After wicking away excess solution and air drying for 1 minute, samples were then negatively stained with uranyl acetate (2% w/v) for 30 seconds prior to TEM imaging.
2.2.7. *In vitro* drug release studies

*In vitro* PTX release from the prepared NPs was conducted using Na$_2$S$_2$O$_4$ as a reducing agent. An amount of PTX-loaded NPs-3b containing 10 µg of PTX was suspended in 3 mL of PBS buffer (pH 7.4) containing 0.8 M of sodium salicylic acid. The drug release was initiated by an addition of Na$_2$S$_2$O$_4$ to the NP suspension. A 200-fold molar excess of the reducing agent to QPA groups in the polymer was employed. Then, the mixture was incubated in a shaking water bath (100 rpm) at 37 °C for 24 hours. At designated times, a 150 µL aliquot of the NP sample was withdrawn from a vial after spinning down the NPs and an equal volume of fresh reducing medium was added and stirred into the NP solution. Samples were diluted with 150 µL of acetonitrile and the supernatant samples obtained with centrifugation (13,000 rpm, 3 min) were injected into the HPLC for analysis. The amounts of released PTX in the collected samples were determined by the same HPLC method as described in 2.5 except for external standard. The calibration curve was obtained from different concentrations of PTX standard solutions in 50% (v/v) aqueous acetonitrile. For the quantitative determination of lactone reduced from QPA groups, synthesized lactone was applied as external standard for HPLC analysis as previously reported. The retention times of released lactone and PTX were 4.98 min and 6.68 min, respectively, at 227 nm with an UV detector.

The drug release from NPs in cell media was also studied in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with/without 5% fetal bovine serum (FBS). PTX-loaded NPs-3b (0.72 mg) were suspended into 1 mL of cell media incubating at 37 °C. At designated time point over 48 hours, 150 µL NP solution was withdrawn from the
system and centrifuged at 10,000 rpm for 5 min to spin down NPs. Aliquots of 110 µL NP solution from supernatant media were mixed to the same amount of acetonitrile (110 µL) to remove protein precipitate. The remaining 40 µL solution including precipitated NPs was suspended with the same volume of fresh media and the suspension was added to the system to keep constant volume. After completing the collecting samples, the NP solution was mixed with the same volume of acetonitrile (1 mL) to measure the amount of PTX remaining in the NPs. To determine the amount of PTX, all collected samples were centrifuged to remove protein precipitate for the preparation of samples and HPLC method was used in the same manner as for the measurement of the amount of drug encapsulated in NPs.

2.2.8. Cell viability assay

Human breast tumor T47D and MDA-MB-231 cells obtained (ATCC, Manassas, VA) were grown in DMEM/F12 medium containing 2.5 mM L-glutamine (Mediatech), supplemented with fetal bovine serum (FBS, 10% v/v final concentration, Hyclone), penicillin G (sodium salt, 50 units mL⁻¹) and streptomycin sulfate (50 µg mL⁻¹) (BioWhittaker). Exponentially grown cells were plated at the density of 30,000 cells per well into 96-well plates in a volume of 100 µL culture media and incubated at 37 °C in a humidified environment (95% air, 5% CO₂), as previously described.⁶¹ PTX was dissolved in DMSO to obtain a 20 µM stock solution. PTX loaded NPs and blank NPs were prepared fresh as 100 µM stock solutions in PBS (pH 7.4). After compound treatment at the specified concentrations for 48 h, the cells were further incubated and
cell viability was determined by the sulforhodamine B (SRB) method. Optical density (OD) was measured at 490 nm with background absorption at 630 nm. Data were normalized to the untreated control and presented as ‘% of control,’ using the formula.

\[
\text{Cell viability [\% of Control] = \left( \frac{\text{OD}_{\text{treated}}}{\text{OD}_{\text{control}}} \right) \times 100}
\]

2.2.9. Statistical analysis

Experimental measurements were triplicated for each sample. The results are presented as mean ± standard deviation. The statistical analysis of experimental data utilized the student’s t-test and statistical significance was considered for \(P\)-values < 0.05.

2.3. RESULTS AND DISCUSSION

2.3.1. Computational analysis and polymer synthesis

Molecular dynamics and molecular docking have been widely used in the process of drug development as means to predict and rationalize the design of drug delivery systems. Theoretical calculation of logP provides rapid evaluation of lipophilicity and cellular penetration, which is widely used as a rational tool in the drug design process. Two polymers were constructed by combinations of previously synthesized QPAMN and dicarboxylic acids, glutaric acid and adipic acid. The main reason to select the two dicarboxylic acids was that NPs from the adipic acid-based redox-sensitive polymer have demonstrated redox-triggered content release by solubility reversal upon polymer reduction. Two polymer molecular weights, 3000 and 5000 Daltons, were considered to
Table 2.1. Calculated logP values of QPA-based redox-responsive polymers at different degrees of reduction. Two polymer molecular weights, 3000 Da and 5000 Da, were chosen for comparison.

<table>
<thead>
<tr>
<th></th>
<th>MW ~5000</th>
<th></th>
<th></th>
<th>MW ~3000</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Non</td>
<td>Partially</td>
<td>Fully</td>
<td>Non</td>
<td>Partially</td>
<td>Fully</td>
</tr>
<tr>
<td></td>
<td>reduced</td>
<td>reduced</td>
<td>reduced</td>
<td>reduced</td>
<td>reduced</td>
<td>reduced</td>
</tr>
<tr>
<td>Polymer 3a</td>
<td>27.81</td>
<td>10.30</td>
<td>-6.29</td>
<td>18.87</td>
<td>7.21</td>
<td>-4.01</td>
</tr>
<tr>
<td>Polymer 3b</td>
<td>32.46</td>
<td>16.27</td>
<td>-0.55</td>
<td>19.72</td>
<td>10.53</td>
<td>-0.39</td>
</tr>
</tbody>
</table>

test lipophilicities using ChemAxon. The logP calculated from the arithmetic average of the three ChemAxon logP methods including VG, KLOP and PHYSPROP showed the lower values in the reduced forms. As shown in Table 2.1., fully reduced polymer with molecular weight of < 5000 Da showed the decrease of logP values of 3a and 3b from 27.81 to -6.29 and from 32.46 to -0.39, respectively. The logP values of polymer with MW < 3000 Da were similar and were lowered from 18.87 and 19.72 to -4.01 and -0.39 with the increase in reduction of polymers 3a and 3b, respectively. Reduction-dependent logP change in the polymers is probably due to the exposure of hydrophilic amine group upon removal of QPA. Especially, exposed primary amine groups would further lower polymer logP by protonation.

2.3.2. Synthesis of monomer and polymers

Redox-sensitive polymer was designed from the monomer with a QPA trigger. Initially, benzoquinone carboxylic acid (β,β,2,4,5-pentamethyl-3,6-dioxo-1,4-
cyclohexadiene-1-propanoic acid) activated with N-hydroxysuccinimide was synthesized according to the previous report. The activated compound was coupled with serinol (2-amino-1,3-propanediol) as a redox-sensitive diol monomer to yield serinol-derived polyester upon esterification with diacyl chloride. Serinol was selected for polymerization because of proven biocompatibility of the serinol-derived polyesters. A coupling reaction between trimethyl-lock benzoquinone succinimidyl ester and serinol in a basic condition, successfully yielded diol monomer containing a QPA (QPAMN), which was confirmed by $^1$H and $^{13}$C NMR spectroscopy and elemental analysis. The polymers 3a and 3b, designed for redox-triggered NPs were synthesized as illustrated in Scheme 2.3. The molar ratio of QPAMN to diacyl chlorides was 1.0 to achieve the highest possible polymer molecular weight which has reached 9,000 Da previously. Polymer 3a synthesis yielded orange-yellow powders with a number-average molecular weight ($M_n$) of 6980 Da (PDI = 1.5). Throughout this study, polymer synthesis has been reproducible to show a very narrow molecular weight variation with a standard deviation within 3% of polymer molecular weight. The proton NMR of polymer 3a in CDCl$_3$ showed all the characteristic peaks and splitting in Figure 2.2. The chemical shift of $\text{-CH}_2\text{-OH}$ protons in QPAMN appearing at 3.32 ppm moved downfield to $\delta = 4.12$ ppm and 4.06 ppm, indicating ester formation upon polymerization. Furthermore, characteristic methyl protons in QPAMN appearing at 1.41, 2.13 and 2.33 ppm were also found in the polymer NMR spectrum, which showed the incorporation of the intact QPA pendant group in the polymer backbone. Compared to the peak shapes in monomers, corresponding peaks in the polymer were also broadened after the polymerization as
expected. The integration ratios of proton peaks on $^1$H-NMR spectrum were consistent with the theoretical ratios of proton numbers in the polymer (Figure 2.3.)
Figure 2.2. $^1$H NMR spectrum of the redox-responsive polymer 3a.
2.3.3. Preparation of redox-responsive NPs

Redox-responsive NPs were prepared in the presence or absence of PTX from the synthesized polymers. An emulsion method using PVA was employed to obtain NPs with controlled particle sizes. Although particles smaller than 500 nm can be passively accumulated in tumor by the enhanced permeability and retention (EPR) effect, NPs with particle sizes are less than 200 nm are generally considered as ideal for targeted drug delivery.\textsuperscript{64} Emulsifying hydrophobic polymers and subsequent solvent evaporation have
frequently yielded NPs suitable for tumor-targeted drug delivery.\textsuperscript{65} The mean size of NPs-3a was determined to be 246.0 nm (PDI = 0.12) and 238.8 nm (PDI = 0.15), for the blank NPs and PTX-loaded NPs, respectively, as summarized in Table 2.2. It was found that the use of surfactants for the preparation of redox-responsive NPs affected particle size. Tween 80 and Pluronic F68 indeed resulted in particle sizes ranging from 400 nm to 830 nm (data not shown). The nonionic stabilizers might differently affect the stability of the oil-in-water emulsions of the redox-responsive polymers. Droplet sizes of the emulsions could be an important factor for the size of final hardened polymer NPs. The sizes of our redox-responsive NPs were comparable to the particle sizes obtained with poly(D,L-lactide-co-glycolid) (PLGA).\textsuperscript{66} The size of PLGA (MW 75,000-120,000) NPs prepared with Pluronic F68 was 461 ± 10 nm, which was larger than the 284 ± 6 nm obtained with PVA.\textsuperscript{66} This result indicated that PVA in CH\textsubscript{2}Cl\textsubscript{2} could decrease interfacial tension more than Pluronic F68 did and, thus, lead to smaller particles size. The increase in size of obtained NPs was observed due to the aggregation of NPs when using Tween 80 and similar observations were also reported on the formation of poly(D,L-lactic acid) NPs emulsified with Tween 80.\textsuperscript{66}
PTX loading into NPs-3a was determined to be 45% (w/w) as summarized in Table 2.3. However, NPs-3b resulted in an enhanced drug loading of 77% (w/w). It looked as though an additional carbon in the polymer repeating unit in polymer 3b might be attributed to the difference in drug loading in two kinds of NPs. As previously observed, drug loading into polymer particles is closely related to drug solubility in the polymer. It is speculated that polymer 3b may have greater miscibility with PTX than
polymer 3a because of greater hydrophobicity as predicted by theoretical logP calculations (Table 2.1.).

Aggregation of hydrophobic NPs during freeze drying has commonly been observed.67 Carbohydrate stabilizers such as sucrose, lactose, glucose, sorbitol, and mannitol have been routinely employed to prevent the product from undergoing the stress that could destabilize colloidal suspension, which is generated during the freeze drying process.68 For the freeze-drying of redox-sensitive polymer NPs prepared from QPA-based polymer, mannitol was added to minimize particle aggregation and improve the handling properties of the dried NPs. It should be noted that mannitol has been widely used as a cryoprotectant in the formulations of biotechnology products which are currently in the market.69 It has also been known that mannitol tends to crystallize during freeze-drying process resulting in powdery and dry products, which usually improves handling property.70 The protective effect of mannitol during freeze drying was examined by particle size measurements before and after nanoparticle drying. The size of redox-responsive NPs-3b before drying was determined to be 220.60 ± 21.56 nm (PDI = 0.16 ± 0.07). Nanoparticle size slightly increased to 269.83 ± 21.56 nm (PDI = 0.24 ± 0.05) after reconstitution of the dried NPs into water (summarized in Table 2.2). This slight particle size increase may be due to hydration of NPs. The redox-responsive polymer NPs seemed to be aggregated by inter-particular hydrophobic interactions during freeze drying without mannitol.71 It appears that nanoparticle aggregation was prevented by the presence of mannitol which can hold water and fill spaces between NPs during the drying process. Drug loading did not noticeably diminish the protective role of mannitol to
hinder particle aggregation. In the case of PTX-loaded NPs-3b, particle size was marginally increased from 256.47 ± 9.55 nm (PDI = 0.08 ± 0.03) to 293.17 ± 13.21 (PDI = 0.17 ± 0.06) for wet NPs before drying and reconstituted particles after freeze-drying, respectively, in the presence of mannitol (Table 2.2). In addition, lyophilized NPs have maintained their stability over an eight week period (summarized in Figure 2.5). Reconstituted PTX-loaded NPs-3b have shown a modest particle size increase from 293.2 nm to 346.4 nm over an eight week incubation at 37 °C (Figure 2.5). Interestingly, a noticeable change in particle size modification was observed with blank NPs, wherein the size of NPs increased from 269.8 nm to 558.5 nm in 4 weeks. The particle size further increased to 682.8 nm in another 4 weeks. The hydrophobic drug might have contributed to an improved stability of PTX-loaded NPs.

Table 2.2. Mean sizes of prepared redox-responsive polymer nanoparticles and drug loading efficiency into the nanoparticles. Mannitol was used to stabilize the particles during freeze-drying. Paclitaxel was used as a model drug. Data presented as mean ± standard deviation of three independent experiments.

<table>
<thead>
<tr>
<th>NPs</th>
<th>Size (nm)</th>
<th>Drug loading efficiency (w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mannitol-free</td>
<td>drying with mannitol</td>
</tr>
<tr>
<td>Blank NPs-3a</td>
<td>246.03 ± 42.58</td>
<td>322.07 ± 28.59</td>
</tr>
<tr>
<td>PTX loaded NPs-3a</td>
<td>238.8 ± 20.49</td>
<td>257.67 ± 43.89</td>
</tr>
<tr>
<td>Blank NPs-3b</td>
<td>220.60 ± 21.56</td>
<td>269.83 ± 26.57</td>
</tr>
<tr>
<td>PTX loaded NPs-3b</td>
<td>256.47 ± 9.55</td>
<td>293.17 ± 13.21</td>
</tr>
</tbody>
</table>
Figure 2.5. Size distribution of blank NPs-3b (☐) and PTX-loaded NPs-3b (■) suspended in DI water and incubated at 37 °C over eight weeks. Data shown as mean ± SD (n = 3).

Other NPs prepared from gelatin, poly(D,L lactide-glycolide) and lipids have also demonstrated a similar mannitol-mediated protective effect with no significant particle size alteration after freeze drying. In addition, as shown in Figure 2.6., mannitol was well integrated with the redox-responsive NPs-3b. The morphologies of NPs with or without PTX loading after drying in the presence of mannitol were different from that of freeze-dried mannitol as confirmed by SEM images. SEM images of dried mannitol revealed spherical particles with relatively uniformed size as well as rounded morphology (Figure 2.6.(C)). On the other hand, the microscopic images of dry NPs (Figure. 2.6.(A) and (B)) showed that mannitol has been integrated well with polymer NPs by showing chunks rather than segregated mannitol spheres. Mannitol integrated with the NPs might
be distributed between particles as a space filler or as a coat on their surface, which prevents inter-particular hydrophobic interactions between NPs.

**Figure 2.6.** Scanning electron microscopy images of freezing dried nanoparticles prepared with mannitol (A) PTX-loaded NPs-3b and (B) Blank NPs-3b, and (C) Freeze-dried mannitol.
**Figure 2.7.** The TEM images of blank nanoparticles (left) and paclitaxel incorporated polymeric nanoparticles (right)

2.3.4. *In vitro* paclitaxel release

The effect of redox stress on the nanoparticles was tested with a chemical reductant, sodium dithionite (Na$_2$S$_2$O$_4$). It was expected that sodium dithionite would reduce QPA to leave free amine groups on the polymer, which would result in physico-chemical changes in nanoparticles that would induce nanoparticle swelling or dissolution at pH 7.4. Sodium dithionite-induced reduction dramatically increased the hydrodynamic diameter of the nanoparticles from 178 nm in a turbid solution to 29.3 µm in a transparent solution, a size beyond the measurable limit by the Zetasizer as shown in Fig. 2.8. The size changes might have resulted from the protonation of free amine groups in serinol of which pK$_a$ is around 9.15.
Figure 2.8. Change in nanoparticle size in the presence of sodium dithionite determined by DLS.

PTX release from the redox-responsive NPs was studied under a redox state simulated with a chemical reducing agent, sodium dithionite. Sodium dithionite has been frequently adopted in physiology experiments as a means to lower solutions’ redox potential ($E^\circ \approx -0.66V$ at pH 7 at 0.35 nM$^{73}$) and to mimic a reductive environment. Figure 2.9.(A) shows that QPA-based redox-responsive NPs were able to release incorporated drug upon pendant QPA group reduction. Under a redox state simulated with sodium dithionite, the amounts of released PTX were significantly greater than PTX release in PBS ($P < 0.05$ for NPs-3a and NPs-3b at 15 min, $P < 0.01$ at 3 hours, in Figure 2.9.(A)). Cumulative percentage of PTX released from two different NPs was more than 80% after a 24 h incubation under the redox state. However, only 41.2% and 26.5% of incorporated PTX were released from the NPs in the absence of the reducing agent. It was interesting
to note that the NP from adipic acid-based redox-responsive polymer 3b showed PTX release comparable to that from NPs-3a.$^{57}$ Lactone released from reduced QPA was consistent with PTX release indicating PTX release was mediated by lactone release. Facilitated PTX release under the redox state can be attributed to QPA reduction which deprotects amine groups in the polymer backbone. Free amine groups exposed upon intramolecular cyclization of QPA could increase polymer solubility upon protonation at pH 7.4, which might result in nanoparticle swelling. Theoretical calculations of hydrophobicity reduction upon polymer reduction, from 27.81 to -6.29 and from 32.46 to -0.55 for polymers 3a and 3b, respectively (Table 2.1), also led to the prediction of polymer solubility increasing upon polymer reduction. In addition, exposed amine groups might contribute to the hydrolysis of the polyester backbone in a base-catalyzed manner.$^{74}$ As expected, NPs in the control release medium containing no reducing agent did not release quinone lactone at all (Figure 2.9.(B). It is worthwhile to mention that NPs-3b showed lower initial PTX release than NPs-3a. The difference in burst PTX release from the NPs can be attributed to the polymer solubility difference. As previous theoretical calculations of polymer property predicted, polymer 3a is less hydrophobic than polymer 3b which has one more carbon in polymer repeating unit. Predicted logP values (Table 2.1) for non-reduced polymer 3a and 3b were 27.81 and 32.46, respectively at a molecular weight of 5000 Da. It seemed that more hydrophobic polymer NPs-3b demonstrate the lower initial burst. Considering theoretical polymer property prediction and experimental drug release under a simulated redox state, NPs-3b would be more suitable for drug delivery applications, with a low burst drug release but maximized
content release upon polymer reduction. However, drug release from the NPs by cancer cell-overexpressing redox enzymes should be further tested and confirmed before the implementation of the novel redox-responsive polymer NPs for tumor-targeted drug delivery.

**Figure 2.9.** (A) *In vitro* redox-responsive release of PTX and (B) release of lactone from reduced QPA when incubated in the reductive media including \( \text{Na}_2\text{S}_2\text{O}_4 \) (● and ■) and at control media (○, □ and x) at 37 °C from NPs-3a (● and ○) and from NPs-3b (■ and □). The results represent the mean ± SD (n = 3).

### 2.3.5. *In vitro* cytotoxicity

Concentration-dependent cell cytotoxicity studies were performed to determine the effects of PTX–loaded redox-responsive NPs on the proliferation/viability of human breast tumor T47D and MDA-MB-231 cells. PTX-loaded and blank NPs-3a were used as positive and negative controls for the study, respectively. At the lower concentration range (0.01 µM or less), almost no cytotoxicity for both cell lines could be detected as
shown in Figure 2.10. When the concentration was at 1 μM, the cell viability of T47D cell line with the incubation of PTX and PTX loaded NPs were 69% and 73%, respectively, while the result of cytotoxicity with blank NPs showed its less toxicity as 91% of cell viability in Figure 2.10 (A). The cell experiment for MDA-MB-231 cell line in Figure 2.10 (B) also showed similar cell viability, in which 48%, 50%, and 97% of cell viability were observed for plain drug, PTX loaded NPs, and blank NPs, respectively. PTX loaded NPs inhibited cell proliferation and viability to the comparable extent as that observed in the presence of PTX. The particle itself did not affect cell proliferation/viability at all concentrations tested indicating that all incorporated PTX has been released from NPs to limit cell growth.

Figure 2.10. Effects of PTX-loaded NPs on human breast tumor cell proliferation/viability determined by SRB method: (A) T47D cells. (B) MDA-MB-231 cells. Exponentially grown T47D and MDA-MB-231 cells were exposed to the different concentration of PTX-loaded NPs, blank NPs, and PTX for 48 h under normoxic condition (95% air: 5% CO₂). The percentage of viable cells was normalized to values
obtained from untreated control under normoxia conditions, and presented as “% Control”. Data shown are average ± standard deviation (n = 3).

Aside from in vitro cellular study, in vitro drug release from the NPs was examined to confirm culture cancer cell-mediated drug release from NPs using serum-free cell culture media and culture media supplemented with 5% FBS presented in Figure 2.11. The amounts of drug released from the NPs over 48 h ranged from 12.58% to 15.71% and from 10.89% to 14.66% in serum free DMEM and in DMEM with 5% FBS, respectively. In addition, the analysis of remaining NPs showed considerable amounts of PTX left in the NPs, indicating that FBS might contribute to drug release in part but not all. The amounts of drug found in the NPs were 86.05% and 53.67% in serum free DMEM and DMEM with 5% FBS, respectively. The reason for less drug retention in the presence of FBS might be the enzymes in FBS. It has been known that FBS contains a mixture of enzymes including esterases and proteases which are able to degrade polyester backbone in the QPA-based redox-responsive polymer. Proteins in FBS, 23 g L$^{-1}$ of albumin and 38 g L$^{-1}$ of total protein, may also be able to affect polymer degradation, nanoparticle stability or PTX protein binding. Indeed, PTX was reported to significantly bind to protein in cell culture media, which was PTX concentration dependent. PTX was known to result in 78.6 ± 2.7% protein binding at a concentration of 0.5 µg mL$^{-1}$ in cell culture medium with 9% FBS and PTX binding to proteins decreased to 20.2 ± 1.4 % as concentration of PTX increased to 15 µg mL$^{-1}$. The PTX protein binding might overestimate PTX release in FBS containing DMEM. However, taking cell cytotoxicity
results and in vitro drug release in culture media together, cultured cancer cells could release PTX from the redox-responsive NPs.

![Graph showing cumulative drug released over time](image)

**Figure 2.11.** In vitro PTX release from NPs-3b in different cell culture media: serum-free cell culture media (○) and the media supplemented with 5% FBS (●). Data shown as mean ± SD (n = 3).

### 2.4. CONCLUSIONS

Novel redox-sensitive polymeric nanoparticles were prepared from a synthesized monomer containing quinone propionic acid as a redox sensitive group. A hydrophobic cancer drug, paclitaxel, was incorporated into the polymeric nanoparticles and released by sodium dithionite-mediated reduction in a triggered manner. Cytotoxicity assay using cultured human breast tumor cell lines demonstrated that cancer cells could release
incorporated drug from the NPs. These redox-sensitive NPs may be useful as delivery platforms for cytotoxic cancer drugs.
CHAPTER 3

REDOX-RESPONSIVE POLYMERIC MICELLES FOR DRUG DELIVERY
ABSTRACT

A novel redox-responsive amphiphilic polymer was synthesized with bioreductive trimethyl-locked quinone propionic acid for a potential triggered drug delivery application. The aim of this study was to synthesize and characterize the redox-responsive amphiphilic block copolymer micelles containing pendant bioreductive quinone propionic acid (QPA) switches. The redox-responsive hydrophobic block (polyQPA), synthesized from QPA-serinol and adipoyl chloride, was end-capped with methoxy poly(ethylene glycol) of molecular weight 750 (mPEG$_{750}$) to achieve a redox-responsive amphiphilic block copolymer, polyQPA-mPEG$_{750}$. PolyQPA-mPEG$_{750}$ was able to self-assemble as micelles to show a critical micelle concentration (CMC) of 0.039 % w/v (0.39 mg mL$^{-1}$, 0.107 mM) determined by a dye solubilization method using 1,6-diphenyl-1,3,5-hexatriene (DPH) in phosphate buffered saline. The mean diameter of polymeric micelles was found to be 27.50 nm (PI = 0.064) by dynamic light scattering.

Furthermore, redox-triggered destabilization of the polymeric micelles was confirmed by $^1$H-NMR spectroscopy and particle size measurements in a simulated redox state. PolyQPA-mPEG$_{750}$ underwent triggered reduction to shed pendant redox-responsive QPA groups and its polymeric micelles were swollen to be dissembled in the presence of a reducing agent, thereby enabling the release of loaded model drug, paclitaxel. The redox-responsive polyQPA-mPEG$_{750}$ polymer micelles would be useful as a drug
delivery system allowing triggered drug release in an altered redox state such as tumor microenvironments with an altered redox potential and/or redox enzyme upregulation.
3.1. INTRODUCTION

Drug delivery system holds great promise as a tool to improve pharmacokinetics and therapeutic efficacy of drugs\textsuperscript{76}. In particular, anti-cancer drugs have been an active subject of targeted delivery aiming at microenvironment changes occurring at tumor sites\textsuperscript{77}. Nanotechnology is emerging as a promising tool for cancer targeted drug delivery system because of their favorable distribution at tumor sites based on enhanced permeability and retention \textsuperscript{78}. However, important challenges associated with drug delivery systems such as target tissue specificity and drug release rate at systemic subcellular levels to achieve maximum therapeutic efficacy but minimal toxicity often remain as obstacles to their successful translation into clinical outcomes. One approach to overcome these limitations would be designing multifunctional materials with consideration of meaningful pathophysiological changes occurring at a target site. In recent years, various drug delivery systems were explored for their properties to release encapsulated drug in a triggered manner in response to abnormal tumor microenvironments such as acidic pH\textsuperscript{79}, over-expressed enzymes\textsuperscript{80}, hyperthermia\textsuperscript{81} and altered redox states\textsuperscript{82} compared to normal tissues.

In cellular systems, redox homeostasis plays pivotal roles in the maintenance of cellular functions. Intracellular compartments maintain a certain redox gradient compared to the extracellular environment, providing an exciting target for selective intracellular chemical release. The redox gradient between intracellular and extracellular
compartments in a wounded site is significantly greater than that in healthy tissues, which is modulated by intricate networks of redox signaling pathways adapting internal environment to the extracellular changes. Redox agents expressed during the intracellular redox-regulatory processes serve as indicators of the redox status. Redox switch materials inspired by these redox agents are appealing tools to be exploited into drug delivery systems which enable to release drugs by triggering chemical/physical changes upon exposure to an altered reductive environment in cancer.

Various redox-switches utilized to create multifunctional materials for drug delivery applications have frequently been based on diselenide and disulfide bonds. Trimethyl locked quinone propionic acid (QPA) group has also been of interest, as they can be reduced to form lactone by intramolecular cyclization of hydroquinone via the two electron reduction. The reduction of the QPA subunits associated with synthetic materials has been demonstrated by the adoption of the simulated redox states not only with chemical agents such as dithionite and borohydride, but also with reductases such as DT-diaphorase (NAD(P)H:quinone oxidoreductase (NQO1), EC 1.6.99.2). It is interesting to note that DT-diaphorase, a two electron reductase, is highly expressed in several cancerous tissues. Tumors such as non-small cell lung cancer and pancreatic cancers have shown to express high levels of DT-diaphorase relative to normal tissue, as much as a 20-fold increase. Hence, QPA-based materials provide a wide range of redox-triggered architectures to tune the cleavage of the redox-switch for targeted drug delivery to tumors. Volpato and coworkers prepared an aniline mustard prodrug containing QPA group to prove the prodrug activation in vitro selectively by DT-
diaphorase, comparing to other reductase such as cytochrome p450 reductase. Their study has demonstrated that active aniline mustard could be released from the prodrug via DT-diaphorase-mediated bioreduction. Redox-sensitive liposomes comprising a QPA head group and dioleoyl phosphatidylethanolamine (DOPE) lipids have also been prepared by Ong et al. The researchers have demonstrated that rapid lysis of the liposomes resulted in the release of incorporated calcein via liposomal structural destabilization upon reduction of QPA head group by dithionite. Recently our group has developed redox-responsive polymeric nanoparticles (NPs) with pendant QPA redox triggers which aqueous solubility could be reversed upon QPA reduction. These QPA-based redox-responsive NPs were able to release incorporated paclitaxel under a redox state simulated with dithionite. Additionally, in vitro cytotoxicity study has confirmed that these NPs could release paclitaxel in the presence of human breast tumor T47D and MDA-MD-231 cells. Although these redox-responsive NPs offer advantages as a tumor targeted drug delivery system, they were noticeably aggregated in aqueous environment because of hydrophobic nature of the polymer. It has been found that hydrophobic polymer nanoparticles have limitations including poor stability in blood during circulation and high cytotoxicity for their potential in vivo applications. To overcome these limitations, we intended to obtain the PEG grafted polymeric drug delivery system as the alternative approach mainly because of well-established non-fouling property of PEG that prevent the adsorption of plasma protein or the opsonization.

In this paper, we describe a novel amphiphilic block copolymer which is designed to possess QPA redox switches in the hydrophobic block while hydrophilic PEG block is
expected to increase physical stability of resultant NPs. Considering the capability of QPA reduction in the presence of meaningful tumor-associated redox changes, we anticipate that polymer micelles consisting of QPA redox switches can be destabilized by a solubility reversal occurring in the hydrophobic polymer block of the copolymer due to the release of pendant QPA groups. To our knowledge, this is the first study reporting the incorporation of QPA redox switches in the polymeric micellar system for a drug delivery application.

3.2. EXPERIMENTAL

3.2.1. Materials

Methanesulfonic acid, 2,3,5-trimethyl hydroquinone, methyl β,β-dimethylacrylate, 2-amino-1,3-propanediol, and adipoyl chloride were obtained from Alfa Aesar (Ward Hill, MA). Adipoyl chloride was distilled under reduced pressure before the reaction. Paclitaxel (PTX) was purchased from LC Laboratories® (Woburn, MA). Methoxy poly(ethylene glycol) of molecular weight 750 (mPEG750) from Sigma-Aldrich (St. Louis, MO) was used after distillation. Acetone-\(d_6\) (99.9%, Cambridge Isotope Laboratories, Inc., Andover, MA) and deuterium oxide (99.8%, Acros) were used for NMR measurements as received. Human DT-diaphorase and reduced dipotassium salt β-nicotinamide adenine dinucleotide (β-NADH) were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals purchased from Fisher Scientific (Pittsburg, PA) were used as received.
Scheme 3.1. Reaction scheme for the synthesis of redox-responsive amphiphilic block copolymer, polyQPA-mPEG$_{750}$, with QPA redox-switches.

3.2.2. Synthesis of amphiphilic redox-sensitive polymer (polyQPA-mPEG$_{750}$)

PolyQPA-mPEG$_{750}$ was synthesized in a two-step reactions shown in Scheme 3.1. First, redox-responsive hydrophobic block, polyQPA, was synthesized as reported previously$^{82,90}$. Briefly, fresh distilled adipoyl chloride diluted in anhydrous THF was slowly added into a solution of the monomer, QPA-serinol, at a molar ratio of 1:1 in pyridine (10 times molar amount of adipoyl chloride) at room temperature and stirred for
After further polymerization under dry nitrogen at room temperature overnight, an additional amount of adipoyl chloride (another 10% of initial molar amount) in THF was charged to the reaction mixture and stirred for another six hours. Next, mPEG\textsubscript{750} was dried by azeotropic distillation using toluene to end-cap polyQPA. Dried mPEG\textsubscript{750} in THF was added to polyQPA at a molar ratio of 10:1 and the end-capping reaction was proceeded for another 16 h. Resultant polyQPA-mPEG\textsubscript{750} was precipitated in an excess amount of ether and the crude polymer was purified further by rinsing three times with methanol to remove unreacted mPEG. The final product polyQPA-mPEG\textsubscript{750} was yielded as yellow solid (62.1%).

\(^1\text{H}-\text{NMR (CDCl}_3\): } \delta \text{ ppm: 4.37 (14H, s), 4.12 (28H, s), 4.06 (28H, s), 3.64 (90H, s), 3.30 (6H, s), 2.83 (28H, s), 2.36 (56H, t), 2.12 (42H, s), 1.95 (56H, m), 1.39 (14H, s).}

3.2.3. Characterization of polyQPA-mPEG750

The chemical structures of the polyQPA-mPEG\textsubscript{750} were identified with \(^1\text{H}-\text{NMR spectrum using a Bruker Avance 400 MHz spectrometer. The NMR spectra were measured in CDCl}_3\). The molecular weight and polydispersity index (PDI) of the synthesized copolymer were determined with a Waters gel permeation chromatography (GPC) system (Waters, Milford, MA). The GPC instrument is equipped with a binary pump (Waters 1525), refractive index detector (waters 2414), and a Styragel HR4E column (300 × 7.8 mm ID, 5 \(\mu\)m particle size). HPLC grade tetrahydrofuran (THF) was run as a mobile phase at a flow rate of 1.0 mL min\(^{-1}\) at 25 °C. Number (\(M_n\)) and weight average (\(M_w\)) molar masses were determined using Breeze software. The molecular
weight of the copolymer was calculated using a calibration curve obtained from polystyrene standards (Polysciences, Inc., Warrington, PA) of molecular weights ranging from 600 to 50,000 g mol\(^{-1}\) in THF.

3.2.4. Preparation of polymeric micelles

Drug-free and PTX-loaded polymeric micelles were prepared by a method of a solid dispersion technique with a minor modification\(^{93}\). Briefly, PTX (32.2 mg) and polyQPA-mPEG\(_{750}\) (150 mg) were completely dissolved in 2 mL of acetonitrile. After evaporation of the organic solvent using a rotary evaporator, remaining organic solvent was further removed by a vacuum pump overnight to obtain the molecularly dispersed PTX in polymer matrix. Three milliliters of phosphate buffered saline (PBS, pH 7.4) were added to the mixture to obtain micellar solution by hydration. After being stirred overnight in dark at room temperature, the solution was filtered through a 0.2 µm filter to remove the unincorporated drug aggregates, followed by lyophilization. The blank micelles were prepared by the same procedure without PTX.

The loading efficiency of PTX in the micelles was determined by the HPLC method previously reported to analyze the amount of PTX in QPA polymer NPs\(^{90}\). The drug loading efficiency (%) was calculated according to the following formula.

\[
\text{PTX loading} \% = \left(\frac{\text{amount of loaded PTX}}{\text{amount of initially added PTX}}\right) \times 100
\]
3.2.4. CMC measurement

The critical micelle concentration (CMC) of the polymer was determined by a dye solubilization method using 1,6-diphenyl-1,3,5-hexatriene (DPH)\textsuperscript{34}. Briefly, serially diluted polymer solutions in a concentration range of $1.0 \times 10^{-4}$ to 1.0 wt.% were prepared with PBS. Next, 25 µL of 0.4 mM DPH solution in methanol was added into 2.5 mL of each polymer solution. The mixture was sonicated for 1 min and equilibrated for 24 h in a dark place. Measurements of UV absorption for each solution were taken on a Genesys 6 UV-Visible scanning spectrophotometer (Thermo Scientific, Massachusetts) at wavelengths of 377 and 391 nm. The absorbance difference at 377 and 391 nm was plotted against polymer concentration. The CMC of polyQPA-mPEG\textsubscript{750} was determined by reading the polymer concentration at which the extrapolated two lines cross over.

3.2.5. Particle size measurements and redox-responsive polymer degradation

The Z-average diameter and polydispersity index (PI) of polyQPA-mPEG\textsubscript{750} micelles were determined by dynamic light scattering using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). A polyQPA-mPEG\textsubscript{750} solution at a concentration above its CMC was prepared to allow self-assembling into micelles in PBS.

The effect of QPA reduction on the micellar structure was tested with a chemical reducing agent, sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$). QPA lactone release was identified by $^1$H-NMR spectrum. A polyQPA-mPEG\textsubscript{750} solution at a concentration of 1.5% (w/v) was prepared for the NMR measurement using a co-solvent of acetone-$d_6$ and deuterium oxide.
at a volume ratio of 1:2. The NMR spectrum of release QPA lactone was measured on the NMR spectrophotometer and compared with that of QPA lactone control synthesized during polymer synthesis. The NMR spectra were periodically taken up to 3 h after the addition of sodium dithionite.

3.2.6. Redox-triggered QPA lactone release from polyQPA-mPEG750 micelles

QPA lactone release from the micelles was examined by challenging polyQPA-mPEG\textsubscript{750} micelles with two different redox-triggers, sodium dithionite and DT-diaphorase. A polyQPA-mPEG\textsubscript{750} stock solution at a concentration of 10 mg mL\textsuperscript{-1} (1%) in PBS was prepared and diluted to obtain polymer solutions at concentrations of 0.01% and 0.5%. Fifteen milligrams of sodium dithionite resulting in a final concentration of 29 mM was added to 3 mL of each polyQPA-mPEG\textsubscript{750} solution. In the case of enzymatic reduction of QPA, five units of DT-diaphorase and bovine serum albumin (0.007%) were added to each PolyQPA-mPEG\textsubscript{750} solution. After an addition of an amount of β-NADH to achieve a final concentration of 2 mM, 150 µL of the medium was collected and mixed into 150 µL of acetonitrile at predetermined time points. All samples were tested in triplicate and the study was carried out in a water bath equilibrated at 37 °C. Amounts of QPA lactone released upon reduction were analyzed using the HPLC method previously reported. A HPLC system equipped with a Luna C18(2) HPLC column (Phenomenex, 150 x 4.6 mm, 5 µm), a binary pump (Waters 1525), and an auto-sampler (Water 717) was used to run a mixture of acetonitrile and water at 55/45% v/v as a mobile phase at a flow rate of 1 mL min\textsuperscript{-1} after injecting a 20 µL sample. Released QPA lactone was
detected at a wavelength of 227 nm on a UV detector (Water 2487). The amount of released lactone was calculated using a calibration curve obtained from known concentrations of QPA lactone solutions in acetonitrile.

3.2.7. In vitro drug release study

In vitro drug release from polyQPA-mPEG<sub>750</sub> micelles was examined by challenging the micellar solution with sodium dithionite to reduce QPA groups after loading PTX in micelles as a model drug. Based on a HPLC measurement performed prior to drug release study, it was shown that one milligram of polyQPA-mPEG<sub>750</sub> micelle contained 177.4 µg of PTX. One hundred microliter of 0.5% of PTX loaded polymer micelles containing 88.7 µg of PTX was used for the study. Forty milliliters of PBS including sodium salicylate at a concentration of 0.8 M were used for redox-triggered drug release. After placing polyQPA-mPEG<sub>750</sub> micelle in a dialysis membrane of MWCO 1,000 Da (Spectrum Laboratories), the dialysis membrane was immersed in drug release media equilibrated at 37 °C and the release media were continuously agitated by magnetic stirring. For redox-triggered PTX release from polyQPA-mPEG<sub>750</sub> micelles, an amount of sodium dithionite to achieve a final concentration of 29 mM was added in the medium. In addition, the solution consisting of PTX (88.7 µg) was utilized as a control. Samples were withdrawn from the release medium at predetermined time points and were analyzed by HPLC.
3.2.8. Cell culture

Human breast tumor T47D and MDA-MB-231 cells lines were purchased from ATCC, Manassas, VA and used in this work. These cell lines were cultured in DMEM/F12 medium containing 2.5 mM L-glutamine (Mediatech), supplemented with fetal bovine serum (FBS, 10% v/v final concentration, Hyclone), penicillin G (sodium salt, 50 units mL\(^{-1}\)) and streptomycin sulfate (50 µg mL\(^{-1}\)) (BioWhittaker). Exponentially grown cells were plated at the density of 30,000 cells per well into 96-well plates in a volume of 100 µL culture media and incubated at 37 °C in a humidified environment (95% air, 5% CO\(_2\)) as previously reported \(^{61}\).

3.2.9. In vitro antitumor activity of polyQPA-mPEG750 micelles

The cytotoxicity of PTX formulated in polyQPA-mPEG\(_{750}\) was evaluated in human breast tumor T47D and MDA-MB-231 cells lines, in comparison to free PTX. Briefly, after seeding in 96-well plates followed by overnight attachment, the cells were treated by PTX-loaded micelles with varied concentrations. Free PTX, at concentrations equivalent to the PTX incorporated in micelles, was also added into cells. After treatment for 24 h, the cells were further incubated and cell viability was determined by a sulforhodamine B (SRB) method \(^{62}\). Optical density (OD) was measured at 490 nm with background absorption at 630 nm. Data were normalized to the untreated control and presented as ‘% of control,’ using the formula.

\[
\text{Cell viability (% of Control)} = \left( \frac{\text{OD}_{\text{treated}}}{\text{OD}_{\text{control}}} \right) \times 100
\]
3.2.10. Statistical analysis

Experimental measurements were triplicated for each sample. The results are presented as mean ± standard deviation. The statistical analysis of experimental data utilized the student’s t-test and statistical significance was considered for $P$-values < 0.05.

3.3. RESULTS

3.3.1. Synthesis and Characterization of polyQPA-mPEG750

PolyQPA-mPEG$_{750}$, designed for redox-responsive polymer micelles, were synthesized as illustrated. After synthesis of hydrophobic block, polyQPA, as presented in Scheme 3.1, the following reaction with an additional amount of adipoyl chloride provided reactive end groups in polyQPA. The reactive polyQPA underwent end-capping with mPEG of molecular weights 750 to achieve redox-sensitive block copolymers. The $^1$H-NMR spectrum of polyQPA-mPEG$_{750}$ in Figure 3.1. showed the peaks of mPEG at $\delta$ = 3.64 ppm (-CH$_2$CH$_2$O-) and 3.37 ppm (CH$_3$O-) in CDCl$_3$. Moreover, three methyl (CH$_3$-) protons of quinone was presented at 1.95-1.96 and 2.12 ppm. Resultant polyQPA-mPEG$_{750}$ was obtained as a yellow solid exhibited good water solubility.

Number average molecular weight ($M_n$) and polydispersity (PDI) of the redox-sensitive block copolymer were determined to be 3640 and 1.65, respectively, by GPC. Clear shift in the molar mass distribution to shorter retention times was observed. Before the end-capping of the PEG at the end of the hydrophobic group, the molecular weight of hydrophobic polymer was calculated as 2482 with PDI of 1.37 by GPC measurements.
Figure 3.1. $^1$H-NMR spectrum of polyQPA-mPEG$_{750}$ in CDCl$_3$. The letters denote peak assignments in $^1$H-NMR spectra.

3.3.2. Micelle formulation

The ability of polyQPA-mPEG$_{750}$ to self-assemble into micelles was examined by a CMC measurement. The CMC of polyQPA-mPEG$_{750}$ was determined by a method based on hydrophobic dye solubilization as shown in Figure 3.2. At a concentration below CMC, amphiphilic polyQPA-mPEG$_{750}$ was not able to increase DPH solubility, resulting in inadequate UV absorbance. A dramatic increase in UV absorbance was observed at a polymer concentration greater than CMC. The CMC was determined by a
graphical extrapolation of two phases of UV absorbance changes and reading out the polymer concentration at which they crossed over. The CMC of polyQPA-mPEG$_{750}$ in PBS was found to be 0.039% w/v (0.39 mg mL$^{-1}$, 0.107 mM) at 25 °C.

**Figure 3.2.** (A) Determination of the critical micelle concentration of polyQPA-mPEG$_{750}$ using DPH. The difference in the absorbance intensities of DPH at wavelengths of 377 and 391 nm was plotted as a function of polyQPA-mPEG$_{750}$ concentration. CMC was determined by reading out the point, A, at which two extrapolated lines were crossed. (B) A representative particle size distribution of drug-free micelles was obtained by DLS measurement at 25 ± 1 °C. Determined average particles size was 27.50 nm with a PI of 0.064.

Both PTX-free and PTX-loaded polymeric micelles were prepared by a solid dispersion technique which was also applied to prepare for PTX-loaded polymer micelles from monomethoxy poly(ethylene glycol)-block-poly(D,L-lactide) (mPEG-PDLLA)$^{93}$. During the process of micelle preparation, the amount of residual acetonitrile, the solvent to dissolve polyQPA-mPEG$_{750}$ and PTX, was under the detection limit by $^1$H NMR
spectroscopy measurements after vacuum drying. A simple hydration of the mixture of polyQPA-mPEG$_{750}$ and PTX with PBS enabled to produce micelle formulations.

The mean diameter of resultant PTX-free micelles was determined to be 27.50 nm with a PI of 0.064 at 25 ± 1 °C by DLS as presented in Figure 3.2. However, the average size of PTX-loaded micelles was 79.92 nm, which was noticeably larger than that of PTX-free micelles. The amount of PTX loaded in 1 mg of polyQPA-mPEG$_{750}$ micelles was found to be 177.4 µg indicating a loading content of 17.74% while drug loading efficiency was found to be 82.5%.

3.3.3. Redox-responsive polymer degradation
PolyQPA-mPEG$_{750}$ was examined for its reduction in the presence of a redox chemical. Since we are interested in redox-triggered drug release from polyQPA-mPEG$_{750}$ micelles, we primarily focused on the characterization of dimensional changes occurring to the micelles in the presence of a redox chemical. This would be eventually translated into drug release. As shown in Figure 3.3., the micellar size based on averaged volume has significantly changed upon the addition of a redox chemical, sodium dithionite. The size of polyQPA-mPEG$_{750}$ micelles rapidly increased from 28 nm to 64 nm within 5 min after the addition of sodium dithionite while no noticeable change in micellar size was observed from the control without sodium dithionite. The average size of micelles further increased to reach 3338 nm at a time point of 30 min. The increase in polyQPA-mPEG$_{750}$ micelle size in the presence of sodium dithionite is attributed to the solubility reversal occurred to the hydrophobic polyQPA core which would become hydrophilic after the
removal of hydrophobic pendant QPA groups via intramolecular cyclization. In addition, PI of the NPs increased over three hours indicating more heterogeneous particle population.

Figure 3.3. Redox-triggered particle size change in polyQPA-mPEG$_{750}$ micelles was monitored by dynamic light scattering measurements in the presence or absence of sodium dithionite.

The effect of sodium dithionite on polyQPA-mPEG$_{750}$ was directly examined by NMR measurement. Figure 3.4.(A) has shown reduction mechanism of QPA switches in polyQPA-mPEG$_{750}$ copolymer with sodium dithionite and the letters in Figure 3.4.(A) indicate peak assignments in $^1$H-NMR spectra. After dissolving polyQPA-mPEG$_{750}$ in a
co-solvent of acetone-$d_6$ : D$_2$O (2:1, v/v) to warrant the solubility of both lactone and copolymer, NMR spectra were periodically recorded with or without sodium dithionite shown in Figure 3.4 (B 1-3).
**Figure 3.4.** (A) Reduction mechanism of redox-switches in polyQPA-mPEG<sub>750</sub> copolymer using sodium dithionite and release of QPA lactone. The letters denote peak assignments in \(^1\)H-NMR spectra. (B) \(^1\)H-NMR spectra (400 MHz) of polyQPA-mPEG<sub>750</sub> were recorded in acetone-\(d_6\) and deuterium oxide (1.5%, 1:2, w/v) before (B-1) and after (B-2, 30 min, and B-3, 3 h) an addition of sodium dithionite at a concentration of 29 mM. The NMR spectrum of lactone (B-4) is also measured for comparison.

Figure 3.4. (B) also includes the NMR spectrum of QPA lactone as control to compare with (Figure 3.4. (B-4)). When sodium dithionite was added into micelle solution, all proton peaks in NMR spectra (Figure 3.4. (B)) were slightly shifted (< 0.25 ppm) compared to the one recorded before adding sodium dithionite. This result is because of ionic strength changes in the NMR sample solution and the similar result were presented in the reported publication<sup>96</sup>. The addition of sodium dithionite also resulted in the appearance of proton peaks attributed to six protons of methyl groups in QPA lactone at \(\delta = 1.33\) ppm. In addition, 30 min after the initiation of reduction, the proton peak from QPA pendant groups in polyQPA-mPEG<sub>750</sub> (-COCH<sub>2</sub>C-) has shifted from \(\delta = 2.75\) to 2.51 ppm, indicating the QPA lactone formation by intramolecular cyclization.

**3.3.4. Redox-triggered QPA lactone release from polyQPA-mPEG750 micelles**

In order to quantitatively analyze QPA reduction in polyQPA-mPEG<sub>750</sub> micelles, the amount of QPA lactone released from polyQPA-mPEG<sub>750</sub> was measured as a function of time in the presence and absence of the redox agent. As shown in Figure 3.5, there was
no noticeable QPA lactone release from the control which did not contain the redox agent. QPA reduction in polyQPA-mPEG_{750} micelles was rapid enough to release 60.04% and 67.04% of QPA groups at polymer concentrations of 0.01 and 0.50%, respectively in 30 min after the initiation of the reduction. Cumulative QPA lactone release reached to 85.30% and 72.62% within 24 hours at polymer concentrations of 0.01% and 0.50%, respectively. At 48 hours after the addition of sodium dithionite, QPA lactone generated from 0.01% of polyQPA-mPEG was 88.09% which reveals no statistically significant differences (two-tailed t-test, p > 0.05) even after 48 hours of incubation.
Figure 3.5. Redox-triggered QPA lactone release from polyQPA-mPEG$_{750}$ solution in PBS buffer (pH 7.4) at 37 °C. Cumulative QPA lactone release from polyQPA-mPEG$_{750}$ at concentrations of 0.50% (A) and 0.01% (B) was plotted as a function of time after an addition of each reducing agent, sodium dithionite (●) or DT-diaphorase/β-NADH (△). The control (×) was copolymers without those reducing agents.

In addition, polyQPA-mPEG$_{750}$ micelles were also able to release QPA lactone via redox enzyme-mediated bioreduction. Indeed, DT-diaphorase could trigger QPA reduction to result in spontaneous QPA lactone release from hydrophobic cores of polyQPA-mPEG$_{750}$ micelles. DT-diaphorase-mediated QPA reduction, however, was dependent on polymer concentration. Cumulative amounts of QPA lactone released by 5 units of DT-diaphorase in the presence of 2 mM of a cofactor β-NADH at 37 °C reached to 61.84% and 20.87% at polymer concentrations of 0.01% (Figure 3.5. (B)), a concentration below CMC, and 0.50% (Figure 3.5. (A)), a concentration above CMC,
respectively. This result indicated that the supramolecular structure of polyQPA-mPEG\textsubscript{750} assembly could affect the efficiency of DT-diaphorase-mediated QPA reduction.

3.3.5. In vitro PTX release from polyQPA-mPEG\textsubscript{750} micelles

*In vitro* PTX release from polyQPA-mPEG\textsubscript{750} micelles, examined by a dialysis method in the presence of sodium dithionite, demonstrated that QPA reduction in polyQPA-mPEG\textsubscript{750} micelles could trigger the release of incorporated PTX. As seen in Figure 3.6, polyQPA-mPEG\textsubscript{750} micelles have released only 17.08\% of loaded PTX over a period of 36 h in the absence of sodium dithionite. An addition of the redox agent into the polyQPA-mPEG\textsubscript{750} micellar solution has dramatically increased the release of PTX resulting in a significant increase in cumulative release of PTX from 18.20\% to 52.53\% between time points 3 h and 12 h, respectively ($p < 0.05$). The cumulative PTX release has further increased to 65.73\% at 36 h after triggering QPA reduction. Triggered PTX release from polyQPA-mPEG\textsubscript{750} micelles was comparable to the PTX release profile from pure PTX solution.
Figure 3.6. Redox-triggered PTX release from polyQPA-mPEG$_{750}$ micelles in the presence (■) and absence (○) of sodium dithionite (29 mM) at 37 °C. An addition of PTX solution with sodium dithionite was used as the control to compare with (×).

3.3.6. *In vitro* antitumor activity of polyQPA-mPEG$_{750}$ micelles

Concentration-dependent cell cytotoxicity studies were performed to determine the effects of PTX-loaded polyQPA-mPEG micelles on the proliferation/viability of human breast tumor cells. As shown in Figure 3.7. (A), at a drug dose of 0.1 µM, cell viabilities of 20% and 24% were observed for MDA-MB-231 cell line following 24 h of incubation with free PTX and PTX-loaded micelles, respectively. The cell experiment for T47D cell line in Figure 3.7. (B) also showed similar cell viability, in which 22% and
26% of cell viability were observed for plain drug, PTX loaded micelles, respectively. The IC$_{50}$ (i.e., inhibitory concentration to produce 50% cell death) of PTX-loaded micelles, were 217.6 µM and 277.0 µM for MDA-MB-231 and T47D cell lines, respectively, while those of free PTX were 323.3 µM and 296.1 µM.

![Graphs showing cell viability vs. concentration]  

**Figure 3.7.** Effects of paclitaxel loaded micelles on human breast tumor cell proliferation/viability determined by SRB method: (A) MDA-MB-231 cells (B) T47D cells. Exponentially grown T47D and MDA-MB-231 cells were exposed to the different concentration of PTX-loaded micelles (•) and PTX (×) for 24 h under normoxic condition (95% air : 5% CO$_2$). Data shown are average standard deviation (n=3).

### 3.4. DISCUSSION

We have previously reported QPA-based redox-responsive polymeric NPs enabling drug release under a simulated redox state. In this study, we developed a redox-responsive amphiphilic block copolymer containing QPA triggers, polyQPA-mPEG$_{750}$,
which is able to self-assemble into micelles, load a hydrophobic drug, and release the drug in response to a redox change. Redox-responsive copolymers were synthesized in a two-step reaction. In the first step in Scheme 3.1, hydrophobic polyQPA block was synthesized by esterification between QPA-serinol and adipoyl chloride as previously reported.\textsuperscript{82,90} The esterification was occurred by the reaction between two hydroxyls in QPA-serinol and reactive two acyl chloride groups in adipoyl chloride. For the following end-capping of polyQPA with mPEG, both terminal functional groups needed to be identically reactive towards the hydroxyl group of mPEG. Since terminal functional groups of polyQPA could be either hydroxyl or acyl chloride, an additional amount of adipoyl chloride was added to secure reactive acyl chloride groups at both ends. The activated polyQPA was reacted with dried mPEG\textsubscript{750} to obtain redox-sensitive amphiphilic polyQPA-mPEG. Based on molecular weight analysis, molecular weights of polyQPA-mPEG\textsubscript{750} and polyQPA, 3640 and 2482 respectively, may indicate that polyQPA-mPEG\textsubscript{750} possesses mixture of tri-block copolymer and diblock copolymer with consideration of mPEG molecular weight 750.

Self-assembling of polyQPA-mPEG\textsubscript{750} into micelles was supported by demonstration of CMC. The CMC of polyQPA-mPEG\textsubscript{750} was 0.039\% w/v, which is lower than commercially available amphiphilic tri-block copolymers, poly(ethylene glycol)-poly(propylene glycol)-poly(ethylene glycol) (Pluronic\textsuperscript{®}). CMCs of Pluronic\textsuperscript{®} polymers range from 0.6\% to 4\% \textsuperscript{97}. Particulary, Pluronic\textsuperscript{®} L64 (MW of 3400 and a PEG content of 40\%), which has molecular characteristics similar to polyQPA-mPEG\textsubscript{750} (MW of 3600 and a PEG content of 41\%), has a CMC of 4\% w/v. It is well established
that self-assembling of amphiphilic block copolymers into micelles is primarily dependent on the nature and length of hydrophobic block.\textsuperscript{97-98} The determined CMC of polyQPA-\textit{mPEG}\textsubscript{750} lower than that of Pluronic\textsuperscript{®} L64 may indicate that polyQPA is more hydrophobic than poly(propylene glycol).

Particle size of polyQPA-\textit{mPEG}\textsubscript{750} measured by dynamic light scattering has shown that micellar sizes of drug-free and drug-loaded micelles were in a range between 20 nm and 100 nm. It should also be noted that the size of PTX-free polyQPA-\textit{mPEG}\textsubscript{750} micelles was smaller than that of drug-loaded micelles. It has been reported that loading a hydrophobic drug in the core of polymer micelle often expands micelles and increases micelle size.\textsuperscript{99} PolyQPA-\textit{mPEG}\textsubscript{750} micelles, both drug-free and drug-loaded, were smaller than other nanocarriers with QPA redox switches such as liposome\textsuperscript{34} and nanoparticles\textsuperscript{90} of which particles sizes were \textasciitilde100 nm and 249.8 nm, respectively. It has been suggested that drug carriers within a size range between 10 nm and 200 nm would avoid reticuloendothelial system (RES)-mediated particle clearance, which would be beneficial for a prolonged systemic circulation.\textsuperscript{100} Considering particle size and protective PEG brushes on surface, polyQPA-\textit{mPEG}\textsubscript{750} micelles would be useful for drug delivery applications.

Generally, hydrophobic drug loading into micellar structures is affected by molecular interactions between a drug and hydrophobic micelle core and steric factors\textsuperscript{101}. In this study paclitaxel was utilized as a model hydrophobic drug to assess the application of prepared micelles to drug delivery. Our previous publication reported that the Flory-Huggins interaction parameter ($\chi$) between polyQPA and PTX was estimated to
be 0.031 by solubility parameters calculated from the Molecular Dynamic simulations, suggesting a good miscibility. Indeed, a relatively high PTX loading efficiency of 82.5% as well as a drug loading content of 18% was achieved with polyQPA-mPEG<sub>750</sub> micelles. Such favorable drug loading might cause more than two-fold increase in micelle size from 27.50 nm to 79.92 nm, which is comparable to other biodegradable polymer micelles incorporated with hydrophobic drugs.

**Scheme 3.2.** Schematic illustration of drug release from redox-responsive polyQPA-mPEG<sub>750</sub> micelles upon redox-triggered QPA reduction.

For a successful application of polyQPA-mPEG<sub>750</sub> micelles as a drug delivery system, redox-triggered dissembling of the micelles and subsequent drug release are very important. Therefore, polyQPA-mPEG<sub>750</sub> micelles were tested for their redox-triggered dimensional changes and drug release. As illustrated in **Scheme 3.2**, drug-incorporated micelles are stable in the absence of sodium dithionite. However, in the presence of sodium dithionite these micelles are expected to expand upon initiation of QPA reduction in the hydrophobic core thereby releasing the entrapped drug. It has been previously
reported that hydrophobic polyQPA undergoes a solubility reversal by exposing free amine groups after QPA lactone release via spontaneous intra molecular cyclization in the presence of the redox trigger \(^{82,90}\). With protonation of exposed free amine groups in pH 7.4, the size of polymeric micelles should increase by swelling due to the repulsive ionic interactions introduced after the removal of QPA lactone. As expected, polyQPA-mPEG\(_{750}\) micelles started to rapidly swell to reach 3338 nm (PI~1.0) within 30 min after the initiation of QPA reduction, indicating that micelle cores were swollen because of polymer hydration induced by the solubility reversal occurred in the polyQPA block. Swollen micelles with loosely interwoven polymer chains in the core would be able to facilitate the release of incorporated PTX. This unique property of redox-triggered dimensional changes in polyQPA-mPEG\(_{750}\) micelles may be useful for targeted drug delivery to the tissues with altered redox state such as tumors.

Redox-triggered structural change in polyQPA-mPEG\(_{750}\) was also examined by NMR measurements to mechanistically correlate the effect of the redox trigger on the molecular property changes in the polymer which could be related to drug release. Yellow polyQPA-mPEG\(_{750}\) solution became colorless implying that QPA groups were reduced to hydroquinone. The NMR spectrum recorded at 30 min after the addition of sodium dithionite showed characteristic proton peaks of QPA lactone, reduced polyQPA-mPEG\(_{750}\) backbone and partially reduced quinone (Figure 3.4.(B-2)). In particular, the characteristic QPA lactone peaks (Figure 3.4. (B-3)) recorded in 3 h appeared after polymer reduction match with the peaks in the NMR spectrum measured with QPA lactone control (Figure 3.4. (B-4)), which indicates successful QPA group reduction.
Combining the experimental results of redox-triggered dimensional changes in micelles and structural changes in polyQPA-mPEG\textsubscript{750}, the redox-responsive polyQPA-mPEG\textsubscript{750} micelles could be swollen by redox-triggered QPA lactone release within 30 min as presented in Scheme 3.2.

Quantitative analysis of lactone was assessed to get further informative insight into the underlying physical and chemical reduction processes of redox-responsive micelles, which can be eventually translated into drug release. The QPA lactone release profile in Figure 3.5. has clearly demonstrated that most QPA in the core of polyQPA-mPEG\textsubscript{750} micelles was as a lactone form in the presence of sodium dithionite regardless of polymer concentration. This result is consistent with QPA reduction on polyQPA-mPEG\textsubscript{750} characterized by NMR measurements (Figure 3.4). It also worth mentioning that PEG block attachment did not affect QPA lactone release from polyQPA. QPA lactone release from polyQPA-mPEG\textsubscript{750} micelles was similar to that from polyQPA NPs.

Considering the degradation of polyQPA-mPEG\textsubscript{750} backbone chain, a change of its hydrophobicity due to carboxylic acid ends liberated by cleavage of their ester bonds will facilitate the reduction of QPA group which leads to an immediate QPA lactone release (Figure 3.5.). As shown in Figure 3.5., enzymatic reduction of polyQPA-mPEG\textsubscript{750} was noticeably different from that with sodium dithionite. A redox enzyme often upregulated in tumors, DT-diaphorase, could release QPA lactone from polyQPA-mPEG\textsubscript{750}. However, the enzymatic QPA reduction on polyQPA-mPEG\textsubscript{750} was clearly affected by polymer concentration, indicating that a steric factor is involved in DT-diaphorase-mediated QPA reduction from polyQPA-mPEG\textsubscript{750} as seen in Figure 3.5. DT-diaphorase
was able to reduce QPA on polyQPA-mPEG\textsubscript{750} when the polymer concentration, 0.01%, was below CMC, 0.039% w/v. When polymer concentration, 0.5%, increased to above CMC, however, DT-diaphorase was not effective in reducing QPA groups. This result indicates that the enzyme had a limited access to QPA groups on polyQPA-mPEG\textsubscript{750} when the polymer assembled into micelles. The QPA groups in the hydrophobic block would be hidden inside the micelle core and also masked by the PEG layer on micelle surface. This explanation may be valid based on the reported work with N-(2-hydroxypropyl)methacrylamide micelles containing hydrophobic oligopeptide side chains. Ulbrich \textit{et al}. demonstrated that the penetration of enzyme into micelle core was impeded by structural hindrance, thereby preventing the effective enzyme binding to substrate \textsuperscript{102}. However, hydrophilic PEG blocks might contribute to increasing water solubility of polyQPA and facilitating the binding between DT-diaphorase and QPA groups on polyQPA-mPEG\textsubscript{750} under a polymer concentration below CMC since most polymer molecules exist as a unimer instead of being associated. The collective results also indicated the process of QPA lactonization plays an important role during the destabilization process of micelle, thereby enabling the release of loaded drug.

\textit{In vitro} PTX release kinetics, indeed, was well correlated with QPA reduction and QPA lactone release previously discussed. PTX release from polyQPA-mPEG\textsubscript{750} micelles in the presence of sodium dithionite was significantly (p < 0.01) greater than drug release in the absence of the redox chemical (Figure 3.6.). PTX release from the micelles was even very similar to that from the control, PTX solution itself (two-tailed t-test, p > 0.2) assuring that most of incorporated drug was released from the polyQPA-mPEG\textsubscript{750}
micelles over a period of experiment time in the presence of the redox chemical. Overall a cumulative PTX release of greater than 80% was comparable to that reported from our previous work with polyQPA NPs. Based on the results presented in Figures 3.5 and 3.6, PTX-loaded redox-responsive polyQPA-mPEG<sub>750</sub> micelles were stable in aqueous environments and able to release the incorporated PTX by redox-triggered QPA reduction which disrupted micelle structure.

The antitumor activity of polyQPA-mPEG<sub>750</sub> micelles was studied in human breast tumor T47D and MDA-MB-231 cells to determine cancer cell mediated drug release. Free PTX was used as positive controls for the study. As shown in Figure 3.7, a concentration-dependent cell-killing effect was shown for PTX-loaded micelles. These PTX-loaded micelles inhibited cell proliferation and viability to the comparable extent as that observed in the presence of PTX. This result may indicate an increased cellular uptake of PTX-loaded polyQPA-mPEG micelles owing to hydrophilic PEG chain on the surface and release all incorporated PTX from micelles to limit tumor cell growth. Indeed, the similar result was presented in the reported publication. When doxorubicin was delivered by poly(DL-lactic-co-glycolic acid)-mPEG<sub>2000</sub> micelles exhibited greater uptake by HepG2 cells, compared to free drug because there is no specific affinity between the outer PEG chains of micelles and negatively charged plasma membrane of the cells. It seemed that the PEG-grafted redox-responsive polymeric micelle would resist opsonization and phagocytic clearance and enable to release the loaded drug to limit tumor cell growth. The antitumor activity of these polyQPA-mPEG<sub>750</sub> micelles may be boosted by decorating the hydroxyl end of redox-responsive micelle, polyQPA-
mPEG\textsubscript{750}, with a targeting ligand such as antibody fragment and folic acid which improve its specificity for drug delivery through active pathway.\textsuperscript{104} These polyQPA-mPEG\textsubscript{750} micelles have integrated features including relatively high drug loading, micellar stability, redox-triggered dimensional change, and redox-mediated drug release, which collectively provide a useful delivery platform for cytotoxic cancer drugs.

### 3.5. CONCLUSIONS

A novel redox-responsive amphiphilic polymer comprising QPA redox switches and hydrophilic mPEG, polyQPA-mPEG\textsubscript{750}, was successfully synthesized and characterized. Coupling of mPEG to hydrophobic polyQPA provided polyQPA-mPEG\textsubscript{750} with improvement of water-solubility as well as self-assembling capability into micelles. Furthermore, incorporation of QPA redox switches into amphiphilic polymer led to unique micelles which enable to rapidly undergo dimensional changes upon QPA reduction and thus release incorporated drug in a redox-triggered manner. PTX-loaded polyQPA-mPEG\textsubscript{750} micelles, indeed, has demonstrated a redox-responsive drug release. Redox-responsive polyQPA-mPEG\textsubscript{750} micelles may be useful as a drug delivery system targeting tumor microenvironments which frequently show altered redox state and/or redox enzyme upregulation.
CHAPTER 4

NITROREDUCTASE-TRIGGERED ACTIVATION OF A NOVEL CAGED FLUORESCENT PROBE OBTAINED FROM METHYLENE BLUE
A novel NIR fluorescent probe, \( p \)-nitrobenzyl 3,7-bis(dimethylamino)-10\( H \)-phenothiazine-10-carboxylate was developed from methylene blue for the selective imaging and therapy. Conjugating methylene blue with a \( p \)-nitrobenzyl moiety redox switch enables it to quench electron transfer within the molecules, thereby leading to improvements in achievable target-to-background auto-fluorescence ratio. Moreover the redox trigger caged probe is readily activated by nitroreductase-catalyzed 1,6-elimination, resulting in the release of active methylene blue fluorophore. This fluorescent probe exhibits neither significant NIR fluorescence intensity nor absorbance between 500 nm to 800 nm. However, it was activated to release the strong fluorescence (\( \lambda_{\text{max}} = 680 \) nm) with the excitation at 580 nm upon nitroreductase-mediated two-electron reduction of the redox switch. In addition, the fluorescence emission was induced by incubation with live Escherichia coli bacteria. It was demonstrated that the NIR fluorescent probe generated singlet oxygen after nitroreductase mediated reduction with a laser irradiation at a wavelength of 634 nm. The redox trigger caged NIR fluorescent probe would be a suitable imaging sensor to detect bacteria expressing NTR. In addition, \( p \)-NBMB also possesses potential as an MB prodrug which can be applied for the treatment of various diseases.
4.1. INTRODUCTION

Nitroreductase (NTR), a flavin mononucleotide (FMN) cofactor dependent protein expressed in Escherichia coli (E. coli), catalyzes reduction of a nitro group to hydroxylamine, which is subsequently converted to an amine in the presence of nicotinamide adenine dinucleotide phosphate (NAD(P)H) as a cofactor.\textsuperscript{105} The large electronic change resulting from conversion of the electron-withdrawing nitro group to the electron-donating hydroxylamino group provides a selective ‘switch’ mechanism for the activation of an inert compound which leads to the subsequent release of the active agent. Based on this switch mechanism, various prodrug or activatable imaging probes have been designed with nitro groups as substrates to be triggered upon reduction by NTR.\textsuperscript{106} The dicoumarin carbonate-based latent fluorophore was devised for the application in imaging to improve selectivity for NTR and aqueous solubility.\textsuperscript{106b} Furthermore, NTR was employed in development of gene-directed enzyme prodrug cancer therapies (GDEPT), antibody-directed enzyme prodrug therapy (ADEPT) and drug screening as exemplified with 2,4-dinitrobenzamide (CB1954).\textsuperscript{107} The basis of CB1954 activation has been fully elucidated and the molecule has advanced into clinical trials.\textsuperscript{35}

Despite the previous extensive investigation about the use of nitrobenzyl redox switches for NTR activatable materials, these switches have not been applied for the development of bifunctional compounds, which can serve as a tool for selective imaging
and therapy. To design NTR activatable agents with combined imaging and therapeutic function, the selection of an appropriate reporter is of great importance. Among various reporters, fluorophores with emission in the far red and near-infrared (NIR), in the range of 600-1000 nm, are widely utilized in relevant research settings to overcome the limitation of poor tissue penetration owing to the interference from background autofluorescence from biomolecules. In addition, NIR fluorophores offer several additional advantages over conventional imaging agents including high resolution, sensitivity and non-invasiveness. In particular, methylene blue (MB) has been recognized as a sensible starting point for the development of a better NIR fluorophore given MB’s strong absorption of broadband red light (550-700 nm, maximum at 664 nm), a wavelength at which absorption and autofluorescence are minimal. Moreover, MB has been applied for clinical therapeutic purposes including treating methemoglobinemia, Barrett’s esophagus, and cervical cancer. Nitroreductase (NTR), a flavin mononucleotide (FMN) cofactor dependent protein expressed in Escherichia coli (E. coli), catalyzes reduction of a nitro group to hydroxylamine, which is subsequently converted to an amine in the presence of nicotinamide adenine dinucleotide phosphate (NAD(P)H) as a cofactor. The large electronic change resulting from conversion of the electron-withdrawing nitro group to the electron-donating hydroxylamino group provides a selective ‘switch’ mechanism for the activation of an inert compound which leads to the subsequent release of the active agent. Based on this switch mechanism, various prodrug or activatable imaging probes have been designed with nitro groups as substrates to be triggered upon reduction by NTR. The dicoumarin carbonate-based latent fluorophore
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Despite the previous extensive investigation about the use of nitrobenzyl redox switches for NTR activatable materials, these switches have not been applied for the development of bifunctional compounds, which can serve as a tool for selective imaging and therapy.\textsuperscript{2-4} To design NTR activatable agents with combined imaging and therapeutic function, the selection of an appropriate reporter is of great importance. Among various reporters, fluorophores with emission in the far red and near-infrared (NIR), in the range of 600-1000 nm, are widely utilized in relevant research settings to overcome the limitation of poor tissue penetration owing to the interference from background autofluorescence from biomolecules.\textsuperscript{8-9} In addition, NIR fluorophores offer several additional advantages over conventional imaging agents including high resolution, sensitivity and non-invasiveness.\textsuperscript{108} In particular, methylene blue (MB) has been recognized as a sensible starting point for the development of a better NIR fluorophore given MB’s strong absorption of broadband red light (550-700 nm, maximum at 664 nm), a wavelength at which absorption and autofluorescence are minimal.\textsuperscript{109} Moreover, MB has been applied for clinical therapeutic purposes including treating methemoglobinemia, Barrett’s esophagus, and cervical cancer.\textsuperscript{110} MB is also known to be photodynamically
active so that it can inactivate viruses, destroy bacteria, and inhibit cancer cell growth.\textsuperscript{109b, 111}

Given its advantageous properties, MB may be an ideal compound to use in design of an NTR-switchable probe with both imaging and therapeutic capability. In this study, we describe a novel NIR fluorescent probe, $p$-nitrobenzyl 3,7-bis(dimethylamino)-10$H$-phenothiazine-10-carboxylate ($p$-NBMB) which can be triggered by NTR. To obtain a MB-based latent fluorescent probe, which is activatable, a $p$-nitrobenzyl moiety as an NTR switch was conjugated to cage MB at the nitrogen of the phenothiazine ring through a carbamate bond, thereby quenching electron transfer within the molecule (Scheme 4.1). This ultimately leads to improvements in achievable target-to-background auto-fluorescence ratios. In the presence of NTR, the resultant caged fluorescent probe is

\textbf{Scheme 4.1.} Mechanism of switching on the fluorescence probe, $p$-NBMB, upon reduction of a $p$-nitrobenzyl moiety after nitroreductase-mediated activation.
intended to be turned back on undergoing reduction of the aryl nitro group. Subsequently, the benzyl moiety will be removed by a rapid 1,6-elimination (Scheme 4.1.). We primarily assessed the feasibility of whether a novel \( p \)-NBMB can be developed into an NTR selective activatable agent. Additionally, a potential therapeutic application of the compound in photodynamic therapy was also addressed.

4.2. EXPERIMENTAL

4.2.1. Materials

4-Nitrobenzyl chloroformate, nitroreductase from *Escherichia coli* (*E. coli*), human DT-diaphorase, \( \beta \)-nicotinamide adenine dinucleotide, reduced dipotassium salt hydrate (NAD(P)H), and \( p \)-nitrosodimethylaniline were purchased from Sigma-Aldrich (Saint Louis, MO). Certified grade methylene blue (MB) was obtained from Acros Organics (Pittsburgh, PA). All other chemicals were purchased from Fisher Scientific (Pittsburgh, PA) and used as received.

4.2.2. Synthesis of \( p \)-nitrobenzyl 3,7-bis(dimethylamino)-10\( H \)-phenothiazine-10-carboxylate (\( p \)-NBMB)

Methylene blue (MB, 373.9 mg, 1 mmol) was dissolved in 10 mL of deionized water. Toluene (40 mL), sodium bicarbonate (292.4 mg, 3.48 mmol) and sodium dithionate (496.2 mg, 2.85 mmol) were added to the solution of MB, stirring at 50 °C. When the mixture turned to yellow, after 30 min, the toluene phase containing leuco-MB was transferred dropwise over 60 min into a solution of \( p \)-nitrobenzyl chloroformate
dissolved in 40 mL of toluene, maintained in an ice bath. After overnight reaction at room temperature, the mixture was collected after washing with water three times. The obtained organic phase was evaporated under reduced pressure and then was precipitated into cold methanol. The precipitates were purified by recrystallization from acetonitrile yielding orange solid powder: yield (52.25 mg, 11.24%): ¹H NMR (400 MHz, CDCl₃) δ 8.21 (d, J = 8.4 Hz, 2H), 7.47 (d, J = 8.3 Hz, 2H), 7.36 (d, J = 8.6 Hz, 2H), 6.70 (d, J = 2.5 Hz, 2H), 6.64 (dd, J = 8.9, 2.3 Hz, 2H), 5.32 (s, 2H), 2.96 (s, 12H), 1.57 (s, 2H). Found C, 61.88; H, 5.10; N, 12.06. Calcd for C₂₄H₂₄N₄O₄S: C, 62.05; H, 5.21; N, 12.06

4.2.3. Spectroscopic assessment

All spectroscopic measurements were performed in 10 mM phosphate buffered saline solution (PBS, pH 7.4, 1% DMSO). For the spectrometer measurements, a quartz cuvette with 1 cm path length was used to hold each sample. The absorbance of the solution was measured using a Genesys 6 spectrophotometer (Thermo Fisher Scientific, Inc., USA). Fluorescence spectra were acquired both using a PerkinElmer LS55 fluorescence spectrometer (Massachusetts) and an inverted microscope setup. A wavelength of 580 nm was chosen as the excitation source and fluorescence was detected in a wavelength range between 640 and 800 nm. The heterogeneity of the sample was probed using an inverted Nikon TE200U microscope with a 10x objective (NA = 1.4). The 514.5 nm laser line of an Ar⁺ ion laser (~500 µW) was used to excite the sample, which was deposited on plasma cleaned glass coverslips. An Acton SP2500 spectrograph with a 150 grooves mm⁻¹ grating was employed to disperse the emission and a Princeton
Instruments ProEM 1024 EMCCD camera was used to record the fluorescence emission between 620 and 800 nm.

4.2.4. Spectral analysis of $p$-NBMB

The solutions of $p$-NBMB (1 µM) in deionized water (1% DMSO) were prepared to assess spectral analysis of $p$-NBMB. The fluorescence and absorbance of $p$-NBMB was evaluated using a UV-Visible spectrophotometer and fluorescence spectrometer, respectively, along with MB (1 µM) as a reference.

4.2.5. Nitroreductase activity assay

$p$-NBMB was dissolved in DMSO to obtain a stock solution of 10 mM and the stock solution was diluted to achieve concentrations ranging from 1 µM to 100 µM. Nitroreductase was mixed to dilute $p$-NBMB solution with an enzyme assay buffer (1 mM NAD(P)H, PBS) containing 1% DMSO to achieve final enzyme concentrations of 1 unit mL$^{-1}$ and 2 units mL$^{-1}$. All prepared samples were incubated at 37 °C. Fluorescence spectra were measured with/without nitroreductase, changing the concentration of imaging probe and enzyme. Fluorescent images were obtained using IVIS Spectrum (Caliper Life Sciences, Perkin Elmer). Excitation light of 640 +/- 17 nm was used, and fluorescence emission was collected at 800 +/- 10 nm (Auto exposure, F stop 2, small binning). Mean fluorescence radiant efficiency was obtained for each well using Living Image Software (Caliper Life Sciences).
4.2.6. *E. coli* assay

*E. coli* XL1-Blue were grown in 3 mL of terrific broth without antibiotic overnight at 37 °C. At the end of the incubation, the bacteria were centrifuged at 2000xg for 10 minutes at 4 °C. The bacterial pellet was resuspended in 1 mL of PBS containing 1% DMSO. To investigate the fluorescent response of p-NBMB to intact *E. coli*, bacteria (approximately $10^9$ CFU mL$^{-1}$) were added to each well of a black 96 well plate. Afterward, p-NBMB was added to a final concentration of 1 – 50 µM to each well of the 96 well plate. In addition, fluorescence response of p-NBMB to serial number of *E. coli* was examined. The bacterial suspension (approximately $10^9$ CFU mL$^{-1}$) was serially diluted in PBS containing 1% DMSO in the following concentrations: undiluted, 1/10, 1/10$^2$, and 1/10$^3$. 100 µL of PBS containing 1% DMSO, undiluted, and diluted bacterial suspension were transferred to a black 96 well plate containing 50 µM of p-NBMB and incubated at 37 °C for 4 hours. After the treatment, these well plates were incubated at 37 °C for 4 hours. At the end of the incubation, the fluorescence intensity was monitored in the same manner as for the measurement in section “Fluorescence imaging”. To calculate the bacterial number, the same bacterial suspension was used. 100 µL from each dilution was spread on agar plates without antibiotics and incubated at 37 °C overnight. The colonies were counted from each plate, and colony numbers were used to calculate colony forming units (CFU) per mL in the original bacterial suspension.
4.2.7. Singlet oxygen (\(^{1}\text{O}_2\)) generation

Singlet oxygen generated from \(p\)-NBMB solution was monitored by oxidation of \(p\)-nitrosodimethylaniline (RNO).\(^1\) It is known that RNO is bleached in the presence of singlet oxygen, implying that the change in the intensity of RNO absorbance indicates \(^{1}\text{O}_2\) generation.\(^1\) A solution of \(p\)-NBMB (10 \(\mu\)M) was prepared with the enzyme assay buffer containing 2 unit \(\text{mL}^{-1}\) of NTR and 1 mM of NAD(P)H. The buffer solution without the redox enzyme was used as a negative control. After incubation of all prepared samples for various time intervals ranging from 3 h to 24 h at 37 °C, RNO (13.2 \(\mu\)M) and histidine (21 mM) were added to the solutions. Those solutions were irradiated with a He-Ne laser (632 nm, 110 mW/cm\(^2\), Hughes Research Laboratories, Culver City, CA). Various irradiation times ranging from 10 to 75 min were used to determine the effect of irradiation time on \(^{1}\text{O}_2\). The optical density at 440 nm (\(\lambda_{\text{max}}\) of RNO) was monitored using a UV-visible spectrophotometer.

4.3. RESULT AND DISCUSSION

The caged NIR fluorescent probe \(p\)-NBMB was synthesized in straightforward steps using MB and \(p\)-nitrobenzyl as shown in Scheme 4.2. Initially, MB in toluene (80% v/v in water) was reduced to obtain the activated leuco form of MB (leuco-MB). The yellow toluene phase containing leuco-MB was transferred into 4-nitrobenzyl chloroformate in toluene to afford the caged \(p\)-NBMB. The orange solid resultant \(p\)-NBMB was characterized by elemental analysis and \(^{1}\text{H}\)-NMR spectroscopy. The proton NMR of \(p\)-NBMB in CDCl\(_3\) in Fig. 4.1. showed all the characteristic peaks and splitting,
which indicated successful $p$-NBMB synthesis. The chemical shift value of the two protons after the reaction was deshielded from 4.82 ppm to 5.32 ppm, which is indicative of the formation of the carbamate bond.

**Scheme 4.2.** Synthetic schemes of fluorescent probe $p$-NBMB. Reagents and conditions: (i) methylene blue, Sodium dithionate, Sodium bicarbonate, H2O/Toluene, 50 °C, 1 h; (ii) Leuco Methylene blue, 4-nitrobenzyl chloroformate, dry toluene,(1) 0 °C, (2) Overnight.
The spectral analysis of \( p \)-NBMB was compared with that of a fluorophore MB as a reference. In spectroscopic evaluation of MB and \( p \)-NBMB, MB exhibits maximal absorbance at 665 nm and fluorescence at 685 nm (\( \lambda_{ex} = 665 \) nm) (Fig. 4.2). In contrast,
$p$-NBMB displayed neither significant absorbance nor fluorescence excitation at 665 nm. This phenomenon appears to be caused by the interruption of $\pi$-conjugation system bridging the three rings of MB, which quenches the absorbance between 500 nm to 800 nm. This result indicated that $p$-NBMB exhibits no fluorescence when the signal compound MB is caged with the nitrobenzyl moiety redox switch as illustrated in Scheme 4.1, which should lead to diminished noise in clinical imaging applications.

![Absorbance spectra](image)

**Figure 4.2.** Absorbance spectra of imaging probe $p$-NBMB (dashed line) and MB (solid line) as a reference by UV spectroscopy.
Figure 4. 3. Change in fluorescence intensity of \( p \)-NBMB (10 \( \mu \)M) after 3, 6, 9, 12, and 24 hours of incubation with NTR (1 unit \( \text{mL}^{-1} \))/NAD(P)H (1 mM). The negative control did not include the enzyme and NAD(P)H. Spectra were acquired in 10 mM PBS, pH 7.4 with the excitation at 580 nm.
The selective uncaging via nitro reduction was assessed using NfsA, which is the major NTR from *E. coli*. As expected, *p*-NBMB exhibited a selective response for NTR, shown in Figure 4.3. The fluorescence spectrum of the control revealed no emission between 640 and 800 nm when excited by either 580 nm light using a fluorescence spectrometer or by 514.5 nm laser light, unless NTR is added in the solution. Heterogeneities of reduced *p*-NBMB were further observed with thin film fluorescence emission spectra as shown in Fig. 4.4. After reduction of *p*-NBMB by NTR, several different peaks were recovered in addition to the dominant 680 nm peak in the spectrum (Fig. 4.4.). These results might be due to the formation of aggregates and excimers when the reduced *p*-NBMB interacts with the surface of a glass cover slip. A previous reported

**Figure 4.4.** Heterogeneity of *p*-NBMB in addition of NTR (1 unit) with NAD(P)H (1 mM). Thin film fluorescence emission spectra were obtained at 541 nm excitation.

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study also showed that the fluorophore MB forms dimers in dilute aqueous solutions and aggregates at increasing concentration due to the strong π-interactions among MB molecules. Thus, peaks from dimer and aggregates would appear on the longer wavelength at 712 nm and 728 nm due to the transition dipole moment from the ground state to the lower excited state, called the N-branch. Considering the increase in fluorescence intensity in the presence of NTR, we anticipate that uncaging p-NBMB is dependent on nitroreductase-mediated two-electron reduction of the nitrobenzyl moiety redox switch.

To verify whether there is a fluorescence response to other redox enzymes, which catalyze two-electron reduction, p-NBMB emission was investigated using DT-diaphorase. As shown in Fig. 4.5., DT-diaphorase did not induce any noticeable fluorescence signal enhancement from p-NBMB compared to the negative control without the enzyme, over an incubation period up to 48 h. According to previous studies on triggerable fluorescent probes, nitrobenzimidazoles containing aryl nitro groups could be an efficient redox switch that can be turned on by DT-diaphorase. In addition, CB1954, the aforementioned prodrug carrying aryl nitro groups, was found to be activated by both DT-diaphorase and NTR but NTR catalyzed the reduction of CB1954 60 times faster than DT-diaphorase. However, caged p-NBMB with the p-nitrobenzyl modification on the phenothiazine ring was not activated by DT-diaphorase. The result indicated that p-NBMB can be selectively reduced in the presence of NTR and hence can be used for NTR detection.
**Figure 4.5.** Fluorescence intensity of \(p\)-NBMB (10 \(\mu\)M) treated with redox enzymes.

Negative control did not include enzymes and NAD(P)H
Figure 4.6. Fluorescence spectra of $p$-NBMB (1 µM, 10 µM, 100 µM) with NTR (1 unit) in the presence of NAD(P)H (1 mM) and the negative control without NTR. Spectra were acquired in 10 mM PBS, pH 7.4 with the excitation at 580 nm.

We also examined changes in fluorescence intensity as a function of concentration of $p$-NBMB, following incubation with one unit per mL of NTR. An increase in fluorescence intensity was observed when the concentration of $p$-NBMB was increased from 1 to 10 to 100 µM (Fig. 4.6.). This result indicates that fluorescence enhancement is dependent on reduction of a $p$-nitrobenzyl redox switch by NTR. However, based on qualitative interpretation, the fluorescence intensity was not proportional to the concentration of $p$-NBMB within the concentration range. From this result, we inferred that the fluorescence enhancement would be more related to the amount of NTR. Hence, two concentrations of NTR, 1 unit mL$^{-1}$ and 2 units mL$^{-1}$, were tested to observe the redox enzyme capacity in releasing fluorophore MB from $p$-NBMB.
At the lowest p-NBMB concentration, 1 µM, the higher fluorescence emission intensity was similar, regardless of NTR concentrations. This implies that p-NBMB might show a detection limit at p-NBMB concentrations higher than 1 µM. For better visualization, we investigated the imaging capacity of p-NBMB using an in vivo imaging system, a Xenogen IVIS® Spectrum. As expected from the result showing NTR dependent fluorescence release in Fig. 4.8., images obtained from IVIS® are consistent. When the fluorescence intensity was compared by the released radiance efficiency in vivo, there were no statistically significant differences between the two p-NBMB concentrations. These results suggest that fluorescence emission occurring by p-NBMB
reduction was predominantly dependent on the reduction capacity of NTR in the presence of NAD(P)H, providing its potential use for monitoring NTR.

Figure 4.8. Fluorescence response of p-NBMB to nitroreductase. (a) IVIS fluorescence images of p-NBMB (10 µM, 100 µM) with nitroreductase (1 and 2 unit mL⁻¹, 1 mM NAD(P)H) and the control without nitroreductase. (b) Quantitation of fluorescence from each group. Data shown as mean ± SD (n = 3). Spectra were acquired after incubation at 37 °C overnight in 10 mM PBS (pH 7.4).

Since we are interested in p-NBMB application not only in NTR activatable imaging in vitro but also for therapeutic purposes, we primarily focused on the characterization of the NTR selectivity of p-NBMB. This can be eventually translated into therapeutic advances in photodynamic therapy using fluorophores. Hence, we further examined the fluorescence response of p-NBMB to intact E. coli K12 strain XL1-Blue. When incubated with 10⁹ CFU mL⁻¹ of E. coli, the NIR fluorescent probe p-NBMB was
activated and released a fluorophore showing emission as indicated in the images in Fig. 4.9 (A). In addition, \( p \)-NBMB demonstrated a concentration dependent increase in fluorescence intensity in the presence of bacteria. When the \( p \)-NBMB concentration is 1 \( \mu \)M as this bacterial concentration, no noticeable change in fluorescence emission was observed, indicating that the detection limit is 1 \( \mu \)M. This result is consistent with Fig. 4.7. We also used a serial dilution of a bacterial suspension to determine \textit{in vitro} sensitivity of \( p \)-NBMB fluorescence to bacterial number. As shown in Fig. 4.9., the raw fluorescence images and the quantified data indicated that fluorescence intensity from \( p \)-NBMB is correlated with an increase in bacterial number. At the concentration and incubation time shown, the detection limit ranged between \( 10^7 \) and \( 10^8 \) CFU mL\(^{-1}\). However, optimizing concentration of \( p \)-NBMB and incubation time could likely increase this limit. These observations implied that \textit{E. coli} expressing NTR activates reduction of a redox switch in \( p \)-NBMB to facilitate release of a fluorophore, MB.
Figure 4.9. (A) IVIS fluorescence images of p-NBMB (1 - 50 µM) incubated with intact *E. coli* K12 strain XL1-Blue ($10^9$ CFU mL$^{-1}$) at 37 °C for 4 hours. (B) Sensitivity of p-NBMB fluorescence to bacterial number. IVIS fluorescence images of p-NBMB (50 µM) incubated with a serial dilution of the intact *E. coli* K12 strain XL1-Blue suspension at 37 °C for 4 hours.
Figure 4.10. Singlet oxygen ($^1$O$_2$) generation from $p$-NBMB solution (A) with or (B) without NTR/NAD(P)H was monitored using $p$-nitrosodimethylaniline (RNO) upon laser light irradiation at a wavelength of 632 nm. Various irradiation times ranging from 10 to 75 min were used to determine the effect of irradiation time on singlet oxygen production. The concentration of $p$-NBMB was 10 $\mu$M in the enzyme assay buffer containing NTR at a concentration of 2 units mL$^{-1}$ and NAD(P)H at a concentration of 1 mM. The intensity of RNO absorbance decreased in the $p$-NBMB solution containing NTR/NAD(P)H while the solution without the redox enzyme did not affect RNO absorbance.

Along with the capacity of $p$-NBMB for imaging anaerobic bacteria expressing NTR, $p$-NBMB might be useful for the detection of hypoxia in application for tumor diagnosis based on the recently reported studies demonstrating that molecular probes caged with nitrobenzyl group showed fluorescence enhancement in hypoxia condition.
Moreover, since the fluorophore methylene blue is an effective photosensitizer for photodynamic therapy, the generation of singlet oxygen ($^1\text{O}_2$) from $p$-NBMB was tested using $p$-nitrosodimethylaniline (RNO) before and after activation by NTR. It is known that RNO is bleached in the presence of single oxygen, implying that the change in RNO absorbance intensity indicates the generation of $^1\text{O}_2$. Figure 4.10.(A) shows that the $p$-NBMB incubated with NTR/NAD(P)H exhibited a decrease in RNO absorbance after laser irradiation at a wavelength of 634 nm (110 mW/cm$^2$, Hughes Research Laboratories, Culver City, CA). In contrast, no significant change in RNO absorbance was observed from the control after laser exposure (Fig. 4.10.(B)). This result indicated that free MB released from $p$-NBMB reduction was able to generate $^1\text{O}_2$ upon an irradiation in a NIR range. Based on the work done by Komine et al. demonstrating that $^1\text{O}_2$ generated from MB (0.01%) with a laser irradiation for 5 min killed *E. faecalis* (>99.9%)$^{117}$, $p$-NBMB will be beneficial both for targeted diagnostic and therapeutic applications.

**4.4. CONCLUSIONS**

We have developed a new NIR fluorescent probe $p$-NBMB equipped with a $p$-nitrobenzyl redox switch that enables selective switching on of fluorescence by NTR. Whereas $p$-NBMB shows no fluorescence emission in PBS buffer at pH 7.4, it exhibits remarkable NIR fluorescence intensity after undergoing selective reduction by NTR. Furthermore, $p$-NBMB fluorescence was induced by incubation with live *E. coli* bacteria,
indicating that endogenous NTR can activate the nitrobenzyl moiety switch to generate the fluorophore. This collectively indicates that \( p \)-NBMB would be a suitable imaging sensor to detect bacteria expressing NTR. In addition, \( p \)-NBMB also possesses potential as an MB prodrug which can be applied for the treatment of various diseases.
CHAPTER 5

CONCLUSIONS
Incorporation of redox-responsive functionality, which are a QPA trigger and a nitrobenzyl trigger, into polymer and fluorophore, respectively, yield the materials with a redox-responsive property. We have accomplished to obtain the novel redox-responsive polymeric nanoparticles and micelles using a QPA redox trigger for targeted drug delivery. We also achieved the synthesis of the new NTR activatable NIR fluorescent probe caged by nitrobenzyl redox trigger for its selective imaging and therapy. In this chapter, we summarize dissertation research work consisting of those two materials prepared from redox triggers and discuss their challenges and future research directions.

Redox-responsive polymer was synthesized with a QPA trigger and utilized for the preparation of redox-responsive NPs. These NPs enabled to release incorporated drug triggered by stimulated redox-state using sodium dithionite. These NPs, however, were noticeably aggregated in aqueous environment because of hydrophobic nature of the polymer. Since PEG grafting has been established as a vital alternative to overcome various in vitro/in vivo stability issues of NPs, we adopted this approach to yield redox-responsive amphiphilic copolymer to improve the polymeric NPs. After successful mPEG grafting to the redox-sensitive hydrophobic polymer, polyQPA-mPEG became to be water soluble as well as self-assembled as micelles. In vitro PTX release kinetics has shown that PTX-loaded redox-responsive polyQPA-mPEG micelles were stable in aqueous environments and able to release the loaded PTX by redox-triggered QPA reduction which disrupted micelle structure. During the study on reducing enzyme-
mediated lactone release from redox-responsive micelles, we found that hydrophilic PEG blocks might contribute to increasing water solubility of polyQPA, thereby facilitating the binding between the enzyme and QPA triggers on the polymer. Therefore, the relationships between the structures of the QPA based polymer/monomer and its reduction activity as substrates of DT-diaphorase are needed to be explored for the optimization. The modification of polymers for further application in targeted delivery might be more easily achieved afterward.

We also successfully synthesized a nitroreductase-triggered near infrared fluorescent probe. Conjugating methylene blue with a redox trigger enables the fluorescent probe to be activated by 1,6-elimination in the presence of nitroreductase, resulting in the release of methylene blue fluorophore. We also found that MB release after the reduction of a probe could produce singlet oxygen, indicating that its potential use as a therapeutic tool. In the future, kinetics of photoxygeneration of this probe needs to be evaluated. The photosensitizing efficacy of this probe on viruses, yeasts, and protozoa also can be conducted for a its wide variety of applications.


104. (a) Xiong, J.; Meng, F.; Wang, C.; Cheng, R.; Liu, Z.; Zhong, Z., Folate-conjugated crosslinked biodegradable micelles for receptor-mediated delivery of


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HONORS and FELLOWSHIPS

- Summer Research Assistantship Awards, University of Mississippi 2015
- Dissertation Fellowship Award, University of Mississippi 2015
- The Mississippi Biophysical Consortium Annual Meeting Travel award 2013
- Initiation of Rho Chi Honors society 2011
PUBLICATIONS


J Bae; A Maurya; S Zia; SN Murthy; S Jo, "Novel redox-responsive amphiphilic copolymer micelles for drug delivery: Synthesis and characterization", AAPS Journal, 17(6), 2015, 1357-68.
