KINETICS STUDY OF DEGRADATION OF MALEIMIDE-THIOL CONJUGATES IN REDUCING ENVIRONMENTS

by

Haocheng Wu

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Materials Science and Engineering

Fall 2017

© 2017 Haocheng Wu
All Rights Reserved
KINETICS STUDY OF DEGRADATION OF MALEIMIDE-THIOL CONJUGATES IN REDUCING ENVIRONMENTS

by

Haocheng Wu

Approved:

Kristi L. Kiick, Ph.D.
Professor in charge of thesis on behalf of the Advisory Committee

Approved:

Darrin J. Pochan, Ph.D.
Chair of the Department of Materials Science and Engineering

Approved:

Babatunde A. Ogunnaike, Ph.D.
Dean of the College of Engineering

Approved:

Ann L. Ardis, Ph.D.
Senior Vice Provost for Graduate and Professional Education
ACKNOWLEDGMENTS

I wish to express my warmest gratitude to all those persons who have left a mark on this work, which could not be accomplished without their comments, questions, criticism, support and encouragement. Frist and foremost, I would like to thank my academic advisor, Dr. Kristi Kiick, for giving me mentorship and the opportunity to work on such an exciting project. She has always given me intellectual freedom in my work, engaged me in new ideas, and demanded a high quality of work in all my endeavors. I would also like to express my special thanks for her time, patience and understanding. She has always reassured me when I needed it, given me confidence and motivated me to improve.

My gratitude also goes to my friends and colleagues at UD for their enthusiastic help and support. I am extremely grateful to all current and past members of the Kiick group, for creating such a supportive and inspiring research platform. I would like to thank Yingkai Liang, Prathamesh Kharkar, and Tianzhi Luo, for their helpful suggestions and conversations regarding my project. I am thankful to Dr. Shi Bai for his instruction on NMR spectroscopy and the access to the advanced instruments. I’m grateful to Dr. Shuang Liu and Tianzhi Luo for their assistance in HPLC analysis experiments. I would also like to express my gratitude to all my friends and colleagues for the great friendship and support they’ve offered during my stays and making my research experience in graduate school a wonderful journey: Changhao Liu, He Zhang, Bradford Paik, Morgan Urello, Yu Tian, Eric Fowler, Lucas Dunshee, Ming Fan, Jingya Qin, Rebecca Scott, Hang Kuen Lau, Olivia George,
Shuyu Xu, Dongxia Wei, Ying Hao, Lingqing Li, and Michael Haider. Regrettably, but inevitably, the above list of names will be incomplete, and I hope that those missing on the list will still accept my sincere appreciation of their influence on my work and me.

Furthermore, I would like to acknowledge the Department of Materials Science at UD, where I spent three academic year as a graduate student, for providing supportive and stimulating working environments. I am very thankful to all current and past staff members in the Department of Materials Science, especially Charles Garbini, Kathy Forwood, Christine Williamson, for securing lab safety and providing administrative assistance. I am so privileged at MSEG department to have someone like them helping all the students.

Lastly, but most importantly, I want to thank my parents, my family and loved ones for their unconditional support and love through this long process. They are the ones that shaped me as the person I am today and gave me all the love and confidence. I am so grateful to them for everything I have. My gratitude to them is beyond words.
# TABLE OF CONTENTS

LIST OF TABLES .................................................................................................................. vii
LIST OF FIGURES ................................................................................................................ viii
ABSTRACT ............................................................................................................................ xiii

Chapter

1 INTRODUCTION .............................................................................................................. 1

1.1 Hydrogels in Drug Delivery ..................................................................................... 1

1.2 Chemical Degradation Approaches for Covalently Crosslinked Hydrogels ........ 2

1.2.1 Hydrolytic Degradation .................................................................................... 3

1.2.2 Enzymatic Degradation ................................................................................... 4

1.2.3 Photo-Triggered Degradation ......................................................................... 6

1.2.4 Thiol-Mediated Degradation ........................................................................... 7

1.2.4.1 Thiol-Disulfide Exchange Reaction ......................................................... 7

1.2.4.2 Retro Michael-type Addition Reaction .................................................... 8

1.3 Thesis Summary ..................................................................................................... 11

REFERENCES .................................................................................................................... 12

2 INTERPLAY OF ELECTRONIC EFFECTS AND THIOL PKA VALUES ON DEGRADATION KINETICS OF MALEIMIDE-THIOL CONJUGATES IN REDUCING ENVIRONMENTS ..................................................... 16

2.1 Introduction .......................................................................................................... 16

2.2 Materials and Methods ....................................................................................... 21

2.2.1 Materials ........................................................................................................ 21

2.2.2 Synthesis of Succinimide Thioether Conjugates ........................................... 21

2.2.3 NMR Analysis of MPA-Maleimide Retro Reactions .................................... 22

2.2.4 HPLC Evaluation of Reaction Kinetics ......................................................... 22

2.3 Results and Discussion ....................................................................................... 24
2.3.1 Synthesis of Succinimide Thioether Conjugates ....................... 24
2.3.2 NMR Analysis of MPA-Maleimide Retro Reactions ................ 34
2.3.3 HPLC Evaluation of Reaction Kinetics ............................... 39

2.4 Conclusions ........................................................................... 60

REFERENCES .............................................................................. 62

Appendix

COPYRIGHT PERMISSION FOR REPRINT OF PUBLISHED ARTICLES ........................................................................... 66
LIST OF TABLES

Table 2.1 Pseudo first-order rate constants and respective half-lives for retro and ring-opening reactions of MPA-maleimide conjugates.......................... 47

Table 2.2 Pseudo first-order rate constants and respective half-lives for retro and ring-opening reactions of MPP-maleimide conjugates ....................... 52

Table 2.3 Pseudo first-order rate constants and respective half-lives for retro and ring-opening reactions of NAC-maleimide conjugates .......................... 53

Table 2.4 Pseudo first-order rate constants and respective half-lives for retro and ring-opening reactions of thiol-NEM conjugates in GSSG and NDC thiol traps ........................................................................................................ 58

Table 2.5 Pseudo first-order rate constants and respective half-lives for retro and ring-opening reactions of MPA-NEM incubated at different pH........... 59
LIST OF FIGURES

Figure 1.1 Hydrolytically and enzymatically degradable hydrogels with properties that change with time. (a) Hydrolytically degradable hydrogels that can control the release of therapeutics via hydrolysis over time. (b) Proteolytically degradable hydrogels that can be selectively triggered by cell-secreted enzymes such as matrix metalloproteinases (MMPs), releasing the encapsulated therapeutics within the matrix. Reprinted by permission from Macmillan Publishers Ltd: Nature Communications (Ref 30), copyright (2012). .................................................. 5

Figure 1.2 Photo-triggered release of green fluorescent protein (GFP) from dextran-based photodegradable hydrogels via the incorporation of the photolabile o-nitrobenzyl moiety. Reproduced from Ref 36 with permission of The Royal Society of Chemistry. .......................... 6

Figure 1.3 Examples of glutathione-sensitive hydrogels for drug delivery applications. (a) Glutathione-sensitive PEG-heparin hydrogels for heparin release. (b) Dually degradable click hydrogels for controlled degradation and protein release. (c) Liposome-crosslinked hybrid hydrogels for glutathione-triggered delivery of multiple cargos molecules. Reproduced from (a) Ref 36 and (b) Ref 40 with permission of The Royal Society of Chemistry. Reprinted with permission from (c) Ref 46, copyright (2016) American Chemical Society. ............................................. 10

Figure 2.1 The order of reactivity of various vinyl groups (Michael acceptors) in thiol Michael-type addition reaction. .................................................. 17

Figure 2.2 Schematic of mechanism for the glutathione-mediated retro Michael-type addition reaction for a succinimide thioether adduct. .................. 18

Figure 2.3 The conversion of various succinimide thioethers with increased reactivity on thiol moiety to glutathione adducts via retro Michael-type addition and exchange reaction. Reprinted with permission from Ref 22, copyright (2011) American Chemical Society. ......................... 20

Figure 2.4 Simplified reaction model and rate constants of the pseudo first-order kinetics................................................................. 23
Figure 2.5 $^1$H NMR spectrum for MPA-NEM conjugate. $^1$H NMR (CD$_3$CN): $\delta$ 0.87 (t, 3H), 2.48–2.51 (dd, 1H), 3.03–3.07 (dd, 1H), 3.28 (q, 2H), 3.51 (s, 2H), 4.00–4.02 (dd, 1H), 7.18 (d, 2H), 7.36 (d, 2H). ........................................ 26

Figure 2.6 $^1$H NMR spectrum for MPA-NPM conjugate. $^1$H NMR (CD$_3$CN): $\delta$ 2.72–2.76 (dd, 1H), 3.25–3.30 (dd, 1H), 3.55 (s, 2H), 4.16–4.18 (dd, 1H), 6.92 (d, 2H), 7.22 (d, 2H), 7.29–7.33 (m, 1H), 7.36 (t, 2H), 7.43 (d, 2H). ........................................................................ 27

Figure 2.7 $^1$H NMR spectrum for MPA-NAEM conjugate. $^1$H NMR (D$_2$O): $\delta$ 2.80–2.84 (dd, 1H), 2.88–2.91 (m, 1H), 2.95–2.99 (m, 1H), 3.29–3.34 (dd, 1H), 3.52–3.60 (m, 2H), 3.68 (s, 2H), 4.25–4.27 (dd, 1H), 7.27 (d, 2H), 7.45 (d, 2H). ........................................................................ 28

Figure 2.8 $^1$H NMR spectrum for MPP-NEM conjugate. $^1$H NMR (CD$_3$CN): $\delta$ 0.85 (t, 3H), 2.48 (t, 2H), 2.47–2.51 (dd, 1H), 2.78 (t, 2H), 3.01–3.06 (dd, 1H), 3.25 (q, 2H), 3.96–3.98 (dd, 1H), 7.14 (d, 2H), 7.32 (d, 2H).............. 29

Figure 2.9 $^1$H NMR spectrum for MPP-NPM conjugate. $^1$H NMR (CD$_3$CN): $\delta$ 2.52 (t, 2H), 2.71–2.75 (dd, 1H), 2.82 (t, 2H), 3.24–3.28 (dd, 1H), 4.13–4.15 (dd, 1H), 6.91 (d, 2H), 7.19 (d, 2H), 7.31–7.34 (m, 1H), 7.37 (t, 2H), 7.39 (d, 2H). .................................................. 30

Figure 2.10 $^1$H NMR spectrum for MPP-NAEM conjugate. $^1$H NMR (D$_2$O): $\delta$ 2.59 (t, 2H), 2.82–2.85 (dd, 1H), 2.87 (t, 2H), 2.85–2.89 (m, 1H), 2.93–2.97 (m, 1H), 3.30–3.35 (dd, 1H), 3.52–3.57 (m, 2H), 4.22–4.24 (dd, 1H), 7.26 (d, 2H), 7.41 (d, 2H)............................................. 31

Figure 2.11 $^1$H NMR spectrum for NAC-NEM conjugate. $^1$H NMR (CDCl$_3$): $\delta$ 1.19 (t, 3H), 2.13 (d, 3H), 2.49–2.56 (m, 1H), 3.09–3.14 (m, 1H), 3.17–3.22 (m, 1H), 3.43–3.46 (dd, 0.5H), 3.56–3.61 (m, 2.5H), 3.77–3.80 (dd, 0.5H), 3.93–3.95 (dd, 0.5H), 4.81–4.89 (m, 1H)................. 32

Figure 2.12 $^1$H NMR spectrum for NAC-NPM conjugate. $^1$H NMR (CDCl$_3$): $\delta$ 2.10 (d, 3H), 2.65–2.73 (m, 1H), 3.11–3.20 (m, 1H), 3.33–3.39 (m, 1H), 3.50–3.63 (m, 1H), 3.95–3.98 (dd, 0.5H), 4.11–4.14 (dd, 0.5H), 4.85–4.91 (m, 1H), 7.28 (d, 2H), 7.41–7.44 (m, 1H), 7.49 (t, 2H).............. 33

Figure 2.13 $^1$H NMR spectrum for NAC-NAEM conjugate. $^1$H NMR (D$_2$O): $\delta$ 2.02 (d, 3H), 2.68–2.73 (m, 1H), 3.01–3.05 (m, 0.5H), 3.14–3.17 (m, 0.5H), 3.17–3.21 (m, 2H), 3.25–3.39 (m, 2H), 3.78–3.85 (m, 2H), 4.07–4.10 (m, 1H), 4.59–4.64 (m, 1H).................................................. 34
Figure 2.14 $^1$H NMR of aromatic protons of (A) MPA, (B) after addition of NEM to MPA, (C) immediately after addition of GSH to (MPA+NEM), and after incubation of (C) @37 $^\circ$C for (D) 24 h, (E) 5 d and (F) 10 d. Evident in (D) is free MPA and in (F) are small amounts of ring-opened MPA-NEM (7.14, 7.33 ppm) and GS-MPA mixed disulfide (7.45, 7.18 ppm).

Figure 2.15 $^1$H NMR of aromatic protons of (A) MPA, (B) after addition of NPM to MPA, (C) immediately after addition of GSH to (MPA+NPM), and after incubation of (C) @37 $^\circ$C for (D) 24 h, (E) 5 d and (F) 10 d. Evident in (D) is free MPA and in (F) are large amounts of ring-opened MPA-NPM (7.11, 7.35 ppm) and GS-MPA mixed disulfide (7.45, 7.18 ppm).

Figure 2.16 $^1$H NMR of aromatic protons of (A) MPA, (B) after addition of NAEM to MPA, (C) immediately after addition of GSH to (MPA+NAEM), and after incubation of (C) @37 $^\circ$C for (D) 24 h, (E) 5 d and (F) 10 d. Evident in (D) is free MPA and in (F) are large amounts of ring-opened MPA-NAEM (7.15, 7.34 ppm) and GS-MPA mixed disulfide (7.45, 7.18 ppm).

Figure 2.17 HPLC traces of degradation of 1 (MPA-NEM) in 50 mM phosphate buffer with 10 mM GSSG @37$^\circ$C over a period of 3 d. The peak area for 1 decreases with time as peak areas for GS-MPA and 1RO increase with time, and peak area for 3 increases first and then decreases, indicating the occurrence of the retro Michael-type reaction. Arrows indicate the direction of peak area growth or decline with time. Peak 3 consists of two equal peaks representing two diastereomers.

Figure 2.18 HPLC traces of degradation of 1 (MPA-NPM) in 50 mM phosphate buffer with 10 mM GSSG @37$^\circ$C over a period of 3 d. The peak area for 1 decreases with time as peak areas for GS-MPA and 1RO increase with time, and peak area for 3 increases first and then decreases, indicating the occurrence of the retro Michael-type reaction. Arrows indicate the direction of peak area growth or decline with time. Peak 3 consists of two equal peaks representing two diastereomers. The two split peaks of 1RO have higher intensity on the right. The four split peaks of 3RO have higher intensity on the right two.
Figure 2.19 HPLC traces of degradation of 1 (MPA-NAEM) in 50 mM phosphate buffer with 10 mM GSSG @37°C over a period of 6 h. The peak area for 1 decreases with time as peak areas for GS-MPA and 1RO increase with time, indicating the occurrence of the retro Michael-type reaction. Arrows indicate the direction of peak area growth or decline with time. The two split peaks of 1RO have higher intensity on the left. 3 was poorly retained in the column and thus was not observed. .......................... 42

Figure 2.20 Schematic of four configurations of the ring-opened products generated from two diastereomers of succinimide thioethers........................................... 43

Figure 2.21 Complete retro and ring-opening reactions of succinimide thioether conjugates in solution with glutathione.............................................................. 44

Figure 2.22 Fractional conversion to the thioether succinimide, over time, for (A) MPA-NEM, (B) MPA-NPM and (C) MPA-NAEM. Relative concentrations were measured via HPLC and the curves constructed using the derived rate constants and integrated fractional concentration equations for 1, 1RO, 3 and 3RO. .................................................. 47

Figure 2.23 The conversion of succinimide thioethers with various N-substituted groups on the maleimide moiety (MPA-NEM, MPA-NPM and MPA-NAEM) to glutathione adducts via retro Michael-type addition and exchange reaction. (EDG: electron-donating group, EWG: electron-withdrawing group)........................................................................................................... 50

Figure 2.24 Fractional conversion to the thioether succinimide, over time, for (A) MPP-NEM, (B) MPP-NPM and (C) MPP-NAEM. Relative concentrations were measured via HPLC and the curves constructed using the derived rate constants and integrated fractional concentration equations for 1, 1RO, 3 and 3RO. .................................................. 51

Figure 2.25 Fractional conversion to the thioether succinimide, over time, for (A) NAC-NEM, (B) NAC-NPM and (C) NAC-NAEM. Relative concentrations were measured via HPLC and the curves constructed using the derived rate constants and integrated fractional concentration equations for 1, 1RO, 3 and 3RO. .................................................. 52

Figure 2.26 Pseudo first-order rate constants and half-lives for selected succinimide thioether adducts. (A) Ring-opening rate constant k1, (B) retro and exchange reaction rate constant k2, (C) conversion ratio of initial adduct to glutathione adduct: k2/(k1 + k2), (D) half-lives for k1, (E) half-lives for k2 ........................................................................................................................................ 55
Figure 2.27 Fractional conversion to the thioether succinimide, over time, for (A)(B) MPA-NEM, (C)(D) MPP-NEM and (E)(F) NAC-NEM. Relative concentrations were measured via HPLC and the curves constructed using the derived rate constants and integrated fractional concentration equations for $1, 1_{RO}, 3$ and $3_{RO}$. (A)(C)(E) were tested in the presence of excess 5 mM GSSG; (B)(D)(F) were tested in the presence of excess 10 mM NDC.

Figure 2.28 Fractional conversion to the thioether succinimide, over time, for MPA-NEM incubated at (A) pH 7.4, (B) pH 6.0 and (C) pH 5.0. Relative concentrations were measured via HPLC and the curves constructed using the derived rate constants and integrated fractional concentration equations for $1, 1_{RO}, 3$ and $3_{RO}$. 
ABSTRACT

The retro-Michael addition and thiol exchange of thioether succinimide click linkages in response to thiol-containing environments offers a novel and well-established strategy for the design of glutathione-sensitive degradable hydrogels for controlled drug delivery. Here we demonstrate that the kinetics and extent of the retro Michael-type addition and thiol exchange are significantly dependent on the nature of both thiols and N-substituents of maleimides. A series of N-substituted succinimide thioethers were prepared through typical Michael-type addition. Model studies (¹H NMR, HPLC) of 4-mercaptophenylacetic acid (MPA, pKₐ 6.6) conjugated to N-ethyl maleimide (NEM), N-phenyl maleimide (NPM) or N-aminoethyl maleimide (NAEM) incubated with glutathione showed half-lives of conversion from 3.1 h to 18 h, with extents of conversion from 12.3% to 89.5%. Rapid thiol exchange is attributed to the resonance effect of the N-phenyl group for NPM conjugates, while the electron-withdrawing inductive effect of groups such as protonated N-aminoethyl group favors a higher level of ring-opening than retro reaction. Further model studies of 4-mercaptohydrocinnamic acid (MPP, pKₐ 7.0), N-acetyl-L-cysteine (NAC, pKₐ 9.5) conjugated to selected N-substituted maleimides incubated with glutathione showed half-lives of conversion from 3.6 h to 258 h, with extents of conversion from 0.8% to 90.7%. Higher pKₐ of thiols decreased the rate of the exchange reaction and limited the impact of other electronic effects of N-substituents on the extents of conversion. Additional factors affecting the degradation kinetics were studied on NEM conjugates. The kinetics of retro and exchange reaction was not hindered in thiol traps of lower
pKₐ but retarded in conditions of lower pH. These studies shed light into details of thiol and maleimide design that could be used to tune the rates of degradation of drug and polymer conjugates for a variety of applications.
Chapter 1
INTRODUCTION

1.1 Hydrogels in Drug Delivery

Hydrogels are three-dimensional, hydrophilic, crosslinked networks that imbibe and retain large amounts of water.\(^1\) They are generally considered as biocompatible materials due to their high water content and mechanical softness, similar to natural extracellular matrices.\(^2\) Hydrogels are of interest in the fields of chemistry and biomedical engineering, especially for applications in tissue engineering, wound healing, and drug delivery because of their biocompatibility, tunable biodegradability, and controllable mechanical properties.\(^3\) The porous structures and tunable viscoelasticity of hydrogels have offered substantial opportunities for the encapsulation of hydrophilic therapeutics (e.g. peptides and proteins), allowing the protection of bioactive cargos against degradation and permitting their release in bioactive form from hydrogels over prolonged periods of time.\(^4\) The delivery of therapeutics at a controlled rate to a targeted site affords opportunities to both improve treatment efficacy and reduce total treatment costs.

Based on the formation mechanisms, hydrogels can be divided into two groups: physically and chemically crosslinked hydrogels.\(^5\) Physically crosslinking via non-covalent interactions, including ionic, electrostatic or hydrophobic interactions between macromers, renders hydrogels suitable for injectable formulations, including shear thinning for injection, dynamic crosslinks for gel dissolution and drug release, and \textit{in situ} formation in the absence of initiators or catalysts.\(^6\) While covalently
crosslinked hydrogels provide better control over crosslink density and allow easier incorporation of labile functional groups for stimuli-responsive degradability of and release from the delivery vehicle. Orthogonal chemistries of exceptional efficiency and functional group versatility have been almost universally applied for the modular design of sophisticated bioconjugates with high levels of precision and control. The well-known alkyne–azide cycloaddition, Diels–Alder reaction, radical-mediated thiol–ene chemistry, Michael-type addition, and hydrazone/oxime chemistry have all been extensively explored for therapeutic delivery applications due to their fast reaction kinetics under mild conditions, allowing rapid formation in situ in the presence of cargo molecules and living cells.

There have been significant accomplishments in the development, characterization, and applications of chemically crosslinked hydrogels, and substantial effort has been directed to their controlled and/or on-demand degradation. Continued interest in and study of the controlled degradation of hydrogel materials will enable new strategies to sustain release profiles in order to expand the therapeutic window and efficacy of known drugs.

1.2 Chemical Degradation Approaches for Covalently Crosslinked Hydrogels

Hydrogel degradation permits systematic variations in the permeability of the network via engineering of the degradation rates and modes, offering great opportunities for the on-demand/target release of bioactive cargos. Over the past several decades, a variety of stimuli-responsive hydrogels have been developed via the incorporation of degradable functional groups in the backbones, crosslinks, and pendant groups. Programmed degradation of covalently cross-linked hydrogels is
commonly accomplished by incorporation of cleavable moieties that undergo ester hydrolysis or enzymatic degradation (Figure 1.1). Recently, many chemical degradation approaches have also been used to control materials-based drug delivery including photolytic degradation, thiol-disulfide exchange, and retro-Michael reaction mechanisms. The covalent bond cleavage kinetics will influence the overall rate of hydrogel degradation. To engineer hydrogel degradation properties, it is thus essential to understand the types of cleavable groups and degradation modes, byproducts and factors affecting degradation rates. These chemical degradation chemistries will be discussed in detail in the following sections.

1.2.1 Hydrolytic Degradation

Hydrolytically degradable hydrogels incorporated with labile ester linkages in the polymer backbone or crosslinker provide the simplest approach to introduce temporal changes in hydrogels (Figure 1.1a). One simple example of a degradable hydrogel features chains of poly(α-hydroxy esters), such as poly(lactic acid), introduced at the ends of PEG molecules before crosslinking with other reactive groups. Other examples include materials based on poly(vinyl alcohol), poly(propylene fumarates), dextrans and hyaluronic acid. The rate of nonspecific chemical hydrolysis depends mainly on aqueous pH and temperature, as well as on the hydrophobicity of the environment around the hydrolytically labile group. Hydrolysis usually occurs at preprogrammed rate throughout the bulk of a material, which can be tuned in efforts to mimic the rate of matrix remodeling in vivo.
1.2.2 Enzymatic Degradation

In an alternative mechanism, the peptide sequences that can be cleaved by cell-produced proteases, such as matrix metalloproteinases (MMPs), are now routinely incorporated into hydrogel crosslinks, to address the need for cell-mediated modification of biomaterials (Figure 1.1b). This approach has been used in a myriad of cases to permit cell-mediated degradation of hydrogels. 27 PEG-peptide hydrogels, pioneered by Hubbell and co-workers and employed creatively by many research groups, include protease-degradable cross-links to constructs for tissues such as bone and vascular structures.28-29 The appealing advantage of the protease-sensitive hydrogels is that the rate of their degradation can be selectively modulated in pathologies where protease activity is altered, such as rheumatoid arthritis, cancer, and after myocardial infarction.30
Figure 1.1 Hydrolytically and enzymatically degradable hydrogels with properties that change with time. (a) Hydrolytically degradable hydrogels that can control the release of therapeutics via hydrolysis over time. (b) Proteolytically degradable hydrogels that can be selectively triggered by cell-secreted enzymes such as matrix metalloproteinases (MMPs), releasing the encapsulated therapeutics within the matrix. Reprinted by permission from Macmillan Publishers Ltd: Nature Communications (Ref 30), copyright (2012).
1.2.3 Photo-Triggered Degradation

Photolabile monomers and polymers engineered to cleave under cytocompatible irradiation conditions permit spatiotemporal control of hydrogel degradation and in situ property tuning.\textsuperscript{31} Pioneered by Anseth\textsuperscript{15,32-33} and others,\textsuperscript{16,34-35} Light sensitivity of the hydrogel was introduced by placing a non-toxic photolabile o-nitrobenzyl moiety in between the dextran backbone and acrylate groups under physiological conditions. The hydrogels degraded when irradiated with cytocompatible doses of long wavelength UV, visible, or two-photon IR light (365, 405, and 740 nm, respectively), enabling precise control over hydrogel degradation profiles in situ. Photocleavable degradation based on photolabile o-nitrobenzyl derivatives allows real-time manipulation of the materials properties and photocontrolled, on-demand release of proteins.

Figure 1.2 Photo-triggered release of green fluorescent protein (GFP) from dextran-based photodegradable hydrogels via the incorporation of the photolabile o-nitrobenzyl moiety. Reproduced from Ref 36 with permission of The Royal Society of Chemistry.
1.2.4 Thiol-Mediated Degradation

Biological systems comprise multiple thiol-containing molecules, including glutathione (GSH), which acts as a cellular reducing agent and is found at different concentrations in intracellular (≈10 mM) and extracellular compartments (<10 μM) in living cells. The largely different redox potential between intracellular and extracellular compartments, as well as the further elevated concentration of GSH in cancer cells, have been a target of particular interest for the promotion of stimuli-responsive materials. The incorporation of GSH-sensitive linkages in biomaterials can permit selective degradation in the presence of GSH. These thiol-sensitive hydrogels can undergo cleavage reactions (disulfide cleavage or retro-Michael reaction) that lead to matrix degradation upon exposure to GSH, allowing the targeted release and stimuli-triggered delivery of therapeutic molecules relevant to cancer applications. Since the rate of degradation and release of cargo molecules from these gels depend upon the local reducing environment, this degradation strategy is promising for intracellular or site-specific controlled drug delivery.

1.2.4.1 Thiol-Disulfide Exchange Reaction

The thiol–disulfide exchange reaction is key to a number of important biological processes, such as the formation and cleavage of structural cysteine disulfide bonds and disulfidemediated redox reactions. The reversible cross-links are typically incorporated into the polymer chains via reductively cleavable disulfide groups. The thiol–disulfide exchange reaction is driven using an excess of thiolate and its pKa. Disulfide bonds have relatively short half-lives (< 1 h) in highly reductive environments, while maintaining a degree of stability in circulation. Nonetheless, the window for the cleavage kinetics to be engineered is considerably limited even if
by altering the disulfides’ neighboring chemical substituents (half-lives ranging from 8 to 45 min), temporally limiting the delivery process (ca. 12-24 h). \(^{44}\)

### 1.2.4.2 Retro Michael-type Addition Reaction

Alternatively, another class of thiol-mediated degradable hydrogels is based on the thiol-maleimide Michael addition reaction, which is selective in aqueous environments (at physiological pH, amines react generally 1 order of magnitude slower than thiols). \(^{45}\) The stimuli-responsive degradation mechanism, retro-Michael-type addition reaction and thiol exchange, involves covalent bond transfer from the initial succinimide thioether compound to a stable GSH conjugate in the presence of excess glutathione. In contrast to thiol-disulfide exchange based hydrogels, novel GSH-sensitive hydrogels developed by our group (Figure 1.3a), in which degradation is mediated by retro-Michael-type addition and subsequent thiol exchange, have demonstrated increased stability against GSH with 10-fold slower rates of degradation. \(^{18}\) The use of cleavable click linkages formed by Michael-type addition reactions in conjunction with hydrolytically cleavable functionalities for the degradation of injectable hydrogels by dual mechanisms for controlled protein release (Figure 1.3b), has demonstrated a control over the degradation rate within a reducing microenvironment resulted in ~2.5 fold differences in the release profile of cargo molecules by employing different combinations of the thiol functional groups. \(^{40}\) The use of liposome-crosslinked, multi-component hydrogel systems (Figure 1.3c) may further extend the lifetime of the hydrogels, owing to the steric hindrance as well as the local hydrophobic environment of the arylthioether succinimide crosslinks at the polymer-liposome interface. \(^{46}\) The enhanced stability allows release of multiple
encapsulated cargo molecules over extended time scales (ca. 3-6 days), indicating promising application for tailoring cargos release within tumor microenvironments.
Figure 1.3 Examples of glutathione-sensitive hydrogels for drug delivery applications. (a) Glutathione-sensitive PEG-heparin hydrogels for heparin release. (b) Dually degradable click hydrogels for controlled degradation and protein release. (c) Liposome-crosslinked hybrid hydrogels for glutathione-triggered delivery of multiple cargos molecules. Reproduced from (a) Ref 36 and (b) Ref 40 with permission of The Royal Society of Chemistry. Reprinted with permission from (c) Ref 46, copyright (2016) American Chemical Society.
1.3 Thesis Summary

The different hydrogel degradation chemistries introduced above (i.e., thiol-disulfide reaction, retro-Michael-type reaction) provide a versatile synthetic toolbox for producing stimuli-responsive degradable hydrogels that degrade in a controlled manner. Particularly, the retro Michael-type addition reaction offers a promising strategy for the reduction-mediated degradation or release with increased blood stability and prolonged drug delivery timescales, compared to disulfide moieties. Continued investigation of these degradation approaches as well as the development of new chemical reactions will open doors to other avenues of on-demand degradation/target release and expand the application space for these materials. In Chapter 2, a library of maleimide-thiol conjugate reagents were developed. Series of kinetics study on the degradation of succinimide thioether adducts in the presence of glutathione at physiological pH and temperature, including NMR analysis and HPLC evaluation approaches, were conducted to explore the dependence of kinetics of retro and ring-opening reactions, on the nature of both thiol substituents and N-substituents of maleimides. Rate constants and half-lives describing the reaction kinetics were calculated and analyzed. Additional factors affecting the degradation and hydrolysis kinetics including the presence of thiol traps and variations of pH values were also investigated and discussed. The aim of our study is to provide valuable insight into the retro Michael-type addition reaction and further explore its possibilities for longer-term delivery of drugs and degradation of materials in reducing environments.
REFERENCES


Chapter 2

INTERPLAY OF ELECTRONIC EFFECTS AND THIOL PKA VALUES ON DEGRADATION KINETICS OF MALEIMIDE-THIOL CONJUGATES IN REDUCING ENVIRONMENTS

2.1 Introduction

Reduction-sensitive biodegradable polymers and bioconjugates serving as intracellular triggered drug and gene delivery systems have attracted much attention.\textsuperscript{1-3} By introducing reduction-sensitive bonds in general, hydrogel degradation and/or drug release takes advantage of naturally existing reducing agents, such as glutathione (GSH).\textsuperscript{4} Typically, the extracellular concentration of glutathione in plasma is around 2–20 µM; whereas the concentration in cytosol, mitochondria and cellular nuclei is well above that, in the range of 0.5–10 mM,\textsuperscript{5} with an elevated level of GSH content in carcinoma cells in particular.\textsuperscript{6} The concentration gradient facilitates the disruption of conjugates and hydrogels intracellularly while offers a degree of stability in circulation.\textsuperscript{5-7} A common motif in the design of reduction-sensitive bioconjugates involves the incorporation of disulfides, which can undergo thiol-disulfide exchange under reductive environments to achieve rapid cleavage, at a time scale from minutes to hours.\textsuperscript{8-9} Nonetheless, the window for the cleavage kinetics to be engineered is considerably limited even if by altering the disulfide’s neighboring chemical substituents.\textsuperscript{10}

In contrast to disulfide-based bioconjugation strategy, the thiol-maleimide Michael addition reaction has been widely implemented in biological systems,
primarily due to the selectivity of the thiol-maleimide reaction in aqueous environments, the rapid kinetics associated with the reaction, and the stability of the thiol-maleimide product. N-substituted maleimides are most frequently utilized as they react quickly as Michael acceptor (Figure 2.1), give no by-products and have high thiol specificity; the nitrogen atom in the ring allows facile functionalization and many such functionalized maleimides are commercially available. The applications of N-substituted maleimides range from performing measurements of thiols in biological fluids, in vivo imaging studies, structural and functional studies of proteins and synthesis of protein-drug conjugates, to the development of bioconjugates such as artificial metalloenzymes and biosensors. The thiol-maleimide reaction has been employed in the cross-linking of hydrogels, the fluorescent labeling of molecules and, more recently, for the ability of the succinimide thioether bonds to undergo retro Michael-type addition reaction in reducing environments.

![Figure 2.1](image.png)

Figure 2.1 The order of reactivity of various vinyl groups (Michael acceptors) in thiol Michael-type addition reaction.
As reported, select succinimide thioethers can undergo retro Michael-type addition and subsequent thiol exchange reaction in the presence of other thiol compounds such as glutathione at physiological pH and temperature. Figure 2.2 shows the reversibility of the Michael-type addition reaction between maleimide and thiols under certain conditions as a potential controlled degradation mechanism. The first step is a retro Michael-type addition to regenerate maleimide 2, which can then react with other thiols in the system, generally glutathione. The equilibrium is significantly shifted to favor the thioether succinimides 1 and 3. Meanwhile, both thioether succinimides 1 and 3 can undergo hydrolysis and the products respectively can no longer undergo the retro Michael-type addition.

Figure 2.2 Schematic of mechanism for the glutathione-mediated retro Michael-type addition reaction for a succinimide thioether adduct.

Baldwin et al. studied the kinetics of the retro Michael-type addition and exchange reactions undergone by succinimide thioethers in excess glutathione, highlighting the potential of employing the strategy for controlled release of drugs or degradation of materials. The study concludes that the kinetics and extent of the retro Michael-type addition and thiol exchange is significantly dependent on the nucleophilicity of the Michael donor (Figure 2.3). For example, aryl thiol substituents
such as 4-mercaptophenylacetic acid (pKₐ 6.6, blue trace in Figure 2.3) will favor the retro and exchange reaction while alkyl thiol substituents such as 3-mercaptopropionic acid (pKₐ 10.3, green trace in Figure 2.3) render the succinimide thioethers fully stable under the conditions tested. The succinimide thioethers with thiol substituents of intermediate pKₐs, such as N-acetyl-cysteine (pKₐ 9.5, red trace in Figure 2.3) will undergo retro and exchange reaction but at a slower rate compared to the one bearing less acidic substituents. Highlighted in the study, the arylthioether-succinimide adducts have a cleavage rate 10–100× lower than that reported for the similar cleavage of disulfides while more rapid than that of the cleavage of certain cysteine-maleimide adducts, providing similar and complementary applications to the disulfide-mediated drug release in reducing environments.²³⁻²⁴ The reversible chemistry for reaction of arylthioether-succinimide adducts with GSH yields a more stable thioether product, the conversion of which reaches approximately 90% in few days. The labile succinimide-thioether linkages formed via the Michael-type addition of aromatic thiols to maleimides, with tunable sensitivity to physiologically relevant reducing potentials, offers a promising strategy for the reduction-mediated degradation or release with increased blood stability and prolonged drug delivery timescales to disulfide moieties.²⁵
In conclusion, degradation strategies that exploit the retro Michael-type reaction of thioether succinimides may find biomedical applications when a slower release or degradation profile is required. Without disregarding the wide scope of the transformations, the competing hydrolysis reaction reduces its versatility, as the ring-opened product is stabilized toward cleavage. Shaun et al.\textsuperscript{26} studied the rates of ring-opening hydrolysis and thiol exchange of a range of N-substituted succinimide thioethers formed by maleimide–thiol conjugation, suggesting conjugates made with electron-withdrawing maleimides can be purposefully hydrolyzed rapidly in vitro to ensure in vivo stability. The research focus on either utilizing or avoiding retro Michael-type addition and exchange reaction of succinimide thioethers indicates a dependence of kinetics of the competing reactions—thiol exchange and hydrolysis—on the nature of both thiol substituents and N-substituents of maleimides. Thus, this chapter gives a thorough understanding of how reagents involved in retro Michael-
type addition and exchange reaction give a combined effect on the degradation kinetics by altering $pK_a$s of thiols, thiol traps and the strength of the electronic effect of N-substituted groups on succinimide, and to enable the design of thiol-mediated degradable materials for specific advanced applications.

2.2 Materials and Methods

2.2.1 Materials

N-ethylmaleimide (NEM), N-phenylmaleimide (NPM), N-(2-aminoethyl)maleimide (NAEM), 4-mercaptophenylacetic acid (MPA), N-acetyl-L-cysteine (NAC), N-diethyl-cysteamine (NDC) were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification. 4-mercaptohydrocinnamic acid (MPP) was purchased from TCI America (Portland, OR, USA). All other reagents and materials including glutathione (GSH) and oxidized glutathione (GSSG) were purchased from Fisher Scientific (Pittsburgh, PA, USA) unless otherwise noted. $^1$H NMR spectra were acquired under standard quantitative conditions at ambient temperature on a Bruker AV400 NMR spectrometer (Billerica, MA, USA).

2.2.2 Synthesis of Succinimide Thioether Conjugates

The thiols including the mercapto-acids, MPA and MPP, and cysteine derivative NAC were dissolved at a concentration of 50 mg/mL in 1 mL acetonitrile and reacted with molar equivalents of the maleimides, NEM, NPM and NAEM. A catalytic amount of triethylamine (0.01×) was added to the mixture. The reaction was stirred for 1 h at room temperature. The crude product was diluted to 5 mL with water and purified via reverse-phase HPLC (Waters Inc., Milford, MA) on a Waters Xbridge
BEH130 Prep C-18 column. Purified fractions were collected and freeze–dried, with approximately 90% yield for all reactions.

2.2.3 NMR Analysis of MPA-Maleimide Retro Reactions

$^1$H NMR spectroscopy with W5 water suppression$^{27}$ was used to monitor retro reactions of MPA-maleimide conjugates. Samples of MPA were dissolved at a concentration of 3 mg/mL in 0.2 M phosphate buffer pH 7.4 with 10% D$_2$O. High buffer concentrations were needed (relative to those employed in the HPLC experiments below) in order to maintain a constant pH throughout the experiment, owing to the high concentration of MPA necessary for NMR investigation. A molar equivalent of maleimide (NEM, NPM, NAEM) was added to each vessel and the NMR spectrum was recorded. Molar equivalents of GSH or GSSG were added to the samples, as the oxidation state of GSH does not significantly impact the rate of thiol-exchange.$^{22}$ After addition of compounds, the pH was adjusted to 7.4 with 0.2 M NaOH if necessary. Samples were incubated at 37°C and spectra were recorded at time 0, 24 h, 5 d and 10 d.

2.2.4 HPLC Evaluation of Reaction Kinetics

Synthesized conjugates were dissolved at a concentration of 0.1 mM in 50 mM phosphate buffers (pH 7.4) containing 5 mM GSSG. A GSSG thiol trap rather than a GSH diluent was used in these studies for the convenience of not having to avoid thiol oxidation over long periods while gave the same rates of hydrolysis and thiol release.$^{22}$ Lower buffer concentrations were employed to permit quantitative detection by HPLC, and these lower concentrations were sufficient to maintain a constant pH throughout the experiment. The kinetics of succinimide ring-opening were measured by
monitoring reactions incubated without reductant. The pH values of all samples were verified and adjusted to 7.4 with 0.2 M NaOH if needed before incubation at 37 °C. 150 µL samples were collected periodically and added to 150 µL of 0.5% formic acid solution to reduce the pH and quench the retro and ring-opening reactions. Samples were stored at −20 °C until analyzed. RP-HPLC injections were carried out under the above-defined conditions and areas of peaks were integrated to calculate conversion curves. The identities of the compounds present in each peak were determined using LC-MS. The same reactions of certain synthesized conjugates were then investigated when the only change of condition is: 5 mM GSSG thiol trap being replaced by 10 mM NDC; pH being maintained at 5.0 (50 mM acetate buffer, pH 5) and 6.0 (50 mM phosphate buffer, pH 6) respectively throughout the reactions.

The kinetics model applied to the measured data was simplified to the combination of a consecutive and parallel reactions (Figure 2.4). Pseudo first-order rate constants $k_1$ and $k_3$ were defined for the ring-opening of 1 and 3, and $k_2$ defined for the retro and exchange reaction of 1 to yield 3 in the presence of excess thiol.

![Figure 2.4 Simplified reaction model and rate constants of the pseudo first-order kinetics.](image)

Based on the simplified kinetics model, reaction rate equations (eqs 1–4)
\[
\frac{d[1]}{dt} = -k_1[1] - k_2[1]
\]  
(1)

\[
\frac{d[1_{RO}]}{dt} = k_1[1]
\]  
(2)

\[
\frac{d[3]}{dt} = k_2[1] - k_3[3]
\]  
(3)

\[
\frac{d[3_{RO}]}{dt} = k_3[3]
\]  
(4)

can be defined and converted to integrated rate laws (eqs 5–8)

\[
[1] = [1]_0 e^{-(k_1+k_2)t}
\]  
(5)

\[
[1_{RO}] = \frac{k_1[1]_0}{k_1 + k_2} \left(1 - e^{-(k_1+k_2)t}\right)
\]  
(6)

\[
[3] = \frac{k_2[1]_0}{k_3 - k_2 - k_1} \left(e^{-(k_1+k_2)t} - e^{-k_3t}\right)
\]  
(7)

\[
[3_{RO}] = \frac{k_3k_2[1]_0}{k_3 - k_2 - k_1} \left(\frac{e^{-(k_1+k_2)t} - 1}{-k_1 - k_2} + \frac{e^{-k_3t} - 1}{k_3}\right)
\]  
(8)

The equations were employed to fit the data measured by HPLC, yielding \( k_1 \), \( k_2 \) and \( k_3 \) with corresponding half-lives. Curves to plot the fractional concentrations of the compounds as a function of time were constructed, by input of the rate constants into eqs 5–8.

2.3 Results and Discussion

2.3.1 Synthesis of Succinimide Thioether Conjugates

The succinimide thioether conjugates were successfully prepared through a typical Michael-type addition reaction with the base-initiated mechanism: A catalytic
amount of weak base TEA was used to deprotonate some quantity of available thiol. The resulting thiolate anion, a strong nucleophile, attacks the π-bond of maleimide, resulting in a strongly basic enolate intermediate. This intermediate deprotonates an additional equivalent of thiol, giving the desired addition product as well as another equivalent of thiolate that can perpetuate the catalytic cycle. Three identities of thiols MPA (pKₐ 6.6), MPP (pKₐ 7.0) and NAC (pKₐ 9.5) were selected as Michael donor reagents, and three identities of maleimides NEM, NPM and NAEM with different electronic effects on N-substituents were selected as Michael acceptor reagents. The nine categories of succinimide thioether conjugate products were respectively synthesized with a yield of approximately 90% for all reactions and confirmed by ¹H NMR (Figure 2.5-2.13).
Figure 2.5 $^1$H NMR spectrum for MPA-NEM conjugate. $^1$H NMR (CD$_3$CN): $\delta$ 0.87 (t, 3H), 2.48–2.51 (dd, 1H), 3.03–3.07 (dd, 1H), 3.28 (q, 2H), 3.51 (s, 2H), 4.00–4.02 (dd, 1H), 7.18 (d, 2H), 7.36 (d, 2H).
Figure 2.6 $^1$H NMR spectrum for MPA-NPM conjugate. $^1$H NMR (CD$_3$CN): $\delta$ 2.72–2.76 (dd, 1H), 3.25–3.30 (dd, 1H), 3.55 (s, 2H), 4.16–4.18 (dd, 1H), 6.92 (d, 2H), 7.22 (d, 2H), 7.29–7.33 (m, 1H), 7.36 (t, 2H), 7.43 (d, 2H).
Figure 2.7 $^1$H NMR spectrum for MPA-NAEM conjugate. $^1$H NMR (D$_2$O): $\delta$ 2.80–2.84 (dd, 1H), 2.88–2.91 (m, 1H), 2.95–2.99 (m, 1H), 3.29–3.34 (dd, 1H), 3.52–3.60 (m, 2H), 3.68 (s, 2H), 4.25–4.27 (dd, 1H), 7.27 (d, 2H), 7.45 (d, 2H).
Figure 2.8 $^1$H NMR spectrum for MPP-NEM conjugate. $^1$H NMR (CD$_3$CN): $\delta$ 0.85 (t, 3H), 2.48 (t, 2H), 2.47–2.51 (dd, 1H), 2.78 (t, 2H), 3.01–3.06 (dd, 1H), 3.25 (q, 2H), 3.96–3.98 (dd, 1H), 7.14 (d, 2H), 7.32 (d, 2H).
Figure 2.9 \(^1\)H NMR spectrum for MPP-NPM conjugate. \(^1\)H NMR (CD\(_3\)CN): \(\delta\) 2.52 (t, 2H), 2.71–2.75 (dd, 1H), 2.82 (t, 2H), 3.24–3.28 (dd, 1H), 4.13–4.15 (dd, 1H), 6.91 (d, 2H), 7.19 (d, 2H), 7.31–7.34 (m, 1H), 7.37 (t, 2H), 7.39 (d, 2H).
Figure 2.10 $^1$H NMR spectrum for MPP-NAEM conjugate. $^1$H NMR (D$_2$O): $\delta$ 2.59 (t, 2H), 2.82–2.85 (dd, 1H), 2.87 (t, 2H), 2.85–2.89 (m, 1H), 2.93–2.97 (m, 1H), 3.30–3.35 (dd, 1H), 3.52–3.57 (m, 2H), 4.22–4.24 (dd, 1H), 7.26 (d, 2H), 7.41 (d, 2H).
Figure 2.11 $^1$H NMR spectrum for NAC-NEM conjugate. $^1$H NMR (CDCl$_3$): $\delta$ 1.19 (t, 3H), 2.13 (d, 3H), 2.49–2.56 (m, 1H), 3.09–3.14 (m, 1H), 3.17–3.22 (m, 1H), 3.43–3.46 (dd, 0.5H), 3.56–3.61 (m, 2.5H), 3.77–3.80 (dd, 0.5H), 3.93–3.95 (dd, 0.5H), 4.81–4.89 (m, 1H).
Figure 2.12 $^1$H NMR spectrum for NAC-NPM conjugate. $^1$H NMR (CDCl$_3$): δ 2.10 (d, 3H), 2.65–2.73 (m, 1H), 3.11–3.20 (m, 1H), 3.33–3.39 (m, 1H), 3.50–3.63 (m, 1H), 3.95–3.98 (dd, 0.5H), 4.11–4.14 (dd, 0.5H), 4.85–4.91 (m, 1H), 7.28 (d, 2H), 7.41–7.44 (m, 1H), 7.49 (t, 2H).
Figure 2.13 $^1$H NMR spectrum for NAC-NAEM conjugate. $^1$H NMR (D$_2$O): $\delta$ 2.02 (d, 3H), 2.68–2.73 (m, 1H), 3.01–3.05 (m, 0.5H), 3.14–3.17 (m, 0.5H), 3.17–3.21 (m, 2H), 3.25–3.39 (m, 2H), 3.78–3.85 (m, 2H), 4.07–4.10 (m, 1H), 4.59–4.64 (m, 1H).

2.3.2 NMR Analysis of MPA-Maleimide Retro Reactions

After obtaining the series of succinimide thioether conjugates, we sought to validate that retro Michael-type additions were a significant reaction for select succinimide thioethers in reducing environments and to determine the difference in thiol exchange reactions when the N-substituents on the maleimide moiety were varied. MPA was selected as the standard thiol moiety. Kinetics experiments were performed via $^1$H NMR spectroscopy, by monitoring the intensity of the protons in the aromatic
region (6.9-7.5 ppm). The formation of the Michael-type adducts and their degradation products were easily observed in the NMR spectra.

Figure 2.14 shows the $^1$H NMR analysis for the reaction of MPA with NEM, with subsequent incubation with GSH. Figure 2.14A shows the $^1$H NMR spectrum for MPA in solution. A majority of MPA in the reduced state was detected with chemical shifts centered at 6.92 and 7.18 ppm. Minor peaks centered at 7.14 and 7.42 ppm were contributed from the oxidized MPA. After addition of a molar equivalent of NEM to the solution, the peaks for reduced MPA totally disappeared and the aromatic protons shifted downfield to peaks centered at 7.18 and 7.36 ppm (Figure 2.14B), indicating the formation of MPA-NEM. The small fraction of oxidized MPA was immediately reduced when a molar equivalent of GSH was added, indicated by a shift of peaks from 7.14 and 7.42 ppm to 6.92 and 7.18 ppm (Figure 2.14C). After 24 h incubation at 37°C, much more free MPA in the reduced state was liberated from the conjugate as indicated by the increased intensity of aromatic protons centered at 6.92 and 7.18 ppm (Figure 2.14D). After 5 d incubation (Figure 2.14E), a minor amount of ring-opened MPA-NEM was detected at 7.14 and 7.33 ppm. Meanwhile, oxidized MPA (7.14 and 7.42 ppm) and GS-MPA (7.18 and 7.45 ppm) started to form. After 10 d incubation, more ring-opened adduct and GS-MPA were accumulated (Figure 2.14F), while the low intensity of peaks for ring-opened adduct indicates this occurs at a significantly slower rate than the retro reaction. With more and more GSH being consumed for the retro reaction and MPA reduction, the free MPA liberated from the adduct via retro reaction was again converted to its oxidized form and GS-MPA. These results are comparable to the kinetics studies performed by Baldwin et al.\textsuperscript{22} Retro Michael-type
addition can be a significant reaction compared to hydrolysis for select succinimide thioethers in reducing environments near physiological conditions.

Figure 2.15 and Figure 2.16 show a very similar process of conjugate formation, retro reaction and hydrolysis for MPA-NPM and MPA-NAEM. But for MPA-NPM (Figure 2.15), the ring-opened adduct (7.11 and 7.35 ppm) represented a large proportion of the final products, indicating a fast hydrolysis in solution. The higher intensity of peaks for free MPA (6.92 and 7.18 ppm) in Figure 2.15D also indicates a faster retro reaction compared to that in Figure 2.14D. In contrast to MPA-NEM, both the retro reaction and hydrolysis in MPA-NPM system occurred more rapidly. For MPA-NAEM (Figure 2.16), the hydrolysis process was even more dominant in solution. The peaks of ring-opened adduct centered at 7.15 and 7.34 ppm have obviously much higher intensity and proportion than any other peak species after incubation for more than 24 h.
Figure 2.14 $^1$H NMR of aromatic protons of (A) MPA, (B) after addition of NEM to MPA, (C) immediately after addition of GSH to (MPA+NEM), and after incubation of (C) @37°C for (D) 24 h, (E) 5 d and (F) 10 d. Evident in (D) is free MPA and in (F) are small amounts of ring-opened MPA-NEM (7.14, 7.33 ppm) and GS-MPA mixed disulfide (7.45, 7.18 ppm).
Figure 2.15 $^1$H NMR of aromatic protons of (A) MPA, (B) after addition of NPM to MPA, (C) immediately after addition of GSH to (MPA+NPM), and after incubation of (C) @37 °C for (D) 24 h, (E) 5 d and (F) 10 d. Evident in (D) is free MPA and in (F) are large amounts of ring-opened MPA-NPM (7.11, 7.35 ppm) and GS-MPA mixed disulfide (7.45, 7.18 ppm).
Figure 2.16 $^1$H NMR of aromatic protons of (A) MPA, (B) after addition of NAEM to MPA, (C) immediately after addition of GSH to (MPA+NAEM), and after incubation of (C) @37 °C for (D) 24 h, (E) 5 d and (F) 10 d. Evident in (D) is free MPA and in (F) are large amounts of ring-opened MPA-NAEM (7.15, 7.34 ppm) and GS-MPA mixed disulfide (7.45, 7.18 ppm).

2.3.3 HPLC Evaluation of Reaction Kinetics

HPLC and LC/MS were used for quantification and identification in the study of reaction kinetics. These maleimides with different N-substituents (ethyl, phenyl, aminoethyl, respectively) were selected to determine how the rate of exchange might
vary with the electronic effects of N-substituents on maleimides. 0.1 mM of 1 was incubated in 10 mM GSSG in phosphate buffer at pH 7.4 and 37 °C. The ring-opening rates of succinimide were determined from solution 1 without addition of GSSG. Reaction kinetics for the formation of 3 was determined by monitoring the changes of peak area in HPLC traces, and the identity of chemicals in each fraction was confirmed by LC/MS.

Figure 2.17 represents a typical set of HPLC traces monitoring the incubation of 1 (MPA-NEM) in excess GSSG, showing the retention time and intensity of all peaks. Arrows indicate the direction of peak growth or decline with time. At time zero, 1 was observed as a single peak. Over time, the peak for 1 decreased while peaks for GS-MPA and 1_{RO} (ring-opened 1) all increased. The peak for 3 increased first as a result of its generation from the retro reaction of 1, and then decreased due to its hydrolysis throughout the time. Peak 3 is split as a result of two diastereomers formed from the Michael-type addition of GSH and NEM. The peak for 1_{RO} is split depending on the side of succinimide ring-opening in relation to the thioether. Figure 2.18 shows that the HPLC traces of 1 (MPA-NPM) incubated under same conditions are pretty similar to the traces for MPA-NEM. The peak 3_{RO} could also be observed since the phenyl group decreased the polarity of the molecule. The unequal split of peak 1_{RO} (higher intensity on the right split peak) indicates a preferred configuration of ring-opened succinimide thioethers 1, which is favored as a result of the resonance effect and steric effect from the phenyl group. 3_{RO} was evenly splitted into four peaks from the two diastereomers of 3, with two preferred configurations as well. The schematic of the four configurations of 3_{RO} is shown in Figure 2.20. Figure 2.19 shows the HPLC traces of 1 (MPA-NAEM) incubated under the same condition. Since the
protonated amine groups at neutral pH increase the polarity of the molecule, 3 and \( 3_{RO} \) are so poorly retained by the column that their peaks were not observed. The unequal split of peak \( 1_{RO} \) (higher intensity on the left split peak) indicates a preferred configuration of ring-opened succinimide thioethers 1, which is favored as a result of the electron-withdrawing effect from the protonated aminoethyl group.

![HPLC traces of degradation of 1 (MPA-NEM) in 50 mM phosphate buffer with 10 mM GSSG @37°C over a period of 3 d. The peak area for 1 decreases with time as peak areas for GS-MPA and \( 1_{RO} \) increase with time, and peak area for 3 increases first and then decreases, indicating the occurrence of the retro Michael-type reaction. Arrows indicate the direction of peak area growth or decline with time. Peak 3 consists of two equal peaks representing two diastereomers.](image)

Figure 2.17 HPLC traces of degradation of 1 (MPA-NEM) in 50 mM phosphate buffer with 10 mM GSSG @37°C over a period of 3 d. The peak area for 1 decreases with time as peak areas for GS-MPA and \( 1_{RO} \) increase with time, and peak area for 3 increases first and then decreases, indicating the occurrence of the retro Michael-type reaction. Arrows indicate the direction of peak area growth or decline with time. Peak 3 consists of two equal peaks representing two diastereomers.
Figure 2.18 HPLC traces of degradation of 1 (MPA-NPM) in 50 mM phosphate buffer with 10 mM GSSG @37°C over a period of 3 d. The peak area for 1 decreases with time as peak areas for GS-MPA and 1RO increase with time, and peak area for 3 increases first and then decreases, indicating the occurrence of the retro Michael-type reaction. Arrows indicate the direction of peak area growth or decline with time. Peak 3 consists of two equal peaks representing two diastereomers. The two split peaks of 1RO have higher intensity on the right. The four split peaks of 3RO have higher intensity on the right two.

Figure 2.19 HPLC traces of degradation of 1 (MPA-NAEM) in 50 mM phosphate buffer with 10 mM GSSG @37°C over a period of 6 h. The peak area for 1 decreases with time as peak areas for GS-MPA and 1RO increase with time, indicating the occurrence of the retro Michael-type reaction. Arrows indicate the direction of peak area growth or decline with time. The two split peaks of 1RO have higher intensity on the left. 3 was poorly retained in the column and thus was not observed.
Figure 2.20 Schematic of four configurations of the ring-opened products generated from two diastereomers of succinimide thioethers.

Figure 2.21 illustrates the complete reactions for an initial succinimide thioether conjugate 1 in the presence of excess GSH, showing ring-opening of the thioether succinimides as well as the exchange with GSH. Throughout these experiments, no measurable amount of free maleimide 2 was detected, confirming that equilibrium favors the succinimide thioether greatly under these experimental conditions. The fate of the succinimide thioether conjugate 1 can be simplified to the combination of hydrolysis (1 → 1RO) and retro and exchange reaction (1 → 3), followed by the ring-opening of 3 (3 → 3RO). Pseudo first-order rate constants, $k_1$ can be defined for the ring-opening of 1. Since a large excess of the thiol trap (10 mM GSH or 5 mM GSSG), $k_2$ can be defined as the pseudo first-order rate constant for the retro and exchange reaction of 1.
Figure 2.21 Complete retro and ring-opening reactions of succinimide thioether conjugates in solution with glutathione.

Fractional concentrations of $1$, $1_{RO}$, $3$, $3_{RO}$ (converted to the initial thioether succinimide linkage of $1$) measured by HPLC were plotted as a function of time for MPA-NEM (Figure 2.22A), MPA-NPM (Figure 2.22B) and MPA-NAEM (Figure 2.22C). Pseudo first-order rate equations were employed to fit the data ($R^2 > 0.98$) and rate constants were then obtained. The values of ring-opening rate constant $k_1$ and retro reaction rate constant $k_2$ with their corresponding half-lives were shown in Table 2.1. By input of rate constants into equations, curves were constructed to plot the fractional concentration of each compound as a function of time in Figure 2.22. The fits show perfect agreement with data points for all compounds. Only the sum of
fractional concentration of 3 and 3_{RO} are shown in Figure 2.22C as the corresponding peaks for each were not observed in HPLC. The fractional concentration of 1 decreased as a function of time and was eventually all consumed. The fractional concentration of 3 was illustrated by an initial increase and subsequent decrease, depending on the extent of retro reaction and hydrolysis. When all reactions reach equilibrium, the end products 1_{RO} and 3_{RO} were completely stable in solution. The final fractional concentrations of 1_{RO} (red plateau in Figure 2.22) and 3_{RO} (green/purple plateau in Figure 2.22) were directly related to \( k_1 \) and \( k_2 \): \([3_{RO}]_f/[1_{RO}]_i = k_2/k_1\), of which the ratio indicates that either the retro and exchange reaction or the ring-opening of 1 predominates in solution. In the reactions of MPA-NAEM, 1_{RO} is apparently the major product at equilibrium, with rate constant \( k_1 \) of 0.9828 h\(^{-1}\) and respective half-life of 0.7 h. While in comparison, the ring-opening of MPA-NEM is three orders of magnitude slower with an apparent rate constant \( k_1 \) of 0.0045 h\(^{-1}\) and respective half-life of 154 h, along with the ring-opening of MPA-NPM one order of magnitude slower. The dramatic differences in the ring-opening rates of the three succinimide thioethers are most likely attributable to the inductive effects of the N-substituents of the maleimide moieties. The protonated aminoethyl groups at physiological pH have large electron-withdrawing inductive effect, which facilitates rapid hydrolysis of MPA-NAEM, while alkyl N-substituted groups such as the ethyl group are weak electron-donating groups and retard the ring-opening of MPA-NEM. The ring-opening of MPA-NPM is only 6-fold faster than that of MPA-NEM, however, as the electron-withdrawing effect of phenyl groups is weak. Our previous report has shown similar pseudo first-order rate constants \( k_1 \) for the ring-opening of NEM conjugates (NEM conjugated to MPA, NAC and 3-mercaptopropionic acid),
which range from 0.0032 h\(^{-1}\) to 0.0044 h\(^{-1}\).\(^{22}\) Kanaoka and coworkers have reported that the hydrolysis half-live of NPM (1.1 min) determined at pH 7.0 and 30 °C is more than one order of magnitude smaller than that of NEM (25 min), suggesting that electronic factors introduced by aromatic substitution on the imide nitrogen is reflected in the accelerated rate of hydrolysis.\(^{29}\) Lyon et al.\(^{30}\) reported the hydrolytic enhancement of the ring opening of an NAEM by a primary amine closely positioned to the succinimide, and proposed a mechanism involving intramolecular base catalysis by the amine. Shaun et al.\(^{26}\) have shown recently the ring-opening of a series of succinimide thioethers N-substituted by protonated amines incubated at pH 7.4 and 25 °C, with half-lives ranging from 0.41 h (NAC-NAEM) to several hours, indicating that ring-opening rates are greatly accelerated by electron-withdrawing N-substituents. Consistent with these reports, our results suggest that the rate of hydrolysis could be enhanced due to an electron withdrawing inductive effect and be reduced by N-alkyl substituents with electron donating character.
Figure 2.22 Fractional conversion to the thioether succinimide, over time, for (A) MPA-NEM, (B) MPA-NPM and (C) MPA-NAEM. Relative concentrations were measured via HPLC and the curves constructed using the derived rate constants and integrated fractional concentration equations for $1, 1_{RO}, 3$ and $3_{RO}$.

Table 2.1 Pseudo first-order rate constants and respective half-lives for retro and ring-opening reactions of MPA-maleimide conjugates

<table>
<thead>
<tr>
<th></th>
<th>$k_1$ (h$^{-1}$)</th>
<th>half-life (h)</th>
<th>$k_2$ (h$^{-1}$)</th>
<th>half-life (h)</th>
<th>$k_2/k_1^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPA-NEM</td>
<td>0.0045</td>
<td>154</td>
<td>0.0382</td>
<td>18</td>
<td>8.5</td>
</tr>
<tr>
<td>MPA-NPM</td>
<td>0.1638</td>
<td>4.2</td>
<td>0.2215</td>
<td>3.1</td>
<td>1.4</td>
</tr>
<tr>
<td>MPA-NAEM</td>
<td>0.9728</td>
<td>0.7</td>
<td>0.1392</td>
<td>5.0</td>
<td>0.14</td>
</tr>
</tbody>
</table>

* $k_2/k_1 > 1$ indicates exchange reaction dominates in the solution; $k_2/k_1 < 1$ indicates ring-opening reaction dominates in the solution.
Apart from hydrolysis, we noticed that the retro and exchange reactions were also impacted depending on the electronic character of the N-substituents. In the reactions of MPA-NEM, $3_{RO}$ is the major product at equilibrium, with a retro reaction rate constant $k_2$ of 0.0382 h$^{-1}$ and a respective half-life of 18 h. By contrast, the rate constant $k_2$ of MPA-NPM and MPA-NAEM are one order of magnitude larger, an expected result when considering the mechanism of the reaction. The retro-Michael reaction is a $\beta$-elimination reaction involving proton abstraction and $\beta$-thiolate release from the succinimide thioether.\textsuperscript{26} The electron-withdrawing effects of N-substituents might be expected to increase the acidity of the dissociable C–H bond, and thus the rate of $\beta$-elimination. Also of note, the retro and exchange reaction of MPA-NPM was even 1.6-fold faster than that of MPA-NAEM, which possesses a stronger electron-withdrawing group. This is probably due to the resonance effect of the phenyl group, which stabilizes the thiolate ions and promotes $\beta$-elimination. The conversion of the three MPA-maleimide conjugates to glutathione adducts is shown in Figure 2.23. The fraction of 3 generated from 1 is determined by the progress of two competing reactions and thus is equal to $k_2/(k_1 + k_2)$. The MPA-NEM supported the greatest conversion ratio of the initial adduct, reaching maximum conversion of 89.5% after approximately 110 h. The MPA-NPM exhibited the next greatest extent of conversion (58.3%), and also the most rapid kinetics, achieving a maximum conversion within 12 h. The MPA-NAEM supported the lowest conversion ratio and medium exchange rate, achieving a conversion of 12.3% after approximately 4 h. The ratio and rate of conversion of the initial adduct are clearly impacted by the inductive effects of the N-substituents as discussed above. The resonance effect of N-phenyl groups contributes to the most rapid thiol exchange with a half-life of 3.1 h for MPA-NPM, followed by
the electron-withdrawing inductive effect of N-aminoethyl groups supporting a medium exchange rate with a half-life of 5.0 h for MPA-NAEM. Our previous report has shown the similar pseudo first-order rate constant \( k_2 \) for the retro reaction of NEM conjugates with a value of 0.0371 h\(^{-1}\) and half-life of 19 h for MPA-NEM and a value of 0.00207 h\(^{-1}\) and half-life of 337 h for NAC-NEM,\(^{22}\) indicating a slower thiol exchange resulting from the electron-donating inductive effect of N-ethyl groups. Shaun et al.\(^{26}\) have shown the pseudo first-order rate constant \( k_2 \) for the retro reactions of a series of succinimide thioethers N-substituted by protonated amines in the presence of 5 mM GSSG incubated at pH 7.4, 25 °C, the short half-lives of which range from 12 h to 61 h. These data are consistent with our reports and confirm the rate enhancement of the retro and exchange reaction by the electron-withdrawing inductive effects of N-substituents.
We next sought to investigate the combined effects on the retro and ring-opening reactions of succinimide thioethers by altering the pKₐ of the thiols as well as the N-substituents of maleimides (ethyl, phenyl, and aminoethyl substituents). All other experimental conditions were kept the same unless otherwise noted. Fractional concentrations of 1, 1₁RO, 3, 3₁RO (converted to the initial thioether succinimide linkage of 1) measured by HPLC were plotted and curves were constructed as a function of time for MPP-maleimide (Figure 2.24), and NAC-maleimide (Figure 2.25). The values of the ring-opening rate constant $k₁$ and the retro reaction rate constant $k₂$ with their...
corresponding half-lives are shown respectively in Table 2.2 and Table 2.3. The close 
pKₐ of MPA (pKₐ 6.6) and MPP (pKₐ 7.0) contributed to a similar set of kinetics data, 
as illustrated in Figure 2.22 and Figure 2.24, while the retro and exchange reaction for 
NAC (pKₐ 9.5) adducts (Figure 2.25) was apparently slower with longer half-lives up 
to 258 h.

Figure 2.24 Fractional conversion to the thioether succinimide, over time, for (A) 
MPP-NEM, (B) MPP-NPM and (C) MPP-NAEM. Relative concentrations were measured via HPLC and the curves constructed 
using the derived rate constants and integrated fractional concentration 
equations for 1, 1RO, 3 and 3RO.
Figure 2.25 Fractional conversion to the thioether succinimide, over time, for (A) NAC-NEM, (B) NAC-NPM and (C) NAC-NAEM. Relative concentrations were measured via HPLC and the curves constructed using the derived rate constants and integrated fractional concentration equations for $1$, $1_{RO}$, $3$ and $3_{RO}$.

Table 2.2 Pseudo first-order rate constants and respective half-lives for retro and ring-opening reactions of MPP-maleimide conjugates

<table>
<thead>
<tr>
<th></th>
<th>$k_1$ ($h^{-1}$)</th>
<th>half-life (h)</th>
<th>$k_2$ ($h^{-1}$)</th>
<th>half-life (h)</th>
<th>$k_2/k_1^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPP-NEM</td>
<td>0.0035</td>
<td>198</td>
<td>0.0339</td>
<td>20</td>
<td>9.7</td>
</tr>
<tr>
<td>MPP-NPM</td>
<td>0.1264</td>
<td>5.5</td>
<td>0.1904</td>
<td>3.6</td>
<td>1.5</td>
</tr>
<tr>
<td>MPP-NAEM</td>
<td>0.8106</td>
<td>0.9</td>
<td>0.1147</td>
<td>6.0</td>
<td>0.14</td>
</tr>
</tbody>
</table>

* $k_2/k_1 > 1$ indicates exchange reaction dominates in the solution; $k_2/k_1 < 1$ indicates ring-opening reaction dominates in the solution.
Table 2.3 Pseudo first-order rate constants and respective half-lives for retro and ring-opening reactions of NAC-maleimide conjugates

<table>
<thead>
<tr>
<th></th>
<th>$k_1$ (h⁻¹)</th>
<th>half-life (h)</th>
<th>$k_2$ (h⁻¹)</th>
<th>half-life (h)</th>
<th>$k_2/k_1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAC-NEM</td>
<td>0.0056</td>
<td>123</td>
<td>0.0027</td>
<td>258</td>
<td>0.48</td>
</tr>
<tr>
<td>NAC-NPM</td>
<td>0.1914</td>
<td>3.6</td>
<td>0.0162</td>
<td>43</td>
<td>0.085</td>
</tr>
<tr>
<td>NAC-NAEM</td>
<td>0.9537</td>
<td>0.7</td>
<td>0.0075</td>
<td>92</td>
<td>0.0079</td>
</tr>
</tbody>
</table>

* $k_2/k_1 > 1$ indicates exchange reaction dominates in the solution; $k_2/k_1 < 1$ indicates ring-opening reaction dominates in the solution.

A summary of the pseudo first-order rate constants and half-lives for selected succinimide thioethers are shown in Figure 2.26. In each diagram, the three groups of thiol adducts for comparison were fixed based on the identities of the thiols, which were MPA ($pK_a$ 6.6), MPP ($pK_a$ 7.0) and NAC ($pK_a$ 9.5), while the thiol trap GSH has the highest $pK_a$ of 9.65 in solution. For each type of thiol adduct, NEM, NPM and NAEM (respectively refer to the navy, green and gray column) are the maleimide moieties employed for further analysis. Figure 2.26A shows the value of the ring-opening rate constant $k_1$ for the succinimide thioether adducts. When the thiol moiety is fixed, a large electron-withdrawing inductive effect of the maleimide N-substituents dramatically increases the rate of ring-opening. When the maleimide moiety is fixed, the difference in solubility of three thiols: $S_{(NAC)} > S_{(MPA)} > S_{(MPP)}$, is suggested to have a slight impact on the rate of ring-opening; improved solubility of adducts would be expected to facilitate hydrolysis. Thus, the value of $k_1$ mainly depends on the strength of the inductive effect of the N-substituents and is affected slightly by the adducts’ solubility. Figure 2.26B shows the value of $k_2$ for the succinimide thioether adducts. Of the thiol adducts, those adducts with phenyl groups showed the most rapid exchange reaction likely due to resonance effects, followed by the adducts with the
strongly electron-withdrawing aminoethyl group. When the maleimide moiety is fixed, the rate of exchange of the initial adduct is clearly impacted by the thiol $pK_a$, with higher $pK_a$ decreasing the rate. The value of $k_2$ depends greatly on the electronic effects of N-substituents and $pK_a$ of thiols. Figure 2.26C shows the value of $k_2/(k_1 + k_2)$, which also represents the conversion ratio of initial adduct to glutathione adduct. As shown in the figure, the $pK_a$ of thiols determined the tunable range of the value of $k_2$, and thus determined the tunable range of the value of $k_2/(k_1 + k_2)$. The lower the $pK_a$, the wider the tunable range is. The electronic effects of N-substituents had great impact on the values of $k_1$ and $k_2$, and thus differentiated the value of $k_2/(k_1 + k_2)$ in the range, with a stronger electron-withdrawing effect decreasing the conversion ratio. Figure 2.26D shows the half-lives for ring-opening, which range from 0.7 h (NAC-NAEM) to 198 h (MPP-NEM). Figure 2.26E shows the half-lives for the exchange reaction, which range from 3.1 h (MPA-NPM) to 258 h (NAC-NEM). Shaun et al.\textsuperscript{26} have shown comparable results that, for a series of succinimide thioethers with amine substituents, the rates of the retro-Michael reactions span only 30-fold, whereas ring-opening reactions span over 600-fold, which also indicates that the retro reaction is less sensitive than the hydrolysis of thioether succinimide to the inductive effects of N-substituents.
Figure 2.26 Pseudo first-order rate constants and half-lives for selected succinimide thioether adducts. (A) Ring-opening rate constant $k_1$, (B) retro and exchange reaction rate constant $k_2$, (C) conversion ratio of initial adduct to glutathione adduct: $k_2/(k_1 + k_2)$, (D) half-lives for $k_1$, (E) half-lives for $k_2$.

The degradation kinetics of 0.1 mM thiol-NEM adducts in the presence of 5 mM GSSG or 10 mM NDC were studied to help understand the relative impact of thiol moieties and thiol traps in the retro and exchange reactions. The kinetics profiles (Figure 2.27) are similar, as suggested by the comparison of the curves of 1 and 1RO for the same thiol-NEM adduct. The values of $k_1$ and $k_2$ are also on the same order of magnitude. The difference is that 3 (NDC-NEM) generated in the presence of NDC ring-opened faster than 3 (GS-NEM) generated in the presence of GSSG. Degradation
profiles of NAC-NEM in the presence of NDC (Figure 2.27F) indicates that $pK_a$ of the thiol trap does not affect the occurrence of the retro and exchange reaction, even though the $pK_a$ of NDC (7.8) is much lower than that of NAC (9.5). These results are also consistent with the retro-Michael reaction mechanism: an increase in the leaving-group ability of the thiolate ions determines the occurrence of the retro reaction, which is correlated to the acidity ($pK_a$) of the thiol moiety; a stoichiometric excess of a thiol trap drives the equilibrium forward toward thiol exchange. In our previous report, we have demonstrated that the kinetics of the retro-Michael addition and thiol exchange was independent of the use of GSSG or GSH as a thiol trap.\textsuperscript{22} Baker and coworkers have described the bromination of maleimides for reversible conjugation of thiols as a bioconjugate technique.\textsuperscript{31-32} The cysteine-bromomaleimide conjugate was found to be reversible by incubation with excess thiols such as 2-mercaptoethanol or GSH with complete conversion within 4 h.\textsuperscript{32} The results are consistent with our findings that the kinetics of the retro and exchange reaction is independent of the identity of the thiol trap. It also indicates that the reduced reactivity of maleimides compared with bromomaleimides correlates with the rate of the retro reaction.
Figure 2.27 Fractional conversion to the thioether succinimide, over time, for (A)(B) MPA-NEM, (C)(D) MPP-NEM and (E)(F) NAC-NEM. Relative concentrations were measured via HPLC and the curves constructed using the derived rate constants and integrated fractional concentration equations for $1$, $1_{RO}$, $3$ and $3_{RO}$. (A)(C)(E) were tested in the presence of excess 5 mM GSSG; (B)(D)(F) were tested in the presence of excess 10 mM NDC.
Table 2.4 Pseudo first-order rate constants and respective half-lives for retro and ring-opening reactions of thiol-NEM conjugates in GSSG and NDC thiol traps

<table>
<thead>
<tr>
<th></th>
<th>( k_1 (h^{-1}) )</th>
<th>half-life (h)</th>
<th>( k_2 (h^{-1}) )</th>
<th>half-life (h)</th>
<th>( k_2/k_1 )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MPA-NEM</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSSG</td>
<td>0.0045</td>
<td>154</td>
<td>0.0382</td>
<td>18</td>
<td>8.5</td>
</tr>
<tr>
<td>NDC</td>
<td>0.0044</td>
<td>159</td>
<td>0.0398</td>
<td>17</td>
<td>9.0</td>
</tr>
<tr>
<td><strong>MPP-NEM</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSSG</td>
<td>0.0035</td>
<td>198</td>
<td>0.0339</td>
<td>20</td>
<td>9.7</td>
</tr>
<tr>
<td>NDC</td>
<td>0.0040</td>
<td>174</td>
<td>0.0489</td>
<td>14</td>
<td>12.2</td>
</tr>
<tr>
<td><strong>NAC-NEM</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSSG</td>
<td>0.0056</td>
<td>123</td>
<td>0.0027</td>
<td>258</td>
<td>0.48</td>
</tr>
<tr>
<td>NDC</td>
<td>0.0056</td>
<td>123</td>
<td>0.0043</td>
<td>163</td>
<td>0.77</td>
</tr>
</tbody>
</table>

* \( k_2/k_1 > 1 \) indicates exchange reaction dominates in the solution; \( k_2/k_1 < 1 \) indicates ring-opening reaction dominates in the solution.

Additional factors such as the pH of the solvent may further affect the kinetics of the reaction in solution reactions. Here the influence of lower pH on the retro reaction kinetics was studied. Figure 2.28 shows clearly slower retro and ring-opening reaction kinetics for MPA-NEM incubated in solutions of lower pH. The value of \( k_1 \) and \( k_2 \) for MPA-NEM is one order of magnitude smaller at pH 6.0 and two orders of magnitude smaller at pH 5.0, compared to that at physiological pH. We noticed that \( k_2/k_1 \) was kept unchanged at pH 6.0, which indicates the conversion ratio from initial adduct to glutathione adduct remained at a significant level while a longer release or degradation period can be achieved at lower pH. These properties of the retro and thiol exchange reactions suggest their potential use for drug release in the acidic microenvironment (pH 6.5–6.9) of carcinoma cells. Specifically, the glutathione-sensitive thioether succinimide linkages may be employed in either the formation of hydrogels or the coupling of anticancer drugs to synthetic polymers, for the purpose of developing biodegradable polymer systems for controlled release of anticancer drugs. A longer release timescale could improve the efficacy and reduce the side
effects of the drugs, as therapeutic levels of the desired anticancer agents might be maintained locally for prolonged periods while reducing systemic side effects.\textsuperscript{37-38}

Figure 2.28 Fractional conversion to the thioether succinimide, over time, for MPA-NEM incubated at (A) pH 7.4, (B) pH 6.0 and (C) pH 5.0. Relative concentrations were measured via HPLC and the curves constructed using the derived rate constants and integrated fractional concentration equations for $\text{1}_R$, $\text{1}_{\text{RO}}$, 3 and $\text{3}_{\text{RO}}$.

Table 2.5 Pseudo first-order rate constants and respective half-lives for retro and ring-opening reactions of MPA-NEM incubated at different pH

| MPA-NEM | $k_1$ (h\textsuperscript{-1}) | half-life (h) | $k_2$ (h\textsuperscript{-1}) | half-life (h) | $k_2/k_1$  
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>pH = 7.4</td>
<td>0.0045</td>
<td>154</td>
<td>0.0382</td>
<td>18</td>
<td>8.5</td>
</tr>
<tr>
<td>pH = 6.0</td>
<td>2.8E-4</td>
<td>2.5E3</td>
<td>2.4E-3</td>
<td>2.9E2</td>
<td>8.5</td>
</tr>
</tbody>
</table>
2.4 Conclusions

In conclusion, we have shown that the kinetics of the GSH-mediated retro Michael-type addition can be significantly expanded by modifying the N-substituents of the Michael acceptor and the pKₐ values of the Michael donor. Succinimide thioethers with electron-withdrawing groups favor a high level of hydrolysis as well as the enhancement of the exchange reaction to some extent, while the resonance effect of N-phenyl groups contributes to a faster thiol exchange. A low pKₐ of the thiols is suggested to be the primary source of the rate enhancement of the retro and exchange reaction, with a wider tunable range of degradation kinetics; higher pKₐ of thiols can limit the impact, on the extent of conversion, of electronic effects of the N-substituents. Our studies also illustrate that the occurrence and kinetics of the retro and exchange reaction are not affected by the pKₐ of a thiol trap, but can be retarded at lower pH. Our findings suggest the impact of both N-substituents and identity of thiols for manipulating the kinetics of the retro Michael-type addition of maleimides and thiols: (i) the conversion ratio $k_2/(k_1 + k_2)$ must be significantly high to favor conversion; and (ii) the half-lives of $k_{\text{exchange}}$ should match the need of the specific application. In the case of tumor-targeted drug delivery, a prompt destabilization of drug carriers inside cells and rapid release (within hours) of the loaded therapeutics is preferred to enhance drug efficacy, overcome multi-drug resistance (MDR), and minimize drug and carrier-associated side effects;³⁹ while for localized chemotherapy such as a site-specific delivery of doxorubicin (DOX) from hydrogels, a sustained and tunable release (over
weeks) is preferred for a long-term control of cytotoxicity of cancer cells \textit{in vivo}.\textsuperscript{40} By imparting tunable glutathione-sensitive linkages into micelles, vessels, tethered drugs or other bioconjugates, the strategy should be widely applicable in improved applications such as sensing,\textsuperscript{41} tissue engineering\textsuperscript{42} and drug delivery.\textsuperscript{43}
REFERENCES


Appendix

COPYRIGHT PERMISSION FOR REPRINT OF PUBLISHED ARTICLES

Chapter 1

Figure 1.1
Licensee: Haocheng Wu
Order Date: Aug 3, 2017
Order Number: 4161360030441
Publication: Nature Communications
Title: Moving from static to dynamic complexity in hydrogel design
Type of Use: reuse in a dissertation / thesis
Order Total: 0.00 USD

Your order details and publisher terms and conditions are available by clicking the link below:

https://s100.copyright.com/CustomerAdmin/PLF.jsp?ref=64ac9c25-0950-40e6-95c3-8f66cc02c58e

Figure 1.2
Licensee: Haocheng Wu
Order Date: Aug 3, 2017
Order Number: 4161370900814
Publication: Soft Matter
Title: Dextran based photodegradable hydrogels formed via a Michael addition
Type of Use: Thesis/Dissertation
Order Total: 0.00 USD

Your order details and publisher terms and conditions are available by clicking the link below:
https://s100.copyright.com/CustomerAdmin/PLF.jsp?ref=4f6ce68f-fdfe-42b0-a5d0-a04cf30f2fb6

Figure 1.3

Figure 1.3a
Licensee: Haocheng Wu
Order Date: Aug 3, 2017
Order Number: 4161370717703
Publication: Polymer Chemistry
Title: Reversible maleimide–thiol adducts yield glutathione-sensitive poly(ethylene glycol)–heparin hydrogels
Type of Use: Thesis/Dissertation
Order Total: 0.00 USD

Your order details and publisher terms and conditions are available by clicking the link below:
Figure 1.3b
Licensee: Haocheng Wu
Order Date: Aug 3, 2017
Order Number: 4161371089562
Publication: Journal of Materials Chemistry B
Title: Dually degradable click hydrogels for controlled degradation and protein release
Type of Use: Thesis/Dissertation
Order Total: 0.00 USD

Your order details and publisher terms and conditions are available by clicking the link below:
https://s100.copyright.com/CustomerAdmin/PLF.jsp?ref=144a132c-3383-482c-aa45-71b588ff357d

Figure 1.3c
Title: Liposome-Cross-Linked Hybrid Hydrogels for Glutathione-Triggered Delivery of Multiple Cargo Molecules
Author: Yingkai Liang, Kristi L. Kiick
Publication: Biomacromolecules
Chapter 2

Figure 2.3