

Polymer Mediated Therapeutic Delivery for Neural
Interface Applications

A Dissertation

Presented for the

Doctor of Philosophy Degree

The University of Tennessee, Knoxville

Yu Cao

August 2012

Copyright © 2012 by Yu Cao

All rights reserved.

ACKNOWLEDGEMENTS

First of all, I would like to greatly thank my advisor, Dr. Wei He, for her continuous support and guidance on my research during the PhD study. I would also like to express my gratitude to my other committee members, Dr. Roberto S. Benson, Dr. Kevin M. Kit and Dr. Bin Zhao, for their contributions to my PhD study.

I want to thank Dr. Tim Sparer in Department of Microbiology, Mr. Scott Moser in Center for Environmental Biotechnology for providing many experimental materials and instruments.

My sincere thanks also go to all the group members, Ms. Zheng Cao, Ms. Lu Huang, Mr. Jonathan Page, Mr. Kaan Serpersu, Mr. Shuangcheng Tang and Mr. Samuel Goddard, for providing a friendly and helpful environment.

I am also grateful to my friends, Ms. Susan Everett (Michelle), Dr. Xueguang Jiang, Dr. Xiaoming Jiang, Dr. Scott Lenaghan, Dr. Lijin Xia, Mr. Jonathan Horton, Mr. Naixiong Jin, Mr. Weidong Li, Mr. Ming Shao, for all the help on my research and personal life.

Finally, I would like to show my greatest appreciation to my parents, Mr. Daoxing Cao and Mrs. Qizheng Zou for their unconditional love and support.

Abstract

The technology of interfacing neurons with machines through implantable neural electrodes has significant implications. Although there have been studies implanting such electrodes in human to help patients with motor disorders, longevity of these implants remains an unresolved issue. One of the key factors influencing longevity has been adverse tissue response toward the implanted electrodes

The objective of this research is to engineer a comprehensive solution that can manage the response at the cellular level while preserving the electrode functions. Given the complexity of the host response, we hypothesize that a multi-pronged approach would better improve the longevity of the electrodes. Specifically, we emphasize the importance of modulating the inflammatory response from glial cells and directly protecting neurons from oxidative stress induced death. To achieve this goal, a range of polymeric therapeutics (i.e., prodrugs) capable of anti-inflammation and anti-oxidation were designed and synthesized. These polymers were fully characterized for their structural properties and therapeutic effects. Applications of these prodrugs onto the electrodes were achieved using the versatile layer-by-layer (LBL) technique, which enabled the preservation of electrical properties of the electrodes. These in vitro studies laid down the foundations for future in vivo investigations of the efficacy of such a multi-pronged, integrated therapeutic approach for modulating host tissue response. Furthermore, the synthesized prodrugs can be applied for other types of medical implants, where inflammation and oxidative stress are common characteristics of the host response to those implants.

The other approach to achieve therapeutic delivery is the use of stimuli-responsive polymers. Based on the lower critical solution temperature (LCST) behaviors of poly(N-vinyl-2-caprolactam) (PVCL) polymers, its functional derivatives with pH-dependent LCST behaviors were designed via copolymerization with a functional derivative of VCL for smart drug delivery. Sharp and reversible response was observed across a broad range of pH values. PVCL copolymer was demonstrated to be non-cytotoxic at low concentrations. LBL compatibility of the copolymer was also explored. The ultimate goal is to correlate the pH-sensitivity of the PVCL copolymers with the tissue acidosis phenomenon to regulate therapeutic release.

Table of Contents

Chapter 1 Introduction	1
1.1 Neural Interface	2
1.1.1 Physical design of neural electrodes	2
1.1.2 Tissue response to neural electrodes.....	6
1.1.3 Methods to improve biocompatibility of neural electrodes	8
1.2 Layer-by-Layer (LBL)	10
1.3 Polymeric Therapeutics (Prodrug)	12
1.4 Stimuli-Responsive Polymers	14
1.4.1 Thermo-responsive polymers.....	14
1.4.2 Dual responsive polymers.....	18
1.5 Polymer chain conformation [73, 99]	19
1.6 Dissertation Structure.....	21
Chapter 2 Synthesis and Characterization of Glucocorticoid Functionalized Poly(N-vinyl pyrrolidone): A Versatile Prodrug for Neural Interface	23
2.1 Introduction.....	24
2.2 Experimental Section.....	27
2.2.1 Materials	27
2.2.2 Characterization	28
2.2.3 Prodrug synthesis	29
2.2.4 pH sensitive drug release	30
2.2.5 LBL assembly with PNVP prodrug (PNVP-Pred)	31
2.2.6 Bioactivity of prodrug and its released products	32

2.2.7 Prodrug diffusion	34
2.3 Results and Discussion	35
2.3.1 Prodrug synthesis and characterization.....	35
2.3.2 Application of prodrug for LBL assembly.....	45
2.3.3 Bioactivity and biocompatibility of prodrug and its released products	49
2.3.4 Prodrug diffusion in a simulated brain tissue model	57
2.4 Conclusions.....	64
Chapter 3 Water Soluble Antioxidant Derivative Poly(Triethylene Glycol Methyl	
Acrylate-co- α -Tocopheryl Acrylate) as a Potential Prodrug to Enable Localized	
Neuroprotection	65
3.1 Introduction.....	66
3.2 Experimental Section.....	69
3.2.1 Materials	69
3.2.2 General characterization	69
3.2.3 Polymer synthesis	70
3.2.4 LBL assembly	70
3.2.5 Quartz crystal microgravimetry (QCM)	72
3.2.6 In vitro cytotoxicity assay.....	73
3.2.7 In vitro cell protection against oxidative stress.....	74
3.2.8 Free radical scavenging assay.....	74
3.2.9 Impedance measurement.....	75
3.3 Results and Discussion	75
3.3.1 Synthesis and characterization of the antioxidant prodrug.....	76

3.3.2 Application of PVT for LBL assembly.....	87
3.3.3 Cytotoxicity study of PVT	95
3.3.4 Antioxidation study of PVT	97
3.3.5 Electrical property of the modified electrode	103
3.4 Conclusion	106
Chapter 4 Functionalized Biocompatible Poly(<i>N</i> -vinyl-2-caprolactam) with pH- dependent Lower Critical Solution Temperature (LCST) Behaviors	107
4.1 Introduction.....	108
4.2 Experimental Section.....	110
4.2.1 Materials	110
4.2.2 Characterization	110
4.2.3 Synthesis of carboxylated VCL homopolymer (COOH-PVCL-H)	111
4.2.4 Synthesis of carboxylated VCL copolymer (COOH-PVCL).....	111
4.2.5 Reactivity study	113
4.2.6 Cloud point measurement	113
4.2.7 In vitro cytotoxicity study.....	114
4.2.8 LBL assembly of COOH-PVCL/PVCL monitored by Quartz crystal microgravimetry (QCM).....	115
4.3 Results and Discussion	115
4.3.1 Polymer synthesis and characterization	115
4.3.2 LCST behaviors of COOH-PVCLs	122
4.3.3 Cytotoxicity study of COOH-PVCL.....	127
4.3.4 Reactivity study	131

4.3.5 LBL coating consisting of COOH-PVCL	135
4.4 Conclusions	138
Chapter 5 Summary and Future Work	139
References	143
APPENDIX	155
Vita	172

List of Tables

Table 1. Molecular weight and compositional data of PVT prodrugs.....	78
Table 2. Molecular parameters of PAA and PVT.....	94
Table 3. Molecular weight and compositional data of PVCL containing polymers.....	123
Table 4. Calculation for the reactivity of VCL and VP.	156
Table 5. Calculation for the reactivity of TBVCL and VP.	157

List of Figures

Figure 1. Examples of Utah and Michigan type neural electrodes. (a) Scanning electron micrograph of Utah electrode.[4] (b) Various configurations of the Michigan type electrodes.[5].....	3
Figure 2. The general structure of a Michigan type neural electrode.[6]	4
Figure 3. Structure of a micromachined single shank acute probe used in our research (16 recording sites, model: A1×16-5mm-100-177-A16, NeuroNexus Technologies, Inc.)[7]	5
Figure 4. Schematic of brain tissue response to an implanted electrode characterized at the cellular level.[11]	7
Figure 5. Schematic of the LBL assembly process.....	11
Figure 6. Features of polymeric therapeutics.....	13
Figure 7. An example of phase diagram for polymers with LCST behaviors.[73]	15
Figure 8. Overview of the dissertation research.	22
Figure 9. Surface modification of neural electrode with LBL assembly and drug release responsive to tissue reaction.	25
Figure 10. Glucocorticoid prodrug synthesis via conjugation of prednisolone onto functionalized poly(<i>N</i> -vinyl pyrrolidone).	36
Figure 11. ¹ H-NMR spectra of (a) PTBNcoN, (b) COOH-PNVP, (c) PNVP-NH-NH-BOC and (d) PNVP-NH-NH ₂ in D ₂ O.	38
Figure 12. ¹ H-NMR spectra of (a) prodrug PNVP-pred and (b) free drug prednisolone in d ₈ -DMSO.	39

Figure 13. UV spectra of 0.1 mg/mL aqueous solution of (a) prodrug PNVP-pred, (b) free drug prednisolone, and (c) precursor polymer PNVP-NH-NH ₂	40
Figure 14. In vitro cumulative release of prednisolone from prodrug at different pH levels up to 25 days. Standard deviation is less than 5% of the mean value (n=3)......	42
Figure 15. Control reaction for illuminating the reactivity of the two carbonyl groups at positions 3 and 20 in prednisolone.....	44
Figure 16. Assembly of LBL films. (a) Plot of ellipsometric film thickness versus the number of PAA/prodrug bilayers deposited on a silicon substrate. (b) UV-vis absorption spectra of multilayer films of PAA/prodrug after 2, 4, 6 and 8 bilayer depositions. Inset: Absorbance at 193 nm (contribution from PNVP) versus number of assembled bilayers.....	47
Figure 17. NO production by LPS activated macrophages, LPS activated macrophage treated with either polymer PNVP-NH-NH ₂ , prodrug PNVP-pred, or free drug prednisolone, respectively. Non-activated macrophages treated with polymer PNVP-NH-NH ₂ alone were also included. Cells without LPS activation served as control. Data shown are average $\pm$ STD (n = 3).	51
Figure 18. NO production by LPS activated macrophages with and without treatment of prodrug solutions subjected to release for up to 10 days at pH 5.0 and 7.4. Cells without LPS activation served as control. Data shown are average $\pm$ STD (n = 3). * indicates $p < 0.05$	53
Figure 19. (a) In vitro neural cytotoxicity of prodrug PNVP-pred and precursor polymer PNVP-NH-NH ₂ . Data shown are average $\pm$ STD (n = 3). Confocal images show morphology of neurons following 48 h treatment with either (b) prodrug PNVP-	

pred, (c) precursor polymer PNVP-NH-NH ₂ , or (d) non-treated control. Scale bar = 100 μ m.	56
Figure 20. Scheme of prodrug diffusion in the simulated brain model by a block of agarose gel.	58
Figure 21. Fluorescent intensity of prodrug analog as a function of distance away from the Si wafer.	59
Figure 22. Approximation of fluorescent intensity of prodrug analog as a function of distance away from the Si wafer.....	60
Figure 23. Scheme of simplified cube of diffused prodrug away from the Si wafer.....	62
Figure 24. (a) Synthesis of monomer α -tocopheryl acrylate (VeAc). (b) ¹ H NMR characterization of VeAc in CDCl ₃ . Successful acrylation of Ve is confirmed by the observation of peaks at chemical shifts of 6.62, 6.36 and 5.98 ppm, corresponding to the vinyl protons. (c) ¹³ C NMR characterization of VeAc in CDCl ₃ . Characteristic peaks associated with the acrylate carbons are identified.	71
Figure 25. Synthesis of anti-oxidant vitamin E based prodrug PVT.	77
Figure 26. ¹ H NMR characterization of prodrugs (a) PVT4, (b) PVT8, (c) PVT24, and (d) PVT45 in deuterated chloroform. Characteristic peaks from each monomer were labeled.	80
Figure 27. UV spectra of (a) PTEGMA (10 mg/mL), (b) Ve (0.05 mg/mL), (c) PVT4 (1 mg/mL), and (d) PVT8 (1 mg/mL) in ethanol solution.	82
Figure 28. FTIR characterization of (a) Ve, (b) monomer VeAc, and (c) prodrug PVT4.	83

Figure 29. DSC thermograms of prodrugs PVT4, PVT8, PVT24, and PVT45. Glass transition temperature, T_g , for each of the prodrug is -55, -52, -40, and -30 °C, respectively.	84
Figure 30. Full DSC thermograms of prodrugs PVT4 and PVT45.	86
Figure 31. Schematic illustration of proposed use of prodrugs for surface modification of implantable neural MEAs with LBL assembly to enable localized delivery of antioxidants. The antioxidant prodrug PVT can be incorporated in two designs, dissociable layers constructed with poly(acrylic acid) (PAA), and stable layers made with tannic acid (TA) to serve as drug depots.....	89
Figure 32. Schemes of LBL multilayer assembly pair and film characterization. Hydrogen bonds formed between prodrug PVT and (a) PAA or (b) TA drives multilayer growth. (c) Film growth was monitored by ellipsometric measurement of film thickness as a function of number of PAA/PVT or TA/PVT bilayers. (d) Stability of the prodrug containing multilayer films in physiological pH 7.4 phosphate buffer was examined by comparing the film thickness before and after incubation.	90
Figure 33. In situ characterization of multilayer growth on gold crystals using QCM. (a) QCM frequency shifts and calculated film thickness during alternating deposition of PAA and PVT or TA and PVT, and after incubation in PBS to demonstrate film stability. (b), (c) Evolution of adsorbed Sauerbrey mass (Γ) plotted against layer numbers for PAA (or TA), PVT, and total mass deposited.	92

Figure 34. Proposed molecular behavior of polymer chains during LBL process mediated by hydrogen bonding.	96
Figure 35. In vitro cytotoxicity of Ve, antioxidant prodrug PVT, and precursor polymer analog PTEGMA on A172 astrocytes at concentrations of 10 and 500 μ M (equivalent to Ve). Data shown are average $\pm$ STD (n = 3). * $p < 0.05$. Statistical significance is compared with the non-treated control.	98
Figure 36. Representative phase contrast photomicrographs of cultured A172 astrocytes subjected to various treatments for cytotoxicity study. Non-treated control culture is shown for comparison. Scale bar = 100 μ m.....	99
Figure 37. (a) In vitro protection of A172 astrocytes against injurious oxidative stress induced by hydrogen peroxide (H_2O_2). Data shown are average $\pm$ STD (n=3). * $p < 0.05$. (b) Antioxidation activity determined by the DPPH free radical scavenging method. (c)-(f) Representative phase contrast photomicrographs of cultured A172 astrocytes subjected to various treatments. Scale bar = 100 μ m.	101
Figure 38. Photomicrographs of a Michigan electrode before (a) and after (b) deposition of 8 bilayers of (PVT/PAA).....	104
Figure 39. Impedance of recording sites after 8-bilayers (LBL (8)) and 16-bilayers (LBL (16)) of LBL coatings. The value of the bare sites before coating was also shown for comparison.....	105
Figure 40. Synthesis of the monomer 3-(<i>tert</i> -butoxycarbonyl)- <i>N</i> -vinyl-2-caprolactam (TBVCL) and partially or fully carboxylated PVCL polymers. Intramolecular hydrogen bonding was shown in polymers 3 and 5.....	112

Figure 41. ^1H -NMR spectra of (a) monomer TBVCL in CDCl_3 , (b) protected homopolymer COOH-PVCL-BOC-H in CDCl_3 , and (c) deprotected homopolymer COOH-PVCL-H in d_6 -DMSO.....	117
Figure 42. ^1H -NMR spectra of (a) protected copolymer COOH-PVCL-BOC14, (b) acid deprotected COOH-PVCL14, (c) protected copolymer COOH-PVCL-BOC9, and (d) acid deprotected COOH-PVCL9. CDCl_3 was used for all polymers..	118
Figure 43. TGA spectra of protected TBVCL homopolymer COOH-PVCL-BOC-H (a), protected copolymers COOH-PVCL-BOC14 (b) and COOH-PVCL-BOC9 (c).	120
Figure 44. IR spectra of (a) monomer TBVCL, (b) protected homopolymer COOH-PVCL-BOC-H, (c) deprotected homopolymer COOH-PVCL-H, (d) protected copolymer COOH-PVCL-BOC14, and (e) deprotected copolymer COOH-PVCL14.	121
Figure 45. Phase transition profiles of 2.5 mg/mL of (a) COOH-PVCL9 and (b) COOH-PVCL14 in 0.1 M phosphate buffered saline at different pH values. (c) Cloud points (T_c) of various COOH-PVCLs at different pH values. The dash line represents the T_c of PVCL homopolymer at pH 7.4. (d) pH vs degree of ionization for COOH-PVCLs at room temperature.	124
Figure 46. Influence of chemical compositions on the temperature sensitivity of phase transition. Top and bottom bars of each data point represent the temperature at 90% and 10% of the solution transmittance, respectively.	128

Figure 47. NIH/3T3 fibroblast cells viability after overnight incubation with different concentrations of COOH-PVCL copolymers. Cells without polymer treatment served as a negative control. Data shown are average $\pm$ STD (n = 3).	129
Figure 48. Schematic illustration for reactivity value measurements.....	133
Figure 49. A QCM plot showing the change in frequency (proportional to adsorbed mass) as a function of the adsorption and build-up of multilayers of COOH-PVCL/PVCL.	137
Figure 50. Fluorescent images and intensity in the diffused region of the fluorescent prodrug analog.	155
Figure 51. K-T plot for the reactivity study of VCL and VP.....	158
Figure 52 . K-T plot for the reactivity study of TBVCL and VP.....	159
Figure 53. NMR spectra of VCL and VP at a molar feeding ratio of 0.5, before polymerization.	160
Figure 54. NMR spectra of VCL and VP at molar feeding ratio of 0.5, after polymerization at 65 °C for 20 minutes.....	161
Figure 55. NMR spectra of VCL and VP at molar feeding ratio of 1, before polymerization	162
Figure 56. NMR spectra of VCL and VP at molar feeding ratio of 1, after polymerization at 65 °C for 20 minutes.....	163
Figure 57. NMR spectra of VCL and VP at molar feeding ratio of 2, before polymerization	164
Figure 58. NMR spectra of VCL and VP at molar feeding ratio of 2, after polymerization at 65 °C for 20 minutes.....	165

Figure 59. NMR spectra of TBVCL and VP at molar feeding ratio of 0.5, before polymerization.	166
Figure 60. NMR spectra of TBVCL and VP at molar feeding ratio of 0.5, after polymerization at 65 °C for 20 minutes.....	167
Figure 61. NMR spectra of TBVCL and VP at molar feeding ratio of 1, before polymerization.	168
Figure 62. NMR spectra of TBVCL and VP at molar feeding ratio of 1, after polymerization at 65 °C for 20 minutes.....	169
Figure 63. NMR spectra of TBVCL and VP at molar feeding ratio of 2, before polymerization	170
Figure 64. NMR spectra of TBVCL and VP at molar feeding ratio of 2, after polymerization at 65 °C for 20 minutes.....	171

Chapter 1

Introduction

1.1 Neural Interface

Chronically implanted recording electrode arrays have the potential to improve the quality of life of patients suffering from partial or full paralysis.[1-3] Such arrays can be implanted into the cortex of a patient's brain and the extracellular potentials from nearby neurons are recorded, allowing the information encoded to control external devices.

1.1.1 Physical design of neural electrodes

Neural electrodes have evolved from glass to metallic materials over the years. The dimension of neural electrodes has also been decreasing constantly for the ease of handling and minimizing damage to brain tissue, with the help of technology advancement, especially the silicon micromachining development. The most common modern models include Utah and Michigan type electrodes (Figure 1). The Michigan type electrode has been widely used for many years because of its diversity. Hundreds of designs and configurations have been developed and these electrodes could be connected with the Integrated Circuit (IC) technology, realizing large amount of data collection and analysis. The structure of such a micromachined electrode is shown schematically in Figure 2. The bulk of the electrode is based on a silicon substrate. The recording sites are made from conductive metals such as gold. There is one company focusing on developing and manufacturing various types of electrodes for research and clinical applications. The size of the recording sites of the Michigan electrode used in our study is on the order of micrometer (Figure 3).

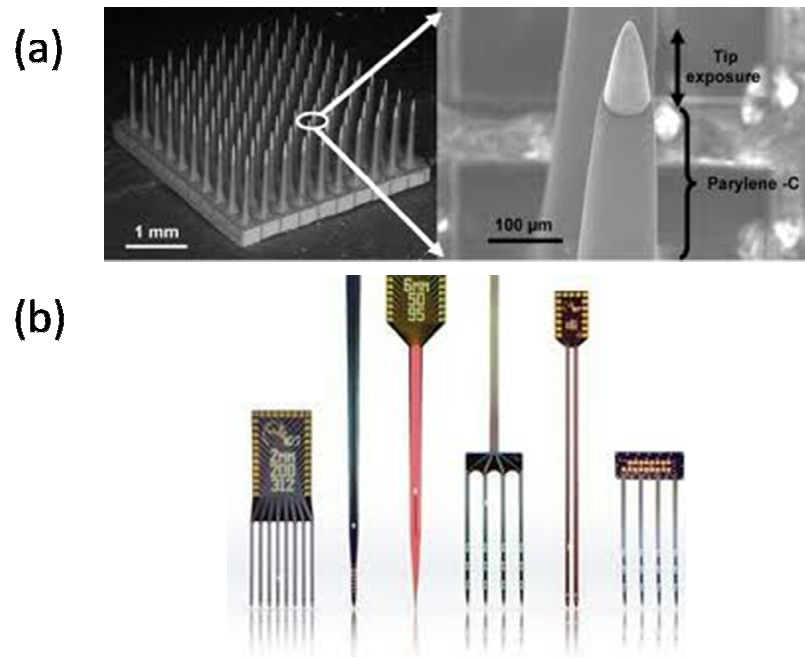


Figure 1. Examples of Utah and Michigan type neural electrodes. (a) Scanning electron micrograph of Utah electrode.[4] (b) Various configurations of the Michigan type electrodes.[5]

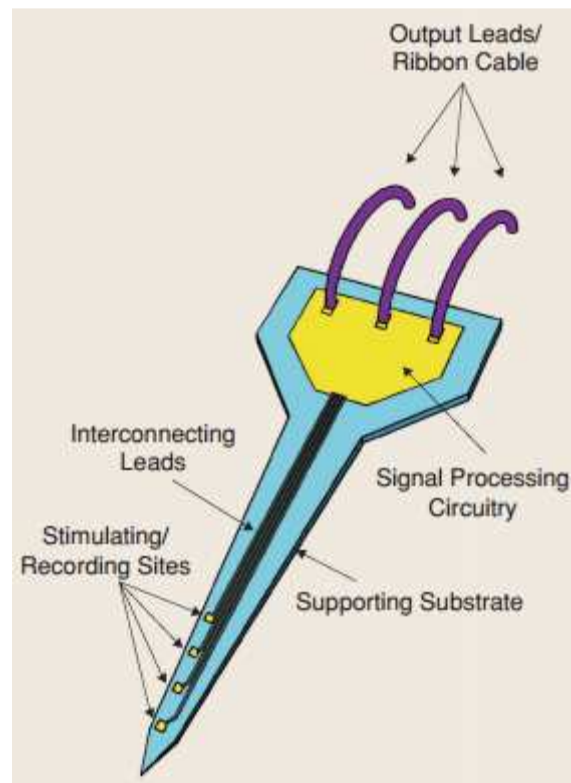


Figure 2. The general structure of a Michigan type neural electrode.[6]

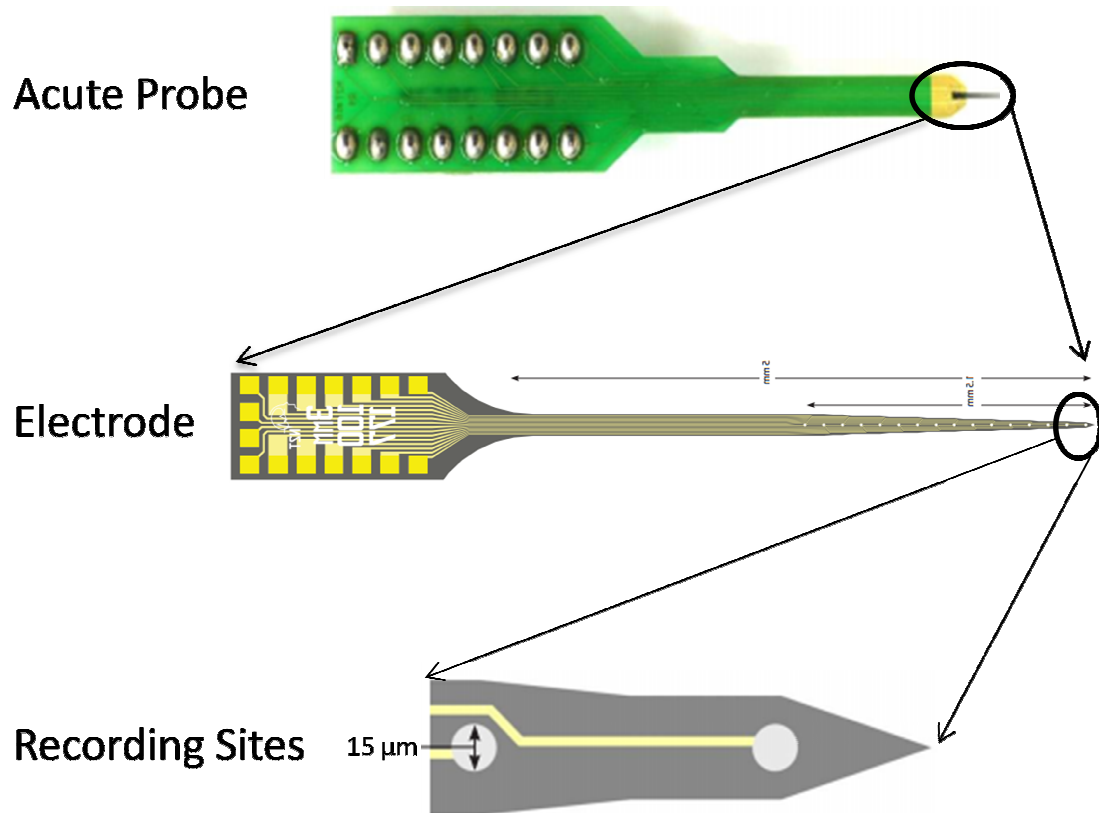


Figure 3. Structure of a micromachined single shank acute probe used in our research (16 recording sites, model: A1×16-5mm-100-177-A16, NeuroNexus Technologies, Inc.)[7]

1.1.2 Tissue response to neural electrodes

Neural electrodes often fail to function reliably in clinically relevant chronic settings. One of the proposed leading causes to such failure is foreign body response in the central nervous system (CNS), resulting in an encapsulation layer referred to as the “glial scar” and accompanied by loss of healthy neurons around the implants (Figure 4).[8-10]

The glial scar is formed as a result of activation of astrocytes and microglia. It encapsulates the electrode as a physical barrier. It has several adverse effects toward normal functioning of the implant. Firstly, this physical barrier is an insulating material between the electrode and nearby neurons. The electrical interaction between brain and machine is thus significantly compromised. Secondly, the tight glial scar takes up the immediate room around the electrode, keeping neurons away from the interface.

Neuronal death is another phenomenon that has been considered causing malfunction of the neural electrode. Neurons are challenged with a variety of toxic environment such as oxidative stress after implantation of the neural electrode. Reactive nitrogen species (RNS) and reactive oxygen species (ROS) are classes of free radicals and related molecules that are capable of exerting oxidative stress on cells when produced in excess. Nitric oxide (NO), a simple yet highly versatile molecule, is a type of RNS/ROS frequently studied and involved in a wide range of physiological as well as pathophysiological mechanisms. In response to injury, the production of NO can overpower the endogenous antioxidants, leading to oxidative stress and cellular damage.

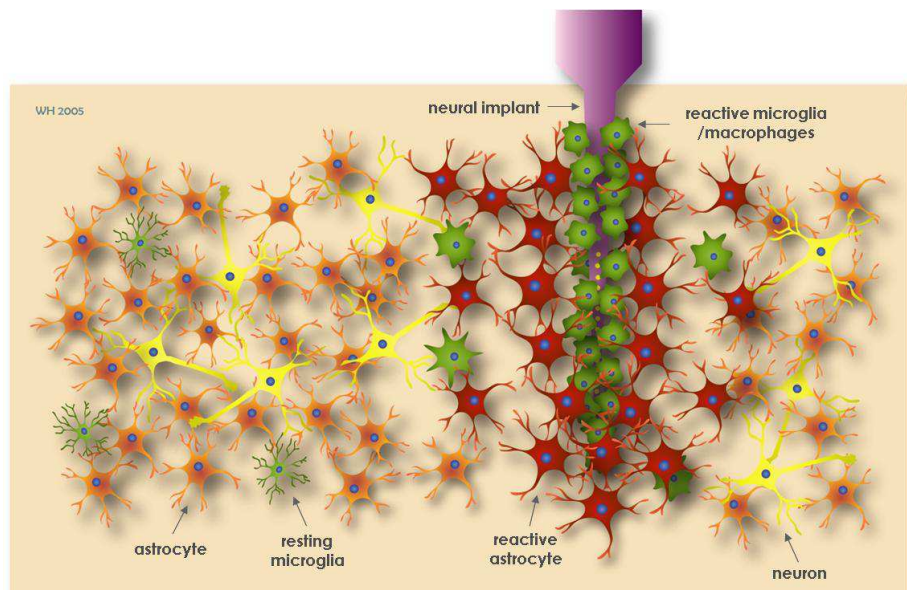


Figure 4. Schematic of brain tissue response to an implanted electrode characterized at the cellular level.[11]

Reactive astrocytes and activated microglia have the capacity to generate NO, and the production can be induced by pro-inflammatory cytokines such as interleukin (IL-1) and tissue necrosis factor (TNF- α). NO is a ubiquitous second messenger[12] that diffuses freely across cell boundaries and can damage neurons by potentiating glutamate excitotoxicity.[13]

1.1.3 Methods to improve biocompatibility of neural electrodes

Improvements on electrode design[14-16] and manipulation of bioactive molecules[17-24] through the tethering and/or controlled release of bioactive molecules from the surface are the two main strategies to reduce the aforementioned tissue response. Advances in electrode designs include not only microfabrication of electrodes with new configurations[25], but also the development of novel polymers at the interface of biology[26, 27], and bioinspired, mechanically dynamic polymers to replace currently used stiff silicon materials[28]. Standard wound healing suppression and immune-suppression techniques are options for minimizing the initial immune response and even the formation of a glial scar.[8] The available methods of using glucocorticoids and other therapeutics to reduce the inflammatory response include systemic administration,[20, 29] localized delivery from coatings deposited on the electrode surface,[30] and direct surface immobilization[11]. However, none of these approaches are ideal. For example, systemic administration of drugs may cause serious side effects, including myopathy and diabetes, and the presence of the blood-brain-barrier (BBB) adds extra hurdles for systemic drug delivery to the CNS.[8, 31-33] Concerns associated with localized delivery of therapeutics from surface coatings include the fine balance between drug loading

capacity and the conductivity of electrode (i.e., potential increase in impedance by the coating), difficulty in controlling drug release rate, and the duration of delivery. Immobilization of bioactive molecules directly onto the surface of electrodes could provide a device with an intrinsically anti-inflammatory surface, but it is still restricted by limited potency as the effect is confined to cells in direct contact with the surface. Although it is possible that this is a design strategy which can be implemented to control the local material-tissue interface while allowing other activated immune cells to participate in local wound healing to “clean” up debris caused by electrode implantation, this hypothesis needs to be confirmed.

A potential solution to pharmacologically manage the tissue response to implanted electrodes is to enable self-regulated drug release based on the severity of tissue response, without interfering with the electrical properties of the electrode.

Additionally, many studies have used localized release of anti-inflammatory agents to control the inflammation against MEAs,[30, 34-37] but few reports have addressed direct protection of cells from oxidative stress. We hypothesized that protecting cells from the injurious oxidative stress could benefit the communication between MEAs and healthy neurons. Astrocytes are one type of glial cells in the brain supportive of neuron functions. It has been shown that astrocytes can protect neurons from oxidant hydrogen peroxide toxicity.[38, 39] Therefore, we suggest that it is rational to shield astrocytes and neurons from oxidative stress associated with implantable MEAs. Such an approach could potentially lead to better survival of neurons that are vital for long-term MEAs functions.

1.2 Layer-by-Layer (LBL)

Layer-by-layer (LBL) assembly technique is a versatile method for surface modification. Traditionally, species with opposite charges are adsorbed alternately to a surface, resulting in the formation of thin films with thickness ranging from nanoscale to microscale (Figure 5).[40-42] On the one hand, the fundamental underlying mechanisms such as the physicochemical aspects are a continuing research area. On the other hand, LBL has been recently studied for applications in a range of fields, including biological applications such as biomimetics, biosensors, drug delivery, protein and cell adhesion, mediation of cellular functions, and implantable materials.[43]

Though the LBL technique has been most widely applied to charged molecules, non-electrostatic forces, such as hydrogen bonding, have been recognized as an alternative mechanism for film growth.[44-53] The common hydrogen bonding acceptor polymers include poly(*N*-vinylpyrrolidone) (PNVP), poly(acrylamide) (PAAM) and poly(ethylene oxide) (PEO), and the hydrogen bonding donor polymers include polyaniline, poly(acrylic acid) (PAA), and poly(methacrylic acid) (PMAA).[54] Tannic acid (TA), a water soluble natural polyphenol has also been reported as hydrogen bonding donor.[55, 56]

In addition to sharing the advantages of conventional electrostatic-based LBL method, the multilayered-films constructed through hydrogen bonding could undergo dissociation upon pH change, which could serve as smart coatings on implants.

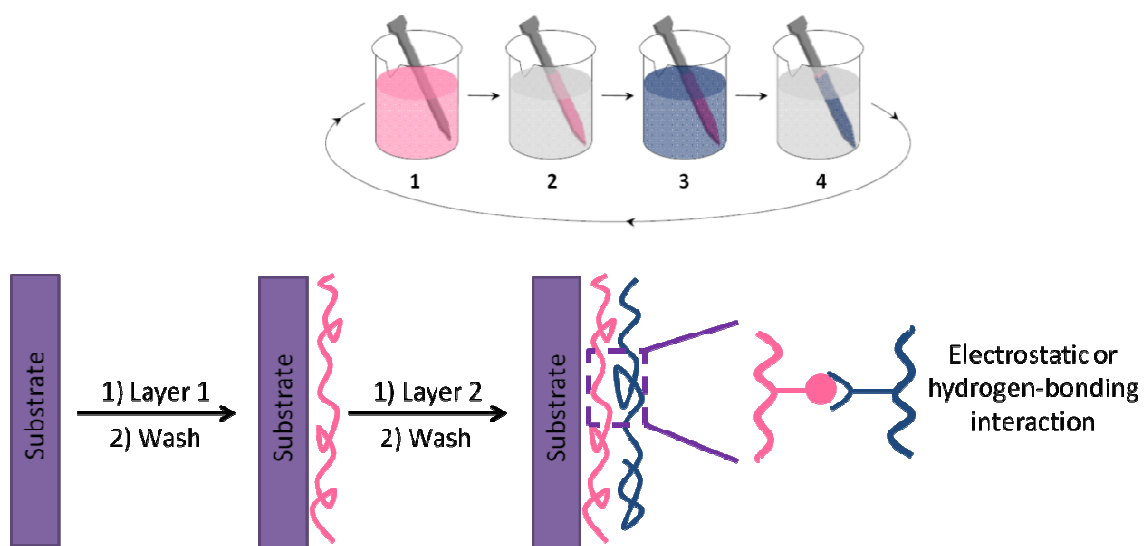


Figure 5. Schematic of the LBL assembly process.

from neural electrodes.[45] To overcome this issue, we propose to build a temporary drug-containing coating on the electrodes using hydrogen bonding based LBL assembly. Upon insertion into the tissue, the layers will quickly disintegrate under physiological pH, revealing the underlying electrode surface and thereby eliminating the concern of coating-induced loss of electrode functions.

1.3 Polymeric Therapeutics (Prodrug)

Polymer-drug conjugation (Figure 6) is a well-known and widely exploited technique that is useful to improve therapeutic properties of peptides, proteins, small molecules or oligonucleotides.[57-61] Polymer-conjugated drugs, sometimes referred to as polymeric therapeutics or prodrugs, generally exhibit prolonged half-life,[62] higher stability, improved water solubility,[63] lower immunogenicity and antigenicity,[64] and can be designed to allow specific targeting to tissues or cells.[65] Besides the above advantages, in this dissertation, polymeric therapeutics are selected to be compatible with the LBL surface modification method to improve performance of the chronic neural electrode.

A wide variety of polymers have been explored for prodrug synthesis. These include synthetic polymers such as *N*-(2-hydroxypropyl) methacryl-amide copolymer (HPMA), polyglutamic acid (PGA), poly(ethylene glycol) (PEG), poly(*N*-vinylpyrrolidone) (PNVP), and poly(ethyleneimine) (PEI). Examples of natural polymers used for this application include dextran, pullulan, chitosan, hyaluronic acid and many others.[66]

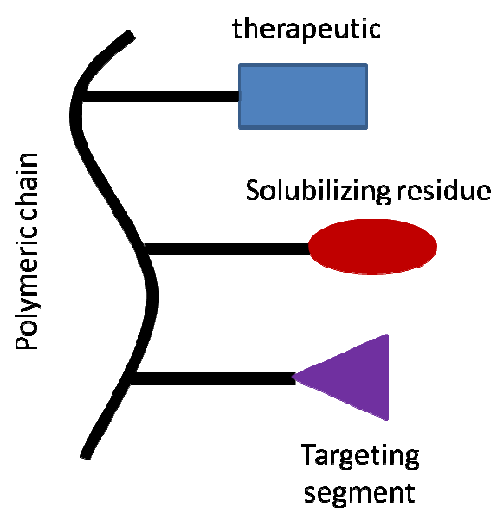


Figure 6. Features of polymeric therapeutics.

In this dissertation, two types of prodrugs were prepared, one anti-inflammatory for glial scar prevention and the other anti-oxidative for direct neural protection. The anti-inflammatory prodrug was built from PNVP. PNVP is a hydrophilic synthetic polymer generally known for its biocompatibility and non-antigenicity, and is considered safe for biological experiments.[67] Soluble PNVP is one of the most versatile and widely used pharmaceutical auxiliaries and topical disinfectants.[68] However, lack of common functional groups in PNVP has limited its further applications such as conjugation with other biological therapeutics. I proposed to overcome such a limitation by functionalizing PNVP with carboxylic acid groups to allow the synthesis of anti-inflammatory prodrugs. For the anti-oxidant prodrugs, I prepared a polymerizable monomer derived from an anti-oxidant and copolymerized it with a biocompatible acrylate monomer with functionalities amenable for LBL assembly.

1.4 Stimuli-Responsive Polymers

1.4.1 Thermo-responsive polymers

Thermo-responsive polymers are among the most intensively investigated smart materials in biomedical fields.[69-71] A key characteristic of these polymers is that they display a lower critical solution temperature (LCST). At temperatures below the LCST, these polymers are soluble in water. Upon raising temperatures above the LCST, such polymers will undergo a conformational transition from loose solvated coils to compact dehydrated globules, collapse and become insoluble in water.[72] Such a phase separation process occurs by either nucleation and growth, or spinodal decomposition (Figure 7).

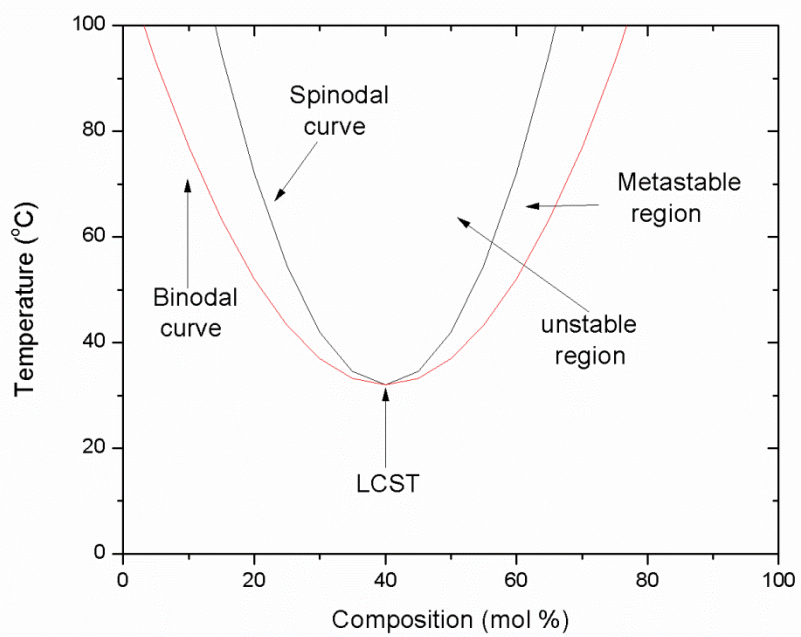


Figure 7. An example of phase diagram for polymers with LCST behaviors.[73]

Nucleation and growth are associated with metastability. Spinodal decomposition, on the other hand, refers to phase separation under conditions in which the energy barrier is negligible, so no activation energy is required. The spinodal line is constructed from the points where,

$$\frac{\partial^2 G}{\partial \phi^2} = 0 \quad 1$$

where G is the free energy, ϕ is the composition.

The binodal curve is related to the equilibrium phase boundary between the single phase and the phase separated region. In a binary system, this is related to the chemical potentials of an individual component being equal in both phases as expressed by the following relationships:

$$\Delta\mu_1^a = \Delta\mu_1^b \quad 2$$

$$\Delta\mu_2^a = \Delta\mu_2^b \quad 3$$

where μ is the chemical potential, 1,2 represent the two components and a,b represent the phases.[73, 74] If the temperature of a mixture is raised above the LCST, that component undergoes thermally induced phase separation by either nucleation and growth or by spinodal decomposition (SD). The minimum value occurring at the intersection of the binodal line and spinodal line is the LCST. Whether nucleation and growth or SD occurs depends on how phase boundary is crossed. If the binodal line is crossed, the component becomes thermodynamically metastable. Under this scenario, phase separation is favorable but only achieved by nucleation and growth. If instead the spinodal line is crossed, SD will occur. The transition points between phase-separation and homogenous solution are therefore dependent on how temperature is changed.

Hysteresis of the reversible transition is related to the difference between spinodal and binodal curves. Note that phase separation is visually accompanied by an increase in turbidity or cloudiness of a solution. The cloud point is the temperature where the solution starts to become cloudy. And LCST is the cloud point at the critical composition. For simplicity, an experimentally determined cloud point is often reported rather than an LCST.

While poly(*N*-isopropylacrylamide) (PNIPAAm) has dominated as the most well-documented thermo-responsive polymer, there are growing interests on other types of polymers with LCST behaviors, such as poly(vinyl methyl ether) (PVME)[75], poly(2-oxazoline)[76], and poly(*N*-vinyl-2-caprolactam) (PVCL)[77].

PVCL is water soluble and biocompatible, with LCST in the vicinity of physiological temperature.[78] Its good biocompatibility is attributed to the fact that the amide group in PVCL is directly bonded to the hydrophobic backbone, thus its hydrolysis will not result in the production of cytotoxic small amide compounds.[79] The unique temperature dependent behavior of PVCL has been employed to prepare thermo-responsive microgels for a variety of applications, including thermal sensitive drug delivery[80] and template for oxidative polymerization of pyrrole.[81, 82] It also has been used to stabilize proteases.[83] Furthermore, thermo-sensitive PVCL was recently employed by Zhuk and coworkers[84] for constructing pH responsive multilayers, which can be delaminated at high pH values.

1.4.2 Dual responsive polymers

In conjunction with temperature, other stimuli including pH have been used in developing dual responsive polymers. For example, researchers have copolymerized NIPAAm with negatively charged carboxylic acid containing monomers such as alkyl acrylic acid,[85-88] *N*-acryloylvaline,[89] and succinic anhydride modified dextran[90]. Alternatively, monomers containing positively charged amino groups have also been commonly polymerized with NIPAAm, such as *N,N*-dimethylaminoethyl methacrylate (DMAEMA),[91] *L*-lysine,[92] and *trans*-4-hydroxyl-*L*-proline.[93] A few other polymers, which are not based on NIPAAm, have been reported with dual-responsive properties, such as copolymers of di(ethylene glycol) methyl ether methacrylate (MEO₂MA) with either methacrylic acid (MAA) or DMAEMA,[94] homopolymer of poly(*N*-ethylpyrrolidine methacrylate),[95] graft copolymers containing PMAA backbone and oligo(2-ethyl-2-oxazoline) (OEtOx) side chains,[96] and poly(amidoamine) dendronized poly(2-hydroxyethyl) methacrylate[97]. These dual responsive polymers have been explored in applications including control of micelle structures,[89, 91, 93] advanced drug delivery systems,[85, 88, 98] fabrication of dual functional nanofluidic devices,[86] and modification of gold nanoparticles.[90]

Based on the LCST behaviors of PVCL polymers, I designed its functional derivative with pH-dependent LCST behaviors for smart drug delivery. The goal is to use the smart polymer as an alternative to enable tissue response specific release of anti-inflammatory drugs.

1.5 Polymer chain conformation [73, 99]

The freely jointed chain model is the simplest mathematical description of a polymer chain. In assumption, a single polymer chain has n bonds, each of length l , joined in a linear sequence with no restrictions on the angles between successive bonds. In this model, the mean-square end-to-end distance (R_f^2) is given by the following equation:

$$R_f^2 = nl^2 \quad 4$$

However, it underestimates the real end-to-end distance of a polymer chain, omitting factors such as bond angle, conformation angle, excluded volume, steric hindrances, etc. The correction is represented by a constant, characteristic ratio C_∞ by the following equation:

$$R_0^2 = C_\infty nl^2 \quad 5$$

The end-to-end distance is difficult to measure. Instead, the radius of gyration, R_g , is often used since this quantity can be measured by a light scattering technique. The radius of gyration is the average distance from the center of the gravity to the chain segment. The mean-square value of the radius of gyration can be related to the mean-square value of the end-to-end distance by the following equation:

$$R_g^2 = \frac{1}{6} R_0^2 = \frac{C_\infty nl^2}{6} \quad 6$$

In a crude approximation, each linear polymer chain occupies a space of a sphere with the dimension of R_g . At low concentrations, these spheres are separated from each other. As the concentration increases, they become congested and eventually touch each other. At the overlap concentration (c^*), the whole volume of the solution is packed with

these spheres. The overlap concentration of the solution is given by the following equation:

$$c^* = \frac{3M}{4\pi N_A R_g^3} \quad 7$$

where M is the molecular weight of the polymer, and N_A is the Avogadro's number.

When polymer concentration is below c^* , the solution is considered dilute. The polymer chains are separated from each other and behave more or less independently. The chain interacts primarily with the solvent molecules. The solution is close to an ideal solution. The situation is different at concentrations above c^* . The solution in this regime is considered semidilute. The polymer chains are overlapped and entangled. Their mobility is greatly reduced compared with the chains in dilute solutions. The thermodynamic properties of the semidilute solutions are greatly different from those of an ideal solution extrapolated to the same concentration. At an even higher concentration, the solution enters the concentrated regime in which each segment of the polymer chain does not have a sufficient space available.

This dissertation studied the molecular behaviors in the LBL assembly by examining polymer chain dimension in aqueous solutions. The assembly process at the molecular level was proposed by correlating thickness of the layer with the polymer chain conformation information.

1.6 Dissertation Structure

The objective of this dissertation research is to develop polymer mediated therapeutic interventions to help improve long-term performance of implantable neural electrodes. Failure of neural electrodes has been mainly attributed to glial scar formation and loss of neurons around the implant. To address each of the causes, I have tailor designed polymer based therapeutic interventions and studied their application toward neural electrodes. Furthermore, a pilot study was carried out to synthesize a dual-responsive biocompatible polymer intended for smart drug delivery at the interface. The content of this dissertation is schematically shown in Figure 8:

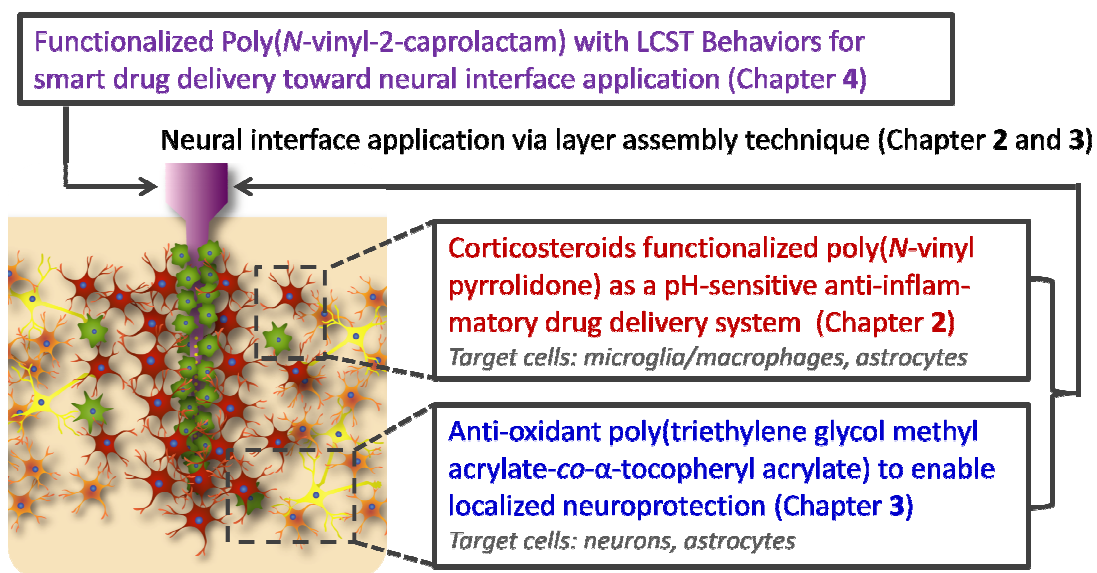


Figure 8. Overview of the dissertation research.

Chapter 2

Synthesis and Characterization of Glucocorticoid Functionalized Poly(N-vinyl pyrrolidone): A Versatile Prodrug for Neural Interface

An article presenting part of the results in this chapter has been published on Biomacromolecules. [Cao Y, He W. Synthesis and Characterization of Glucocorticoid Functionalized Poly(N-vinyl pyrrolidone): A Versatile Prodrug for Neural Interface. Biomacromolecules 2010;11:1298-307.] The first author (Yu Cao) finished all of the experiments in this study. The corresponding author (Dr. Wei He) is advisor of Yu Cao and financially supported this work.

2.1 Introduction

Chronically implanted recording electrode arrays have the potential to improve the quality of life of patients suffering from partial or full paralysis. However, they often fail to function reliably in clinically relevant chronic settings. A potential solution to pharmacologically manage the tissue response to implanted electrodes is to enable self-regulated drug release based on the severity of tissue response, without interfering with the electrical properties of the electrode. Integrating the surface modification method based on LBL assembly and stimuli-responsive drug conjugates, one could accomplish the proposed ‘smart’ and effective drug delivery at the neural interface (Figure 9).

The multilayered-films constructed through hydrogen bonding mediated LBL assembly could undergo dissociation upon pH change, which could serve in the application of smart coatings and be particularly attractive to biosensors such as neural electrodes, where preserving electrical properties significantly limits the characteristics of any coating deposited on the surface.[45]

Specifically, the electrode surface could serve as a temporary support for the drug-containing LBL multilayered film. Upon insertion into the tissue, the layers will

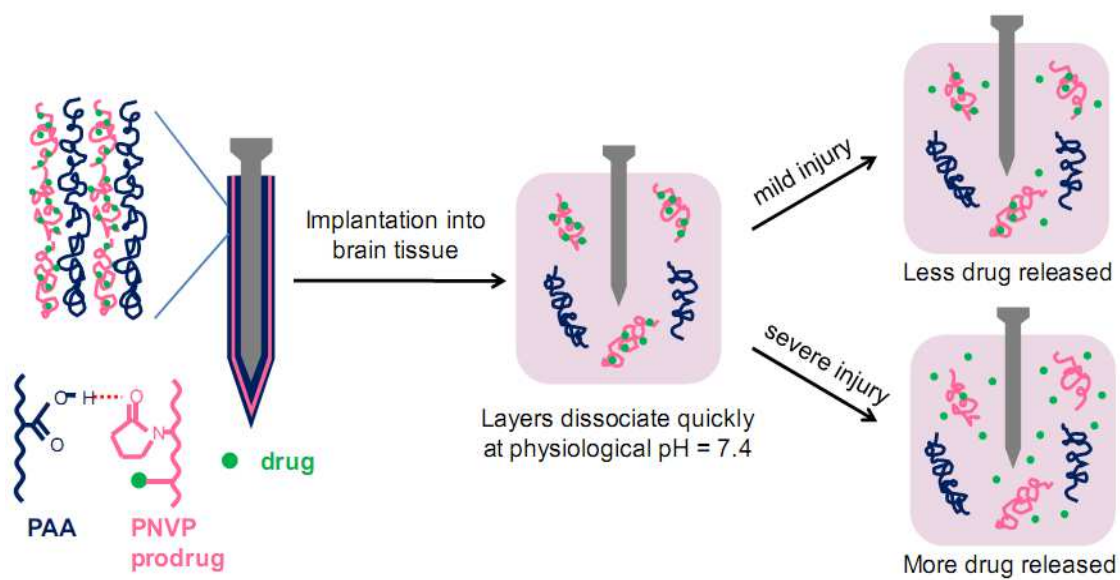


Figure 9. Surface modification of neural electrode with LBL assembly and drug release responsive to tissue reaction.

quickly disintegrate under physiological pH, revealing the original electrode surface and eliminating the concern of coating-induced loss of electrode function (Figure 9).

To directly link drug release with the extent of tissue response, the molecular characteristics of tissue response can be used as the trigger. Tissue acidosis, a decrease of extracellular pH of the tissue, has been observed after brain injury by many researchers.[100-103] Such a decrease in pH is also related to frustrated phagocytosis, a process that involves macrophages actively attacking non-phagocytosable surfaces, leading to the accumulation of degradative substances such as acid at the cell-biomaterial interface.[62, 104] Therefore, a drug conjugate with a pH sensitive moiety will potentially enable tissue response regulated drug release. Prodrug is useful to improve therapeutic properties of peptides, proteins, small molecules or oligonucleotides. In order to realize tissue acidosis controlled drug release, one could easily engineer a pH-sensitive trigger into the molecular structure of the prodrug. Hydrazone is one of the common acid labile linkages used in prodrug applications.[105, 106] It is relatively stable at physiological pH (7.4), but hydrolyzes at acidic pH normally found at pathological sites (below 6.0) such as tumors,[107, 108] infarcts,[109] and inflamed tissue.[110] Therefore, hydrazone bonding was used in this study to form prodrug of anti-inflammatory glucocorticoids.

The other desired feature of the prodrug is its ability to participate in the LBL process based on hydrogen bonding. Thus a functional derivative of the hydrogen bond acceptor PNVP was used for drug conjugation. Several methods have been explored to introduce functional groups such as carboxylic acid and amine onto either the pendant

chain or polymer end.[111-115] The pendant carboxyl groups of PNVP derivative can be used for drug conjugation while the repeating lactam ring provides hydrogen bond accepting function in the LBL process. PNVP is a hydrophilic synthetic polymer generally known for its biocompatibility and non-antigenicity, and is considered safe for biological experiments.[67] Soluble PNVP is one of the most versatile and widely used pharmaceutical auxiliaries and topical disinfectants.[68] However, lack of common functional groups in PNVP limits its further applications such as conjugation with other biological therapeutics.

In this chapter, I have synthesized a glucocorticoid functionalized PNVP prodrug that displayed responsive drug release in vitro under conditions mimicking inflammation. The feasibility of transporting the prodrug to the neural interface using the LBL method without permanently encapsulating the electrode was evaluated. The bioactivity of the prodrug and its released products was also examined in terms of anti-inflammatory potency and neurotoxicity. This newly developed drug delivery system could potentially resolve the dilemma between increasing drug loading capacity and maintaining the conductivity of electrode. Furthermore, it enables direct regulation of drug release by the tissue response, thus allowing more effective and active therapeutic treatment at the neural interface.

2.2 Experimental Section

2.2.1 Materials

Diisopropylamine, azobisisobutyronitrile (AIBN), di-*tert*-butyl dicarbonate, *N*-ethyl-*N*'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), prednisolone, and

polyethyleneimine (PEI) with $M_n = 1,800$ g/mol were obtained from Sigma-Aldrich. Tetrahydrofuran (THF), *N*-vinyl-2-pyrrolidone (NVP), *N,N'*-dimethylformamide (DMF), *n*-butyllithium, *tert*-butyl carbazate (TBC) and trifluoroacetic acid (TFA) were obtained from Acros. Deuterated dimethyl sulfoxide, chloroform, and water were obtained from Cambridge Isotope Laboratories, Inc. Polyacrylic acid (PAA) with $M_w = 450,000$ g/mol was purchased from Polysciences, Inc. Hexanes, ethyl acetate, dichloromethane (DCM) and quartz slides were purchased from Fisher Scientific. Dialysis membrane (MWCO = 500 Da) was obtained from Spectrum Lab, Inc. All reagents were used as received unless otherwise noted.

2.2.2 Characterization

^1H NMR (300 MHz) spectra were recorded on a Varian Mercury 300 NMR spectrometer. Gel permeation chromatography (GPC) was performed at room temperature using PL-GPC 50 plus (an integrated GPC/SEC system from Polymer Laboratories, Inc.) with a refractive index detector. DMF was used as the carrier solvent at a flow rate of 1.0 mL/min. Standard monodisperse polystyrenes (Polymer Laboratories) were used for calibration. UV-vis spectra were measured using a UV-vis spectrometer (Biomate 5 from Thermospectronic). Ellipsometric measurements were performed on dry films in air using an L0116S Stokes ellipsometer (Gaertner scientific corporation) with He/Ne laser (632 nm) illumination at a 70° angle of incidence. A refractive index of 1.465 for the dry film was assumed.

2.2.3 Prodrug synthesis

Synthesis of carboxylated NVP copolymer (COOH-PNVP). Monomer of 3-(*tert*-butoxycarbonyl)-*N*-vinyl-2-pyrrolidone (TBNVP) was synthesized as described previously.[114] NVP (5.328 g, 1.6 mol/L), TBNVP (2.532 g, 0.4 mol/L), AIBN (0.148 g, 0.03 mol/L) and freshly distilled THF (30 mL) were added into a 250 mL flask, and subjected to three freeze-thaw cycles to remove oxygen. Polymerization reaction took place at 65 °C for 22 h. The polymer was then precipitated in hexane, redissolved in THF and reprecipitated in hexane for purification. The obtained polymer (PTBNcoN) was dried in a vacuum oven at 55 °C overnight (6.555 g, 83% yield, Mw: 16,900 g/mol). The carboxyl groups (-COOH) were recovered by removing the *t*-BOC protecting groups using TFA. Briefly, PTBNcoN (6.259 g, 9.55 mmol *t*-BOC) was dissolved in DCM (15 mL) followed by the addition of 15 mL TFA. After reacting for 4 h, the polymer was recovered by solvent evaporation and dialysis against deionized water overnight and lyophilization. The yield was 5.05 g (80%).

Synthesis of NVP-hydrazide copolymer (PNVP-NH-NH₂). COOH-PNVP (3.568 g, 5.9 mmol COOH) and TBC (0.808 g, 5.9 mmol) was dissolved in 25 mL of ultrapure water. Aqueous solution of EDC (1.17 g, 5.9 mmol) was then added dropwise. The reaction mixture was stirred for 24 h at room temperature. Unreacted TBC and EDC were removed by dialysis against deionized water extensively. The product, *t*-BOC protected NVP-hydrazide copolymer (PNVP-NH-NH-BOC) was obtained by lyophilization (3.584 g, 76%). To remove the BOC protecting groups, TFA was used. Briefly, PNVP-NH-NH-BOC (1.171 g) was dissolved in DCM (10 mL) and TFA was added (10 mL). After 4 h of

reaction at room temperature, the solvent was evaporated, and the purified PNVP-hydrazide copolymer (PNVP-NH-NH₂) was obtained by dialysis against deionized water extensively and lyophilization. The yield was 1.058 g (89%).

Synthesis of NVP-glucocorticoid prodrug (PNVP-Pred). PNVP-NH-NH₂ (0.712 g) was mixed with the glucocorticoid drug prednisolone (1.204 g) in DMF and 50 μ L of acetic acid was added as a catalyst for the reaction. The solution was kept stirring for 24 h. The mixture was precipitated in water to remove unreacted prednisolone (limited water solubility 142 mg/L[116]) while the prodrug remained in the aqueous solution. The obtained solution was dialyzed against deionized water extensively and lyophilized to obtain prodrug (0.410 g, 38%).

2.2.4 pH sensitive drug release

Prodrug (10 mg/mL) was dissolved in either an acetate buffer (0.15 M, pH 5.0), or a phosphate buffer (0.15 M, pH 7.4). One millimeter of prodrug solution was then loaded to a dialysis tube (MWCO 500 Da) and incubated in 20 mL of corresponding buffer at 37 °C. At selected time intervals, the solution outside the dialysis membrane (60 μ L) was withdrawn for high-performance liquid chromatography (HPLC) analysis. A Thermo Scientific Finnigan HPLC system coupled with dual wavelength detector was used for the drug release study. A reverse phase C18 (Waters Sysmmetry, 4.6 $\times$ 150 mm, 5 μ m) was used for the analysis with acetonitrile/water as the mobile phase. The flow rate was set constant at 1.0 mL/min. The UV detection wavelength was at 240 nm. The sample injection volume was 20 μ L for all evaluations. A linear external prednisolone calibration curve was established. The amount of prednisolone released was quantified by converting

the UV absorbance at 240 nm against the standard calibration curve. All drug release data are presented as the amount of free prednisolone relative to the total prednisolone content in the prodrug conjugate.

2.2.5 LBL assembly with PNVP prodrug (PNVP-Pred)

The self-assembled films were prepared either on quartz slides or silicon substrates with a 100 nm thick oxide layer (University Wafer), depending on the specific characterization methods used. Before layer deposition, the silicon wafers were cut into small squares (1 x 1 cm). Both substrates were cleaned in a boiling piranha solution (3:1 concentrated H_2SO_4 : 30% H_2O_2 v:v; *piranha solution reacts violently with organics and should be handled with extreme caution*) for 30 min, washed with copious amount of water and dried under nitrogen. The substrates were first immersed in a PEI solution (1.5 mg/mL, pH = 7) for 30 min, followed by rinsing in water for 1 min. The PAA/prodrug multilayers were then assembled by alternating immersion of the substrates in solutions of PAA (0.5 mg/mL, pH = 3) and prodrug (0.5 mg/mL, pH = 3) for 5 min, respectively. Between each step, the excess polymer was removed by rinsing the substrates with HCl solution (pH = 3) for 1 min. All solutions were prepared using ultrapure water (Milli Q-plus system, Millipore) with a resistivity of 18.2 $\text{M}\Omega\cdot\text{cm}$. The assembly process was monitored with UV-vis spectrometer and ellipsometry. The coated substrates were immersed in phosphate buffered saline (PBS, pH = 7.4) for 1 h, and subsequent ellipsometric measurements were carried out to characterize layer desorption.

2.2.6 Bioactivity of prodrug and its released products

In vitro anti-inflammatory study of prodrug. A subclone of RAW264.7 murine macrophages (kindly provided by Dr. Tim Sparer of the University of Tennessee) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 1% L-glutamine and 1% penicillin/streptomycin. Cells were cultured at 37 °C, 5% CO₂ and 95% relative humidity. For anti-inflammatory study, cells were seeded in 48-well tissue culture plates at a density of 1.0×10^5 cells/well and allowed to attach overnight. Culture medium was then aspirated and replaced with fresh medium. Cells were stimulated to generate nitric oxide (NO), a potent inflammatory mediator, with 100 ng/mL of lipopolysaccharide (LPS) (Sigma), and treated with solution of prednisolone, prodrug or precursor polymer (PNVP-NH-NH₂) (concentration equivalent to 1.1 μ M prednisolone) simultaneously. All treatments were done in triplicate. After 24 h of incubation, the amount of stable nitrite accumulated in the culture medium, the end product of NO production, was determined by Griess reagent (Promega).[116]

In vitro anti-inflammatory study of products from prodrug release. Prodrug solution was prepared with either a PBS buffer (pH = 7.4) or an acetate buffer (pH = 5.0), and incubated at 37 °C. At various time points (day 1 and day 10), an aliquot of solution was withdrawn and stored at -20 °C. Prodrug solutions were also collected before incubation and noted as day 0 samples. Macrophages were cultured and activated as described in the previous section, and treated using the incubated prodrug solutions

collected at day 0, 1, and 10, respectively. NO production was analyzed using the Griess assay.

The results for Griess assay study were expressed as percentage to the untreated LPS stimulated control, and analyzed by analysis of variance (ANOVA) using a SAS software. Least squares means were compared by Least Significant Difference (LSD) test. For all analyses, significance was assigned for $p < 0.05$. All cell culture studies were repeated to account for biological variance.

Primary cortical neuron culture. Cortical neurons were obtained from 9-day-old chicken embryos. Forebrains of the embryo were dissected, minced into small pieces, and enzymatically dissociated with 0.25% trypsin in PBS for 20 min at 37 °C, followed by inactivation with medium containing 10% FBS (Invitrogen). A cell pellet was obtained after a brief centrifugation, and mechanical trituration using a fire-polished Pasteur pipet was applied to further dissociate the cells. Cells were then preplated on a collagen-coated Petri dish and incubated for 1 h at 37 °C in a 5% CO₂ atmosphere. This process has been reported to provide culture with 97% of neuron composition.[117] Purified neuronal cells were collected and resuspended for cell culture.

In vitro cytotoxicity study. The cytotoxicity of the prodrug and its polymer precursor PNVP-NH-NH₂ towards neurons was determined. Cortical neurons were seeded into wells of 24-well tissue culture plates precoated with Laminin at a density of 5.0×10^4 cells/cm² and cultured in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, and 1% L-glutamine at 37 °C in a 5% CO₂ atmosphere for 2 days. Cell culture medium was then aspirated and replaced with fresh medium

supplemented with either prodrug or PNVP-NH-NH₂ (100 μM, equivalent to prednisolone). Cells were incubated for another 2 days and cell viability was determined using a WST-1 assay (Roche) according to the manufacturer's instruction. Cell viability was calculated by normalizing the absorbance to that of the controls. Furthermore, a qualitative morphological study was performed simultaneously. Briefly, at the end of culture, cells were live stained with 0.05 v/v Calcein AM (Invitrogen) solution in PBS and incubated at 37 °C in a 5% CO₂ atmosphere for 30 min. Samples were rinsed twice with PBS before and after fluorescence staining. Fluorescent images were collected using a 10× objective lens on a confocal microscope (Leica SP2, Germany).

2.2.7 Prodrug diffusion

Synthesis of fluorescent prodrug analog (FPNVP). The reaction is modified from a previous report.[118] PNVP-NH-NH₂ (0.03 g) was dissolved in sodium carbonate buffer (16 mL) at pH 9. Fluorescein isothiocyanate (FITC) in anhydrous DMSO (0.15 mL, 1 mg/mL) was added to the above PNVP-NH-NH₂ solution. The solution was kept stirring for 10 h. The mixture was quenched by the addition of ammonium chloride (16 mg) to a final concentration of 50 mM. The obtained solution was dialyzed against deionized water extensively in dark and lyophilized to obtain FPNVP (0.012 g, 40%).

Diffusion experiment. The Si substrates were firstly coated with 8 bilayers of FPNVP and PAA using the LBL method mentioned in section 2.2.5. A 0.6 wt% SeaKem agarose solution was poured onto a Si wafer with 8 bilayers of (FPNVP-PAA). Gel was formed within 5 minutes. Then 1 mL PBS was added on top of the gel. The system was

kept in dark overnight at 37 °C. Then fluorescent images were captured. The software of Image J was used to analyze the fluorescent pictures to obtain quantitative data.

2.3 Results and Discussion

2.3.1 Prodrug synthesis and characterization

In this work, the synthesized prodrug was based on PNVP and anti-inflammatory glucocorticoid drug of prednisolone. The prodrug was prepared following procedures outlined in Figure 10. The conjugation scheme can be easily translated to other members of the glucocorticoid family, such as dexamethasone and methylprednisolone. Due to the lack of reactive groups for drug conjugation in monomer (NVP), it is common to copolymerize NVP with other functional vinyl monomers. However, reactivity of NVP differs greatly from those of other monomers, which results in compositional shift from feeding ratio. Therefore, a derivative of NVP was used to copolymerize with NVP in preparing functional PNVP. The derivative, TBNVP, bears a protected carboxyl group at the α -position of NVP, and is expected to show similar radical reactivity with NVP due to the structure similarity between the two monomers. As shown in a, integration of $^3\text{CH}_2$ and $^7\text{CH}_3$ revealed that the molar ratio of TBNVP in the prepared copolymer was 18 mol%, suggesting good agreement with the monomer feeding ratio of 20 mol%. Thus, copolymerization of TBNVP and NVP monomers allows controlled introduction of functional groups into the polymer with little compositional drift.

In order to render the prodrug susceptible to tissue acidosis, an acid labile hydrazone group was used to conjugate prednisolone to the functional PNVP.

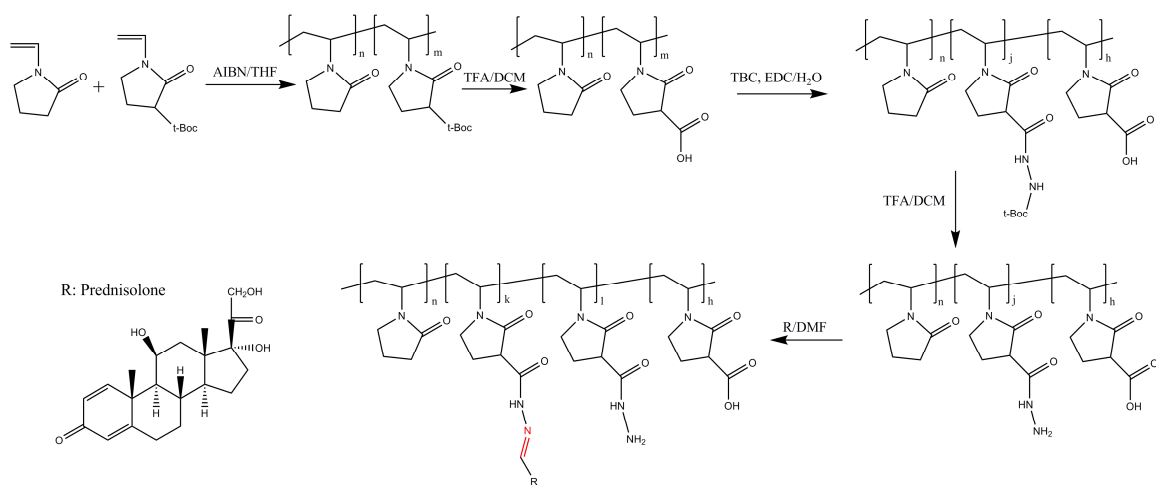


Figure 10. Glucocorticoid prodrug synthesis via conjugation of prednisolone onto functionalized poly(*N*-vinyl pyrrolidone).

Briefly, the carboxyl group at the α -position of PNVP was exposed by acid removal of the *t*-BOC protecting group, and subsequently converted into hydrazide groups by reacting with *tert*-butyl carbazate followed by acid deprotection. The use of TFA for deprotection of *t*-BOC groups is highly efficient as reported in the literature[119] and further confirmed in this study by the disappearance of the characteristic *t*-butyl peak at δ 1.33 in the NMR spectra (Figure 11b and d). Conversion of the functional groups can be obtained using integrations of $^3\text{CH}_2$ and $^7\text{CH}_3$. Briefly, PTBNcoN was fully converted to COOH-PNVP. Subsequently, 88.9% of the -COOH groups were converted to BOC-hydrazide, followed by 100% conversion to unprotected hydrazide. This means that the composition of the copolymer prior to drug conjugation was 82 mol% of NVP, 16 mol% of NVP-NH-NH₂, and 2 mol% of NVP-COOH. Prednisolone was conjugated onto the copolymer through formation of a hydrazone bond between the pendant hydrazide group of the copolymer and the ketone carbonyl group on prednisolone. Because prednisolone has poor solubility in D₂O, d₈-DMSO was chosen as the NMR solvent for comparison between free drug and prodrug, even though the prodrug has good water solubility. As shown from Figure 12, the ^1H -NMR spectrum of prodrug presents signals between δ 4.0 and δ 7.6, attributed to protons of the prednisolone moieties. It indicates success of drug conjugation to the copolymer. The degree of prednisolone conjugation to the polymer was determined by UV absorbance spectroscopy analysis of the prodrug solution. Figure 13 showed UV spectra of 0.1 mg/mL solutions of prednisolone, prodrug, and its polymer precursor PNVP-NH-NH₂.

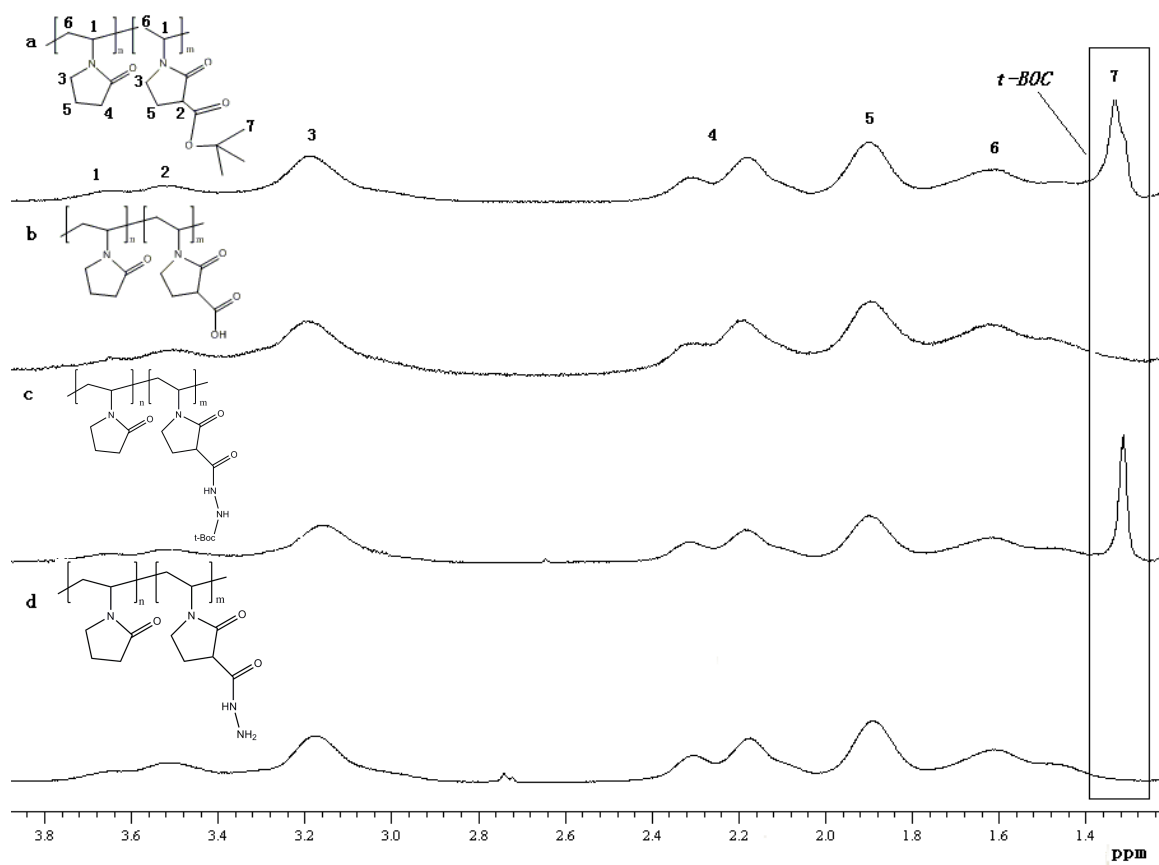


Figure 11. ^1H -NMR spectra of (a) PTBNcoN, (b) COOH-PNVP, (c) PNVP-NH-NH-BOC and (d) PNVP-NH-NH₂ in D_2O .

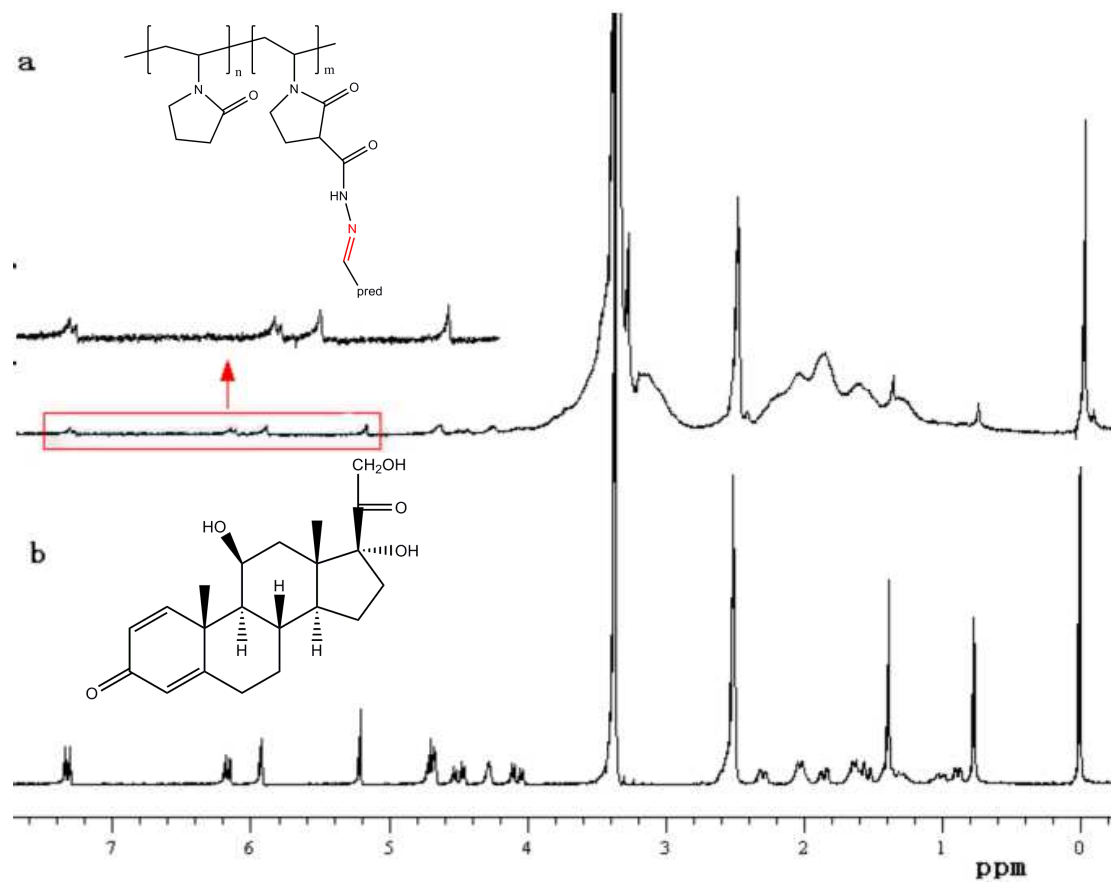


Figure 12. ^1H -NMR spectra of (a) prodrug PNVP-pred and (b) free drug prednisolone in d_8 -DMSO.

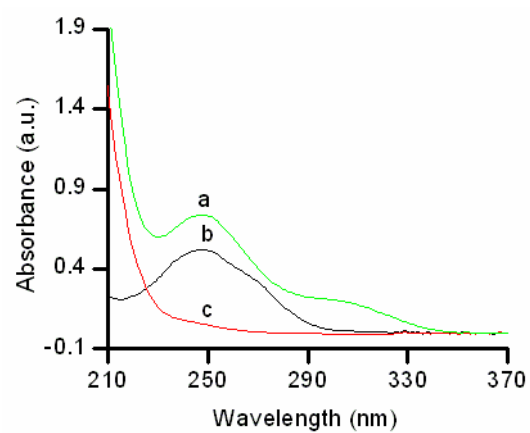


Figure 13. UV spectra of 0.1 mg/mL aqueous solution of (a) prodrug PNVP-pred, (b) free drug prednisolone, and (c) precursor polymer PNVP-NH-NH₂.

The prodrug and prednisolone share the characteristic peak at 247 nm. Prednisolone solutions of various concentrations were prepared and the absorbance at 247 nm was measured to generate a calibration curve for prednisolone content calculation. The amount of prednisolone conjugated to the polymer was 160 mg/g prodrug, determined by comparison of UV absorbance spectrum of prodrug against the prednisolone calibration curve generated in our lab.[120, 121] Composition of NVP-pred in the prodrug was calculated as 6 mol%, indicating that 60% of the hydrazide groups were consumed for the conjugation. The polydispersity of the prodrug was 3.84 as characterized by GPC. Although only one degree of conjugation was studied in the present work as a proof-of-concept, the level of functionalization and drug loading capacity can be easily tuned by varying the initial copolymer composition between NVP and TBNVP. To characterize the release profile of the synthesized prodrug bearing acid-labile functional groups, two release conditions were used. One condition is a pH 7.4 buffer representing normal tissue and the other condition is a pH 5.0 buffer simulating severely inflamed tissue.[122] As expected, the hydrazone-containing prodrug demonstrated pH-sensitive drug release behavior. Within 25 days, the percentage of prednisolone released from the prodrug was always higher at acidic pH than at physiological pH (Figure 14).

The faster drug release rate under acidic condition is characteristic of the hydrazone bond, as its hydrolysis rate has been shown to be accelerated in the presence of an acid.[106, 123] This result suggests that more anti-inflammatory drugs will be released in case of tissue acidosis, therefore potentially enabling more effective and active pharmacological management of inflammatory tissue response at the implant interface.

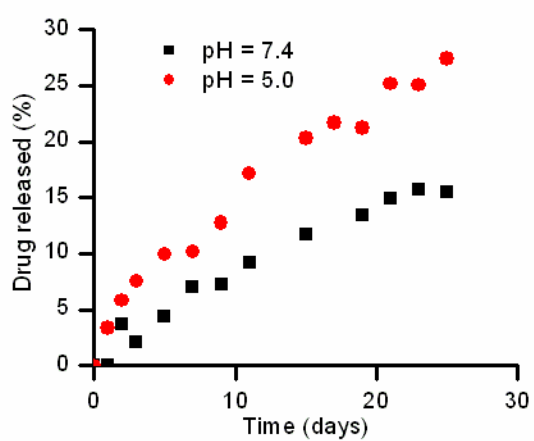


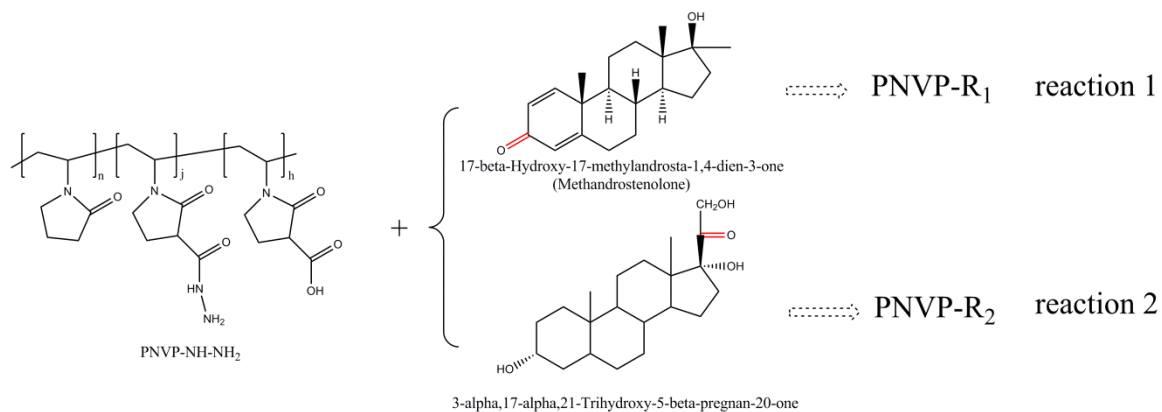
Figure 14. In vitro cumulative release of prednisolone from prodrug at different pH levels up to 25 days. Standard deviation is less than 5% of the mean value (n=3).

There are two carbonyl groups at positions 3 and 20 in the prednisolone structure, as shown in Figure 10. A question related to the reaction mechanism is that which group reacts with PNVP-NH-NH₂ to form the prodrug. Inferred from the fact that no crosslinking occurred during the reaction, it is possible that these two carbonyl groups differ in reactivity. Because the carbonyl group attached to the six-membered ring (position 3) is stabilized by the conjugated structure, I suggest that the other carbonyl group is more reactive. Therefore, the carbonyl group attached to the five-membered ring (position 20) is thought to be the reacting group in the prodrug synthesis step.

In order to directly identify which carbonyl group reacts with PNVP-NH-NH₂, control experiments could be designed. This study can also shed light on the crosslinking concern caused by potential role of prednisolone as a crosslinker due to the presence of two carbonyl groups. Control reactions designed using commercially available chemicals are shown in Figure 15.

The reaction and workup procedures will be the same with that described in section 2.2.3. NMR spectra will be collected to determine chemical structure of product from reaction 1 and 2. Specifically, residual peaks from R₁ or R₂ will be screened to prove if any reaction happened. There are four possible outcomes as show in Figure 15.

Out of these four outcomes, if b or c happens, it will be a clear indication of which carbonyl group on prednisolone reacted with PNVP-NH-NH₂. Result d is unlikely because either reaction 1 or 2 should occur based on the results of the reaction between PNVP-NH-NH₂ and prednisolone.



Possible outcomes from the control reactions:

	Reaction 1 occurred	Reaction 1 not occurred
Reaction 2 occurred	a	b
Reaction 2 not occurred	c	d

Figure 15. Control reaction for illuminating the reactivity of the two carbonyl groups at positions 3 and 20 in prednisolone.

If outcome a is observed, this will mean either carbonyl can react. Further control reaction could be carried out by mixing an equal molar amount of R_1 and R_2 . The amount of R_1 (or R_2) will be kept in excess as compared to the amine group on PNVP-NH-NH₂, such as at a ratio of 3:1. Then the structure of final product could be determined. From here, the reactivity of the two carbonyl groups can be finally confirmed.

2.3.2 Application of prodrug for LBL assembly

The approach of integrating prodrug within self-assembled multilayers has been previously reported. Thierry and co-workers used a hyaluronan ester prodrug of paclitaxel, a potent hydrophobic chemotherapeutic agent, as a polyanion to prepare polyelectrolyte multilayers with a natural polyamine chitosan.[124] In their study, drug release resulted from the hydrolysis of the ester bond and subsequent diffusion of paclitaxel out of the film, while the multilayers remained stable throughout the release study. In the present work, a less stable multilayered film was preferred considering the application towards neural sensors such as electrodes. Therefore, the PNVP hydrazone prodrug of prednisolone was used for LBL assembly based on hydrogen bonding. PAA and PNVP could be deposited on a variety of substrates using the LBL technique through the hydrogen bond between the carboxylic acid groups on PAA and carbonyl groups on PNVP.[40, 45, 125] The stability of hydrogen-bonded PAA/PNVP multilayers is limited to a particular pH range, and layer deconstruction usually occurs under physiological pH (7.4).[126] Such pH-dependent stability is in fact advantageous for neural electrodes. As mentioned earlier, there is a fine balance between effective coating-based localized release of therapeutics and the electrical functionality of the neural electrodes. With this

destructible hydrogen bonding interaction, the neural electrodes can serve as a temporary support for film construction and transport the film to the tissue-electrode interface via implantation. The physiological pH will then quickly release the film off the electrode surface leading to an implanted bare electrode in the neural tissue surrounded by molecular components of the disintegrated film (Figure 9). Those components can exert therapeutic actions when needed if proper release triggers are engineered into the molecular structure. Since prednisolone was conjugated via the pendant functional groups of the PNVP, the prepared prodrug still contained the carbonyl groups, which can form hydrogen bond with PAA. Using the LBL technique, hydrogen-bonded PAA/prodrug multilayers were coated onto silicon substrates, a material commonly used in recording neural electrodes. Ellipsometric measurements (Figure 16a) revealed that film growth was a linear function of the number of PAA/prodrug bilayers deposited (for up to 8 bilayers), with an average bilayer thickness of 27 nm. This trend was also confirmed by UV-vis spectrophotometry of the multilayered films on quartz substrates, as indicated in Figure 16b. The absorbance peak at 193 nm originates from PNVP of the prodrug layer. A linear increase of absorbance at 193 nm in relation to the number of bilayers was observed, suggesting film growth of PAA and prodrug.[127]

To demonstrate that the PAA/prodrug multilayer film can be released from the silicon surface in a physiological environment, the coated substrates were immersed in PBS (pH = 7.4), and subsequent ellipsometric measurement confirmed the destruction of the multilayer coating within 1 h.

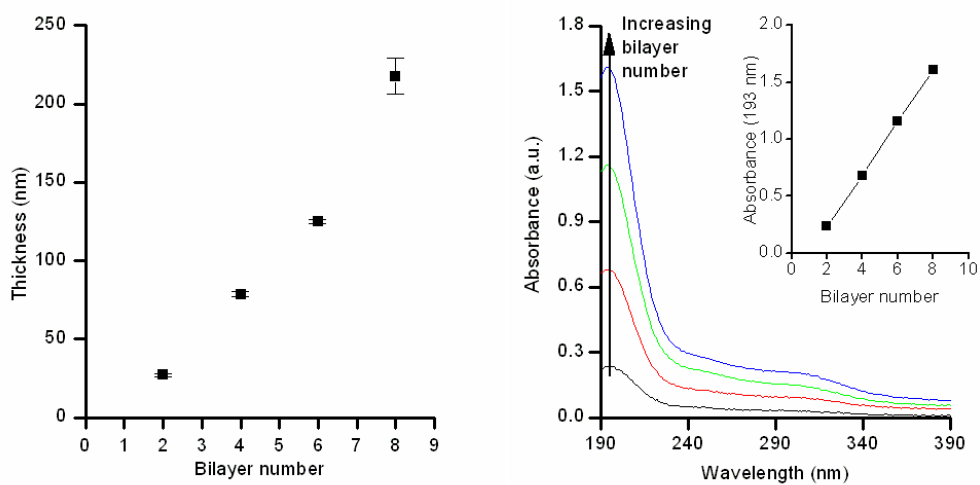


Figure 16. Assembly of LBL films. (a) Plot of ellipsometric film thickness versus the number of PAA/prodrug bilayers deposited on a silicon substrate. (b) UV-vis absorption spectra of multilayer films of PAA/prodrug after 2, 4, 6 and 8 bilayer depositions. Inset: Absorbance at 193 nm (contribution from PNVP) versus number of assembled bilayers.

The pH sensitivity of the hydrogen-bonded PAA/prodrug multilayer was consistent with other reports.[45, 126] This pH sensitive prodrug containing coating could be a simple yet effective approach to modify recording neural electrodes, in which one could incorporate a sufficient amount of prodrug to aid in managing the host response to the implanted electrode without the concern of sacrificing the electrical property of the neural electrode.

Because of the versatility of the LBL technique, such a prodrug system can be easily applied to other implantable sensors where a permanent, drug embedded coating might compromise the sensing function of the device. It is worth mentioning that PEI was used as the base layer to prime the surface for subsequent LBL process. Although toxicity of PEI is a debatable concern for biological applications[128-130], only a minute amount of PEI was present on the substrate (i.e., it has been reported less than 0.1 g/cm^2 of PEI deposited as a base layer for LBL[131]). Furthermore, toxicity of PEI depends on the molecular weight, where 2 kDa PEI was nontoxic even at concentrations as high as 45 g/mL[132]. The PEI used in this study has a molecular weight of 1.8 kDa, and therefore, it is not expected to exert significant adverse effects on neurons.

In this LBL assembly system of PAA/prodrug, PAA is chosen to facilitate deposition of prodrug in the dissociable coating. Actually, other polymers with carboxylic acid group could possibly substitute PAA. Therefore, polymers with good biocompatibility could be tried, which will be further discussed in chapter IV.

2.3.3 Bioactivity and biocompatibility of prodrug and its released products

The prodrug was designed for suppression of host inflammatory response towards recording electrodes inserted in the CNS. Activation of microglia, the only resident phagocytotic cells in the CNS, was observed following injury caused by electrode implantation.[8, 133, 134] The activated microglia, also known as the brain macrophages, continually produce various cytotoxic and neurotoxic factors.[8] It has been suggested that inhibition of microglia activation is a potential strategy to reduce immune response against the electrode.[11, 30] In addition to microglia, electrode implantation inevitably damages the vasculature of the CNS, leading to extravasation of blood borne, monocyte-derived macrophages into the CNS tissue. These macrophages act in concert with activated microglia to defend the CNS against the non-self electrode. Because of the functional similarity, the present study selected the readily available monocyte-derived macrophages instead of isolating primary microglia from neonatal rats to examine the anti-inflammatory effect of the prodrug and its released products. Specifically, the well-established RAW264.7 murine macrophages were used. RAW264.7 murine macrophages are responsive to LPS stimulation and produce nitric oxide (NO) as a marker of activation. NO is a potent inflammation mediator molecule and also is responsible for oxidative stress induced cell death.[13] Studies have shown that if the activated macrophages were responsive to anti-inflammatory therapeutics, the amount of NO production will decrease.[116] Therefore, the extent of NO reduction could be taken as a measurement of anti-inflammatory potency of therapeutic materials. In the present study, the anti-inflammatory effect of prodrug and its released products were determined by

comparing the production of NO between the activated cells treated with these compounds and untreated control. It has been shown that glucocorticoids can exert anti-inflammatory effects within a wide range of dosage, from 0.001 nM to 0.1 mM or higher[135]. In the present work, I selected a dosage in the middle of the range (i.e., 1.1 μ M of prednisolone) to assay NO production.

The inhibitory effect of prodrug and its precursor polymer on NO production in RAW264.7 macrophages were compared (Figure 17). A 40% reduction in NO production was observed when the cells were treated with prodrug, suggesting that the prednisolone-conjugated prodrug has an anti-inflammatory effect against activated macrophages. However, the anti-inflammatory effect of prodrug was less potent compared to activated cells treated with the same amount of free drug, which showed over 60% reduction in NO production.

It is unclear at present how the prodrug exerts its anti-inflammatory action. From the release profile at pH 7.4 (Figure 14) I can rule out the possibility that a significant portion of the conjugated drug was cleaved off the polymer carrier and interacted with the activated cells after 24 h of incubation. Based on the mechanisms of the cellular actions of glucocorticoids reported by Buttgereut et al. [135], I speculate that all the free drug molecules can participate in both the genomic mechanisms and nongenomic mechanisms. However, in the form of prodrug, it is likely that the nongenomic, receptor-mediated mechanisms are dominant. Depending on the polymer chain conformations, it is possible that only a portion of the conjugated drug molecules will be accessible to the receptors. Therefore, the prodrug form is less effective to inhibit NO production than free drug.

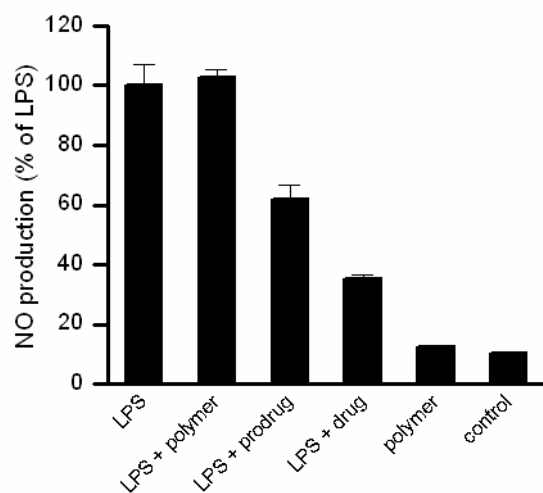


Figure 17. NO production by LPS activated macrophages, LPS activated macrophage treated with either polymer PNVP-NH-NH₂, prodrug PNVP-pred, or free drug prednisolone, respectively. Non-activated macrophages treated with polymer PNVP-NH-NH₂ alone were also included. Cells without LPS activation served as control. Data shown are average $\pm$ STD (n = 3).

It will be informative to elucidate the mechanism underlying the therapeutic effect of the prodrug in a future study. Along with the prodrug, I was also interested to examine whether the polymer precursor (PNVP-NH-NH₂) had any biological activity, since it will be one of the major release products in addition to the conjugated drug. Of particular relevance are whether the polymer has any anti-inflammatory effect, and more importantly, whether the polymer is stimulatory towards macrophages.

As shown in Figure 17, when LPS activated macrophages were treated with the polymer precursor (PNVP-NH-NH₂) at a level equivalent to the prodrug, no significant difference was observed in the amount of NO produced as compared to LPS activation alone, suggesting that the polymer precursor had no anti-inflammatory effect. Furthermore, when non-activated macrophages were exposed to the polymer precursor, the level of NO production remained similar to that of the non-activated negative control. It implies that the polymer will not induce any macrophage activation and thus would be suitable as a drug carrier to treat host inflammatory response to implanted electrodes.

The other important aspect of the bioactivity study was to determine whether the therapeutic nature of the drug was preserved after subjection to the conjugation reaction via the hydrazone linkage. To address this question, prodrug solution was prepared with either a PBS buffer (pH = 7.4, physiological condition) or an acetate buffer (pH = 5.0, inflamed tissue) and incubated at 37 °C. At various time points, an aliquot of solution was withdrawn and stored at -20 °C until the bioactivity study was performed. LPS activated RAW264.7 macrophages were used and treated with the various prodrug solutions mentioned above.

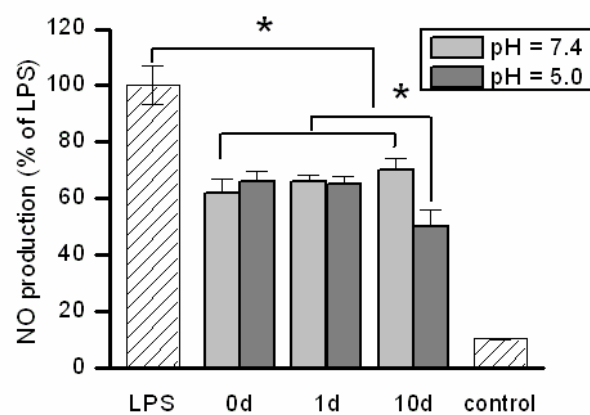


Figure 18. NO production by LPS activated macrophages with and without treatment of prodrug solutions subjected to release for up to 10 days at pH 5.0 and 7.4. Cells without LPS activation served as control. Data shown are average $\pm$ STD ($n = 3$). * indicates $p < 0.05$.

The therapeutic effect was gauged through level of inhibition of NO production. As shown in Figure 18, when cells were treated with prodrug solutions obtained after 1-day incubation, the level of inhibition on NO production was similar to the original day 0 samples, regardless of the buffer pH. However, when treating cells with samples collected after 10-day incubation, a significant improvement ($p < 0.05$) in NO inhibition was observed for the sample prepared in the acidic buffer, where the inhibition level increased from 40% to 50%; but the sample of physiological pH did not show any statistical change.

On one hand, these results confirmed that more drug release occurred under acidic condition where hydrolysis of the hydrazone bond was accelerated, consistent with the HPLC data. On the other hand, these results indicated that the released prednisolone retained its therapeutic activity, suggesting that the conjugation scheme did not compromise the bioactivity of the drug. Though the HPLC data showed drug release at day 10 for the normal physiological pH, it is possible that the amount of free drug in the solution was below its therapeutic concentration, and therefore not able to further reduce NO production. Overall, these cell culture results proved that the synthesized prodrug was sensitive to pH and able to release more potent free drug at lower pH. It could be used as a smart drug delivery system to actively treat inflammation against implanted neural electrodes, that is, a more severe inflammatory response will result in further tissue acidosis which in turn triggers more drug release to counteract on the inflammation.

Besides its therapeutic effect, of equal importance to the neural electrode application is the biocompatibility of the prodrug in the CNS. It is critical that the prodrug and its released product, i.e. the polymer precursor PNVP-NH-NH₂ do not exhibit toxicity towards neurons, which are the sole target of the electrode. Therefore, I studied toxicity both quantitatively and qualitatively. For quantitative analysis, the WST-1 assay was adopted. It is a standard assay used widely for analysis of cytotoxicity. Its working principle is based on the reduction of a tetrazolium salt by cellular mitochondrial dehydrogenases to a water-soluble salt, which can be photometrically quantified at 450 nm. In this assay, the untreated neurons were used as a positive control and all values from the experimental group were compared with this set of data. As shown in Figure 19a, the reduction of WST-1 was unaffected by prodrug or its polymer precursor, and no significant difference was observed between the viability of treated neurons and the control. For qualitative analysis, neuron morphology was observed via a live Calcein AM staining. The confocal images (Figure 19b-d) illustrated that regardless of the treatment, the cortical neurons were viable (good fluorescent intensity) and displayed morphology and neurite extension comparable to the control. Both of the polymers used in the LBL process, that is, PNVP and PAA, are non-biodegradable. One will be concerned about the bioeliminability of these polymers in vivo. Although I used the well-studied high molecular weight PAA in this proof-of-concept study for LBL construction, future studies can use a degradable polymer such as poly(glutamic acid) to replace the non-degradable PAA. For PNVP, even though it is non-degradable, it can be eliminated if the molecular weight is maintained below 100,000 g/mol.

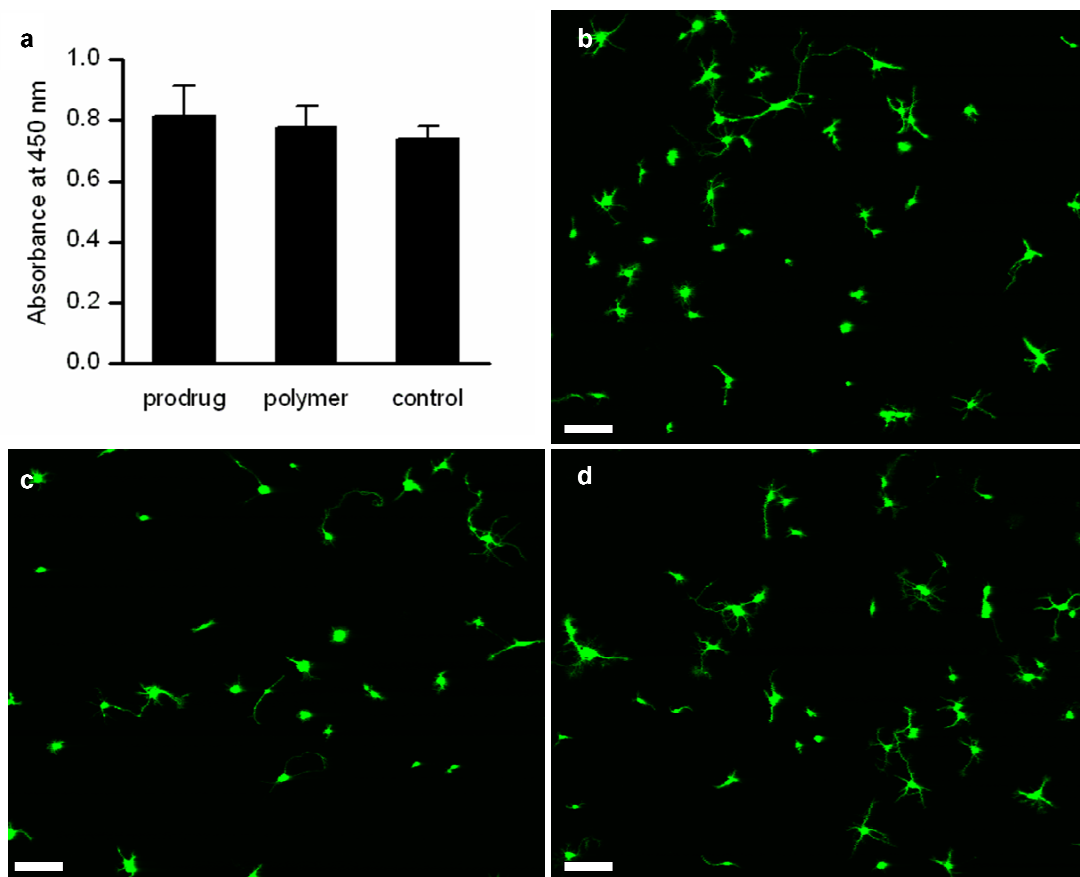


Figure 19. (a) In vitro neural cytotoxicity of prodrug PNVP-pred and precursor polymer PNVP-NH-NH₂. Data shown are average $\pm$ STD ($n = 3$). Confocal images show morphology of neurons following 48 h treatment with either (b) prodrug PNVP-pred, (c) precursor polymer PNVP-NH-NH₂, or (d) non-treated control. Scale bar = 100 μ m.

2.3.4 Prodrug diffusion in a simulated brain tissue model

Distribution of prodrug in the brain tissue around the implanted electrode is an important parameter for clinical applications due to the importance of therapeutic concentrations to combat inflammation. Therefore, a simple diffusion experiment in a simulated brain tissue was designed to provide basic information of the therapeutic concentration.

A silicon wafer was firstly coated with fluorescent prodrug analog. Then the coated silicon was embedded into an agarose hydrogel, simulating the gel-like brain tissue environment. After incubation of the system for a predetermined time, the fluorescent prodrug analog diffused away from the electrode (Figure 20). The fluorescent images were recorded using a fluorescent microscope. The concentration distribution of the prodrug analog around the electrode was therefore calculated.

The raw images were shown in the appendix (Figure 50). The processed fluorescent intensity along the diffusion pathway was shown in Figure 21.

The fluorescence intensity at 4200 μm was firstly subtracted as the background value to obtain corrected fluorescent intensity. To simplify the calculation, the intensity curve was divided into three regimes, with linear distribution in each regime (Figure 22).

The equation for the simplified intensity, $I(x)$, was shown below:

$$I(x) = \begin{cases} 82, & x < 250 \\ -0.04x + 92, & 250 < x < 1800 \\ -0.00083x + 35, & 1800 < x < 4200 \end{cases} \quad 8$$

where x is the diffusion distance from the surface of the Si wafer.

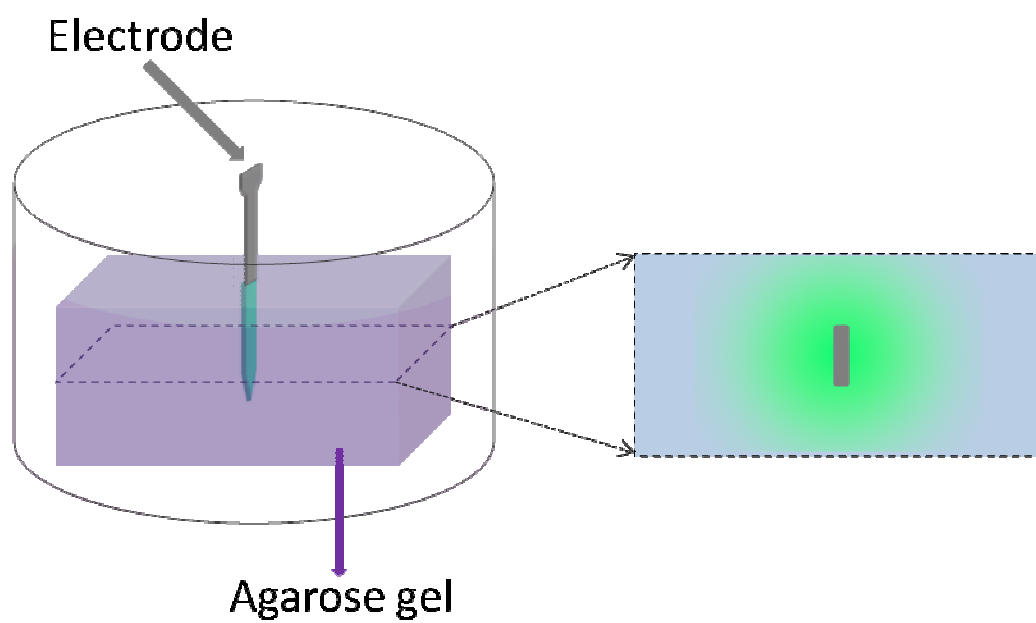


Figure 20. Scheme of prodrug diffusion in the simulated brain model by a block of agarose gel.

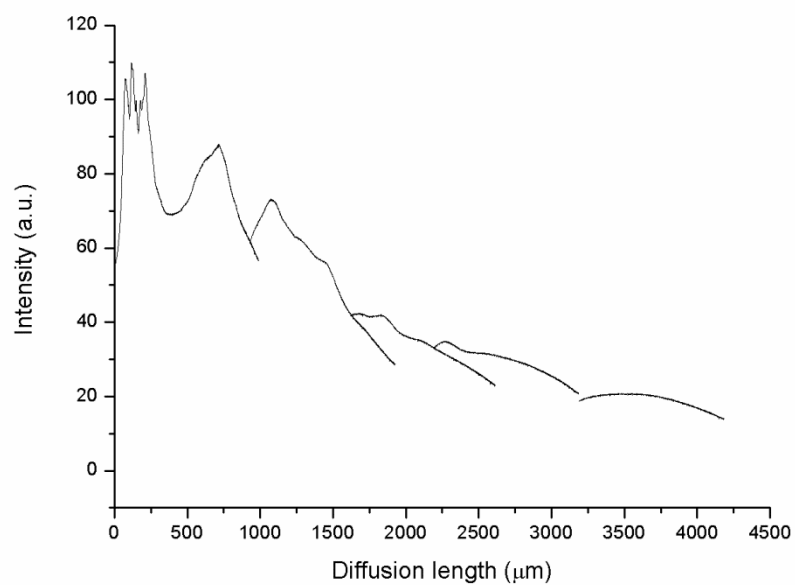


Figure 21. Fluorescent intensity of prodrug analog as a function of distance away from the Si wafer.

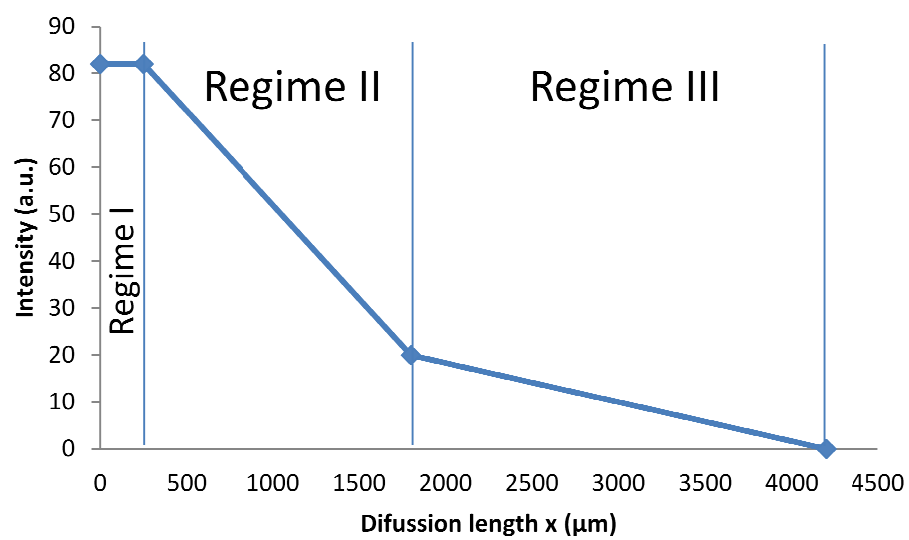


Figure 22. Approximation of fluorescent intensity of prodrug analog as a function of distance away from the Si wafer

The prodrug concentration at distance x , $c(x)$, was obtained based on the assumption of its proportional correlation the fluorescent intensity, as shown in the following equation.

$$c(x) = \sigma I(x) \quad 9$$

where σ is a constant.

In the cube of the diffused prodrug analog (Figure 23), it is reasonable to assume the same concentration along the shank of electrode (z).

Therefore, the total amount of prodrug in the gel block (n_0) could be calculated according to the following equation:

$$\int_0^{4200} c(x) A dx = n_0 \quad 10$$

where A is the area of Si wafer. Furthermore, n_0 can be expressed by the following equation:

$$n_0 = \Gamma N A \varphi \quad 11$$

where Γ is the amount of polymer per layer from LBL assembly. A typical value is $1 \text{ mg/m}^2/\text{layer}$, from many previous reports and my own QCM results. Therefore, $1 \text{ mg/m}^2/\text{layer}$ is used for current calculation.

N is the layer number for the prodrug analog. 8-layer was taken for calculation.

Φ is the drug loading capacity of the prodrug. In the current study, Φ is taken as 160 mg/g , which is the drug loading capacity of PNVP-pred.

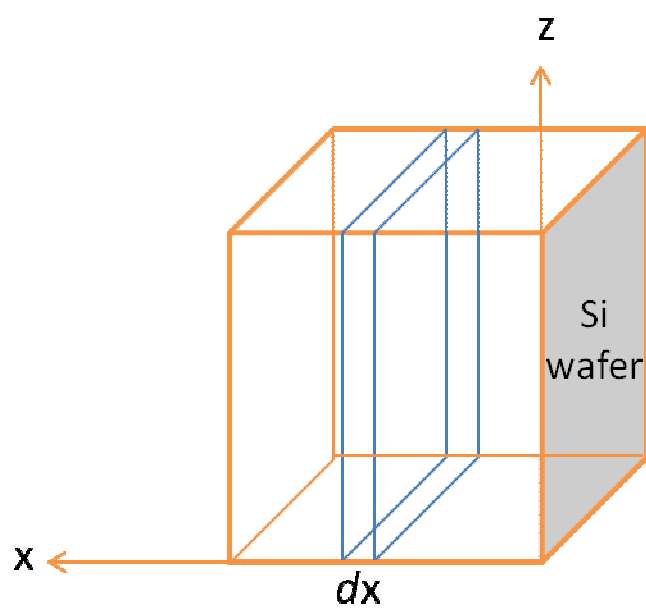


Figure 23. Scheme of simplified cube of diffused prodrug away from the Si wafer.

Finally, the concentration of prodrug could be determined at any location along the substrate. Take the concentration at 100 μm away from the substrate for example, the concentration was calculated as 2.36 μM . The brief calculation procedures are shown below:

$$\begin{aligned}
 \sigma &= \frac{n_0}{\int_0^{4200} I(x) A dx} = \frac{\Gamma N A \phi}{\int_0^{4200} I(x) A dx} \\
 &= \frac{\frac{1 \text{ mg}}{\frac{\text{m}^2}{\text{layer}}} \times 8 \text{ layer} \times \frac{0.16 \text{ g}}{\text{g}} \times A}{A \left[\int_0^{250} 82 dx + \int_{250}^{1800} (-0.04x + 92) dx + \int_{1800}^{4200} (-0.083x + 35) dx \right]} \\
 &= 0.0000104 \text{ kg/m}^3 \\
 c(100 \mu\text{m}) &= \sigma I(100 \mu\text{m}) = \sigma \times 82 \\
 &= 0.00085 \text{ kg/m}^3 \\
 &= 2.36 \mu\text{M predenisolone}
 \end{aligned}$$

The ultimate goal of this experiment is to determine the spatio and temporal distribution of therapeutic at any time period and any location. A more rigorous process will be a combination of theoretical prediction and experimental validation. Experimental results will provide parameters such as the diffusion constants for theoretical calculation and proofs for the theoretical models. Theoretical models will in turn give comprehensive understanding of the drug concentration distribution. The above model needs to be improved in several ways such as the reproducibility, accurate description of the fluorescent intensity curve, etc. Although the current model is very rudimental, it is a simple and first step toward determination of kinetics of drug distribution in the brain tissue.

2.4 Conclusions

A glucocorticoid-polymer prodrug was synthesized by conjugating prednisolone to functionalized PNVP through a hydrazone bond. Drug release from the prodrug was sensitive to pH as hydrolysis of the hydrazone linkage was enhanced under acidic pH. Besides being a drug carrier, the prodrug was also compatible with the LBL technique leading to hydrogen-bonded PAA/prodrug multilayers successfully deposited onto silicon wafers and quartz plates through LBL assembly. The prodrug and its released products demonstrated good biocompatibility with neurons, and the conjugated drug retained its therapeutic effect by successfully inhibiting NO production from activated macrophages. Collectively, these results suggest that the glucocorticoid-PNVP prodrug can serve as a promising and versatile drug delivery system. It can be easily applied to neural electrodes without compromising the electrical property of the implant, and can potentially realize tissue response-regulated drug release based on tissue acidosis. Future studies include in vitro anti-inflammatory study of prodrug on the brain macrophage microglia as well as astrocytes, application of the prodrug on implantable neural electrodes and investigation of the effectiveness of the prodrug in actively managing tissue response to electrodes in a rat model.

Chapter 3

Water Soluble Antioxidant Derivative Poly(Triethylene Glycol Methyl Acrylate-co- α -Tocopheryl Acrylate) as a Potential Prodrug to Enable Localized Neuroprotection

An article presenting part of the results in this chapter has been submitted to *Acta Biomaterialia*. [Cao Y, He W. Water soluble antioxidant derivative poly(triethylene glycol methyl acrylate-co- α -tocopheryl acrylate) as a potential prodrug to enable localized neuroprotection.] The first author (Yu Cao) finished all of the experiments in this study. The corresponding author (Dr. Wei He) is the advisor of Yu Cao and financially supported this work.

3.1 Introduction

Reactive oxygen species (ROS) are a class of oxygen free radicals and related molecules that are capable of exerting oxidative stress on cells when produced in excess. When the amounts of ROS are generated beyond the limit of natural antioxidant defense systems like reductive glutathione (GSH) and antioxidant enzymes, cells start to lose normal functions followed by necrosis[136, 137]. ROS generation has been used to correlate inflammatory cell response to biomaterials in numerous in vitro biocompatibility studies. For example, nanoparticles were shown to trigger an oxidative stress on cells by inducing the production of intracellular ROS, leading to subsequent cellular toxicity cascade reactions [138, 139]. Another example of ROS implication in biomedical material application is the oxidative stress response resulted from degradation products of poly(lactic acid) (PLA) [140, 141]. Such a high relevance of oxidative stress is also demonstrated by recent renewed interest in regulating oxidative stress to modulate the tissue response to biomaterials and improve their biocompatibility.

For biomaterials used in neural tissues, such as MEAs, it is of utmost importance to control oxidative stress. Very often, neuro-inflammation is accompanied by oxidative

stress [142, 143]. Furthermore, the insoluble nature of MEAs stimulates microglia/macrophage cells into a “frustrated” phagocytosis mode. The process of “frustrated” phagocytosis is known to produce excess degradative substances such as hydrogen peroxide in an effort to destroy the implant [144, 145]. The consequence of these molecular actions is cellular damage and death. Although there have been many studies using localized release of anti-inflammatory agents to control inflammation against MEAs, few reports have addressed direct protection of cells from oxidative stress.

Astrocytes are one type of glial cells in the brain supportive of neuron functions. It has been shown that astrocytes can protect neurons from oxidant hydrogen peroxide toxicity [38, 39]. Therefore, we suggest that it is rational to shield astrocytes from oxidative stress associated with implantable MEAs. Such an approach could potentially lead to improving survival of neurons that are vital for long-term MEAs functions.

To protect cells from oxidative stress, a common strategy is to use therapeutic antioxidants. There are a variety of natural antioxidant molecules serving physiological roles of scavenging ROS. These include α -tocopherol (vitamin E, Ve) , trans-ferulic acid [146], ascorbic acid [147], α -lipoic acid [148], as well as proteins such as glutathione peroxidase [149], and myo-inositol hexaphosphoric acid and its derivatives [150-152]. Among these molecules, the antioxidant potency of Ve lies in its capability to scavenge free radicals that cause lipid peroxidation and damage of cell membrane. Ve has been widely selected as a defense against oxidative damage in a biomaterial context. For example, Ve has been used as an additive to stabilize PU elastomer in vivo [153]. New

dialysis membranes coated with Ve have also been developed [154]. In this study, I choose Ve to serve as the antioxidant agent to protect astrocytes from oxidative stress.

Successful localized delivery of Ve around MEAs for in vivo applications needs to overcome several hurdles. First, Ve is highly hydrophobic, which is unfavorable for its solubilization and transportation in the predominantly aqueous environment presented by the physiological tissues [155, 156]. Second, use of Ve may be limited by factors such as unfavorable pharmacokinetics and a short action time due to fast metabolism [157]. Last, the delivery strategy needs to be carefully designed to avoid any compromise of the electrical properties of MEAs. To address the first two concerns, one can adopt a prodrug approach, that is, converting antioxidants into polymeric antioxidants to improve the transport and metabolic stability of antioxidant molecules. Furthermore, the rate of drug degradation can be reduced, ensuring a longer presence than free antioxidants [158, 159]. Such prodrugs can be formed by linking antioxidant molecules to polymeric matrices through covalent bonds [160, 161]. For example, polymer-tocopherol prodrugs were synthesized to treat porcine sperm cell. The intracellular sperm levels of Ve were largely increased. As a result, the endogenous fatty acid degradation under oxidative stress was greatly reduced [161]. For the last concern of maintaining electrical properties of the implant, I will design the prodrug to be compatible with LBL assembly process to minimize any impedance burden to the MEAs.

In this chapter, a synthetic antioxidant polymer derivative of Ve was designed with the goal to enable localized delivery of antioxidants to the neural tissue around the MEAs implant, and to present a pharmacological depot to protect cells from the injurious

oxidative stress. The applicability of using this antioxidant prodrug with MEAs was demonstrated through LBL film assembly on silicon substrates. In vitro astrocyte cultures were carried out to study the cytotoxicity and anti-oxidation potency of the prodrug. To elucidate the protective mechanism against oxidative stress, a free radical scavenging assay was also performed.

3.2 Experimental Section

3.2.1 Materials

Acryloyl chloride, α -tocopherol, triethylamine, azobisisobutyronitrile (AIBN), polyethyleneimine (PEI, Mw = 2,000 g/mol), 2,2-diphenyl-1-picryl-hydrazyl (DPPH) and tannic acid (TA) were obtained from Sigma-Aldrich, USA. Deuterated chloroform was obtained from Cambridge Isotope Laboratories, Inc., USA. Poly(acrylic acid) (PAA, Mw = 450,000 g/mol) was purchased from Polysciences, Inc., USA. Tetrahydrofuran (THF), dichloromethane (DCM), hexane, ethyl acetate, and anisole were purchased from Fisher Scientific, USA. All reagents were used as received unless otherwise noted. Monomer of triethylene glycol methyl acrylate (TEGMA) was kindly provided by Dr. Bin Zhao in Department of Chemistry, UTK.

3.2.2 General characterization

^1H NMR and ^{13}C NMR spectra were recorded on a Varian Mercury 300 NMR spectrometer. Fourier transform infrared spectroscopy (FTIR) was carried out on a Bio-RAD FTS6000 FTIR spectrometer. Gel permeation chromatography (GPC) was performed at room temperature using PL-GPC 50 plus (an integrated GPC/SEC system from Polymer Laboratories, Inc., USA) with a refractive index detector. THF was used as

the carrier solvent at a flow rate of 1.0 mL/min. Standard monodisperse polystyrenes (Polymer Laboratories, USA) were used for calibration. A Q-1000 differential scanning calorimeter (TA Instruments, USA) was used to characterize thermal behavior of the copolymers. UV-vis spectra were measured using a UV-vis spectrophotometer (Biomate 5 from Thermospectronic, USA). Ellipsometric measurements were performed on dry LBL films in air using an L0116S Stokes ellipsometer (Gaertner scientific corporation, USA) with He/Ne laser (632 nm) illumination at a 70° angle of incidence. A refractive index of 1.465 was assumed for the dry film.

3.2.3 Polymer synthesis

The monomer α -tocopherol acrylate (VeAc) was synthesized (Figure 24a) based on prior literature [161]. Copolymerization of VeAc and TEGMA was conducted at different feeding ratios, with molar percentage of VeAc as 5%, 10%, 25%, and 50%. In a typical procedure, VeAc (0.362 g, 0.065 mol/L), TEGMA (3.107 g, 1.234 mol/L), AIBN (0.037 g, 0.019 mol/L), and anisole (12 mL) were added into a 50 mL flask. The mixture was then subjected to three freeze-thaw cycles to remove oxygen. Polymerization reaction took place at 70 °C for 5 h. The polymer was then precipitated in hexane, redissolved in anisole, and reprecipitated in hexane for purification. The obtained polymer (PVT) was dried overnight in a vacuum oven at 70 °C (3.029 g, 87% yield).

3.2.4 LBL assembly

The self-assembled films were prepared on silicon substrates with a 100 nm thick oxide layer (University Wafer, USA). Before layer deposition, the silicon wafers were cut into small squares (1 x 1 cm).

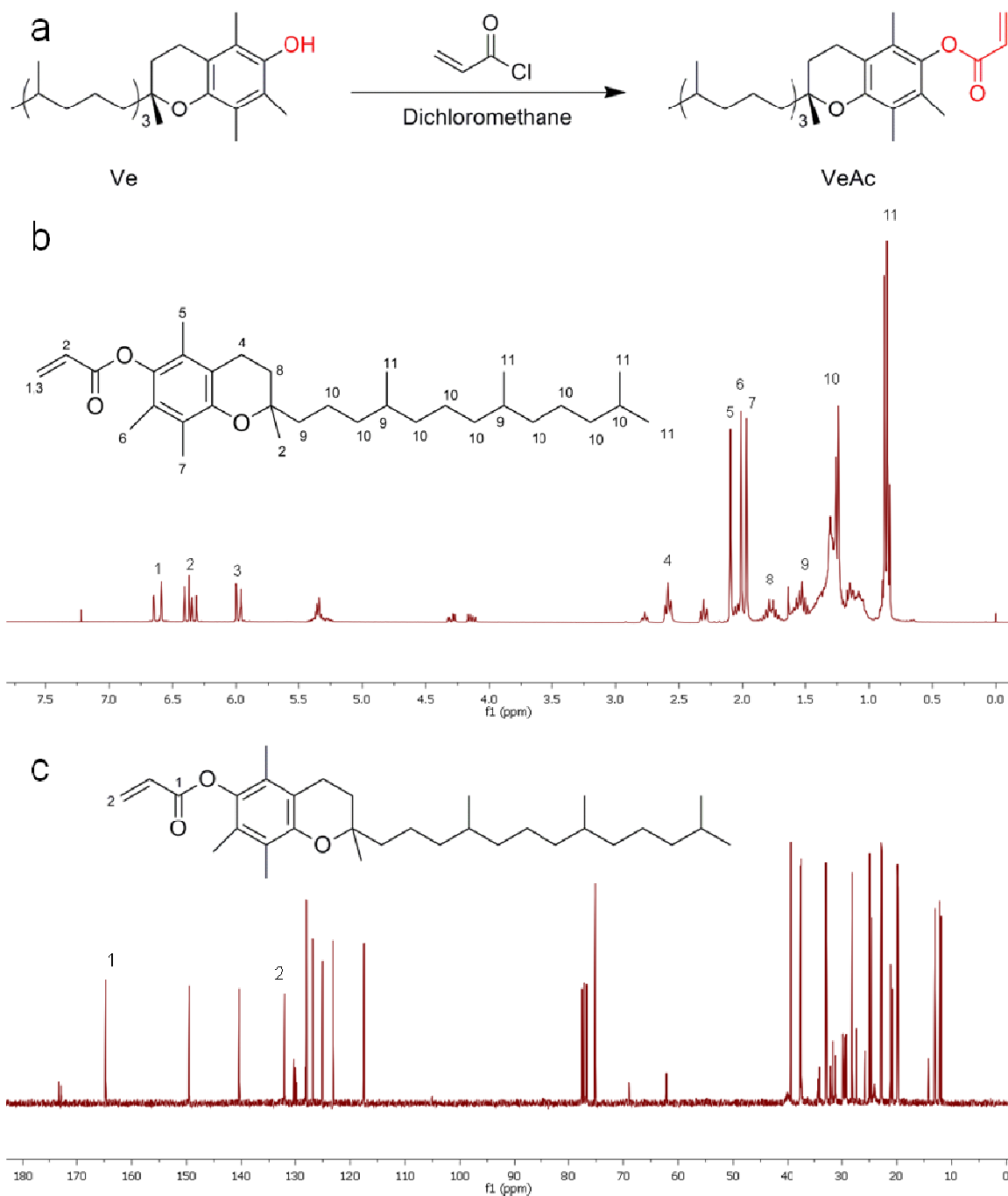


Figure 24. (a) Synthesis of monomer α -tocopheryl acrylate (VeAc). (b) ^1H NMR characterization of VeAc in CDCl_3 . Successful acrylation of Ve is confirmed by the observation of peaks at chemical shifts of 6.62, 6.36 and 5.98 ppm, corresponding to the vinyl protons. (c) ^{13}C NMR characterization of VeAc in CDCl_3 . Characteristic peaks associated with the acrylate carbons are identified.

All substrates were cleaned in a boiling piranha solution (3:1 concentrated H_2SO_4 : 30% H_2O_2 v:v; *piranha solution reacts violently with organics and should be handled with extreme caution*) for 30 min, washed with copious amount of water, and dried under nitrogen. The substrates were first immersed in a PEI solution (1.5 mg/mL) for 30 min, followed by rinsing in water for 1 min. The PAA/PVT (or TA/PVT) multilayers were then assembled by alternating immersion of the substrates in solutions of PAA (or TA) and PVT for 5 min, respectively.

Between each step, the excess material was removed by rinsing the substrates with HCl solution (pH = 3.0) for 1 min. Solutions of PAA, TA, and PVT were prepared to a concentration of 0.5 mg/mL (pH = 3.0) using ultrapure water (Milli Q-plus system, Millipore, USA) with a resistivity of 18.2 $\text{M}\Omega\cdot\text{cm}$. The assembly process was monitored with ellipsometry. To evaluate stability of the multilayers under physiological conditions, silicon substrates coated with PAA/PVT or TA/PVT multilayers were immersed in phosphate buffered saline (PBS, pH = 7.4) for 10 min, and subsequent ellipsometric measurements were carried out to characterize the thickness of remaining films.

3.2.5 Quartz crystal microgravimetry (QCM)

QCM measurements were conducted at room temperature using a Q-sense device (E4) with a flow cell (Q-Sense AB, Västra Frölunda, Sweden). A gold-coated 5 MHz AT-cut crystal was excited at multiple overtones, and the change in the resonance frequency was recorded. After deposition of the initial layer of PEI (1.5 mg/mL), the chamber was washed with water, after which the solution of PAA or TA was introduced into the chamber and allowed to adsorb for 5 min. Subsequently, the chamber was

washed again with HCl (pH = 3.0) and then filled with PVT solution, allowing an adsorption time of 5 min. The multilayer film was then assembled by alternating incubations between PAA (or TA) and PVT.

3.2.6 *In vitro* cytotoxicity assay

In vitro cytotoxicity of the PVT prodrug was examined using the A172 human astrocyte cell line (ATCC, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were maintained at 37 °C in 5% CO₂ with 95% relative humidity. Prior to cytotoxicity test, cells were harvested using a trypsin–ethylenediamine tetraacetic acid (EDTA) solution (0.25% trypsin–0.038% EDTA) and diluted to a density of 5×10^5 cells/mL. The cell suspension was added into 96-well plates at 100 µL/well, and cells were allowed to attach overnight. Cell culture medium was then aspirated the next day and replaced with fresh medium supplemented with PVT at different concentrations (either 10 or 500 µM). Cells were incubated overnight and polymer cytotoxicity was determined using an MTT assay (Promega, CellTiter 96, USA) according to the manufacturer's instruction. Positive (without any treatment) and blank (without cells) controls were also included. Cell viability was examined by comparing the absorbance of treated cells to that of the positive control. Statistical analysis was conducted using Student's *t*-test. Cell morphology was also recorded using an inverted optical microscope (ZEISS Observer A1, USA).

3.2.7 In vitro cell protection against oxidative stress

A172 astrocytes were harvested using trypsin–EDTA solution and diluted to a density of 3.3×10^4 cells/mL. The cell suspension was added into 96-well plates at 100 μ L/well, and cells were allowed to attach overnight. The cultures were then incubated with Ve or PVT at a concentration equivalent to 500 μ M Ve for 1 h before exposure to hydrogen peroxide (500 μ M) to induce toxic oxidative stress. After 16 h, cell viability and morphology were examined using the MTT assay and optical imaging, respectively. The protective effect was inferred from the percentage of cells survived by normalizing the absorbance of treated cells to that of control cells without hydrogen peroxide treatment.

3.2.8 Free radical scavenging assay

Ve was released from PVT by dissolving the prodrug in a saturated KOH solution with saponification at 70 °C for 30 min. Released Ve was extracted by hexane twice, followed by solvent evaporation. The amount of Ve recovered was quantified using an Agilent 1100 HPLC system with a variable wavelength UV detector. A reverse phase C18 column (Phenomenex, 4.6×100 mm, 2.5 μ m) was used for the analysis with acetonitrile and methanol (95:5, v/v) as the mobile phase. The flow rate was set constant at 1.5 mL/min. A sample injection volume of 20 μ L was used for all evaluations. Detection of Ve was made by UV absorption at 280 nm. The antioxidation property of the released Ve was examined using a DPPH free radical scavenging assay following procedures modified from a previous report.[162] Briefly, aqueous solution (1 mL) of various samples was added to 3 mL of freshly prepared DPPH solution (0.025 mg/mL in

methanol). The tested samples included PVT, released Ve from PVT, and commercial grade Ve, and the concentrations were kept the same to be equivalent to 350 μ M Ve. Absorbance at 550 nm was measured on a UV-vis spectrophotometer. The percentage of remaining DPPH free radicals in each experimental sample was calculated by normalizing the corresponding absorbance to that of the control without any sample addition.

3.2.9 Impedance measurement

The effect of the multilayers of (PVT/PAA) on the electrical properties of Si MEAs was evaluated by impedance spectroscopy. A micromachined single shank acute probe (16 recording sites, model: A1 $\times$ 16-5mm-100-177-A16) obtained from NeuroNexus Technologies, Inc. was coated with (PVT/PAA) multilayers, and the impedance of the probe sites was measured before the coating, after 8 bilayers, and 16 bilayers, respectively. Electrochemical impedance spectroscopy was done using Precision impedance analyzer 4294A (Agilent). A solution of 0.1 M PBS buffer was used as the electrolyte. AC sinusoid signal of 5 mV amplitude, with the DC potential set to 0V was used as the input. The value of the impedance was determined around the biologically relevant frequency of 1 kHz.

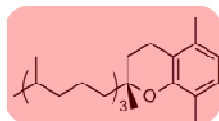
3.3 Results and Discussion

Oxidative stress can impose a significant challenge on cell survival if not controlled properly. Such an issue is of particular relevance to delicate brain tissue as it is highly prone to oxidation damage. In order to ensure the proper function of devices implanted in brain such as recording MEAs, it is reasonable to suggest that a potential

strategy is to maintain a healthy state of brain tissue at the interface through localized delivery of antioxidants. Toward this goal, I proposed to use an antioxidant prodrug. The prodrug was designed to serve two key functions: carrier of antioxidants and readily applicable for MEAs through LBL assembly. These features were achieved through simple copolymerization of α -tocopheryl acrylate (VeAc) with triethylene glycol methyl acrylate (TEGMA) to make poly(triethylene glycol methyl acrylate-co- α -tocopheryl acrylate) (PVT) prodrugs. VeAc is a derivative of the potent natural antioxidant vitamin E (Ve). The phenolic hydroxyl group allows straightforward functionalization of Ve into a polymerizable VeAc monomer. TEGMA is selected as the co-monomer to both increase water solubility of the prodrug and endow LBL compatibility through its hydrogen bond accepting ethylene glycol units.

3.3.1 Synthesis and characterization of the antioxidant prodrug

The antioxidant prodrug PVT was prepared through a conventional free radical copolymerization of monomers TEGMA and VeAc (Figure 25) with AIBN as the initiator. Prior to polymerization, the synthesized monomer VeAc was verified through ^1H NMR and ^{13}C NMR, and the results agreed well with the expected structure (Figure 24b and c). A series of PVT prodrugs were prepared by varying the feed molar ratio of the two monomers. The number average molecular weights M_n of the copolymers were in the range of 14,000-25,000 g/mol with a relatively high polydispersity (), which is typical for acrylate polymers synthesized by free radical polymerizations in solution.



77

Table 1. Molecular weight and compositional data of PVT prodrugs.

Prodrug	VeAc (mol%)		M_n (g/mol)	PDI
	actual	feeding		
PVT4	3.7	5	21,700	3.29
PVT8	7.8	10	24,900	1.87
PVT24	23.7	25	21,700	1.50
PVT45	45.5	50	14,500	1.43

The chemical structure of the PVT prodrug was characterized with ^1H NMR. As shown in Figure 26, a series of signals at chemical shift 3.35-3.75 ppm and around 4.20 ppm corresponded to the alkoxy protons of the ethylene glycol units in the side chain of the TEGMA moiety. The peaks around 0.89 ppm originated from the methyl protons in the alkyl tail of the VeAc component. Based on the characteristic peak from TEGMA (4.20 ppm) and VeAc (0.89 ppm), chemical compositions of PVTs were calculated. The molar percentage of VeAc incorporated into the final copolymers was 3.7%, 7.8%, 23.7%, and 45.5%, respectively.

The use of copolymerization allows the preparation of a series of PVT prodrugs with different therapeutic capacities by controlling the copolymer composition. Since both monomers are in the acrylate family, similar reactivity is expected between the two monomers, and the actual composition of the obtained copolymer should be consistent with that in the initial feeding. Such an expectation is confirmed from the ^1H NMR results, where the initial molar feeding ratio of VeAc ranging from 5% to 50% resulted in a final molar percentage of VeAc incorporated at 3.7% to 45.5%, respectively. Attempts have also been made to synthesize a homopolymer of VeAc. However, no polymerization occurred, which could be attributed to the antioxidant properties of the α -tocopheryl group. Although the exact cause is beyond the scope of this paper, a similar result has been reported showing low efficiency of making methacrylate copolymers with high molar percentage of Ve derived methacrylate [163]

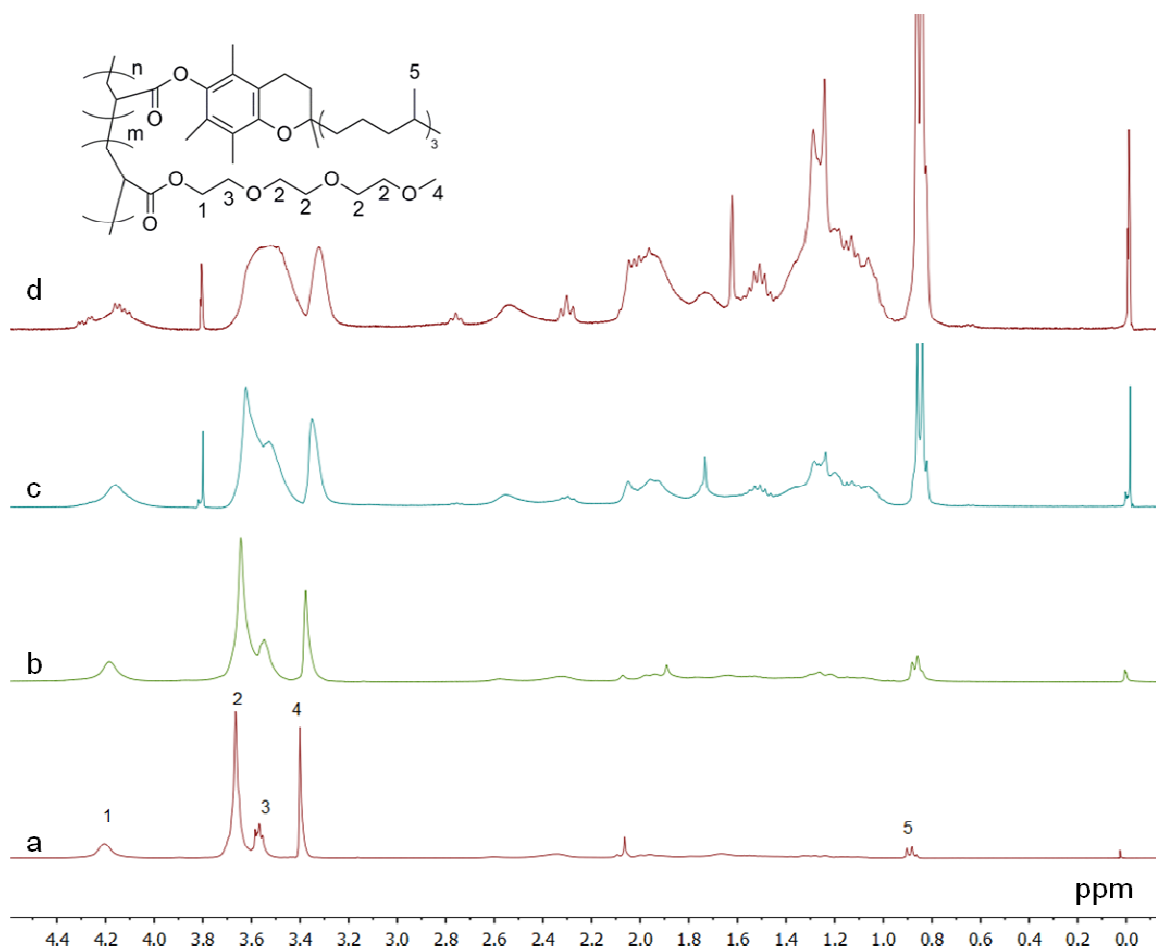


Figure 26. ^1H NMR characterization of prodrugs (a) PVT4, (b) PVT8, (c) PVT24, and (d) PVT45 in deuterated chloroform. Characteristic peaks from each monomer were labeled.

The chemical compositions of PVTs were further confirmed using UV-vis spectroscopy. As shown in Figure 27, λ_{max} of the Ve spectrum appeared at 292 nm, while that of PVT copolymers shifted to 285 nm. Such a blue shift in the peak absorbance can be attributed to the substitution of the phenolic –OH group in Ve with an ester group in the PVT polymer. No significant absorbance was observed between 260-320 nm for the polymer analog of the prodrug (i.e. PTEGMA). Using a predetermined standard curve of Ve absorbance, the molar composition of VeAc in PVT4 and PVT8 were calculated to be 4.3% and 9.0%, respectively. These results were comparable to those obtained from the ^1H NMR spectra.

Chemical structures of the monomer VeAc and PVT copolymer were also characterized by FTIR (Figure 28). The characteristic phenolic –OH group from Ve is absent in both VeAc and PVT since no peak was observed around 3509 cm^{-1} , which corresponds to the –OH stretching. The strong absorbance around 1734 cm^{-1} in VeAc and around 1743 cm^{-1} in PVT corresponded to the stretch vibration of ester C = O group. Furthermore, absorbance peaks around 1637 cm^{-1} and 1580 cm^{-1} in VeAc were due to C = C stretching vibration from both the vinyl group and the phenyl group.

Thermal behavior of the PVT copolymers was characterized using DSC (Figure 29). All PVTs displayed a glass transition temperature (T_g) well below body temperature, ranging from $-55\text{ }^\circ\text{C}$ to $-30\text{ }^\circ\text{C}$ when the fraction of VeAc increased from 4 mol% to 45 mol%.

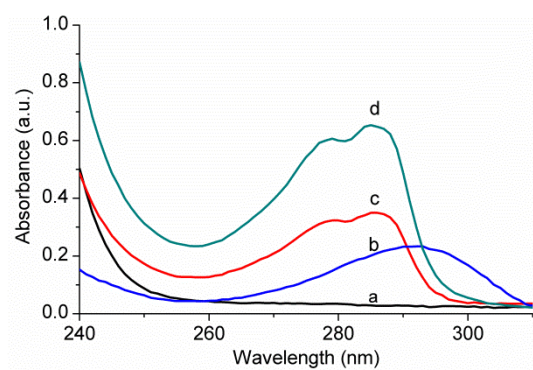


Figure 27. UV spectra of (a) PTEGMA (10 mg/mL), (b) Ve (0.05 mg/mL), (c) PVT4 (1 mg/mL), and (d) PVT8 (1 mg/mL) in ethanol solution.

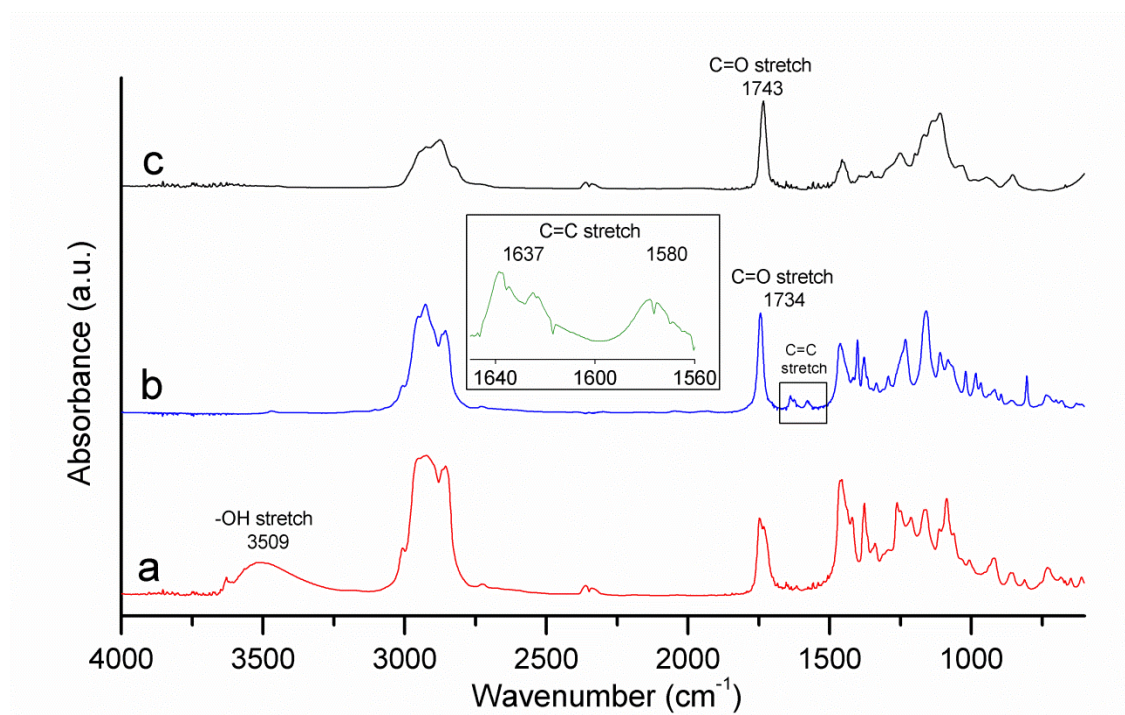


Figure 28. FTIR characterization of (a) Ve, (b) monomer VeAc, and (c) prodrug PVT4.

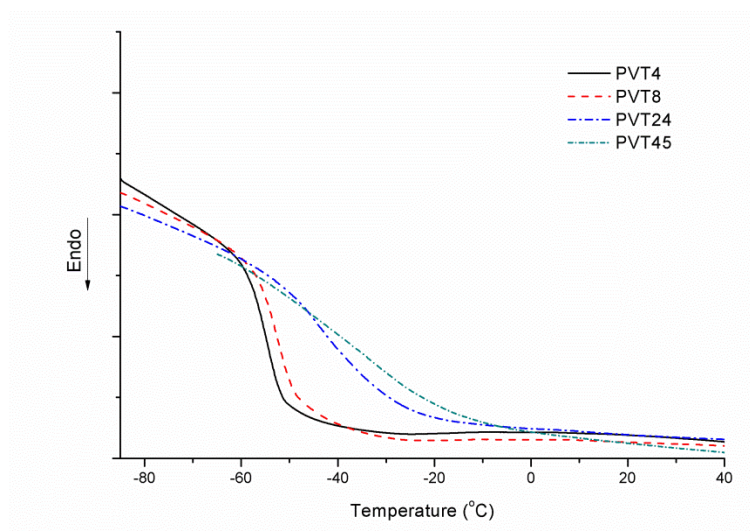


Figure 29. DSC thermograms of prodrugs PVT4, PVT8, PVT24, and PVT45. Glass transition temperature, T_g , for each of the prodrug is -55, -52, -40, and -30 °C, respectively.

Such a correlation is expected as a higher amount of the bulky and rigid Ve pendant group will hinder the ability of the polymer chains to undergo segmental motion, thus a higher T_g . No melting peak was observed when the samples were heated to a temperature as high as 200 °C (Figure 30).

Varying compositions of PVTs also provided us an opportunity to identify the highest antioxidant loading capacity while maintaining reasonable water solubility. As mentioned earlier, the highly hydrophobic characteristic of Ve hinders its transportation through the predominantly aqueous extracellular medium of the physiological tissues. In contrast to the nearly insoluble nature of Ve in water (0.021 mg/mL or 49 μ M) [164], the PVT prodrugs can be formulated to achieve significantly higher solubility. In particular, PVT4 is the most water soluble, at a feasible concentration of 3.1 mg/mL that corresponds to 500 μ M of Ve. PVT8 and PVT24 possessed medium solubility (0.5 mg/mL), while PVT45 exhibited the lowest water solubility (0.02 mg/mL). The trend of water solubility correlates well with our expectation that the more of the hydrophobic VeAc monomer is incorporated into the prodrug, the less water soluble it will become.

Water solubility of the prodrug is also highly relevant for its practical deployment to the tissue-implant interface using the LBL method, since the most commonly used solvent for LBL is water. Typically, I have been using a minimum concentration of 0.5 mg/mL for our LBL assembly processes [34]. Therefore, prodrug PVT45, which has the highest molar percentage of VeAc, is not applicable for use in LBL.

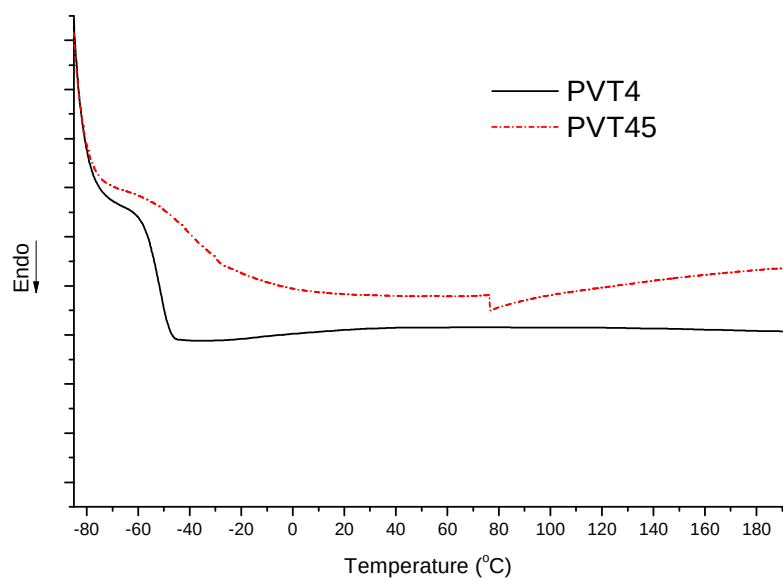


Figure 30. Full DSC thermograms of prodrugs PVT4 and PVT45.

3.3.2 Application of PVT for LBL assembly

LBL is a simple and convenient technique for surface modification of biomedical implants. Hydrogen-bond mediated LBL has been proposed to obtain well-engineered multilayer structures on neural MEAs to resolve the traditional dilemma between drug loading and preservation of electrical property of electrodes [34]. In the current work, I took advantage of the tunable stability of hydrogen bonding and rationally designed LBL parameters to achieve the bonus feature of forming a drug depot around the implant. As proposed schematically in Figure 31, prodrug-embedded detachable multilayers can be prepared on the electrode by incorporating an underlying sacrificial multilayer that is dissociable at physiological pH. Such a design could help confine the polymer therapeutics within the vicinity of the implant-tissue interface and exert therapeutic actions locally.

To realize the design, two types of hydrogen-donating molecules were needed to form hydrogen-bonding with our hydrogen-accepting prodrug PVT. Among the many well-studied hydrogen-donating molecules, I selected poly(acrylic acid) (PAA) that is rich in carboxyl groups, and tannic acid (TA) that is rich in phenolic hydroxyl groups [55, 165] (Figure 32a and b). Intermolecular hydrogen bonds are available between the hydrogen-accepting ether oxygen in the pendant ethylene glycol groups in PVT and the hydrogen-donating carboxyl groups in PAA or phenolic hydroxyl groups in TA. PAA is chosen for physiologically disruptable layers because of its low pKa (4.3), while TA is employed to maintain layer stability due to its high pKa value (8.5). The additional benefit of using TA is to capitalize on its biological properties such as antibacterial and

antioxidant activities [166]. The PAA/PVT and TA/PVT multilayers were constructed on silicon wafers with PEI as a precursor layer. Silicon is selected as the substrate because of its prevalent use for implantable neural MEAs [47, 48].

Ellipsometric measurements showed the layer thickness increased linearly with the layer number, allowing easy control of drug loading capacity (Figure 32c). Eight bilayers of PAA/PVT and TA/PVT were 277 nm and 114 nm thick, respectively. For the same number of layers, a thicker film was obtained using PAA/PVT compared to that with TA/PVT. Such a difference could be due to molecular size disparity, as PAA has a much higher molecular weight (450,000 g/mol) than TA (1701.2 g/mol). To demonstrate the tunable stability of multilayers prepared with the same PVT prodrug, a simple immersion test was done in PBS, simulating the physiological condition of human tissue. The stability was characterized by the change of film thickness before and after immersion for 10 min. As shown in Figure 32d, a drastic decrease in film thickness, from 277 nm to 4 nm, was observed for PAA/PVT multilayers. The remaining 4 nm film can be attributed to the precursor PEI layer along with the adsorbed first PAA layer. It indicates a significant film loss (more than 98% in thickness), which is attributed to the low pKa of PAA (4.3). At physiological pH of 7.4, the carboxyl groups of PAA became deprotonated, resulting in disruption of hydrogen bonding between PAA and PVT, which in turn led to film dissociation. In contrast, the TA/PVT multilayers remained stable as no significant change was noted in the film thickness after exposure to PBS (d).

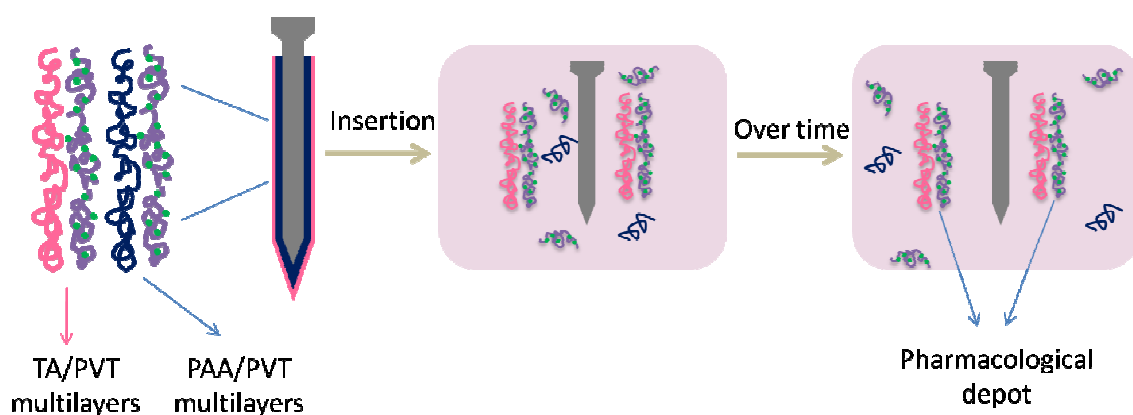


Figure 31. Schematic illustration of proposed use of prodrugs for surface modification of implantable neural MEAs with LBL assembly to enable localized delivery of antioxidants. The antioxidant prodrug PVT can be incorporated in two designs, dissociable layers constructed with poly(acrylic acid) (PAA), and stable layers made with tannic acid (TA) to serve as drug depots.

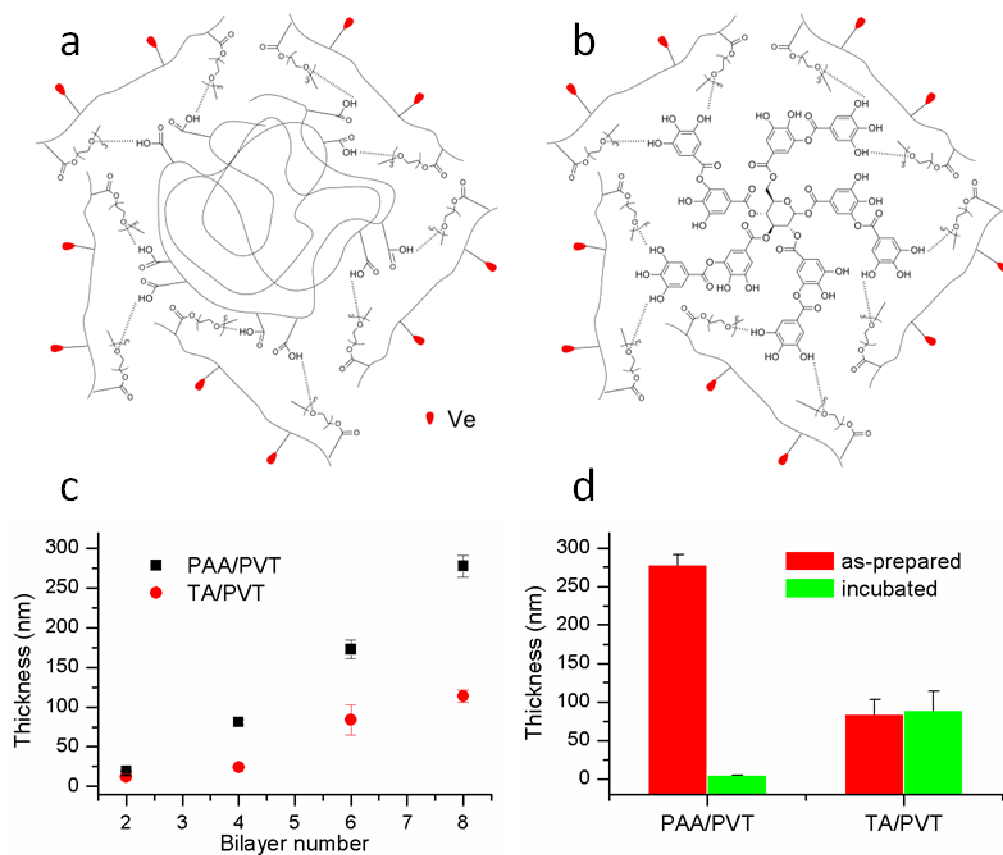


Figure 32. Schemes of LBL multilayer assembly pair and film characterization. Hydrogen bonds formed between prodrug PVT and (a) PAA or (b) TA drives multilayer growth. (c) Film growth was monitored by ellipsometric measurement of film thickness as a function of number of PAA/PVT or TA/PVT bilayers. (d) Stability of the prodrug containing multilayer films in physiological pH 7.4 phosphate buffer was examined by comparing the film thickness before and after incubation.

The high pKa (8.5) of TA provided stability for the TA/PVT multilayers due to the lack of ionization of phenolic groups at physiological pH, therefore, preserving strong hydrogen bonding between TA and PVT.

I also monitored film buildup as a function of the bilayer number in situ by following the frequency change (Δf) with a QCM. As a complementary method to the ellipsometry technique that characterized the dry multilayer films, QCM allowed studies of in situ film growth in a hydrated state. As shown in Figure 32 and Figure 33a, a comparison of the results showed that for eight bilayers of PAA/PVT, the ellipsometric thickness was 277 nm and the QCM thickness was 414 nm. For TA/PVT multilayers, the values were 114 and 342 nm, respectively, indicating an even higher degree of swelling for this layer configuration. Considering that the TA/PVT multilayers are designed to serve as the antioxidant reservoir situated at the periphery of the interface, its ability to be well hydrated is highly desirable to minimize any irritation of the surrounding soft brain tissue.

Another advantage of the QCM technique is that it allows the determination of adsorbed mass by converting the frequency change using the Sauerbrey equation [167]:

$$m_{\text{QCM}} = C \frac{\Delta f_i}{i} \quad 12$$

The mass deposited on the crystal, m_{QCM} , is proportional to the normalized frequency shifts, $\Delta f_i/i$, where i is the overtone number. For crystal sensors with a resonance frequency of 5 MHz, the mass sensitivity constant C is $17.5 \text{ ng Hz cm}^{-2}$, and the frequency shift for the third overtone (i.e. $i = 3$) was used to determine m_{QCM} .

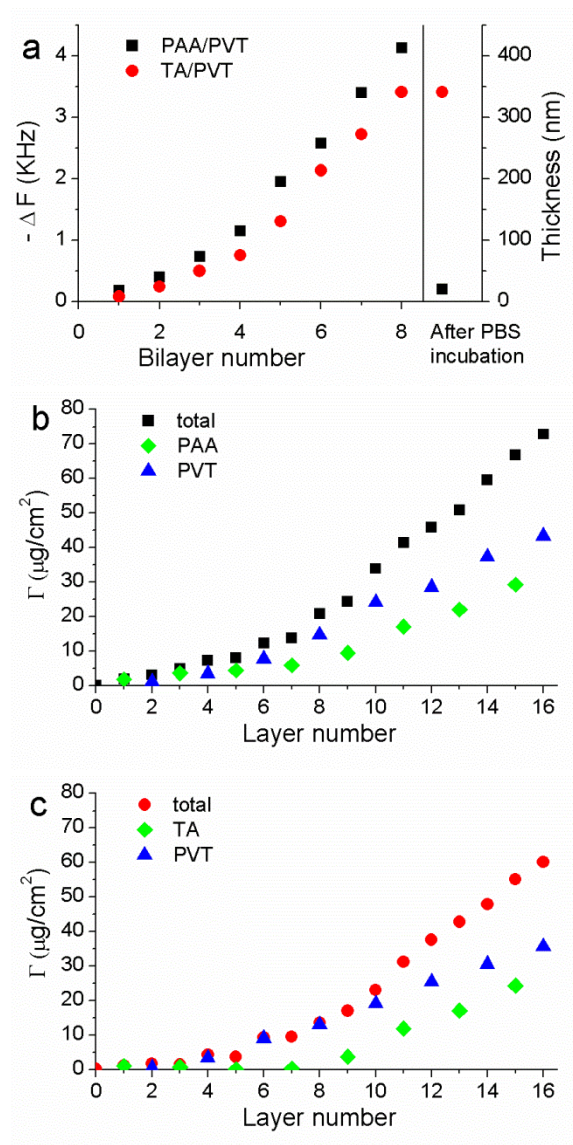


Figure 33. In situ characterization of multilayer growth on gold crystals using QCM. (a) QCM frequency shifts and calculated film thickness during alternating deposition of PAA and PVT or TA and PVT, and after incubation in PBS to demonstrate film stability. (b), (c) Evolution of adsorbed Sauerbrey mass (Γ) plotted against layer numbers for PAA (or TA), PVT, and total mass deposited.

The mass information is essential for in vivo applications, as it forms the rationale for finding the number of layers for a therapeutic concentration of the prodrug following MEAs implantation. The calculated Sauerbrey mass for PAA/PVT and TA/PVT following each layer deposition is shown in Figure 33b and c. For both multilayer configurations, the mass increase per PVT layer appeared to be constant shown by the linear increase in the Sauerbrey mass; whereas for the complementary PAA or TA layer, the mass increase was close to exponential with an initial “slow-growing” regime. For eight bilayers of deposition, the total mass adsorbed for PAA/PVT and TA/PVT was 73 and 60 $\mu\text{g}/\text{cm}^2$, respectively. The mass contribution from PVT layer deposition was higher than that from either PAA or TA layer, accounting for ~60% of the total mass. Furthermore, the difference in multilayer stability in PBS was confirmed with QCM. A significant recovery in the resonance frequency was quickly observed for the PAA/PVT combination (within one minute of PBS incubation), indicating the rapid loss of deposited multilayers (Figure 33a), whereas no change was noted for the TA/PVT multilayer coated crystal.

In order to shed some light on the molecular behavior of the LBL assembly, radius of gyration (R_g) and critical overlap concentration (c^*) of polymers are calculated, according to equations 6-7 shown in section 1.5. Take PAA/PVT system for example, the results are tabulated in .

R_g values of PAA and PVT are 21 nm and 3 nm respectively. c^* values of PAA and PVT are 20 and 336 mg/mL respectively. The polymer concentration for LBL assembly is 0.5 mg/mL, much smaller than c^* .

Table 2. Molecular parameters of PAA and PVT.

	C_{∞}	l (nm)	n	M (g/mol)	R_g (nm)	c^* (mg/ml)
PAA	9 ^a	0.154 ^a	12500	450000	21.08732	19.04073
PVT	11 ^b	0.154 ^c	200	21700	2.948875	335.7574

^a Data are cited from handbook[168]; ^b Data are from a similar polymer, poly (ethyl acrylate) in handbook[168]; ^c The chain length is assumed to be that of acrylic acid.

Therefore, adsorption of polymer is from non-overlapping chains in solutions. According to the QCM results, the average layer thickness is 26 nm. Referred to a previous report,[46] the molecular behaviors of the layer adsorption are proposed in Figure 34. The individual polymer chain is adsorbed onto a surface due to the hydrogen-bonding interaction. Polymer chains are then extended and reorganized to form tails and loops extending from the surface.

It should be noted that in the above calculation process, several assumptions are made for simplicity. Even though many estimations and limitations exist in the calculation, it can provide a semi-quantitative insight on the molecular level of the LBL process.

3.3.3 Cytotoxicity study of PVT

Besides satisfying the requirement of LBL compatibility, successful use of the antioxidant PVT prodrug for MEAs implantation also calls for high biocompatibility. Therefore, cytotoxicity of the prodrug on astrocytes was examined both quantitatively and qualitatively. A commercially available A172 human astrocyte cell line was chosen for this purpose. Since the Ve residue is linked to the acrylic backbone through a labile ester bond, the prodrug could be hydrolyzed in the physiological environment and give off Ve and the corresponding acrylic polymer. For this reason, cytotoxicity of these potential released products, i.e. Ve and the polymer (approximated with a homopolymer PTEGMA), was also tested. Because PVT4 has the highest water solubility among the prepared PVT prodrugs, it was selected for all the in vitro biological studies.

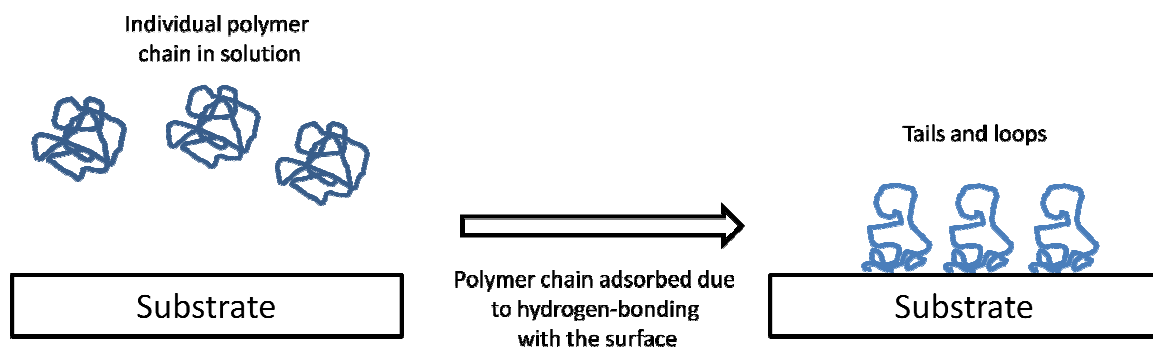


Figure 34. Proposed molecular behavior of polymer chains during LBL process mediated by hydrogen bonding.

Two concentrations equivalent to Ve were used for cell treatment, 10 μ M and 500 μ M. In order to ensure dissolution of Ve at high concentrations, dimethylsulfoxide (DMSO) was introduced as a co-solvent. The highest concentration used for cell culture was equivalent to 500 μ M of Ve, a value consistent with subsequent antioxidation study.

As shown in Figure 35, cells exposed to the PVT prodrug were as healthy as cells in the control culture regardless of the prodrug concentrations. In comparison, for both Ve and PTEGMA, cells showed good viability upon low dose treatment, while a slight decrease in viability (i.e. $\sim$ 80% of the control) was observed for the high dose condition. For the polymer PTEGMA, the slight toxicity could be attributed to residual organic solvents in the polymer. Such an effect was also reflected from the difference in cell morphology as shown by the qualitative phase contrast images (Figure 36). Unlike the polarized spread shape presented by cells in the control culture, a more rounded morphology was observed from cells exposed to the high concentration of PTEGMA.

3.3.4 Antioxidation study of PVT

Antioxidation property of PVT was investigated using A172 cells due to the protective roles of astrocytes to neurons [38, 39, 169]. Hydrogen peroxide was chosen as the oxidant stimulant because of its physiological relevance [170, 171]. Cell viability was used as a caliber to examine whether the PVT prodrug can protect cells from oxidative stress. Proper test conditions, including cell density, cytotoxic concentration of H₂O₂ and protective concentration of Ve toward A172 cells, as well as exposure time were first identified from pilot experiments.

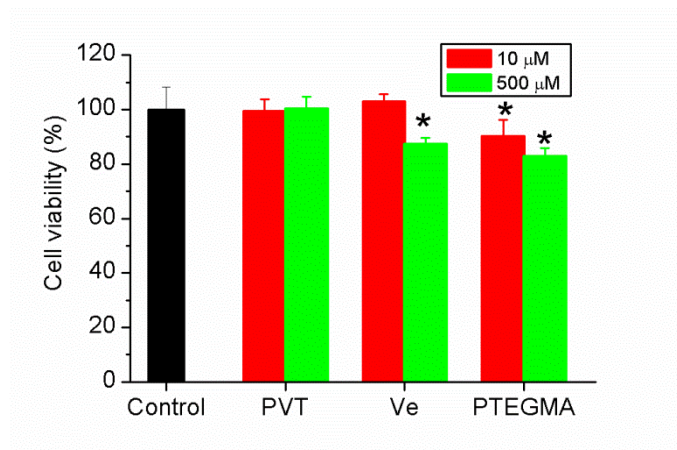


Figure 35. In vitro cytotoxicity of Ve, antioxidant prodrug PVT, and precursor polymer analog PTEGMA on A172 astrocytes at concentrations of 10 and 500 μM (equivalent to Ve). Data shown are average $\pm$ STD ($n = 3$). * $p < 0.05$. Statistical significance is compared with the non-treated control.

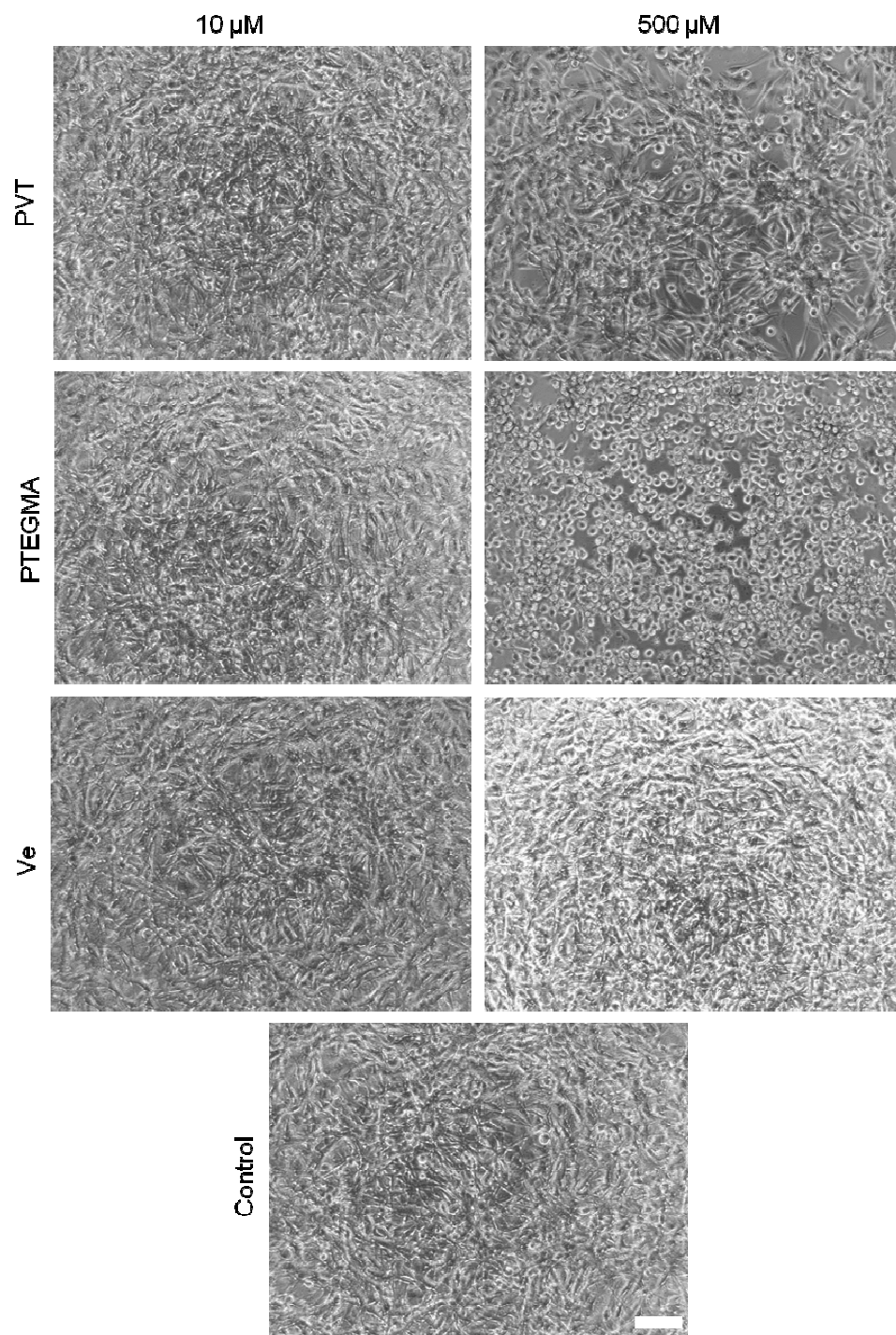


Figure 36. Representative phase contrast photomicrographs of cultured A172 astrocytes subjected to various treatments for cytotoxicity study. Non-treated control culture is shown for comparison. Scale bar = 100 μ m.

A172 cells were challenged with hydrogen peroxide at at 500 μM that is sufficient to induce significant oxidative stress and cause cell death. As shown in Figure 37a, cells treated with hydrogen peroxide alone showed a very low survival rate, less than 10%. When antioxidant Ve was added to the culture, it effectively protected cells from toxic hydrogen peroxide, as the survival rate was improved to 68%. When cells were treated with PVT prodrug at a concentration equivalent to 500 μM Ve, a viability of 43% was obtained, which is indicative of protection. The protective effect is also supported by qualitative morphological observations. In contrast to the characteristic polarized shape with processes shown by healthy astrocytes (Figure 37c), marked morphological change was observed for cells experiencing severe oxidative stress, resembled by strong shrinkage of cell bodies and degeneration of processes (Figure 37d). With Ve or PVT treatment, an intermediate state was observed, where both polarized and degenerated cells were present (e and 7f). A higher percentage of polarized cells were seen in Ve treated culture (Figure 37e) than PVT treated cells (Figure 37f), suggesting a lower degree of cell protection by PVT.

It is summarized from above results that treatment with antioxidant Ve at 500 μM showed substantial cytoprotection. While PVT with an equivalent Ve concentration was also able to reduce cell death from H_2O_2 induced oxidative stress, the extent of protection is lower than Ve supplemented to the culture in its free form. The protective mechanism of Ve has been largely attributed to its capacity to scavenge free radicals that cause lipid peroxidation and damage of cell membrane. Specifically, it can donate the phenolic hydrogen to free radicals.

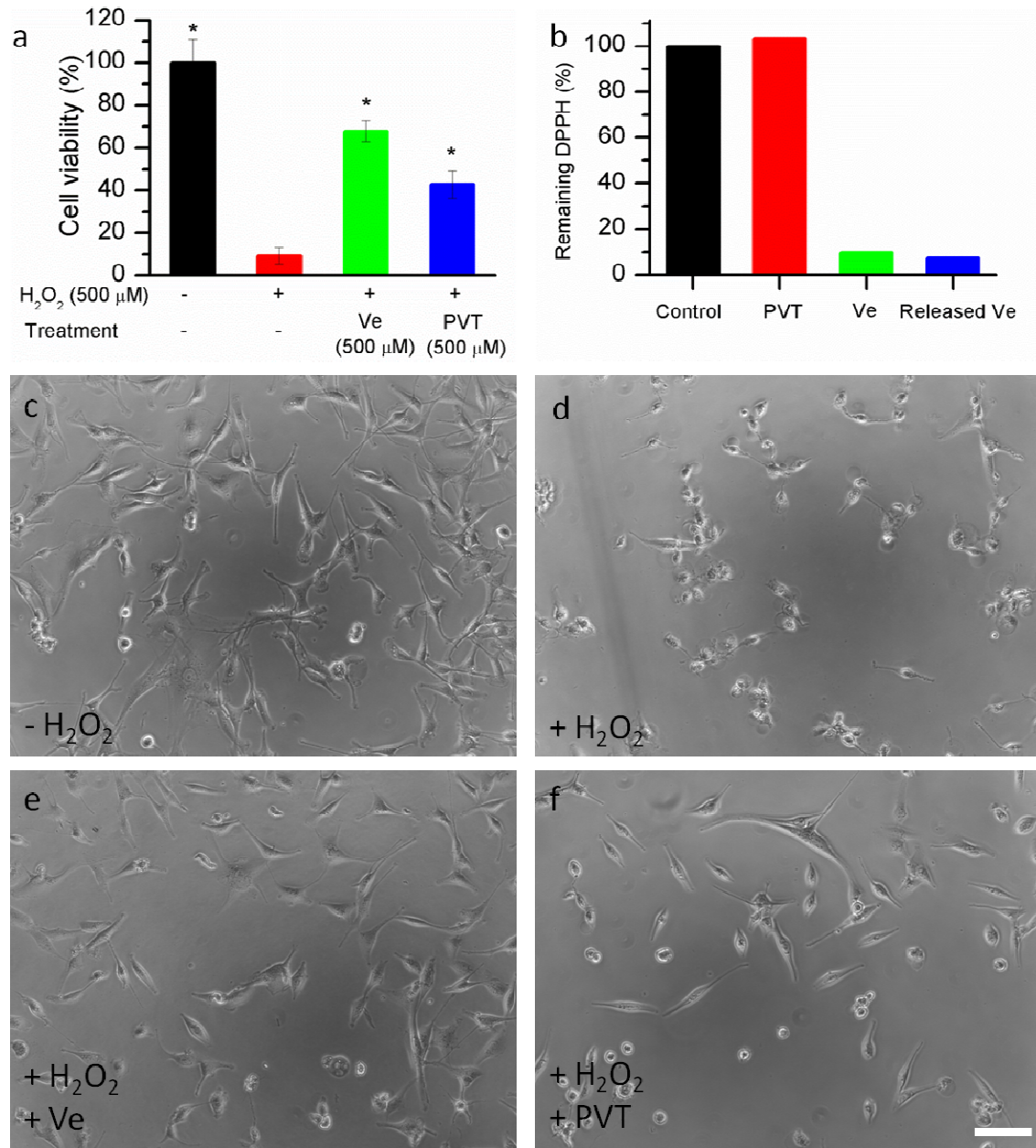


Figure 37. (a) In vitro protection of A172 astrocytes against injurious oxidative stress induced by hydrogen peroxide (H₂O₂). Data shown are average $\pm$ STD (n=3). * $p < 0.05$. (b) Antioxidation activity determined by the DPPH free radical scavenging method. (c)-(f) Representative phase contrast photomicrographs of cultured A172 astrocytes subjected to various treatments. Scale bar = 100 μ m.

Considering the essential role of the phenolic group for antioxidation, I hypothesized that the cytoprotective effect shown by the PVT prodrug was a result of hydrolysis of the labile ester bond between Ve and polymer chain, leading to the release of Ve in its active form.

To test this hypothesis, antioxidant property was further studied using a free radical scavenging method to elucidate the protection mechanism of PVT. DPPH is a stable free radical useful for the investigation of antioxidation activity of compounds. DPPH radical shows a characteristic absorbance peak around 550 nm. When interacting with antioxidants such as Ve, DPPH radical can be reduced through abstraction of transfer labile phenolic hydrogen atoms and the corresponding absorbance at 550 nm decreases. The extent of radical scavenging, which correlates with antioxidant potency, can be measured by the amount of decrease in absorbance.

As a positive control, fresh Ve was added to DPPH solution at a concentration of 350 μ M and 90% of radicals were consumed (Figure 37b). When the prodrug PVT was added at a concentration equivalent to 350 μ M Ve, no free radical scavenging effect was observed with almost 100% radical remaining at the end of reaction. PVT was unable to interact with DPPH free radicals due to the lack of phenolic hydrogen. In contrast, when DPPH was treated with Ve released from the PVT prodrug by disruption of the ester bond, the effect was comparable to that with fresh Ve. This result suggests that the released Ve retained its antioxidant potency and that the PVT protection of cells from oxidative stress was derived from the breakage of the ester bond and release of Ve in an active form. Such a mechanism correlates well with those reported on antioxidation

activity of esterified derivatives of phenolic antioxidants [172]. The lower degree of cytoprotection by PVT can be a result of slower release of Ve from PVT. It is well known that hydrolysis of ester group is closely dependent on water accessibility around the chemical bond [173, 174]. The strong hydrophobicity and steric hindrance of the Ve segment can limit water diffusion and slow down degradation.

3.3.5 Electrical property of the modified electrode

Neural electrodes were coated with (PVT/PAA) layers using the LBL technique, and the electrode impedance was measured before and after coating. This experiment was designed to examine the influence of coatings on the conductivity of the electrode. The thickness of coatings is expected to be the important parameter. Thin layers will not change the impedance, while thick coatings could increase the impedance significantly. In the latter case, the coated electrode will be immersed into PBS and the dissociable property of (PVT/PAA) coatings will be proved from the impedance data of the recording sites. It is expected that after incubation of the coated electrode in PBS, the coatings will dissociate and the conductive recording sites will be exposed, returning the impedance to original values.

The images of the electrode before and after coatings are shown in Figure 38. The electrode was wrapped with some fluffy films around the tip, which could possibly be the polymer complex. After 16 bilayers of deposition, the impedance is still low, comparable to the uncoated electrode (Figure 39). The results demonstrate that current coated layers did not change the electrical property of the electrode.

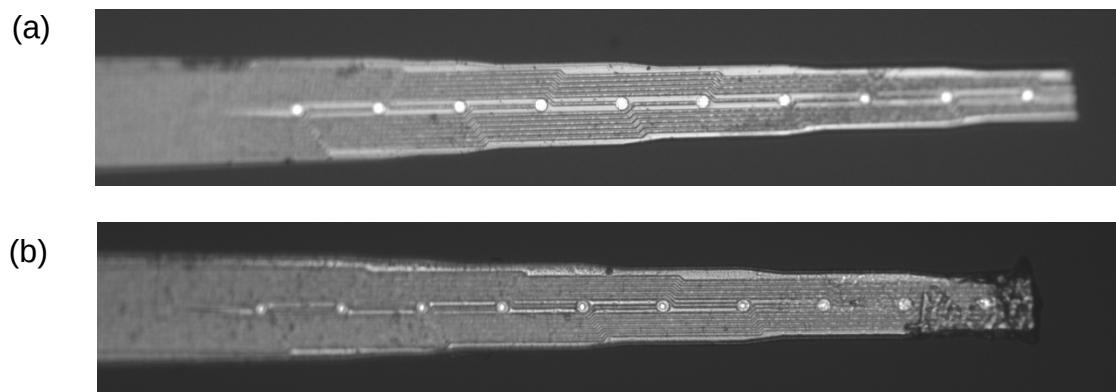


Figure 38. Photomicrographs of a Michigan electrode before (a) and after (b) deposition of 8 bilayers of (PVT/PAA)

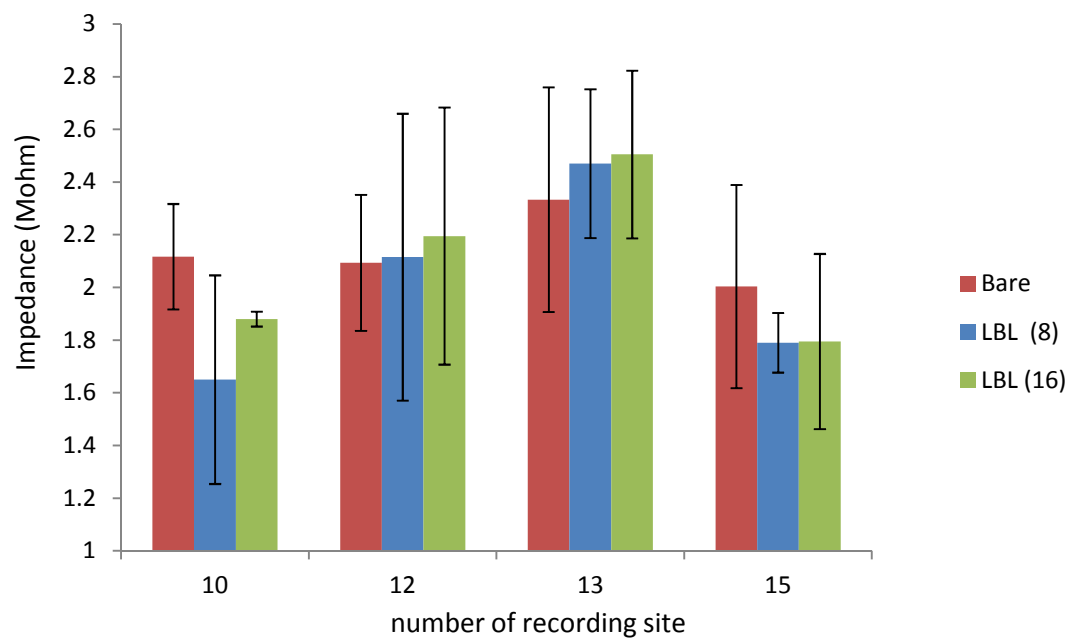


Figure 39. Impedance of recording sites after 8-bilayers (LBL (8)) and 16-bilayers (LBL (16)) of LBL coatings. The value of the bare sites before coating was also shown for comparison.

3.4 Conclusion

Compositionally controllable antioxidant polymeric therapeutics based on Ve were prepared. A significant improvement in the water solubility of Ve was observed. The synthetic PVT prodrug can be used to modify substrates of neural MEAs implants through hydrogen-bond mediated LBL technique. Multilayers with tunable stability at physiological condition could be achieved simply by engineering the layer composition. In vitro biocompatibility study showed that the PVT prodrug was non-cytotoxic toward A172 human astrocytes. Furthermore, the prodrug was able to reduce astrocyte death from hydrogen peroxide induced oxidative stress through the release of Ve in its active form. Future studies include evaluation of protection of cortical neurons from oxidative stress with the PVT prodrug and its effectiveness in improving the long-term function of implantable neural MEAs in vivo. I believe that the strategy of using antioxidant prodrug for direct cell protection from oxidative stress induced cell death provides a new opportunity to improve the pharmacological control of brain tissue response to MEAs. In addition to neural implant applications, our prodrug could also be applied to other medical implants where reducing oxidative stress is essential.

Chapter 4

Functionalized Biocompatible Poly(*N*-vinyl-2-caprolactam) with pH-dependent Lower Critical Solution Temperature (LCST) Behaviors

An article presenting part of the results in this chapter has been published on Macromolecular Chemistry and Physics. [Cao Y, He W. Functionalized biocompatible poly (N-vinyl-2carprolactam) with pH-dependent lower critical solution temperature (LCST) behaviors. Macromolecular Chemistry and Physics, 2011, 212.] The first author (Yu Cao) finished all of the experiments in this study. The corresponding author (Dr. Wei He) is advisor of Yu Cao and financially supported this work.

4.1 Introduction

PVCL is water soluble and biocompatible, with LCST in the vicinity of physiological temperature.[78] In conjunction with temperature, other stimuli including pH have been used in developing dual responsive polymers. These dual responsive polymers have been explored in applications including control of micelle structures,[89, 91, 93] advanced drug delivery systems,[85, 88, 98] fabrication of dual functional nanofluidic devices,[86] and modification of gold nanoparticles.[90]

In order to introduce dual responsiveness to temperature and pH into PVCL based materials, a similar copolymerization approach is commonly used, where VCL monomers are copolymerized with other vinyl monomers such as MAA or acrylic acid (AA). For example, aqueous solutions of linear copolymer of VCL and MAA with variable compositions were prepared and the copolymers exhibited compositionally dependent coil swelling behavior as a function of both pH and temperature.[175] However, similar to its counterpart in the *N*-vinylamide family, such as *N*-vinyl-2-pyrrolidone (NVP), VCL exhibits significantly lower reactivity comparing to methacrylates and acrylates due to the inherently low reactivity of *N*-vinylamide.[82] Such drastic disparity in reactivity

ratio makes compositional control a major concern in copolymerization-based functionalization of VCL polymers. In order to minimize compositional heterogeneity in the copolymers, special techniques such as gradual feeding strategy has been proposed.[176] Alternatively, one could consider copolymerization of structurally similar monomers of VCL that bear functional groups to obtain homogeneous copolymers. For example, Aoyagi and coworkers designed a reactive monomer, 2-carboxyisopropylacrylamide (CIPAAm), a derivative from *N*-isopropylacrylamide (NIPAAm).[177] They showed that copolymerization of CIPAAm with NIPAAm resulted in homogeneous copolymer compositions and improved the phase transition sensitivity.

In this chapter, the same principle was applied by copolymerizing VCL with its functional derivative 3-(*tert*-butoxycarbonyl)-*N*-vinyl-2-caprolactam (TBVCL), and improved compositional control was achieved. The protected carboxyl groups in the TBVCL units were converted to free carboxylic acids in the copolymer. Besides maintaining the intrinsic thermo-sensitivity, the incorporated carboxyl groups enabled pH-modulated LCST behavior. Sharp and reversible response was observed across a broad range of pH values. The carboxyl groups can also provide an easy access for further conjugation of bioactive moieties such as drugs, peptides, and proteins. Using NIH/3T3 fibroblasts, I demonstrated that this PVCL copolymer was non-cytotoxic at low concentrations. This simple PVCL copolymer with multi-characteristics, including thermal and pH sensitivity, and reactivity for conjugation, will further expand the biomedical applications of PVCL and provide a new option to the growing list of dual-

responsive biocompatible materials. Specifically, they could be added in the design toolbox for achieving better control of tissue response to neural electrode. One possible application could be correlating the pH-sensitivity of the PVCL polymers with the tissue acidosis phenomenon following the implantation of the electrode. PVCL polymer chains will be aggregated or expanded in response to pH variation, thereby providing a potential underlying trigger for smart drug delivery.

4.2 Experimental Section

4.2.1 Materials

Diisopropylamine, azobisisobutyronitrile (AIBN), di-*tert*-butyl dicarbonate and *N*-vinyl-2-caprolactam (VCL) were obtained from Sigma-Aldrich. Tetrahydrofuran (THF), *n*-butyllithium and trifluoroacetic acid (TFA) were obtained from Acros. Deuterated dimethyl sulfoxide (d₆-DMSO) and chloroform (CDCl₃) were obtained from Cambridge Isotope Laboratories, Inc. Hexanes and dichloromethane (DCM) were purchased from Fisher Scientific. All reagents were used as received unless otherwise noted.

4.2.2 Characterization

¹H-NMR (300 MHz) spectra were recorded on a Varian Mercury 300 NMR spectrometer. Fourier transform infrared spectroscopy (FT-IR) was carried out using potassium bromide pellets on a Bio-RAD FTS6000 FT-IR spectrometer. Gel permeation chromatography (GPC) was performed at room temperature using PL-GPC 50 Plus (Polymer Laboratories, Inc.) with a refractive index detector. *N,N*-Dimethylformamide was used as the carrier solvent at a flow rate of 1.0 mL/min. Standard monodisperse

polystyrenes (Polymer Laboratories) were used for calibration. Thermogravimetric analysis (TGA) was performed on Q-50 (TA Instrument) at a heating rate of 20 °C/min in a nitrogen atmosphere. The degree of ionization of the copolymer at various pH values was determined from potentiometric titration curves. Polymer solutions (1 mg/mL) were titrated with 0.1 mol/L NaOH. pK_a was estimated from the titration curve. The degree of ionization was then calculated as $\alpha = \frac{1}{1+10^{(pK_a-pH)}}$, $\alpha = [1 / (1 + 10^{(pK_a-pH)})]$.

4.2.3 Synthesis of carboxylated VCL homopolymer (COOH-PVCL-H)

Monomer of 3-(*tert*-butoxycarbonyl)-*N*-vinyl-2-caprolactam (TBVCL) was synthesized as described previously.[178] TBVCL (1.195 g, 5 mmol), AIBN (0.008 g, 0.049 mmol) and freshly distilled THF (5 mL) were added into a 50 mL flask, and subjected to three freeze-thaw cycles to remove oxygen. Polymerization reaction took place at 65 °C for 24 h. The polymer was then precipitated in hexane, redissolved in THF and reprecipitated in hexane for purification. The obtained polymer (COOH-PVCL-BOC-H, 160 mg, 14% yield) was dried in a vacuum oven at 55 °C overnight ($M_n = 5,400$ g/mol). The carboxyl groups (-COOH) were recovered by removing the *t*-BOC protecting groups using TFA. Briefly, PVCL-COOH-BOC-H was dissolved in DCM followed by the addition of TFA. After reacting for 3 h, the polymer was recovered by solvent evaporation and dried in a vacuum oven at 100 °C overnight.

4.2.4 Synthesis of carboxylated VCL copolymer (COOH-PVCL)

In a typical procedure (Figure 40), TBVCL (0.478 g, 2 mmol), VCL (2.5 g, 18 mmol), AIBN (0.049 g, 0.3 mmol) and freshly distilled THF (10 mL) were added into a 35 mL reaction tube.

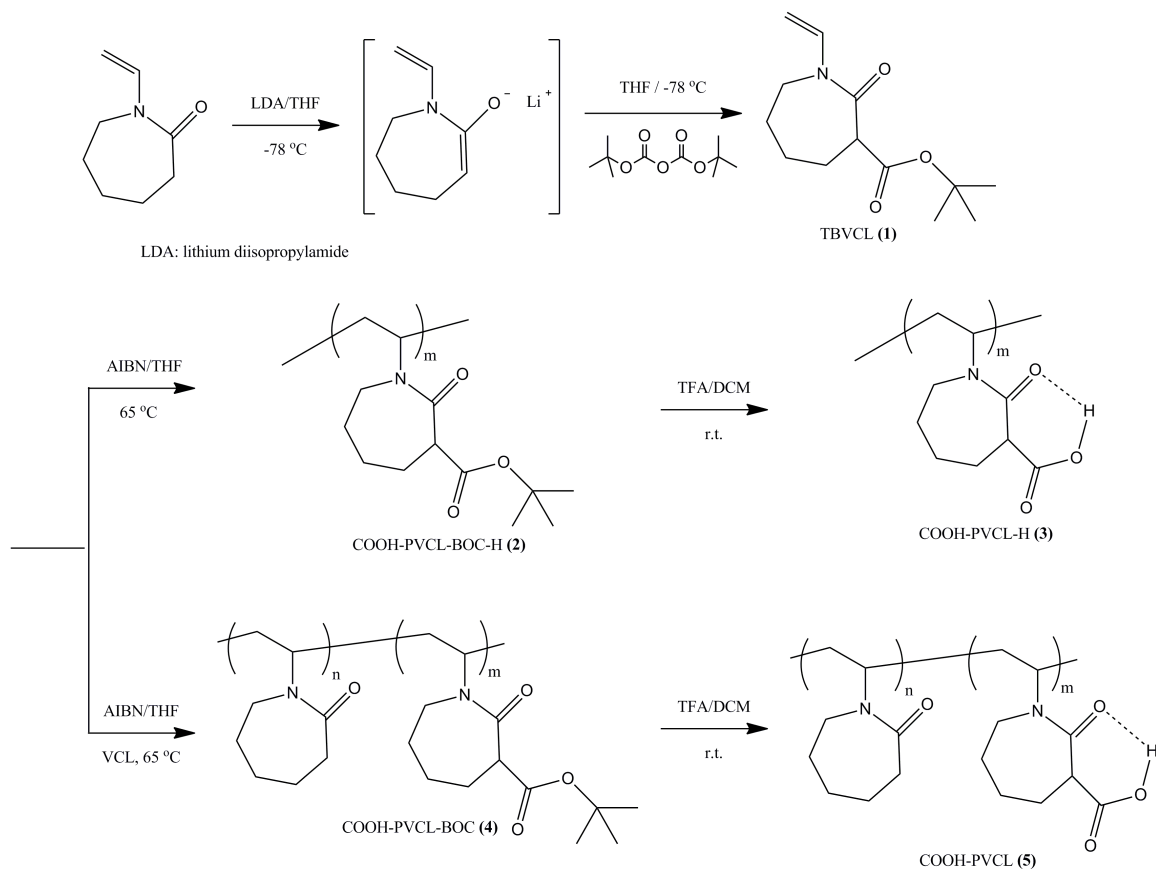


Figure 40. Synthesis of the monomer 3-(*tert*-butoxycarbonyl)-*N*-vinyl-2-caprolactam (TBVCL) and partially or fully carboxylated PVCL polymers. Intramolecular hydrogen bonding was shown in polymers **3** and **5**.

The mixture was then subjected to three freeze-thaw cycles to remove oxygen. Polymerization reaction took place at 65 °C for 24 h. The polymer was then precipitated in hexane for purification. The obtained polymer (COOH-PVCL-BOC) was dried in a vacuum oven at 55 °C overnight. The yields were above 80%. The carboxyl groups (-COOH) were recovered by removing the *t*-BOC protecting groups using TFA. Briefly, COOH-PVCL-BOC was dissolved in DCM followed by the addition of TFA. After 3 h of reaction, the polymer (COOH-PVCL) was recovered by solvent evaporation followed by precipitation in hexane and dried in a vacuum oven overnight.

4.2.5 Reactivity study

A typical experiment is described below. TBVCL (69.73 mg, 0.29 mmol) and VP (35.51 mg, 0.32 mmol) were firstly mixed in CDCl₃ (0.5 mL), with the initiator AIBN (1.5 mg, 0.009 mmol). Pyridine (4.94 mg, 0.06 mmol) was added as an external standard. The mixture in CDCl₃ was then placed in the NMR spectrometer for the initial spectrum collection and frozen in a -20 °C fridge to quench any polymerization at room temperature. After reaction in the oil bath at 65 °C for 20 minutes, the NMR tube was taken out and quenched in a -20 °C fridge before measurement. Similarly, two other NMR tubes with different compositions of TBVCL and VP were prepared and the same procedures were followed. The experiments for determining reactivity values of VCL and VP were also carried out using the same procedures described above.

4.2.6 Cloud point measurement

The transmittance for the aqueous polymer solution was measured with a UV-visible spectrophotometer (Biomate 5 from Thermospectronic) at 540 nm by following

the turbidity of the polymer solution as a function of temperature. Cloud point was defined using the heating curve as the temperature at which the transmittance was 50% of the value at room temperature. A heating/cooling rate of 0.2 °C/min was used. Concentrations of all polymer solutions were 2.5 mg/mL.

4.2.7 In vitro cytotoxicity study

In vitro cytotoxicity of the COOH-PVCL copolymer was examined using the NIH/3T3 fibroblast cell line. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Fetal Clone III, Fisher), 1% L-glutamine and 1% penicillin/streptomycin. Cells were maintained at 37 °C, 5% CO₂ and 95% relative humidity. Prior to cytotoxicity test, cells were harvested using trypsin–ethylenediamine tetraacetic acid (EDTA)–PBS solution (0.25% trypsin–0.038% EDTA) and diluted to a density of 1×10^5 cells/mL. The cell suspension was added into 96-well plates at 100 µL/well, and cells were allowed to attach overnight. Cell culture medium was then aspirated and replaced with fresh medium supplemented with COOH-PVCL at different concentrations ranging from 0.1 to 10 mg/mL. Cells were incubated overnight and polymer cytotoxicity was determined using an MTT assay (Promega, CellTiter 96) according to the manufacturer's instruction. Negative (without COOH-PVCL) and blank (without cells) controls were also included. Statistical analysis was conducted using Student's *t*-test.

4.2.8 LBL assembly of COOH-PVCL/PVCL monitored by Quartz crystal microgravimetry (QCM)

QCM measurements were conducted at room temperature using a Q-sense device (E4) with a flow cell (Q-Sense AB, Västra Frölunda, Sweden). A gold-coated 5 MHz AT-cut crystal was excited at multiple overtones, and the change in the resonance frequency was recorded. After deposition of the initial layer of PEI (1.5 mg/mL), the chamber was washed with water, after which the solution of COOH-PVCL (0.5 mg/mL, pH = 3.0) was introduced into the chamber and allowed to adsorb for 5 min. Subsequently, the chamber was washed again with HCl (pH = 3.0) and then filled with PVCL solution (0.5 mg/mL, pH = 3.0), allowing an adsorption time of 5 min. The multilayer film was then assembled by alternating incubations between COOH-PVCL and PVCL.

4.3 Results and Discussion

4.3.1 Polymer synthesis and characterization

To integrate additional functionalities such as carboxyl groups while maintaining the structural continuity of the caprolactam ring, a derivative of VCL monomer (TBVCL) containing a protected carboxylic acid side group was synthesized via nucleophilic substitution of VCL at the α -position with *tert*-butoxycarbonyl group (Figure 40).[179] This monomer was previously reported specifically for applications as deep UV photoresists for lithography[179-185], and to our best knowledge there is no prior study of its potential as smart biomaterials.

Chemical structures of TBVCL monomer and its homopolymer were confirmed with ^1H -NMR (Figure 41). The characteristic peak of *t*-BOC at 1.46 ppm was observed in the monomer structure (Figure 41a). Homopolymers of carboxylic acid functionalized PVCL (COOH-PVCL-H) were obtained by polymerization of TBVCL followed by TFA deprotection. The polymer preparation process was monitored with ^1H -NMR by following the disappearance of characteristic peaks of the double bond in the monomer (Figure 41b, peaks at chemical shifts 4.41-4.57 and 7.29-7.44 ppm) and the absence of the *t*-BOC peaks in COOH-PVCL-H (Figure 41c).

The TBVCL monomer allowed us to introduce a controllable amount of carboxylic acid groups in the VCL polymer pendent chains to acquire pH-sensitivity, using simple free radical copolymerization of VCL and TBVCL. As TBVCL bears close structural resemblance to that of VCL, it is expected that the reactivities of these two monomers will be similar comparing to that between VCL and methacrylates/acrylates, thus allowing for easier control of the copolymer compositions. Based on the results of ^1H -NMR (Figure 42a, peaks 1 and 4), a molar feed ratio of 9 : 1 VCL : TBVCL resulted in a composition of 5.92:1 of the two monomers in the final copolymer (equivalent to 14.4 mol % TBVCL). This compositional drift is significantly lower than that reported in copolymerization of VCL with MAA, where a 9:1 VCL : MAA molar feed ratio yielded a 1:1 ratio of VCL to MAA in the copolymer (50 mol % MAA).[186] It indicates that: (1) the disparity of reactivity between VCL and its functional analogue TBVCL is much less than that between VCL and MAA;

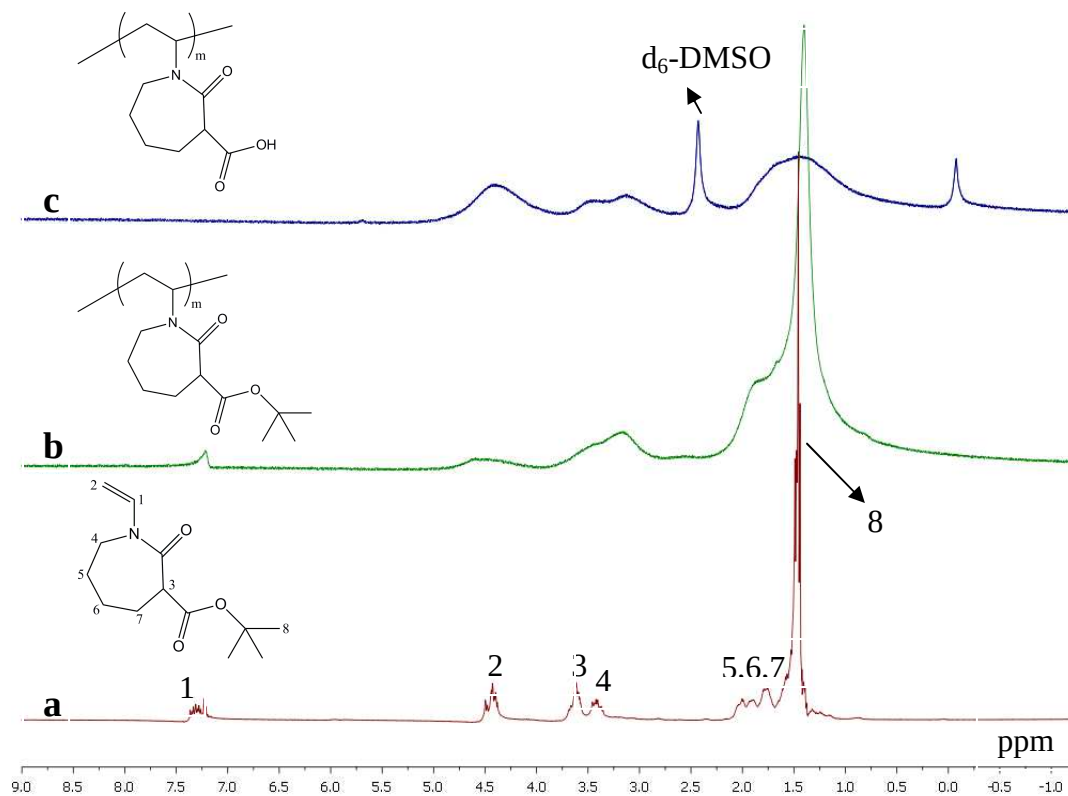


Figure 41. ^1H -NMR spectra of (a) monomer TBVCL in CDCl_3 , (b) protected homopolymer COOH-PVCL-BOC-H in CDCl_3 , and (c) deprotected homopolymer COOH-PVCL-H in d_6 -DMSO.

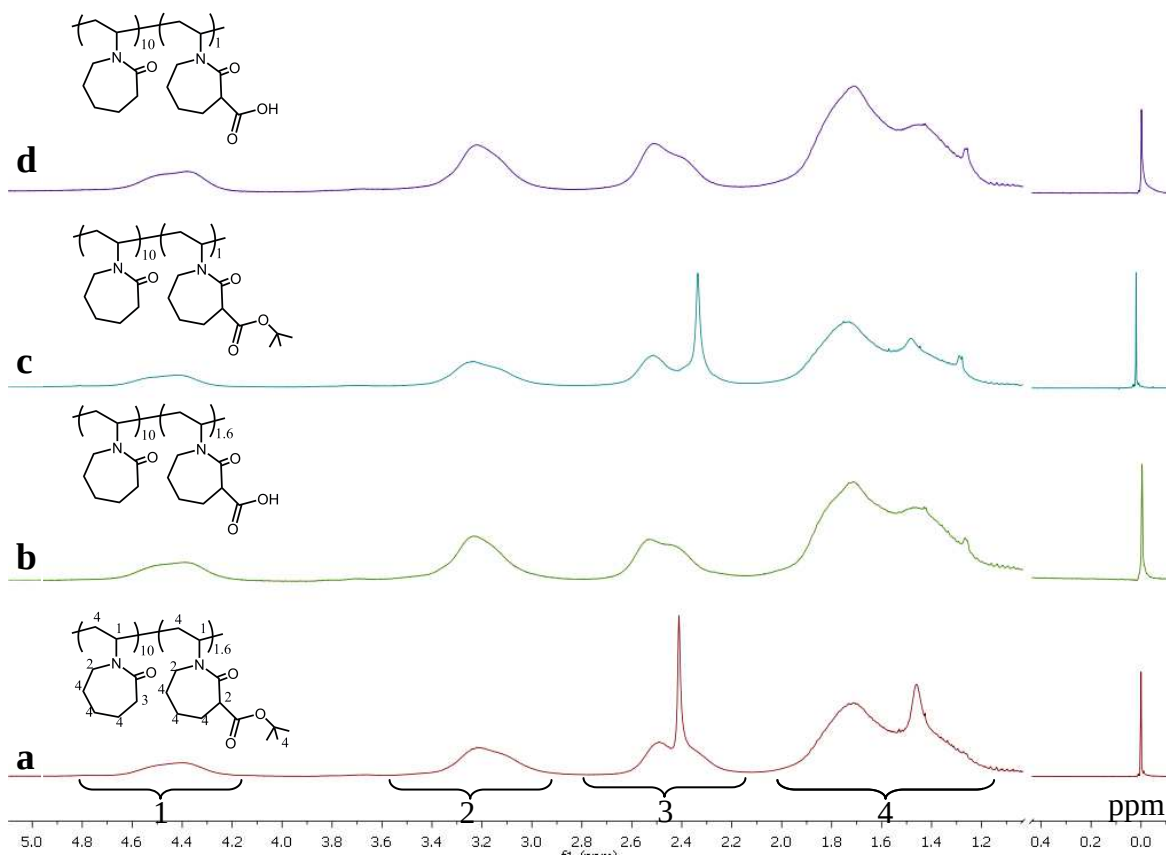


Figure 42. ^1H -NMR spectra of (a) protected copolymer COOH-PVCL-BOC14, (b) acid deprotected COOH-PVCL14, (c) protected copolymer COOH-PVCL-BOC9, and (d) acid deprotected COOH-PVCL9. CDCl_3 was used for all polymers.

and (2) TBVCL is more reactive than VCL, as a higher than theoretical percentage (10 mol %) of TBVCL was incorporated into the copolymer. The latter holds significance on its own, as this could potentially enable further development of functional copolymers containing thermo-sensitive VCL structures. A detailed study to determine the reactivity ratios of TBVCL with VCL is described later.

In addition to ^1H -NMR, the copolymer composition was further confirmed by TGA. As shown in Figure 43a, homopolymer of TBVCL (COOH-PVCL-BOC-H) started to lose weight at 203 °C, characteristic of the spontaneous thermolysis of the *t*-BOC groups into carbon dioxide (CO_2) and isobutylene (C_4H_8). At 400 °C, a typical mass loss from the PVCL structure was observed. The copolymer (COOH-PVCL-BOC14) also possessed the above two regions of mass loss (Figure 43b), and the percentage of *t*-BOC loss was used to calculate molar ratio of TBVCL in the copolymer as 13.6 mol %, which is comparable with the NMR result.

The chemical structures of the homopolymer and copolymers were also confirmed by IR spectra (Figure 44). The characteristic band at 1630 cm^{-1} corresponding to the C=C stretching of the vinyl group in the monomer TBVCL (Figure 44a) disappeared in all polymer structures. The strong absorbance peak around 1637 cm^{-1} in the monomer and all the polymers are assigned to the C=O stretching of the amide carbonyl group. The peak originating from the ester C=O stretching in *t*-BOC around 1735 cm^{-1} was observed in polymers containing *t*-BOC groups (COOH-PVCL-BOC-H (Figure 44b) and COOH-PVCL-BOC (Figure 44d)).

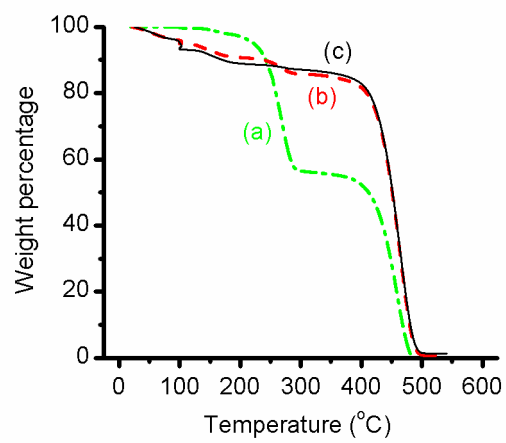


Figure 43. TGA spectra of protected TBVCL homopolymer COOH-PVCL-BOC-H (a), protected copolymers COOH-PVCL-BOC14 (b) and COOH-PVCL-BOC9 (c).

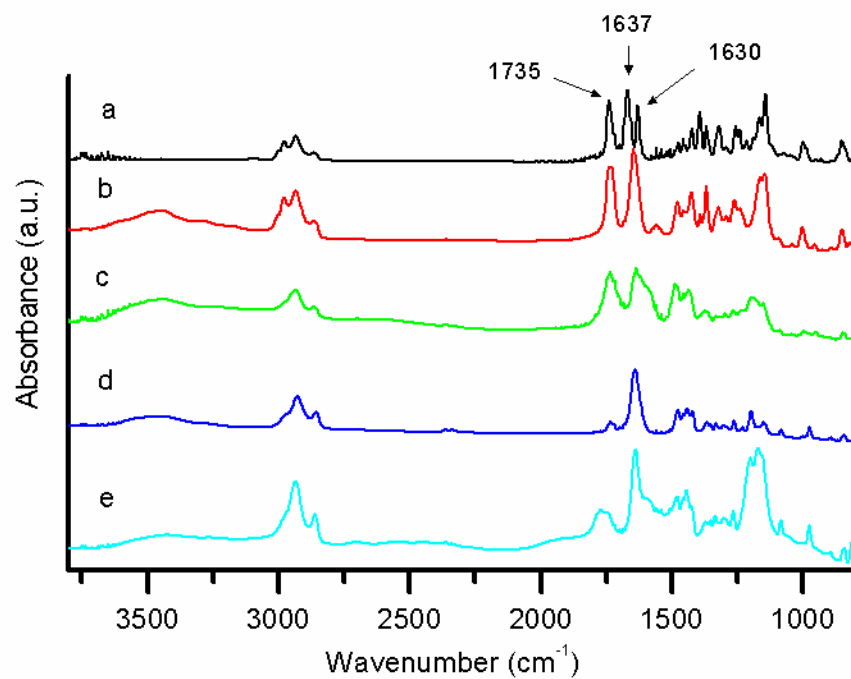


Figure 44. IR spectra of (a) monomer TBVCL, (b) protected homopolymer COOH-PVCL-BOC-H, (c) deprotected homopolymer COOH-PVCL-H, (d) protected copolymer COOH-PVCL-BOC14, and (e) deprotected copolymer COOH-PVCL14.

Broad peaks from 2200 to 3600 cm^{-1} were assigned to the -OH stretching in the -COOH groups of the deprotected homopolymer COOH-PVCL-H (Figure 44c) and copolymer COOH-PVCL (Figure 44e).

4.3.2 LCST behaviors of COOH-PVCLs

The key rationale of introducing carboxylic acid groups in the VCL polymer structure is to modulate its thermo-sensitivity with pH. Transmittance at 540 nm of the carboxylated copolymer solutions at different pH values was studied using a UV-vis spectrometer for cloud point (T_c) measurements. As summarized in , PVCL homopolymer and two PVCL copolymers with comparable molecular weights but different amounts of carboxylic acid groups (COOH-PVCL9 and COOH-PVCL14) were prepared to specifically study the carboxylic acid groups and compositional effects on the thermo-sensitive properties. The molecular weights (M_n) for PVCL, COOH-PVCL-9 and COOH-PVCL14 were 3100, 2600, 2600 g/mol respectively. It is important to maintain the molecular weights of these polymers consistent as studies have shown that the LCST of PVCL is also dependent on molecular weight.[187] Generally, as the molecular weight of PVCL increases, its LCST will decrease.

Figure 45 shows that not only the copolymers retained thermo-sensitivity of PVCL, but its phase transition temperature can also be varied as a function of solution pH by modulating the amount of carboxylic acid groups in the copolymer. Specifically, cloud point of COOH-PVCL9 increased from 35 °C to 44 °C as the pH of solution changed from 2 to 7.4 (Figure 45a).

Table 3. Molecular weight and compositional data of PVCL containing polymers

polymer	TBVCL amount (mol %)			M _n (g/mol)	M _w /M _n
	in feed	in polymer			
PVCL	-	-	-	3,100	3.06
COOH-PVCL9	2.5	8.9 ^a	7.4 ^b	2,600	2.67
COOH-PVCL14	10	13.6 ^a	14.4 ^b	2,600	2.76

^a Estimated from TGA. ^b Estimated from ¹H-NMR.

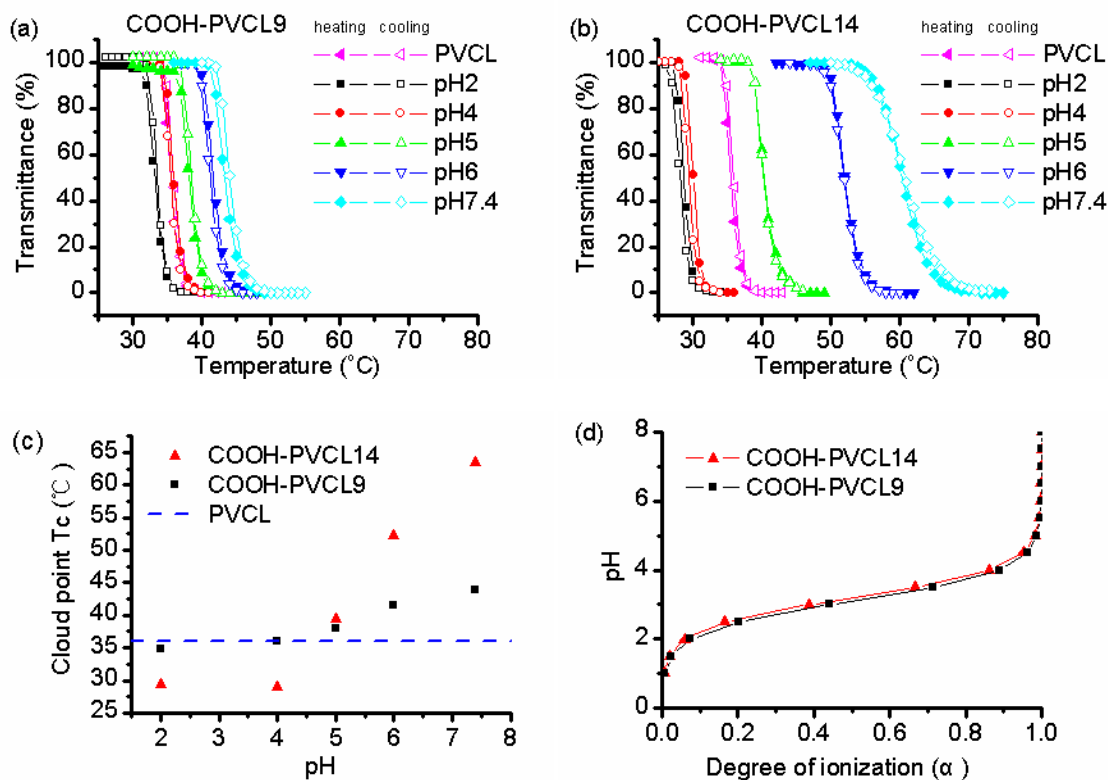


Figure 45. Phase transition profiles of 2.5 mg/mL of (a) COOH-PVCL9 and (b) COOH-PVCL14 in 0.1 M phosphate buffered saline at different pH values. (c) Cloud points (T_c) of various COOH-PVCLs at different pH values. The dash line represents the T_c of PVCL homopolymer at pH 7.4. (d) pH vs degree of ionization for COOH-PVCLs at room temperature.

When the amount of carboxylated VCL increased to 14.4 mol %, a broader range of cloud points was observed (Figure 45b), spanning from 29 °C to 64 °C as the solution pH increased from 2 to 7.4. In addition, the cooling curves (represented by open symbols in Figure 45a and b) overlapped well with the heating curves, demonstrating good reversibility of the thermo-responsive PVCL copolymers with little thermo-hysteresis. It can also be inferred from the thermo-hysteresis results that the positions of the spinodal curve and binodal curve for the PVCL copolymer will be very close to each other on its phase diagram, if it were constructed experimentally.

The observed pH-dependent behavior of the PVCL copolymers can be explained by the hydrophilic/hydrophobic balance between the thermo-dependent hydrophobic lactam unit and the ionizable –COOH groups. Based on the titration curves (d) the pK_a values of COOH-PVCL9 and COOH-PVCL14 were determined to be 3.1 and 3.2, respectively. Thus, at pH 2 the carboxylic acid groups of the copolymers are partially deprotonated, and at pH 7.4 fully deprotonated. Compared with homopolymer PVCL, the carboxylic acid groups could either decrease or increase T_c of the copolymer, depending on whether carboxylate groups (i.e. protonated form of carboxylic acid, -COOH) or deprotonated carboxylic acid groups (-COO⁻) dominated in the polymer structure. On one hand, carboxylate groups lead to more hydrophobic structures from the likely intramolecular hydrogen bonding (shown in Figure 40), resulting in a lower T_c . On the other hand, deprotonated carboxylic acid groups, which increase hydrophilicity of the polymer, would require a higher T_c to compensate for the enhanced hydrophilicity.[87, 188] As shown in Figure 45c, T_c of COOH-PVCL9 at pH 4 was the same as PVCL

homopolymer, suggesting that the hydrophilic effect of the ionized -COO^- groups balanced the hydrophobic effect of the protonated -COOH groups at that pH. However, T_c of COOH-PVCL14 at the same pH was 7 °C lower. Considering that COOH-PVCL14 has a slightly higher pK_a than COOH-PVCL9, I expect that at pH 4 less carboxylic acid groups are ionized/deprotonated for COOH-PVCL14, leading to a higher percentage of hydrophobic -COOH groups and therefore a lower T_c .

Another observation of the thermo-sensitivity studies is that as pH increased, the T_c for both copolymers increased as well, since more -COOH groups were ionizing and the polymer became more hydrophilic. A more dramatic increase was observed for COOH-PVCL14 due to the higher molar percentage of carboxyl groups. Although only two copolymer compositions were studied here, I can postulate that a further increase of the molar percentage of TBVCL in the copolymer will likely lead to a wider range of T_c as a function of pH. I have observed that for the deprotected homopolymer of TBVCL (COOH-PVCL-H), its solution at pH 4 remained cloudy even at 4 °C; and at pH 5, the solution remained clear until the temperature reached 95 °C (data not shown). These observations can be explained by the very high concentration of -COOH groups, which both further improve hydrophobicity at low pH and hydrophilicity at high pH. Additionally, the homopolymer COOH-PVCL-H was not soluble in pure water due to strong prevalence of intra- and intermolecular hydrogen bonding. I also examined the phase behavior of homopolymer PVCL at pH 2 and 5, and the corresponding T_c values were the same as that at pH 7.4, indicating the thermo-responsive behavior of homopolymer PVCL lacks pH sensitivity.

It is also worth noting the sensitivity in the phase transition behavior of the copolymers. As shown in , temperature sensitivity was obtained by measuring the temperature range corresponding to transmittance change from 90% to 10%. Unlike other reported pH-sensitive thermo-responsive polymers,[87, 177] where sharp phase transition is limited to those with a small amount of acidic component (i.e. less than 3-5 mol %) at certain pH values (pH 5.0 – 6.5), our system demonstrated that even at a high 14 mol % of functionalization, these copolymers responded sharply to both pH (from 2.0 to 7.4) and temperature the same way as the PVCL homopolymer (Figure 46). Such improvement could be attributed to the well maintained continuity of the thermo-dependent lactam groups in the polymer structure, allowing sufficient polymer aggregation even in the presence of high amounts of ionic groups. The importance of sharp phase transition is evident as it will boost the sensitivity of intended biomedical applications of these smart biomaterials, such as molecular switching and drug delivery.

4.3.3 Cytotoxicity study of COOH-PVCL

It is well known that PNVP has been widely used in biomedical applications.[68] Considering the structural similarity between PVCL and PNVP, PVCL based polymers have also been proposed for biological applications. It was shown that PVCL homopolymers were well tolerated at various concentrations (0.1-10.0 mg/ml).[189] To study how the incorporation of carboxyl groups affects biocompatibility of PVCL, I used fibroblast cells to measure cytotoxicity of COOH-PVCL copolymers. As shown in Figure 47, COOH-PVCL was non-toxic to the cells at concentrations up to 2 mg/mL.

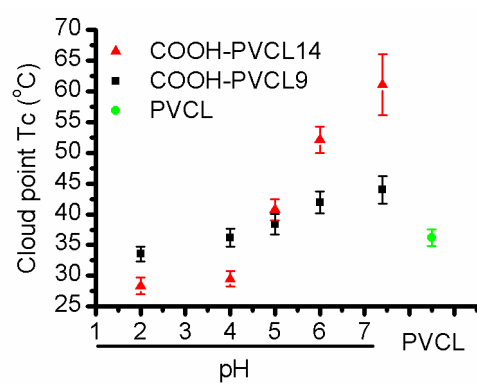


Figure 46. Influence of chemical compositions on the temperature sensitivity of phase transition. Top and bottom bars of each data point represent the temperature at 90% and 10% of the solution transmittance, respectively.

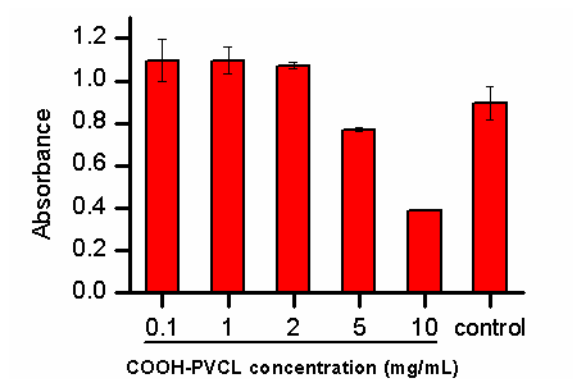


Figure 47. NIH/3T3 fibroblast cells viability after overnight incubation with different concentrations of COOH-PVCL copolymers. Cells without polymer treatment served as a negative control. Data shown are average $\pm$ STD (n = 3).

Upon further increase of the polymer concentration to 10 mg/mL, the cell viability significantly decreased ($p < 0.01$) as shown by the reduction in the MTT assay absorbance, an indication of decrease of mitochondrial activity in cells. It is likely that higher concentrations of the carboxylated polymer lead to acidification of the culture medium indicated by the color change of medium, thus affecting cell growth. These results suggest that the synthesized functional PVCL copolymer is biocompatible depending on the concentrations applied.

Although this new biocompatible PVCL-derived dual-responsive polymers can have various biomedical applications where the use of smart biomaterials are desirable, I am particularly interested in its use for drug delivery from the surface of biomedical implants. As the LCST of this PVCL copolymer at pH 5.0, a pH condition featured in inflamed tissues,[34, 88, 190] is drastically different from that at normal physiological tissue of pH 7.4, I could use such characteristics to engineer multilayers on the surface of biomedical implants and achieve inflammation triggered drug delivery. The idea is to assemble multilayers with this dual-responsive PVCL copolymer as one of the components, and upon inflammation induced tissue acidosis, it leads to the molecular conformational changes due to its associated reduction of LCST, which will subsequently change the properties of the multilayers and enable drug release. With this biocompatible dual-responsive polymer, I hope to improve the correlation and control between drug treatment and the extent of tissue response to biomedical implants in future studies.

4.3.4 Reactivity study

In order to expand the application of TBVCL, copolymerization with other biomaterials-related monomers is a good option. The reactivity values of these monomer pairs are therefore of our interest. There are many mathematical approaches to calculate reactivity values. Among these methods, Kelen and Tudos (K-T) method is chosen for my study due to its relative simplicity and popularity. Depending on the molecular structures, different experimental techniques could be employed including TGA, FTIR, UV, NMR, etc. The common objective for these experiments is to obtain compositions of the two monomers before and after composition. In my case, NMR was used due to the distinctive monomer peaks in the spectra. To test my method, two commercially available monomers (VCL and VP) were firstly studied. Small amounts of VCL and VP were firstly mixed in CDCl_3 at a predetermined feeding ratio. Pyridine was added as an external standard. In a quantitative NMR study, internal standard, which means a peak belonging to one of the reactants, is preferred if possible. However, in this case, there is no such suitable internal standard peak. Therefore, an external standard is necessary and the choice is based on the following conditions. Firstly, the standard peak needs to be clearly distinguished from other peaks. Secondly, the standard chemical cannot react with monomers. Thirdly, the chemical should be stable at the polymerization temperature, 65 °C. Based on the above limits and the spectral information from VCL and VP, one possible region of standard peak was identified at the lower chemical shift. Common solvents were screened and pyridine was finally chosen as the external standard satisfying the above requirements.

The mixture in CDCl_3 was firstly placed in the NMR spectrometer for the initial spectrum collection. After reaction in the oil bath at 65 °C for 20 minutes, the NMR tube was taken out and quenched in a -20 °C fridge before measurement. Similarly, two other NMR tubes with different compositions were prepared and the same procedures were followed. The above data (the NMR spectra are shown in Appendix Figure 53-64) were plotted according to the widely used linear graphic K-T method.[191] The above procedures are also summarized in Figure 48. And the reactivity values were obtained from the slope and intercept. The K-T method is based on the following equation:

$$\eta = \left(r_1 + \frac{r_2}{\alpha}\right)\xi - \frac{r_2}{\alpha} \quad 13$$

where r_1 , r_2 are the reactivity values for monomers. Other parameters are defined by the following equations:

$$X = \frac{M_1}{M_2} \quad 14$$

$$Y = \frac{dM_1}{dM_2} \quad 15$$

$$F = \frac{X^2}{Y} \quad 16$$

$$A = \frac{X(Y-1)}{Y} \quad 17$$

$$\eta = \frac{A}{\alpha + F} \quad 18$$

$$\xi = \frac{F}{\alpha + F} \quad 19$$

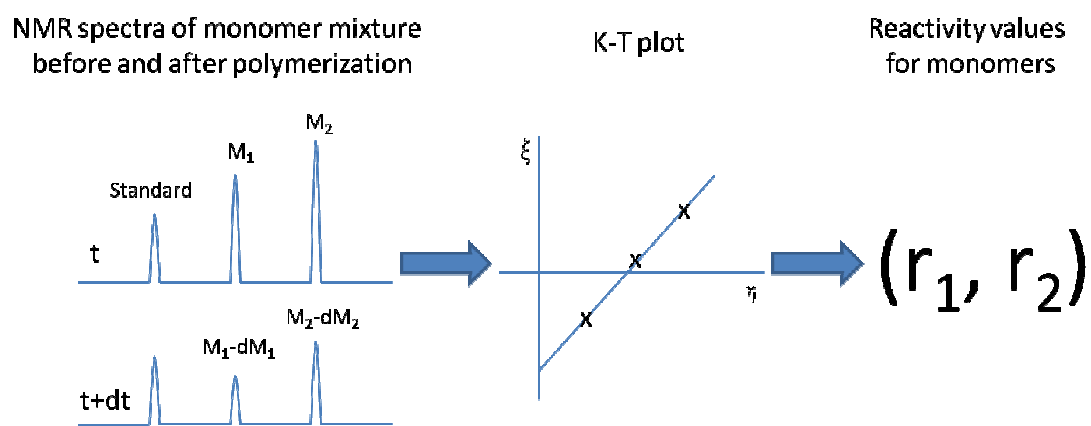


Figure 48. Schematic illustration for reactivity value measurements.

where M_1 , M_2 are the molar amount of the two monomers at the starting point t . dM_1 , dM_2 are the amount of monomers reacted during polymerization period. α is an arbitrary positive constant. A feasible choice is:

$$\alpha = \sqrt{F_m F_M} \quad 20$$

where F_m and F_M are the lowest and highest values of F , respectively.

There is one constraint on the K-T method. It requires the monomer conversion to be rather low ($< 20\%$). In this case, I kept the conversion below 15%. Detailed calculation was carried out and shown in the Appendix (Table 4 and Table 5).

The reactivity values for VP and VCL are calculated to be 4.5 and 0.8 respectively. These data were reported previously in another handbook as 2.8 and 1.7 respectively. My results are consistent with the reference in that the value for VP is larger than VCL. However, the difference could be attributed to the inherent variance between different methods for determining reactivity values.

After the experiment for monomer pair of VP and VCL, I proceed to VP and TBVCL. Using the same method, the reactivity values for VP and TBVCL are determined as 0.7 and 2.1 respectively.

Attempts have also been made to study VCL and TBVCL. However, this method did not work because no distinctive peaks in NMR spectra can be identified for calculation due to the structural similarity between VCL and TBVCL. Even though direct derivation of the values cannot be obtained, an indirectly approach to compare the reactivity of VCL and TBVCL is possible and described as followed.

From the above results, the reactivity values for VP and TBVCL are 0.7 and 2.1 respectively, leading to a conclusion that TBVCL is more reactive than VP. The reactivity values for VP and VCL are calculated as 4.5 and 0.8 using the same method. It is concluded that for the order of reactivity, $TBVCL > VP$ and $VP > VCL$. Therefore, I inferred that TBVCL is more reactive than VCL. This conclusion also coincides with the composition of the copolymer of TBVCL and VCL. For example, when VCL and TBVCL were added at 9:1 molar ratio, the resulting polymer has a molar composition of 6:1. It should be noted that it is still possible to determine the reactivity values of VCL and TBVCL by other methods such as TGA. Such an approach was not pursued further due to the limitation of monomer availability.

4.3.5 LBL coating consisting of COOH-PVCL

The synthesized COOH-PVCL is used as the hydrogen bonding donor component in the LBL process. To simply prove the feasibility of this approach, PVCL was employed as the hydrogen bonding acceptor, which has been demonstrated by other researchers to be able to form LBL films with another polymeric component. QCM was utilized to monitor the layer assembly process. As shown in Figure 49, after deposition of the base layer of PEI, COOH-PVCL could be adsorbed onto the PEI layer. However, further alternately flowing PVCL and COOH-PVCL could not deposit any appreciable amounts of polymers onto the Au surface. The underlying reasons could be the low density of hydrogen bonding donor groups, resulted from low molecular weight of COOH-PVCL or the small amount of carboxylic acid groups available on the polymer

chain. The weak hydrogen bonding interaction from the current polymer pair did not provide sufficient force for stable multilayers formation.

Even though the above exploratory experiment did not result in successful multilayer deposition, further improvements could be concentrated on increasing the polymer molecular weight or percentage of COOH groups to realize biocompatible LBL coatings.

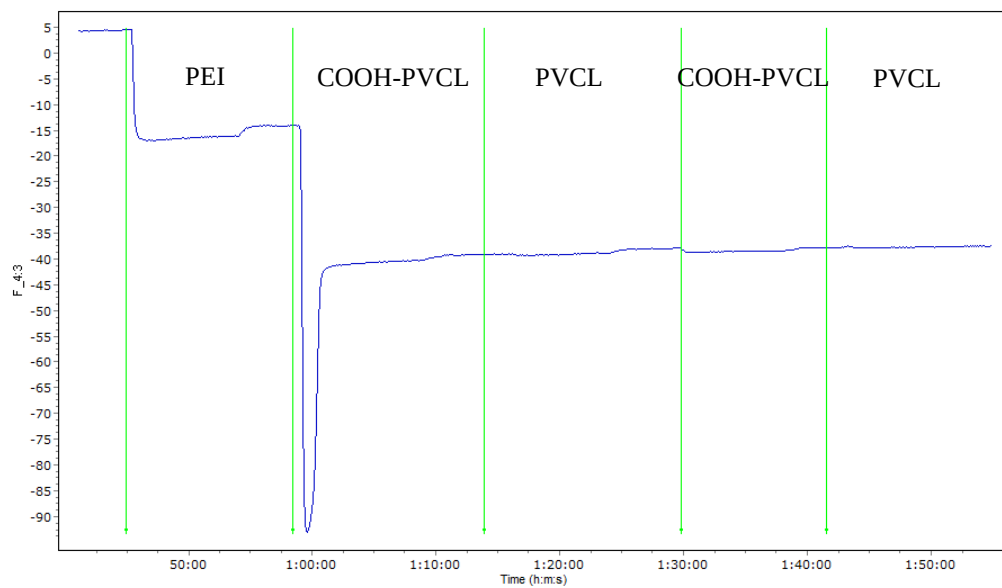


Figure 49. A QCM plot showing the change in frequency (proportional to adsorbed mass) as a function of the adsorption and build-up of multilayers of COOH-PVCL/PVCL.

4.4 Conclusions

I have successfully integrated pH and thermo-sensitivity for PVCL polymers by maintaining the structural continuity of the caprolactam ring while introducing carboxyl groups via copolymerization with TBVCL, a functional derivative of VCL. The cloud point of the copolymer can be tuned from 29 °C to 64 °C by changes of solution pH from 2 to 7.4. Cytotoxicity studies of the copolymer on NIH/3T3 fibroblasts showed good biocompatibility up to a polymer concentration of 2 mg/mL. The high content of the functional carboxyl groups in the copolymer will also enable further conjugation of bioactive molecules for broader applications in biotechnology and drug delivery.

Chapter 5

Summary and Future Work

A multi-functional approach to combat the tissue response from brain to MEAs was designed. Anti-inflammatory and anti-oxidant therapeutics were employed to achieve the objective. Individual polymer therapeutic was developed and their properties were fully characterized. The polymers structure and the therapeutic loading capacity are controllable. The in vitro cell studies showed that they maintained the therapeutic effects. These polymeric therapeutics were further successfully incorporated onto the silicon substrate, as the main materials for the neural implants. Also some key design features were demonstrated such as the pH-responsive release of anti-inflammatory prodrug, dissociable coatings in the brain environment, and complex coatings structure consisting stable and unstable components. Furthermore, a smart polymer responsive to pH and temperature was synthesized and characterized, with the potential to be incorporated in the complex design to realize advanced therapeutic delivery system.

The specific conclusions in this dissertation were listed below:

1. A poly(*N*-vinyl pyrrolidone) based glucocorticoid-polymer prodrug was synthesized by conjugating prednisolone to functionalized PNVP via a hydrazone linkage.
2. pH sensitivity of drug release from the poly(*N*-vinyl pyrrolidone) based prodrug was observed under conditions mimicking inflammation-induced tissue acidosis, as hydrolysis of the hydrazone linkage was enhanced under acidic pH.

3. Poly(*N*-vinyl pyrrolidone) based prodrug and its released products demonstrated good biocompatibility with neurons. Neural culture showed that both the prodrug and its precursor polymer were non-toxic to neurons and did not alter the ability of neurons to maintain neurite extensions, reassuring further application of this prodrug to mitigate inflammation at the neural interface.

4. Bioactivity of the poly(*N*-vinyl pyrrolidone) based prodrug and its released free drug were studied with RAW264.7 macrophages in vitro, where inhibition of nitric oxide production was observed, suggesting the desired anti-inflammatory effect.

5. Poly(*N*-vinyl pyrrolidone) based prodrug was also compatible with the hydrogen bond-driven LBL assembly technique. Multilayers of poly(acrylic acid)/prodrug were successfully deposited onto silicon substrates and quartz plates. The layers were destructible when incubated in physiological pH, which could be beneficial for neural electrodes.

6. Poly(*N*-vinyl pyrrolidone) based prodrug can serve as a promising and versatile drug delivery system. It can be easily applied to neural electrodes without compromising the electrical property of the implant, and can potentially realize tissue response-regulated drug release based on tissue acidosis. This system allows localized pharmacological interventions that deliver anti-inflammatory drugs based on the extent of inflammation without interfering with the electrical properties of the neural electrodes.

7. Compositionally controllable antioxidant polymeric therapeutics PVTs were prepared by copolymerizing monomers of triethylene glycol methyl acrylate (TEGMA) and α -tocopheryl acrylate (VeAc). In contrast to the poor water solubility of

Ve, solubility of the PVT prodrug in water can reach as high as 3.1 mg/mL (equivalent to 500 μ M Ve) by tuning the copolymer composition.

8. The PVT prodrug was non-cytotoxic toward A172 human astrocytes. Furthermore, PVT was able to protect astrocytes against oxidative stress exerted by hydrogen peroxide in vitro. Using a free radical scavenging assay, the protection mechanism was suggested to be attributed to the hydrolysis of the labile ester linkage and release of the active Ve.

9. Multilayers containing PVT with tunable stability at physiological condition could be achieved simply by engineering the layer composition. PVT was successfully deposited on silicon (Si) substrates with poly(acrylic acid) (PAA) or tannic acid (TA) using the LBL technique mediated by hydrogen bonding. Ellipsometry and quartz crystal microbalance (QCM) data showed that the multilayers of PAA/PVT were destructible at physiological pH. In contrast, multilayers of TA/PVT were stable.

10. Functionalized temperature- and pH-sensitive poly(*N*-vinyl-2-caprolactam) polymers were prepared by copolymerizing monomers of VCL and a VCL derivative, TBVCL..

11. Sharp, complete and reversible phase transitions of the copolymers with little hysteresis were shown to be pH-dependent, with cloud points ranging from 35 °C to 44 °C for COOH-PVCL9, and 29 °C to 64 °C for COOH-PVCL14, upon pH change from 2.0 to 7.4.

12. Biocompatibility cytotoxicity assay demonstrates that the functionalized PVCL copolymers were biocompatible with NIH/3T3 up to 2 mg/mL.

13. The functionalized PVCL copolymers hold potential to be used for drug delivery from the surface of biomedical implants. The idea is to assemble multilayers with this dual-responsive PVCL copolymer as one of the components, and upon inflammation induced tissue acidosis, it leads to the molecular conformational changes due to its associated reduction of LCST, which will subsequently change the properties of the multilayers and enable drug release.

Based on above conclusions in this dissertation, future work is proposed, including:

1. In vitro study of anti-inflammatory and anti-oxidant prodrugs on primary cells such as brain derived microglia, astrocytes, and neurons.

2. Validation of the efficacy of different films in improving the biocompatibility of the neural implant in a rat model. These films will include LBL coatings with either anti-inflammatory or anti-oxidant prodrug. Furthermore, possible synergistic effect will be examined when the film contains both prodrugs.

3. Modulation of the molecular weight and composition of functional poly(*N*-vinyl-2-caprolactam), and incorporation of these polymers in multilayered films as a smart drug delivery platform responsive to temperature and pH, toward neural implant applications.

References

- [1] Carmena JM, Lebedev MA, Crist RE, O'Doherty JE, Santucci DM, Dimitrov DF, et al. Learning to control a brain-machine interface for reaching and grasping by primates. *Plos Biology* 2003;1:193-208.
- [2] Hochberg LR, Serruya MD, Friehs GM, Mukand JA, Saleh M, Caplan AH, et al. Neuronal ensemble control of prosthetic devices by a human with tetraplegia. *Nature* 2006;442:164-71.
- [3] Chapin JK, Moxon KA, Markowitz RS, Nicolelis MAL. Real-time control of a robot arm using simultaneously recorded neurons in the motor cortex. *Nature Neuroscience* 1999;2:664-70.
- [4] Negi S, Bhandari R, Rieth L, Van Wagenen R, Solzbacher F. Neural electrode degradation from continuous electrical stimulation: Comparison of sputtered and activated iridium oxide. *Journal of Neuroscience Methods* 2010;186:8-17.
- [5] www.neuronexustech.com/neuroscience-products.
- [6] Wise KD. Silicon microsystems for neuroscience and neural prostheses. *Ieee Engineering in Medicine and Biology Magazine* 2005;24:22-9.
- [7] Product catalog from NeuroNexus.
- [8] Polikov VS, Tresco PA, Reichert WM. Response of brain tissue to chronically implanted neural electrodes. *Journal of Neuroscience Methods* 2005;148:1-18.
- [9] He W, Bellamkonda RV. A Molecular Perspective on Understanding and Modulating the Performance of Chronic Central Nervous System (CNS) Recording Electrodes. In: Reichert WM, editor. *Indwelling neural implants, Strategies for contending with the in vivo environment*: CRC Press; 2008. p. 162.
- [10] Grill WM, Norman SE, Bellamkonda RV. Implanted Neural Interfaces: Biochallenges and Engineered Solutions. *Annu Rev Biomed Eng* 2009;11:1-24.
- [11] He W, McConnell GC, Schneider TM, Bellamkonda RV. A novel anti-inflammatory surface for neural electrodes. *Advanced Materials* 2007;19:3529-33.
- [12] Denninger JW, Marletta MA. Guanylate cyclase and the (NO)-N-/cGMP signaling pathway. *Biochimica Et Biophysica Acta-Bioenergetics* 1999;1411:334-50.
- [13] Hewett SJ, Csernansky CA, Choi DW. Selective potentiation of NMDA-induced neuronal injury following induction of astrocytic iNOS. *Neuron* 1994;13:487-94.
- [14] Szarowski DH, Andersen MD, Retterer S, Spence AJ, Isaacson M, Craighead HG, et al. Brain responses to micro-machined silicon devices. *Brain Research* 2003;983:23-35.
- [15] Rousche PJ, Pellinen DS, Pivin DP, Williams JC, Vetter RJ, Kipke DR. Flexible polyimide-based intracortical electrode arrays with bioactive capability. *IEEE Trans Biomed Eng* 2001;48:361-71.
- [16] Nicolelis MAL, Dimitrov D, Carmena JM, Crist R, Lehew G, Kralik JD, et al. Chronic, multisite, multielectrode recordings in macaque monkeys. *Proc Natl Acad Sci U S A* 2003;100:11041-6.
- [17] Yoon JJ, Kim JH, Park TG. Dexamethasone-releasing biodegradable polymer scaffolds fabricated by a gas-foaming/salt-leaching method. *Biomaterials* 2003;24:2323-9.

- [18] Yang JY, Martin DC. Microporous conducting polymers on neural microelectrode arrays II. Physical characterization. *Sensors and Actuators a-Physical* 2004;113:204-11.
- [19] Singh A, Ehteshami G, Massia S, He JP, Storer RG, Raupp G. Glial cell and fibroblast cytotoxicity study on plasma-deposited diamond-like carbon coatings. *Biomaterials* 2003;24:5083-9.
- [20] Shain W, Spataro L, Dilgen J, Haverstick K, Retterer S, Isaacson M, et al. Controlling cellular reactive responses around neural prosthetic devices using peripheral and local intervention strategies. *Ieee Transactions on Neural Systems and Rehabilitation Engineering* 2003;11:186-8.
- [21] Maynard EM, Fernandez E, Normann RA. A technique to prevent dural adhesions to chronically implanted microelectrode arrays. *J Neurosci Methods* 2000;97:93-101.
- [22] Kennedy PR. The cone electrode - a long-term electrode that records from neurites grown onto its recording surface. *Journal of Neuroscience Methods* 1989;29:181-93.
- [23] Ignatius MJ, Sawhney N, Gupta A, Thibadeau BM, Monteiro OR, Brown IG. Bioactive surface coatings for nanoscale instruments: Effects on CNS neurons. *Journal of Biomedical Materials Research* 1998;40:264-74.
- [24] He W, McConnell GC, Bellamkonda RV. Nanoscale laminin coating modulates cortical scarring response around implanted silicon microelectrode arrays. *Journal of Neural Engineering* 2006;3:316-26.
- [25] Kipke DR, Vetter RJ, Williams JC, Hetke JF. Silicon-substrate intracortical microelectrode arrays for long-term recording of neuronal spike activity in cerebral cortex. *Ieee Transactions on Neural Systems and Rehabilitation Engineering* 2003;11:151-5.
- [26] Abidian MR, Martin DC. Experimental and theoretical characterization of implantable neural microelectrodes modified with conducting polymer nanotubes. *Biomaterials* 2008;29:1273-83.
- [27] Green RA, Ordonez JS, Schuettler M, Poole-Warren LA, Lovell NH, Suaning GJ. Cytotoxicity of implantable microelectrode arrays produced by laser micromachining. *Biomaterials* 2010;31:886-93.
- [28] Capadona JR, Shanmuganathan K, Tyler DJ, Rowan SJ, Weder C. Stimuli-responsive polymer nanocomposites inspired by the sea cucumber dermis. *Science* 2008;319:1370-4.
- [29] Spataro L, Dilgen J, Retterer S, Spence AJ, Isaacson M, Turner JN, et al. Dexamethasone treatment reduces astroglia responses to inserted neuroprosthetic devices in rat neocortex. *Experimental Neurology* 2005;194:289-300.
- [30] Zhong YH, Bellamkonda RV. Dexamethasone-coated neural probes elicit attenuated inflammatory response and neuronal loss compared to uncoated neural probes. *Brain Research* 2007;1148:15-27.
- [31] Koehler PJ. Use of Corticosteroids in Neurooncology. *Anti-Cancer Drugs* 1995;6:19-33.
- [32] Kaal ECA, Vecht CJ. The management of brain edema in brain tumors. *Current Opinion in Oncology* 2004;16:593-600.
- [33] Twycross R. The Risks And Benefits Of Corticosteroids In Advanced Cancer. *Drug Safety* 1994;11:163-78.

- [34] Cao Y, He W. Synthesis and Characterization of Glucocorticoid Functionalized Poly(N-vinyl pyrrolidone): A Versatile Prodrug for Neural Interface. *Biomacromolecules* 2010;11:1298-307.
- [35] Zhong YH, Bellamkonda RV. Controlled release of anti-inflammatory agent alpha-MSH from neural implants. *J Controlled Release* 2005;106:309-18.
- [36] Wadhwa R, Lagenaur CF, Cui XT. Electrochemically controlled release of dexamethasone from conducting polymer polypyrrole coated electrode. *J Controlled Release* 2006;110:531-41.
- [37] Kim DH, Martin DC. Sustained release of dexamethasone from hydrophilic matrices using PLGA nanoparticles for neural drug delivery. *Biomaterials* 2006;27:3031-7.
- [38] Drukarch B, Schepens E, Stoof JC, Langeveld CH, Van Muiswinkel FL. Astrocyte-enhanced neuronal survival is mediated by scavenging of extracellular reactive oxygen species. *Free Radical Biol Med* 1998;25:217-20.
- [39] Desagher S, Glowinski J, Premont J. Astrocytes protect neurons from hydrogen peroxide toxicity. *J Neurosci* 1996;16:2553-62.
- [40] Quinn JF, Johnston APR, Such GK, Zelikin AN, Caruso F. Next generation, sequentially assembled ultrathin films: beyond electrostatics. *Chem Soc Rev* 2007;36:707-18.
- [41] Bertrand P, Jonas A, Laschewsky A, Legras R. Ultrathin polymer coatings by complexation of polyelectrolytes at interfaces: suitable materials, structure and properties. *Macromol Rapid Commun* 2000;21:319-48.
- [42] Decher G. Fuzzy nanoassemblies: Toward layered polymeric multicomposites. *Science* 1997;277:1232-7.
- [43] Tang ZY, Wang Y, Podsiadlo P, Kotov NA. Biomedical applications of layer-by-layer assembly: From biomimetics to tissue engineering. *Advanced Materials* 2006;18:3203-24.
- [44] Wang LY, Wang ZQ, Zhang X, Shen JC, Chi LF, Fuchs H. A new approach for the fabrication of an alternating multilayer film of poly(4-vinylpyridine) and poly(acrylic acid) based on hydrogen bonding. *Macromol Rapid Commun* 1997;18:509-14.
- [45] Sukhishvili SA, Granick S. Layered, erasable, ultrathin polymer films. *Journal of the American Chemical Society* 2000;122:9550-1.
- [46] Stockton WB, Rubner MF. Molecular-level processing of conjugated polymers .4. Layer-by-layer manipulation of polyaniline via hydrogen-bonding interactions. *Macromolecules* 1997;30:2717-25.
- [47] Quinn JF, Caruso F. Thermoresponsive nanoassemblies: Layer-by-layer assembly of hydrophilic-hydrophobic alternating copolymers. *Macromolecules* 2005;38:3414-9.
- [48] Quinn JF, Caruso F. Facile tailoring of film morphology and release properties using layer-by-layer assembly of thermoresponsive materials. *Langmuir* 2004;20:20-2.
- [49] Zelikin AN, Quinn JF, Caruso F. Disulfide cross-linked polymer capsules: En route to biodeconstructible systems. *Biomacromolecules* 2006;7:27-30.
- [50] Yang SY, Rubner MF. Micropatterning of polymer thin films with pH-sensitive and cross-linkable hydrogen-bonded polyelectrolyte multilayers. *Journal of the American Chemical Society* 2002;124:2100-1.

- [51] Wang F, Ma N, Chen QX, Wang WB, Wang LY. Halogen bonding as a new driving force for layer-by-layer assembly. *Langmuir* 2007;23:9540-2.
- [52] Quinn JF, Caruso F. Multivalent-ion-mediated stabilization of hydrogen-bonded multilayers. *Adv Funct Mater* 2006;16:1179-86.
- [53] Chen JY, Cao WX. Fabrication of a covalently attached self-assembly multilayer film via H-bonding attraction and subsequent UV-irradiation. *Chem Commun* 1999;1711-2.
- [54] Kharlampieva E, Sukhishvili SA. Hydrogen-bonded layer-by-layer polymer films. *Polym Rev* 2006;46:377-95.
- [55] Erel-Unal I, Sukhishvili SA. Hydrogen-bonded multilayers of a neutral polymer and a polyphenol. *Macromolecules* 2008;41:3962-70.
- [56] Erel-Unal I, Sukhishvili SA. Hydrogen-Bonded Hybrid Multilayers: Film Architecture Controls Release of Macromolecules. *Macromolecules* 2008;41:8737-44.
- [57] Veronese FM, Morpurgo M. Bioconjugation in pharmaceutical chemistry. *Farmaco* 1999;54:497-516.
- [58] Veronese FM. Peptide and protein PEGylation: a review of problems and solutions. *Biomaterials* 2001;22:405-17.
- [59] Ulbrich K, Subr V. Polymeric anticancer drugs with pH-controlled activation. *Advanced Drug Delivery Reviews* 2004;56:1023-50.
- [60] Ulbrich K, Etrych T, Chytil P, Pechar M, Jelinkova M, Rihova B. Polymeric anticancer drugs with pH-controlled activation. *International Journal of Pharmaceutics* 2004;277:63-72.
- [61] Li C, Wallace S. Polymer-drug conjugates: Recent development in clinical oncology. *Advanced Drug Delivery Reviews* 2008;60:886-98.
- [62] Yamaoka T, Tabata Y, Ikada Y. Fate of Water-Soluble Polymers Administered via Different Routes. *Journal of Pharmaceutical Sciences* 1995;84:349-54.
- [63] Basu A, Yang K, Wang ML, Liu S, Chintala R, Palm T, et al. Structure-function engineering of interferon-beta-1b for improving stability, solubility, potency, immunogenicity, and pharmacokinetic properties by site-selective mono-PEGylation. *Bioconjugate Chem* 2006;17:618-30.
- [64] He XH, Shaw PC, Tam SC. Reducing the immunogenicity and improving the in vivo activity of trichosanthin by site-directed PEGylation. *Life Sci* 1999;65:355-68.
- [65] Berna M, Dalzoppo D, Pasut G, Manunta M, Izzo L, Jones AT, et al. Novel monodisperse PEG-dendrons as new tools for targeted drug delivery: Synthesis, characterization and cellular uptake. *Biomacromolecules* 2006;7:146-53.
- [66] Pasut G, Veronese FM. Polymer-drug conjugation, recent achievements and general strategies. *Progress in Polymer Science* 2007;32:933-61.
- [67] Hamidi M, Azadi A, Rafiei P. Hydrogel nanoparticles in drug delivery. *Advanced Drug Delivery Reviews* 2008;60:1638-49.
- [68] Buhler V. Polyvinylpyrrolidone Excipients for Pharmaceuticals Povidone, Crospovidone and Copovidone. Berlin, Heidelberg: Springer-Verlag Berlin Heidelberg; 2005.
- [69] Jeong B, Gutowska A. Lessons from nature: stimuli-responsive polymers and their biomedical applications. *Trends in Biotechnology* 2002;20:305-11.

- [70] Roy I, Gupta MN. Smart polymeric materials: Emerging biochemical applications. *Chemistry & Biology* 2003;10:1161-71.
- [71] Mano JF. Stimuli-responsive polymeric systems for biomedical applications. *Advanced Engineering Materials* 2008;10:515-27.
- [72] Schild HG. Poly(N-isopropylacrylamide): experiment, theory and application. *Progress in Polymer Science* 1992;17:163-249.
- [73] Sperling LH. Introduction to physical polymer science. 4th ed. Hoboken, N.J.: Wiley; 2006.
- [74] Robeson LM. Polymer blends : a comprehensive review. Cincinnati: Hanser Publications; 2007.
- [75] Maeda Y, Yamamoto H, Ikeda I. Effects of ionization of incorporated imidazole groups on the phase transitions of poly(N-isopropylacrylamide), poly(N,N-diethylacrylamide), and poly(N-vinylcaprolactam) in water. *Langmuir* 2001;17:6855-9.
- [76] Christova D, Velichkova R, Loos W, Goethals EJ, Du Prez F. New thermo-responsive polymer materials based on poly(2-ethyl-2-oxazoline) segments. *Polymer* 2003;44:2255-61.
- [77] Meeussen F, Nies E, Berghmans H, Verbrugghe S, Goethals E, Du Prez F. Phase behaviour of poly(N-vinyl caprolactam) in water. *Polymer* 2000;41:8597-602.
- [78] Kirsh YE, Yanul NA, Kalninskii KK. Structural transformations and water associate interactions in poly-N-vinylcaprolactam-water system. *European Polymer Journal* 1999;35:305-16.
- [79] Lau ACW, Wu C. Thermally sensitive and biocompatible poly(N-vinylcaprolactam): Synthesis and characterization of high molar mass linear chains. *Macromolecules* 1999;32:581-4.
- [80] Vihola H, Laukkanen A, Hirvonen J, Tenhu H. Binding and release of drugs into and from thermosensitive poly(N-vinyl caprolactam) nanoparticles. *Eur J Pharm Sci* 2002;16:69-74.
- [81] Pich A, Lu Y, Boyko V, Arndt KF, Adler HJP. Thermo-sensitive poly(N-vinylcaprolactam-co-acetoacetoxyethyl methacrylate) microgels: 2. Incorporation of polypyrrole. *Polymer* 2003;44:7651-9.
- [82] Boyko V, Pich A, Lu Y, Richter S, Arndt KF, Adler HJP. Thermo-sensitive poly(N-vinylcaprolactam-co-acetoacetoxyethyl methacrylate) microgels: 1 - synthesis and characterization. *Polymer* 2003;44:7821-7.
- [83] Markvicheva EA, Tkachuk NE, Kuptsova SV, Dugina TN, Strukova SM, Kirsh YE, et al. Stabilization of proteases by entrapment in a new composite hydrogel. *Applied Biochemistry and Biotechnology* 1996;61:75-84.
- [84] Zhuk A, Pavlukhina S, Sukhishvili SA. Hydrogen-Bonded Layer-by-Layer Temperature-Triggered Release Films. *Langmuir* 2009;25:14025-9.
- [85] Ta T, Convertine AJ, Reyes CR, Stayton PS, Porter TM. Thermosensitive Liposomes Modified with Poly(N-isopropylacrylamide-co-propylacrylic acid) Copolymers for Triggered Release of Doxorubicin. *Biomacromolecules* 2010;11:1915-20.

- [86] Guo W, Xia HW, Cao LX, Xia F, Wang ST, Zhang GZ, et al. Integrating Ionic Gate and Rectifier Within One Solid-State Nanopore via Modification with Dual-Responsive Copolymer Brushes. *Advanced Functional Materials* 2010;20:3561-7.
- [87] Yin X, Hoffman AS, Stayton PS. Poly(N-isopropylacrylamide-co-propylacrylic acid) copolymers that respond sharply to temperature and pH. *Biomacromolecules* 2006;7:1381-5.
- [88] Garbern JC, Hoffman AS, Stayton PS. Injectable pH- and Temperature-Responsive Poly(N-isopropylacrylamide-co-propylacrylic acid) Copolymers for Delivery of Angiogenic Growth Factors. *Biomacromolecules* 2010;11:1833-9.
- [89] Lokitz BS, York AW, Stempka JE, Treat ND, Li YT, Jarrett WL, et al. Aqueous RAFT synthesis of micelle-forming amphiphilic block copolymers containing N-Acryloylvaline. Dual mode, Temperature/pH responsiveness, and "Locking" of micelle structure through interpolyelectrolyte complexation. *Macromolecules* 2007;40:6473-80.
- [90] Lv WP, Liu SQ, Fan XB, Wang SL, Zhang GL, Zhang FB. Gold Nanoparticles Functionalized by a Dextran-Based pH- and Temperature-Sensitive Polymer. *Macromolecular Rapid Communications* 2010;31:454-8.
- [91] Zhang BY, He WD, Li WT, Li LY, Zhang KR, Zhang H. Preparation of block-brush PEG-b-P(NIPAM-g-DMAEMA) and its dual stimulus-response. *Polymer* 2010;51:3039-46.
- [92] Huang CJ, Chang FC. Polypeptide diblock copolymers: Syntheses and properties of poly(N-isopropylacrylamide)-b-polylysine. *Macromolecules* 2008;41:7041-52.
- [93] Lee RS, Chen WH, Huang YT. Synthesis and characterization of dual stimuli-responsive block copolymers based on poly(N-isopropylacrylamide)-b-poly(pseudoamino acid). *Polymer* 2010;51:5942-51.
- [94] Yamamoto S, Pietrasik J, Matyjaszewski K. Temperature- and pH-responsive dense copolymer brushes prepared by ATRP. *Macromolecules* 2008;41:7013-20.
- [95] Gonzalez N, Elvira C, Roman JS. Novel dual-stimuli-responsive polymers derived from ethylpyrrolidine. *Macromolecules* 2005;38:9298-303.
- [96] Weber C, Becer CR, Guenther W, Hoogenboom R, Schubert US. Dual Responsive Methacrylic Acid and Oligo(2-ethyl-2-oxazoline) Containing Graft Copolymers. *Macromolecules* 2010;43:160-7.
- [97] Gao M, Jia XR, Li Y, Liang DH, Wei Y. Synthesis and Thermo-/pH- Dual Responsive Properties of Poly(amidoamine) Dendronized Poly(2-hydroxyethyl) Methacrylate. *Macromolecules* 2010;43:4314-23.
- [98] He YY, Zhang Y, Xiao Y, Lang MD. Dual-response nanocarrier based on graft copolymers with hydrazone bond linkages for improved drug delivery. *Colloids and Surfaces B-Biointerfaces* 2010;80:145-54.
- [99] Teraoka I. *Polymer solutions : an introduction to physical properties*. New York: Wiley; 2002.
- [100] Clausen T, Khaldi A, Zauner A, Reinert M, Doppenberg E, Menzel M, et al. Cerebral acid-base homeostasis after severe traumatic brain injury. *Journal of Neurosurgery* 2005;103:597-607.

- [101] Gupta AK, Zygun DA, Johnston AJ, Steiner LA, Al-Rawi PG, Chatfield D, et al. Extracellular brain pH and outcome following severe traumatic brain injury. *Journal of Neurotrauma* 2004;21:678-84.
- [102] Johnson MD, Kao OE, Kipke DR. Spatiotemporal pH dynamics following insertion of neural microelectrode arrays. *Journal of Neuroscience Methods* 2007;160:276-87.
- [103] Kaila K, Ransom BR. pH and brain function. New York: Wiley; 1998.
- [104] Henson PM. The Immunologic Release of Constituents from Neutrophil Leukocytes: II. Mechanisms of Release During Phagocytosis, and Adherence to Nonphagocytosable Surfaces. *J Immunol* 1971;107:1547-57.
- [105] D'Souza AJM, Topp EM. Release from polymeric prodrugs: Linkages and their degradation. *Journal of Pharmaceutical Sciences* 2004;93:1962-79.
- [106] Wang D, Miller SC, Liu XM, Anderson B, Wang XS, Goldring SR. Novel dexamethasone-HPMA copolymer conjugate and its potential application in treatment of rheumatoid arthritis. *Arthritis Research & Therapy* 2007;9.
- [107] Gao ZG, Lee DH, Kim DI, Bae YH. Doxorubicin loaded pH-sensitive micelle targeting acidic extracellular pH of human ovarian A2780 tumor in mice. *J Drug Targeting* 2005;13:391-7.
- [108] Lee ES, Oh KT, Kim D, Youn YS, Bae YH. Tumor pH-responsive flower-like micelles of poly(L-lactic acid)-b-poly (ethylene glycol)-b-poly(L-histidine). *J Controlled Release* 2007;123:19-26.
- [109] Steenbergen C, Deleeuw G, Rich T, Williamson JR. Effects of acidosis and ischemia on contractility and intracellular pH of rat heart. *Circ Res* 1977;41:849-58.
- [110] Frunder H. The pH changes of living tissue during activity and inflammation. *Pharmazie* 1949;4:345-55.
- [111] Bencini M, Ranucci E, Ferruti P, Manfredi A. New stimuli responsive poly(1-vinylpyrrolidin-2-one) bearing pendant activated disulfide groups. *Macromol Rapid Commun* 2006;27:1060-6.
- [112] Bencini M, Ranucci E, Ferruti P, Oldani C, Licandro E, Maiorana S. Synthesis of 3,3-di(ethoxycarbonyl)-1-vinylpyrrolidin-2-one and determination of its reactivity ratios with 1-vinylpyrrolidin-2-one. *Macromolecules* 2005;38:8211-9.
- [113] Engstrom JUA, Lindgren LJ, Helgee B. Synthesis of novel monomers and copolymers from 1-vinylpyrrolidin-2-one: Attractive materials for drug delivery systems? *Macromol Chem Phys* 2006;207:536-44.
- [114] He W, Gonsalves KE, Pickett JH, Halberstadt C. Synthesis, characterization, and preliminary biological study of poly(3-(tert-butoxycarbonyl)-N-vinyl-2-pyrrolidone). *Biomacromolecules* 2003;4:75-9.
- [115] Lee HY, Yu SA, Jeong KH, Kim YJ. Poly(vinyl pyrrolidone) conjugated lipid system for the hydrophobic drug delivery. *Macromol Res* 2007;15:547-52.
- [116] Park KK, Ko DH, You Z, Khan MOF, Lee HJ. In vitro anti-inflammatory activities of new steroidal antedugs: [16 alpha,17 alpha-d] isoxazoline and [16 alpha,17 alpha-d]-3'-hydroxy-iminoformyl isoxazoline derivatives of prednisolone and 9 alpha-fluoroprednisolone. *Steroids* 2006;71:183-8.
- [117] Hanson GR, Iversen PL, Partlow LM. Preparation and Partial Characterization of Highly Purified Primary Cultures of Neurons and Nonneuronal (Glial) Cells from

Embryonic Chick Cerebral Hemispheres and Several Other Regions of the Nervous-System. *Dev Brain Res* 1982;3:529-45.

[118] Hermanson GT. *Bioconjugate techniques*. 2nd ed. San Diego: Academic Press; 2008.

[119] Wuts PGM, Greene TW. *Greene's protective groups in organic synthesis*. 4th ed. Hoboken, N.J.: Wiley-Interscience; 2007.

[120] Ihre HR, De Jesus OLP, Szoka FC, Frechet MJ. Polyester dendritic systems for drug delivery applications: Design, synthesis, and characterization. *Bioconjugate Chem* 2002;13:443-52.

[121] Lu DX, Wen XT, Liang J, Gu ZW, Zhang XD, Fan YJ. A pH-Sensitive Nano Drug Delivery System Derived From Pullulan/Doxorubicin Conjugate. *Journal of Biomedical Materials Research Part B-Applied Biomaterials* 2009;89B:177-83.

[122] Silver IA, Maroudas A. Measurement of pH and Ionic Composition of Pericellular Sites. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences* 1975;271:261-72.

[123] Coessens V, Schacht E, Domurado D. Synthesis of polyglutamine and dextran conjugates of streptomycin with an acid-sensitive drug-carrier linkage. *J Controlled Release* 1996;38:141-50.

[124] Thierry B, Kujawa P, Tkaczyk C, Winnik FM, Bilodeau L, Tabrizian M. Delivery platform for hydrophobic drugs: Prodrug approach combined with self-assembled multilayers. *J Am Chem Soc* 2005;127:1626-7.

[125] Wang LY, Fu Y, Wang ZQ, Fan YG, Zhang X. Investigation into an alternating multilayer film of poly(4-vinylpyridine) and poly(acrylic acid) based on hydrogen bonding. *Langmuir* 1999;15:1360-3.

[126] Sukhishvili SA, Granick S. Layered, erasable polymer multilayers formed by hydrogen-bonded sequential self-assembly. *Macromolecules* 2002;35:301-10.

[127] Yang SG, Zhang YJ, Wang L, Hong S, Xu J, Chen YM. Composite thin film by hydrogen-bonding assembly of polymer brush and poly(vinylpyrrolidone). *Langmuir* 2006;22:338-43.

[128] Vleggeert-Lankamp C, Pego AP, Lakke E, Deenen M, Marani E, Thomeer R. Adhesion and proliferation of human Schwann cells on adhesive coatings. *Biomaterials* 2004;25:2741-51.

[129] Lakard S, Herlem G, Propper A, Kastner A, Michel G, Valles-Villarreal N, et al. Adhesion and proliferation of cells on new polymers modified biomaterials. *Bioelectrochemistry* 2004;62:19-27.

[130] Brunot C, Ponsonnet L, Lagneau C, Farge P, Picart C, Grosogeat B. Cytotoxicity of polyethyleneimine (PEI), precursor base layer of polyelectrolyte multilayer films. *Biomaterials* 2007;28:632-40.

[131] Halthur TJ, Elofsson UM. Multilayers of charged polypeptides as studied by in situ ellipsometry and quartz crystal microbalance with dissipation. *Langmuir* 2004;20:1739-45.

[132] Thomas M, Ge Q, Lu JJ, Chen JZ, Klibanov AM. Cross-linked small polyethylenimines: While still nontoxic, deliver DNA efficiently to mammalian cells in vitro and in vivo. *Pharm Res* 2005;22:373-80.

- [133] Biran R, Martin DC, Tresco PA. Neuronal cell loss accompanies the brain tissue response to chronically implanted silicon microelectrode arrays. *Experimental Neurology* 2005;195:115-26.
- [134] Giulian D, Corpuz M, Chapman S, Mansouri M, Robertson C. Reactive Mononuclear Phagocytes Release Neurotoxins After Ischemic And Traumatic Injury To The Central-Nervous-System. *J Neurosci Res* 1993;36:681-93.
- [135] Buttgereit F, Wehling M, Burmester GR. A new hypothesis of modular glucocorticoid actions - Steroid treatment of rheumatic diseases revisited. *Arthritis and Rheumatism* 1998;41:761-7.
- [136] Trachootham D, Lu WQ, Ogasawara MA, Valle NRD, Huang P. Redox regulation of cell survival. *Antioxid Redox Signal* 2008;10:1343-74.
- [137] Sarsour EH, Kumar MG, Chaudhuri L, Kalen AL, Goswami PC. Redox Control of the Cell Cycle in Health and Disease. *Antioxid Redox Signal* 2009;11:2985-3011.
- [138] Xia T, Kovoichich M, Brant J, Hotze M, Sempf J, Oberley T, et al. Comparison of the abilities of ambient and manufactured nanoparticles to induce cellular toxicity according to an oxidative stress paradigm. *Nano Lett* 2006;6:1794-807.
- [139] Lee K, Lee H, Lee KW, Park TG. Optical imaging of intracellular reactive oxygen species for the assessment of the cytotoxicity of nanoparticles. *Biomaterials* 2011;32:2556-65.
- [140] Fu K, Pack DW, Klibanov AM, Langer R. Visual evidence of acidic environment within degrading poly(lactic-co-glycolic acid) (PLGA) microspheres. *Pharm Res* 2000;17:100-6.
- [141] Anderson JM, Shive MS. Biodegradation and biocompatibility of PLA and PLGA microspheres. *Adv Drug Delivery Rev* 1997;28:5-24.
- [142] Wong CHY, Crack PJ. Modulation of neuro-inflammation and vascular response by oxidative stress following cerebral ischemia-reperfusion injury. *Curr Med Chem* 2008;15:1-14.
- [143] Hald A, Lotharius J. Oxidative stress and inflammation in Parkinson's disease: Is there a causal link? *Exp Neurol* 2005;193:279-90.
- [144] Lin HY, Bumgardner JD. Changes in the surface oxide composition of Co-Cr-Mo implant alloy by macrophage cells and their released reactive chemical species. *Biomaterials* 2004;25:1233-8.
- [145] Krause TJ, Robertson FM, Greco RS. Measurement of intracellular hydrogen-peroxide induced by biomaterials implanted in a rodent air pouch. *Journal of Biomedical Materials Research* 1993;27:65-9.
- [146] Kikuzaki H, Hisamoto M, Hirose K, Akiyama K, Taniguchi H. Antioxidant properties of ferulic acid and its related compounds. *J Agric Food Chem* 2002;50:2161-8.
- [147] Aruoma OI, Grootveld M, Halliwell B. The Role of Iron in Ascorbate-Dependent Deoxyribose Degradation - Evidence Consistent With a Site-Specific Hydroxyl Radical Generation Caused by Iron Ions Bound to the Deoxyribose Molecule. *J Inorg Biochem* 1987;29:289-99.
- [148] Pinchuk I, Lichtenberg D. The mechanism of action of antioxidants against lipoprotein peroxidation, evaluation based on kinetic experiments. *Prog Lipid Res* 2002;41:279-314.

- [149] Halliwell B. Free-Radicals and Antioxidants - a Personal View. *Nutrition Reviews* 1994;52:253-65.
- [150] Graf E, Eaton JW. Antioxidant Functions of Phytic Acid. *Free Radical Biol Med* 1990;8:61-9.
- [151] Muraoka S, Miura T. Inhibition of xanthine oxidase by phytic acid and its antioxidative action. *Life Sci* 2004;74:1691-700.
- [152] Raboy V. myo-Inositol-1,2,3,4,5,6-hexakisphosphate. *Phytochemistry* 2003;64:1033-43.
- [153] Schubert MA, Wiggins MJ, DeFife KM, Hiltner A, Anderson JM. Vitamin E as an antioxidant for poly(etherurethane urea): In vivo studies. *J Biomed Mater Res* 1996;32:493-504.
- [154] Baragetti I, Furiani S, Vettoretti S, Raselli S, Maggi FM, Galli F, et al. Role of vitamin E-coated membrane in reducing advanced glycation end products in hemodialysis patients: A pilot study. *Blood Purif* 2006;24:369-76.
- [155] Traber MG, Schiano TD, Steephen AC, Kayden HJ, Shike M. Efficacy of water-soluble vitamin-e in the treatment of vitamin-e malabsorption in short-bowel syndrome. *Am J Clin Nutr* 1994;59:1270-4.
- [156] Traber MG, Kayden HJ, Green JB, Green MH. Absorption of water-miscible forms of vitamin-e in a patient with cholestasis and in thoracic duct-cannulated rats. *Am J Clin Nutr* 1986;44:914-23.
- [157] Vanhenegouwen G, Junginger HE, Devries H. Hydrolysis of RRR-alpha-tocopheryl acetate (vitamin E acetate) in the skin and its UV protecting activity (an in vivo study with the rat). *J Photochem Photobiol B* 1995;29:45-51.
- [158] Pan JQ, Liu NC, Lau WWY. Preparation and properties of new antioxidants with higher MW. *Polym Degrad Stab* 1998;62:165-70.
- [159] Iemma F, Trombino S, Puoci FR, Cirillo G, Spizzirri UG, Muzzalupo R, et al. Synthesis and antioxidant efficiency of a new copolymer containing phosphorylated myo-inositol. *Macromol Biosci* 2005;5:1049-56.
- [160] Williams SR, Lepene BS, Thatcher CD, Long TE. Synthesis and Characterization of Poly(ethylene glycol)-Glutathione Conjugate Self-Assembled Nanoparticles for Antioxidant Delivery. *Biomacromolecules* 2009;10:155-61.
- [161] Fleming C, Maldjian A, Da Costa D, Rullay AK, Haddleton DM, John JS, et al. A carbohydrate-antioxidant hybrid polymer reduces oxidative damage in spermatozoa and enhances fertility. *Nat Chem Biol* 2005;1:270-4.
- [162] Sanchez-Moreno C, Larrauri JA, Saura-Calixto F. A procedure to measure the antiradical efficiency of polyphenols. *Journal of the Science of Food and Agriculture* 1998;76:270-6.
- [163] Ortiz C, Vazquez B, Roman JS. Synthesis, characterization and properties of polyacrylic systems derived from vitamin E. *Polymer* 1998;39:4107-14.
- [164] Dubbs MD, Gupta RB. Solubility of vitamin E (alpha-tocopherol) and vitamin K-3 (menadione) in ethanol-water mixture. *J Chem Eng Data* 1998;43:590-1.
- [165] Kim B-S, Lee H-i, Min Y, Poon Z, Hammond PT. Hydrogen-bonded multilayer of pH-responsive polymeric micelles with tannic acid for surface drug delivery. *Chem Commun* 2009;4194-6.

- [166] Akagawa M, Suyama K. Amine oxidase-like activity of polyphenols - Mechanism and properties. *Eur J Biochem* 2001;268:1953-63.
- [167] Marx KA. Quartz crystal microbalance: A useful tool for studying thin polymer films and complex biomolecular systems at the solution-surface interface. *Biomacromolecules* 2003;4:1099-120.
- [168] Mark JE. Polymer data handbook. 2nd ed. New York: Springer; 1999.
- [169] Mazlan M, Then SM, Top GM, Ngah WZW. Comparative effects of alpha-tocopherol and gamma-tocotrienol against hydrogen peroxide induced apoptosis on primary-cultured astrocytes. *J Neurol Sci* 2006;243:5-12.
- [170] Finkel T, Holbrook NJ. Oxidants, oxidative stress and the biology of ageing. *Nature* 2000;408:239-47.
- [171] Halliwell B, Clement MV, Long LH. Hydrogen peroxide in the human body. *FEBS Lett* 2000;486:10-3.
- [172] Wattamwar PP, Mo YQ, Wan R, Palli R, Zhang QW, Dziubla TD. Antioxidant Activity of Degradable Polymer Poly(trolox ester) to Suppress Oxidative Stress Injury in the Cells. *Adv Funct Mater* 2010;20:147-54.
- [173] Ignatova M, Manolova N, Rashkov I, Sepulchre M, Spassky N. Water-soluble polymers bearing biologically-active residues .3. hydrolysis of polyethers and poly(ether-ester)s bearing 1-naphthylacetyl groups. *Macromol Chem Phys* 1995;196:1663-9.
- [174] Xu R, Go ML. Structure-hydrolyzability relationships in a series of piperidiny and tropinyl esters with antimuscarinic activity. *Chem Pharm Bull* 1997;45:476-81.
- [175] Makhaeva EE, Tenhu H, Khokhlov AR. Behavior of poly(N-vinylcaprolactam-co-methacrylic acid) macromolecules in aqueous solution: Interplay between Coulombic and hydrophobic interaction. *Macromolecules* 2002;35:1870-6.
- [176] Qiu XP, Sukhishvili SA. Copolymerization of N-vinylcaprolactam and glycidyl methacrylate: Reactivity ratio and composition control. *Journal of Polymer Science Part a-Polymer Chemistry* 2006;44:183-91.
- [177] Aoyagi T, Ebara M, Sakai K, Sakurai Y, Okano T. Novel bifunctional polymer with reactivity and temperature sensitivity. *J Biomater Sci-Polym Ed* 2000;11:101-10.
- [178] Faragalla MM, Hill DJT, Whittaker AK. The copolymerization of N-vinyl-2-pyrrolidone with 2-hydroxyethyl methacrylate. *Polym Bull* 2002;47:421-7.
- [179] Kim JB, Jung MH, Chang KH. Synthesis and polymerization of 3-(t-butoxycarbonyl)-1-vinylcaprolactam and application as deep UV resists. *Polym Bull* 1997;38:241-7.
- [180] Kim ST, Kim JB, Jung MH, Ahn KD. Copolymers of camphorsulfonyloxymaleimide and t-BOC protected vinyl lactams for applications as single-component resists. *Polym Bull* 1997;39:423-30.
- [181] Kim J-B, Jung M-H, Cheong J-H, Kim J-Y, Bok C-K, Koh C-W, et al. Chemically amplified resists containing vinyl lactam derivatives. *Journal of Photopolymer Science and Technology* 1997;10:493-502.
- [182] Kim J-B, Choi J-H, Kim H, Kwon Y-G, Jung M-H. Poly(isobornyl methacrylate-co-3-(tert-butoxycarbonyl)-1-vinyl-2-caprolactam) for an environmentally stable chemically amplified resist. *Polym J* 1999;31:695-9.

- [183] Kim J-B, Kwon Y-G, Choi J-H, Jung M-H. Control of photogenerated acid diffusion and evaporation by copolymerization with a basic monomer. *Journal of Vacuum Science & Technology, B: Microelectronics and Nanometer Structures* 1999;17:2097-102.
- [184] Kim J-B, Choi J-H, Kwon Y-G, Jung M-H, Chang K-H. Acid diffusion control in chemically amplified resists. *Polymer* 1999;40:1087-9.
- [185] Kim J-B, Kwon Y-G, Yun H-J, Jung M-H. Post-exposure delay effect in chemically amplified resists. *Journal of Photopolymer Science and Technology* 2001;14:401-6.
- [186] Okhapkin IM, Nasimova IR, Makhaeva EE, Khokhlov AR. Effect of complexation of monomer units on pH- and temperature-sensitive properties of poly(N-vinylcaprolactam-co-methacrylic acid). *Macromolecules* 2003;36:8130-8.
- [187] Kirsh YE. *Water soluble poly-N-vinylamides: synthesis and physiochemical properties*. Chichester: Wiley; 1998.
- [188] Pakuro N, Yakimansky A, Chibirova F, Arest-Yakubovich A. Thermo- and pH-sensitivity of aqueous poly(N-vinylpyrrolidone) solutions in the presence of organic acids. *Polymer* 2009;50:148-53.
- [189] Vihola H, Laukkanen A, Valtola L, Tenhu H, Hirvonen J. Cytotoxicity of thermosensitive polymers poly(N-isopropylacrylamide), poly(N-vinylcaprolactam) and amphiphilically modified poly(N-vinylcaprolactam). *Biomaterials* 2005;26:3055-64.
- [190] Frunder H. *Pharmazie* 1949;4:345-55.
- [191] Kelen T, Tudos F. Analysis of linear methods for determining copolymerization reactivity ratios .1. New improved linear graphic method. *Journal of Macromolecular Science-Chemistry* 1975;A 9:1-27.

APPENDIX

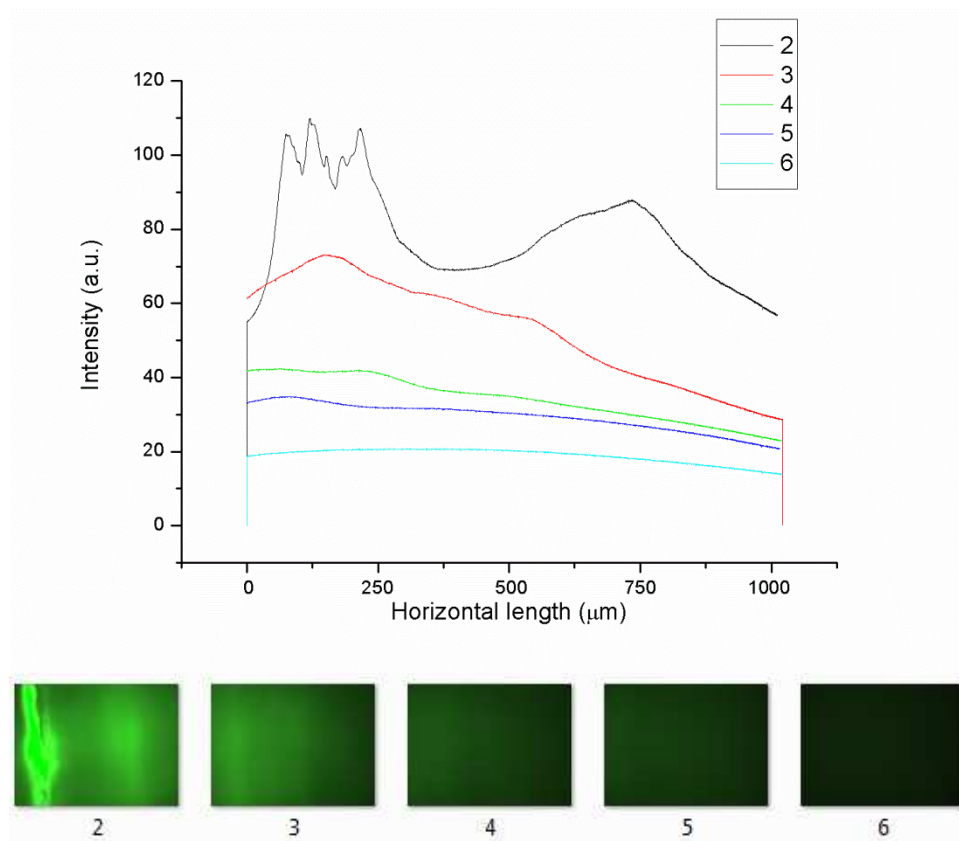


Figure 50. Fluorescent images and intensity in the diffused region of the fluorescent produg analog.

Table 4. Calculation for the reactivity of VCL and VP.

VCLVP0.5			pyrindine	VP	VCL				
	Mw		79	111	139				
	feeding mass (mg)		7.98						
	feeding amount (mmol)		0.101012658						
		integration of VP	integration of VCL	VP amount (mmol)	VCL amount (mmol)	total monomer (mmol)	d(VP) (mmol)	d(VCL) (mmol)	conversion
	before polymerization	367	595	0.370716456	0.601025316	0.971741772			
	after polymerization	325	570	0.328291139	0.575772152	0.904063291	0.0424	0.02525	0.06964657
VCLVP1			pyrindine	VP	VCL				
	Mw		79	111	139				
	feeding mass (mg)		8.16						
	feeding amount (mmol)		0.103291139						
		integration of VP	integration of VCL	VP amount (mmol)	VCL amount (mmol)	total monomer (mmol)	d(VP) (mmol)	d(VCL) (mmol)	conversion
	before polymerization	613	658	0.633174684	0.679655696	1.31283038			
	after polymerization	541	632	0.558805063	0.6528	1.211605063	0.0744	0.02686	0.077104642
VCLVP2			pyrindine	VP	VCL				
	Mw		79	111	139				
	feeding mass (mg)		7.8						
	feeding amount (mmol)		0.098734177						
		integration of VP	integration of VCL	VP amount (mmol)	VCL amount (mmol)	total monomer (mmol)	d(VP) (mmol)	d(VCL) (mmol)	conversion
	before polymerization	1028	548	1.061832911	0.566035443	1.627868354			
	after polymerization	921	532	0.951311392	0.549508861	1.500820253	0.1105	0.01653	0.078045685

	molar amount											
molar ratio	VP	VCL	d(VP)	d(VCL)	X	Y	A	F	α	$\alpha+F$	ξ	η
0.5	0.37072	0.60103	0.04243	0.02525	0.61681	1.68	0.24966	0.22646	1.52436	1.75081	0.12934	0.1426
1	0.63317	0.67966	0.07437	0.02686	0.93161	2.76923	0.5952	0.31341		1.83776	0.17054	0.32387
2	1.06183	0.56604	0.11052	0.01653	1.87591	6.6875	1.5954	0.52621		2.05057	0.25662	0.77803

Table 5. Calculation for the reactivity of TBVCL and VP.

TBVVP0.5			pyrindine	VP	TBVCL							
	Mw		79	111	239							
	feeding mass (mg)		5.09									
	feeding amount (mmol)		0.06443038									
		integration of VP	integration of TBVCL	VP amount (mmol)	TBVCL amount (mmol)	total monomer (mmol)	d(VP) (mmol)	d(TBVCL) (mmol)	conversion			
	before polymerization	250	525	0.161075949	0.338259494	0.499335443						
	after polymerization	229	437	0.14754557	0.281560759	0.429106329	0.0135	0.056698734	0.140645161			
TBVVP1			pyrindine	VP	TBVCL							
	Mw		79	111	239							
	feeding mass (mg)		4.94									
	feeding amount (mmol)		0.062531646									
		integration of VP	integration of TBVCL	VP amount (mmol)	TBVCL amount (mmol)	total monomer (mmol)	d(VP) (mmol)	d(TBVCL) (mmol)	conversion			
	before polymerization	572	545	0.357681013	0.340797468	0.698478481						
	after polymerization	535	483	0.334544304	0.302027848	0.636572152	0.0231	0.03876962	0.08863026			
TBVVP2			pyrindine	VP	TBVCL							
	Mw		79	111	239							
	feeding mass (mg)		4.97									
	feeding amount (mmol)		0.062911392									
		integration of VP	integration of TBVCL	VP amount (mmol)	TBVCL amount (mmol)	total monomer (mmol)	d(VP) (mmol)	d(TBVCL) (mmol)	conversion			
	before polymerization	1173	528	0.737950633	0.332172152	1.070122785						
	after polymerization	1109	476	0.697687342	0.299458228	0.99714557	0.0403	0.032713924	0.068195179			
	molar amount (mmol)											
molar ratio	VP	TBVCL	d(VP)	d(TBVCL)	X	Y	A	F	α	α +F	ξ	η
0.5	0.161076	0.338259	0.01353	0.056699	0.47619	0.238636	-1.51927	0.950221	0.486784	1.437006	0.661251	-1.05725
1	0.357681	0.340797	0.023137	0.03877	1.049541	0.596774	-0.70915	1.845819		2.332603	0.791313	-0.30402
2	0.737951	0.332172	0.040263	0.032714	2.221591	1.230769	0.416548	4.010066		4.496851	0.89175	0.092631

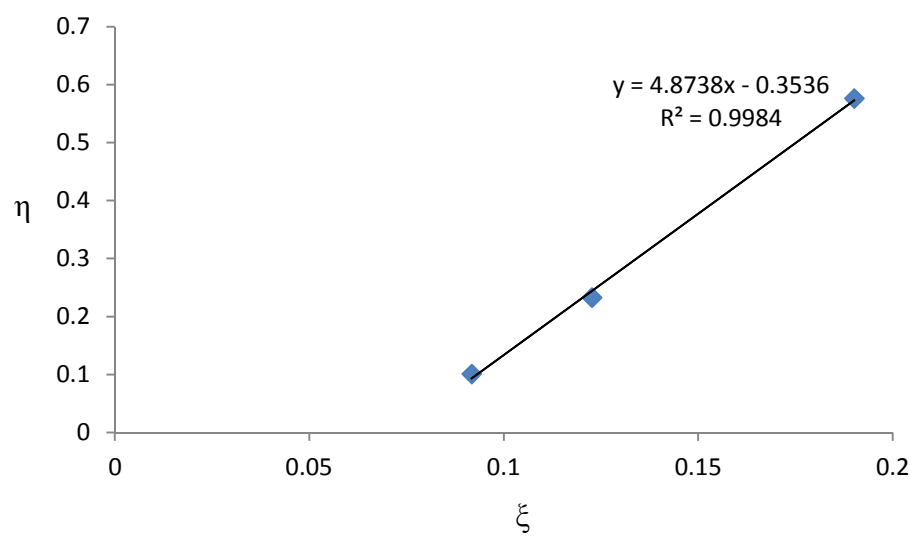


Figure 51. K-T plot for the reactivity study of VCL and VP.

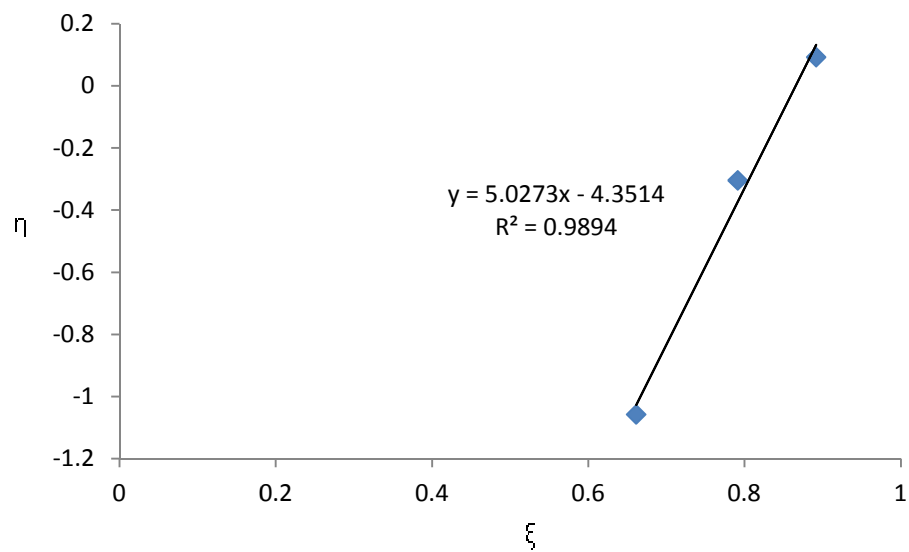


Figure 52. K-T plot for the reactivity study of TBVCL and VP.

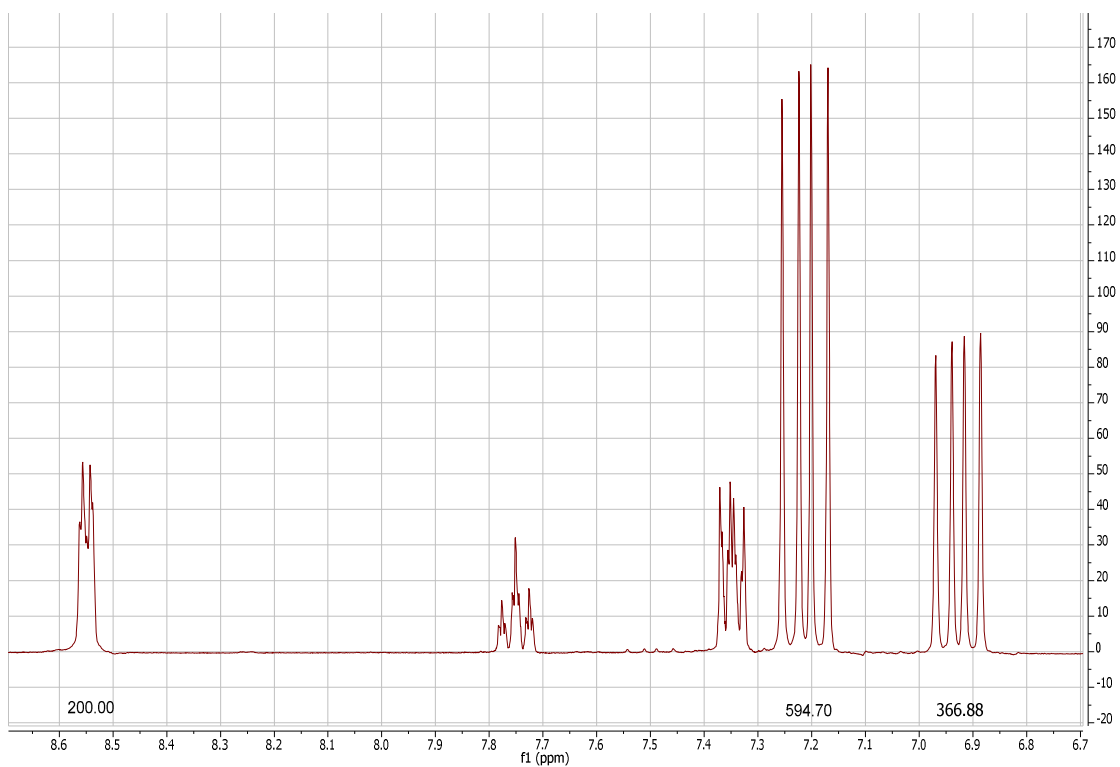


Figure 53. NMR spectra of VCL and VP at a molar feeding ratio of 0.5, before polymerization.

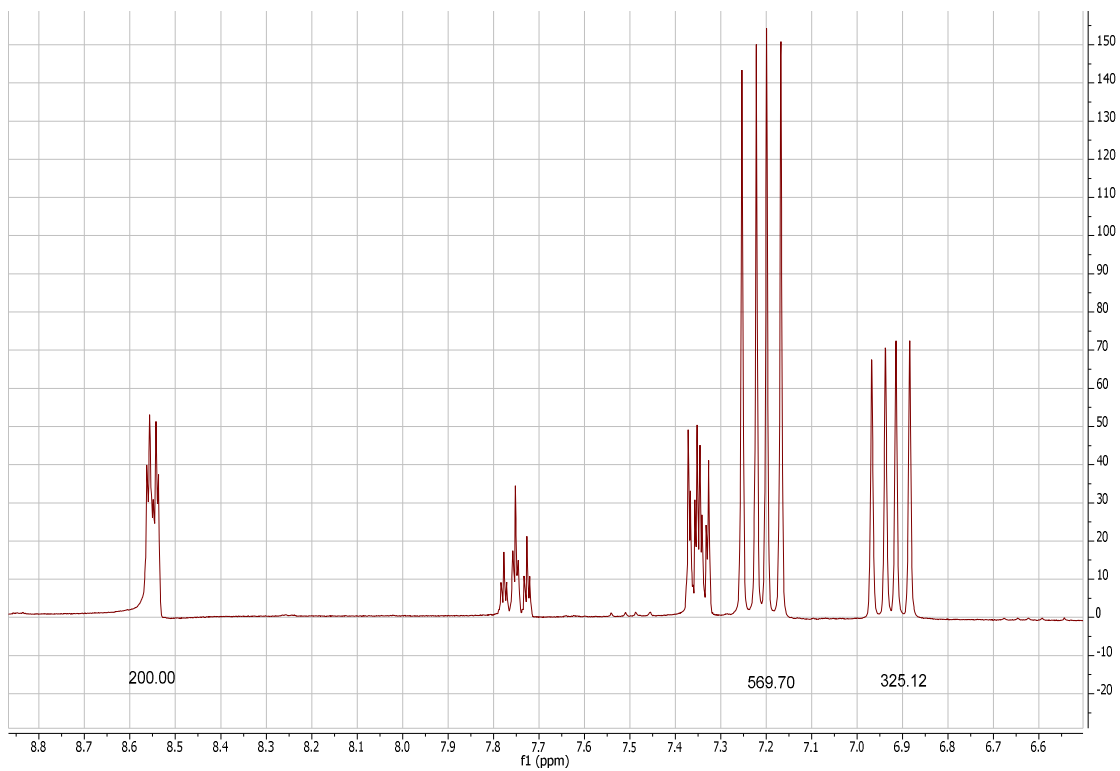


Figure 54. NMR spectra of VCL and VP at molar feeding ratio of 0.5, after polymerization at 65 °C for 20 minutes.

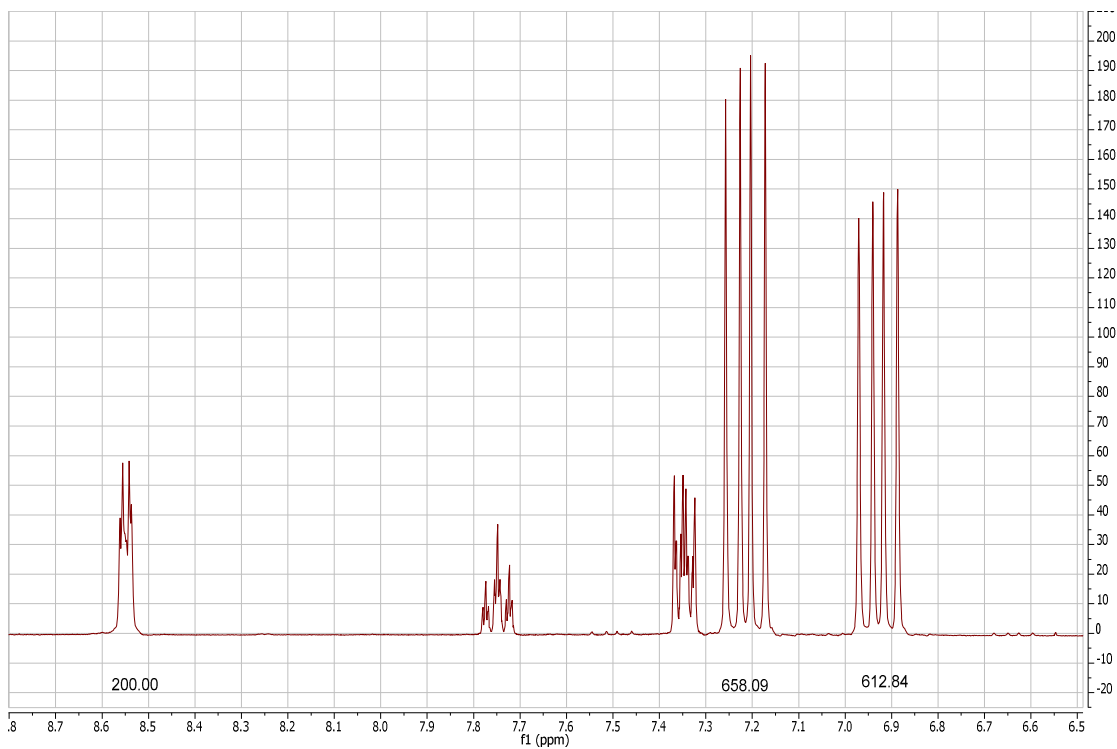


Figure 55. NMR spectra of VCL and VP at molar feeding ratio of 1, before polymerization

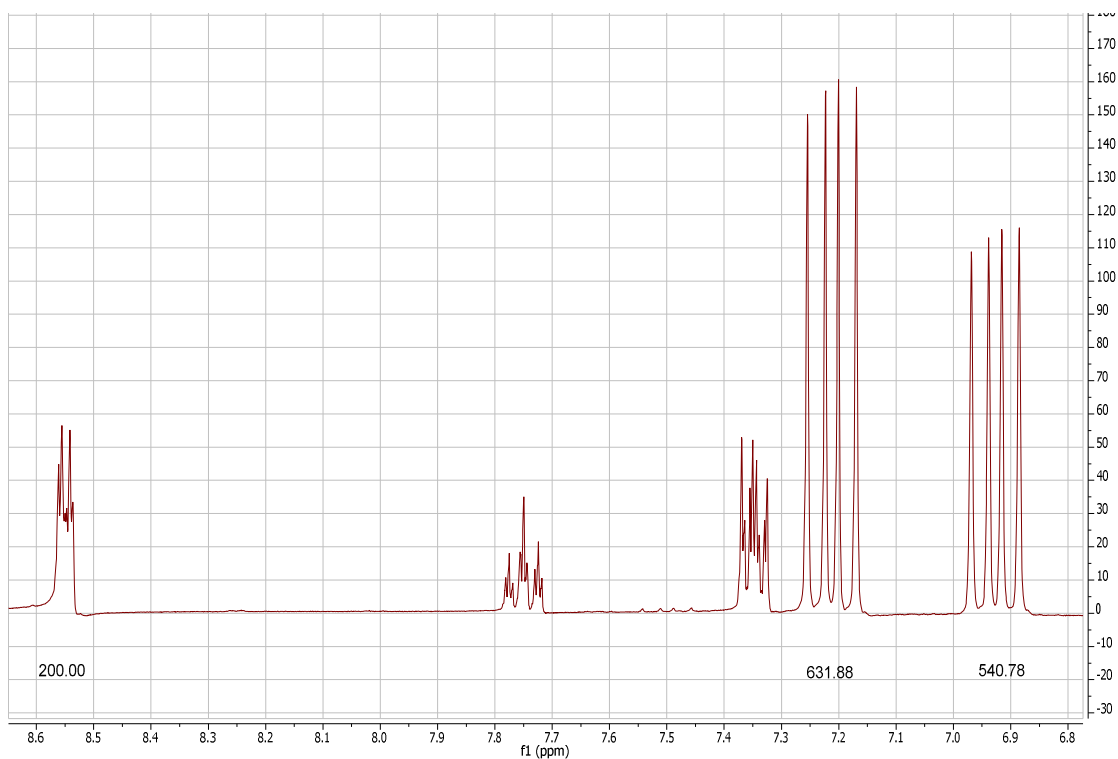


Figure 56. NMR spectra of VCL and VP at molar feeding ratio of 1, after polymerization at 65 °C for 20 minutes.

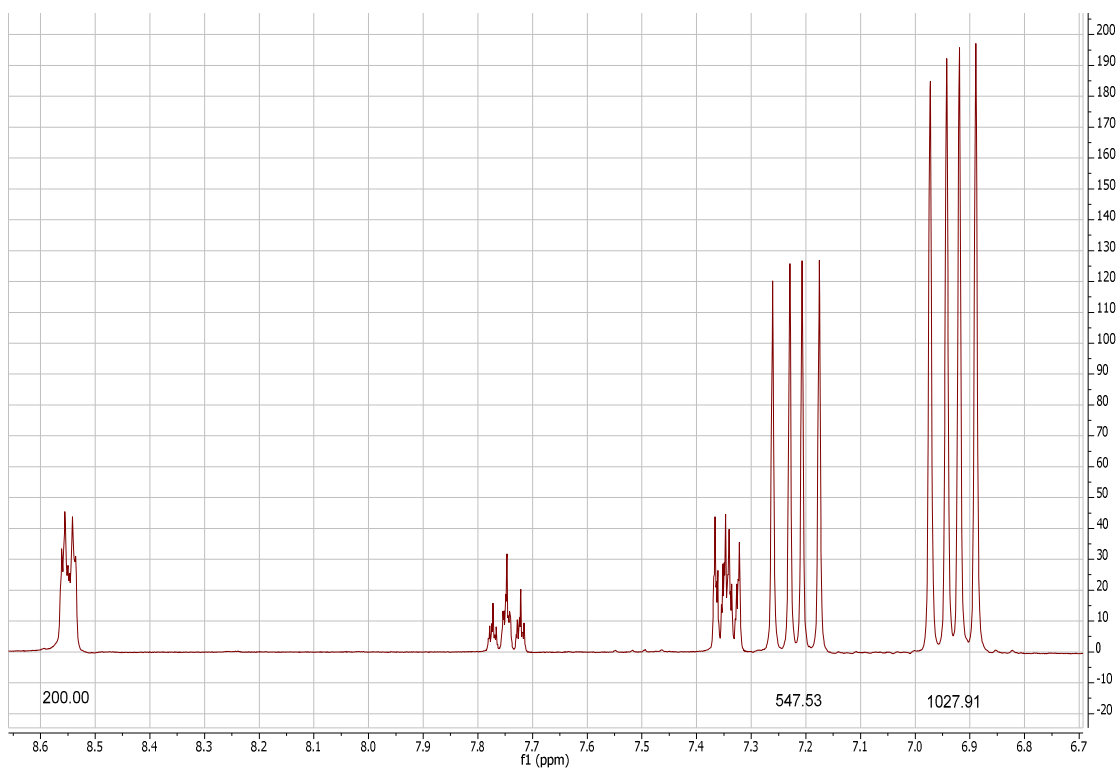


Figure 57. NMR spectra of VCL and VP at molar feeding ratio of 2, before polymerization

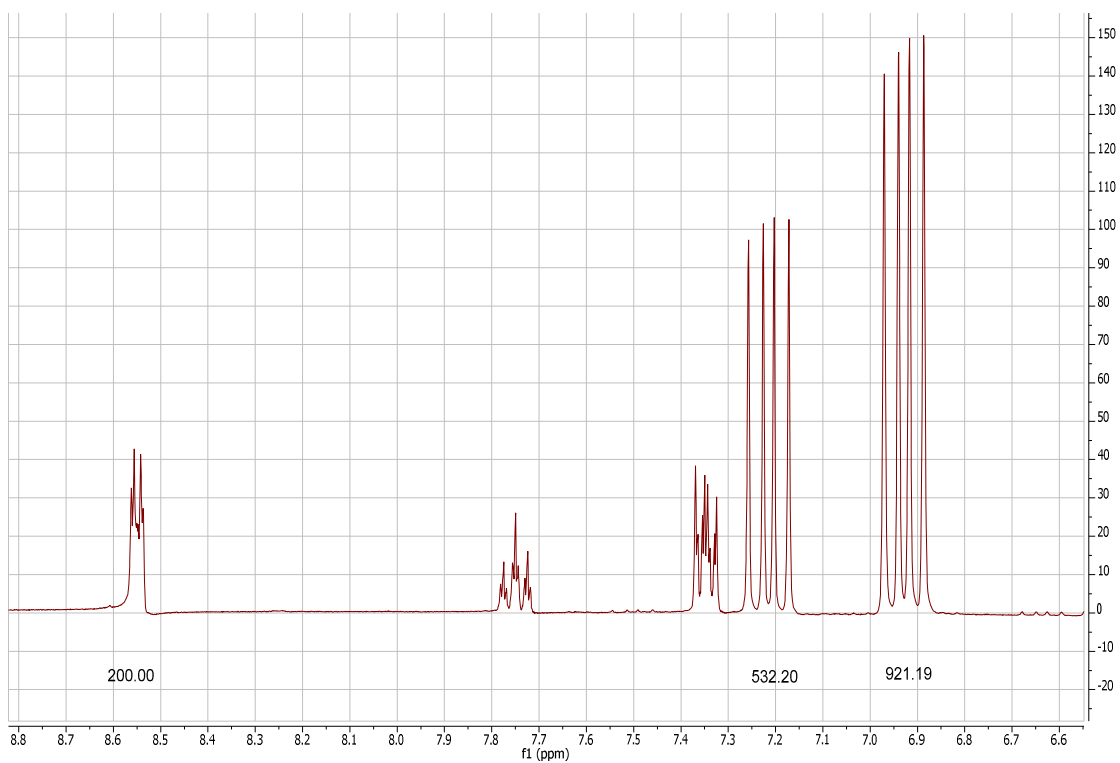


Figure 58. NMR spectra of VCL and VP at molar feeding ratio of 2, after polymerization at 65 °C for 20 minutes.

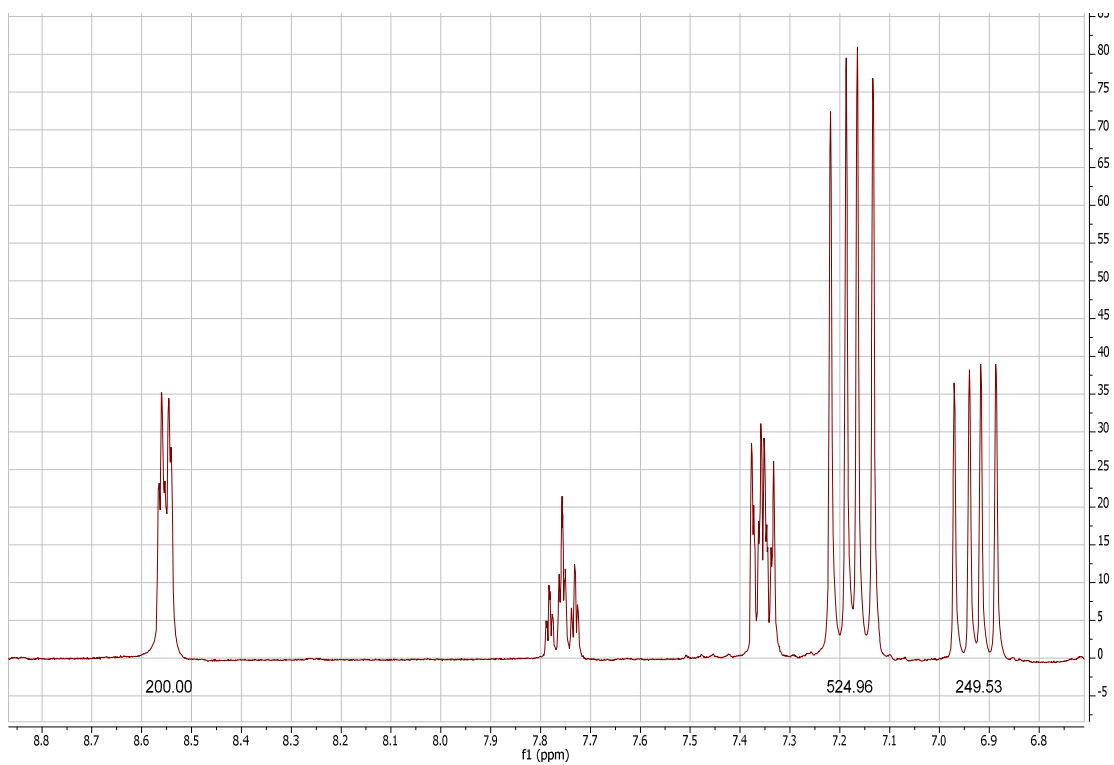


Figure 59. NMR spectra of TBVCL and VP at molar feeding ratio of 0.5, before polymerization.

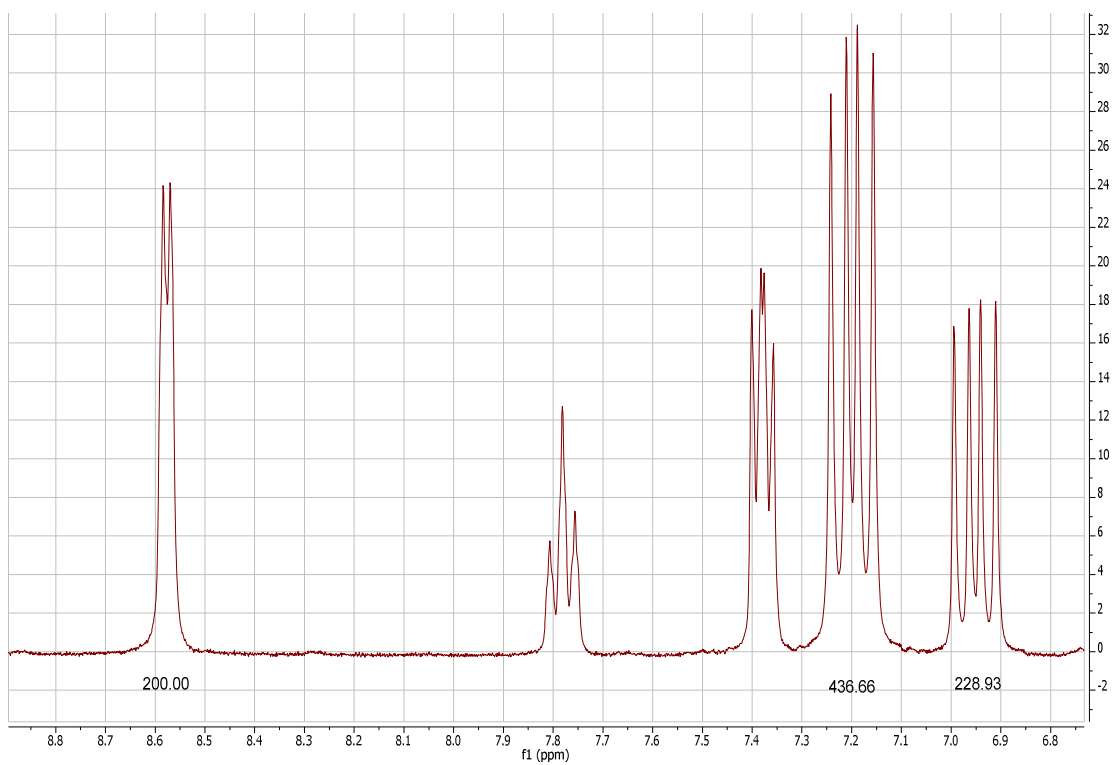


Figure 60. NMR spectra of TBVCL and VP at molar feeding ratio of 0.5, after polymerization at 65 °C for 20 minutes.

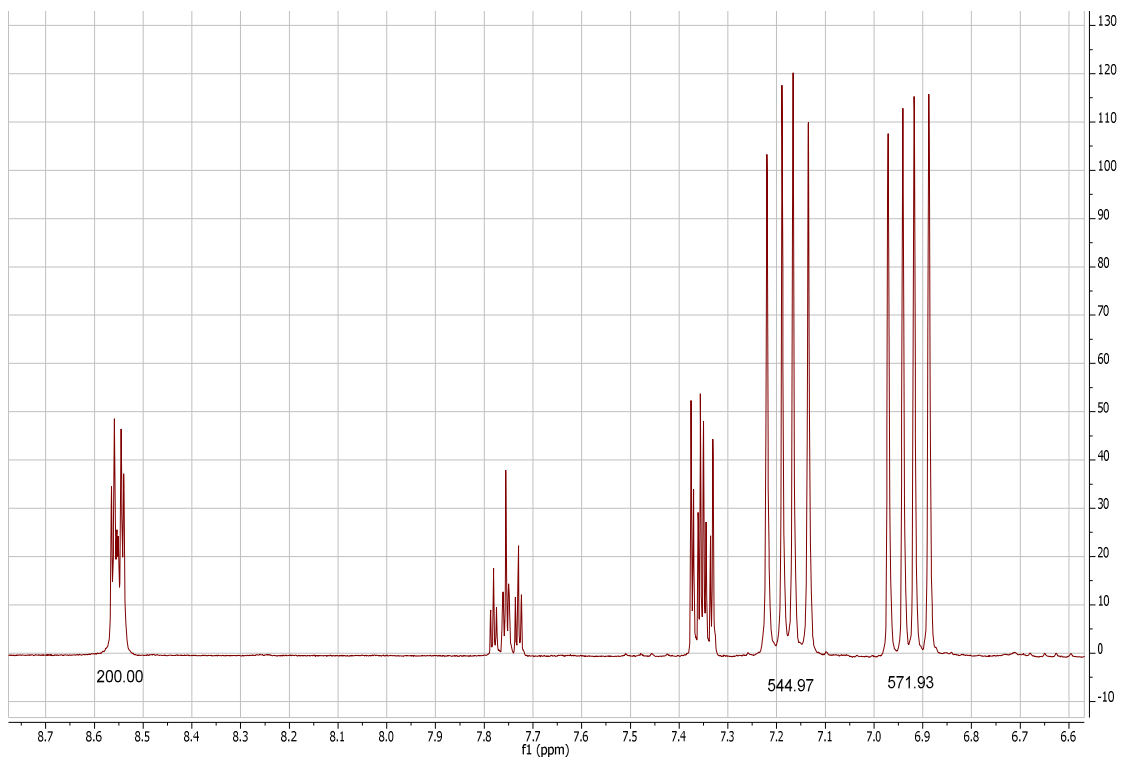


Figure 61. NMR spectra of TBVCL and VP at molar feeding ratio of 1, before polymerization.

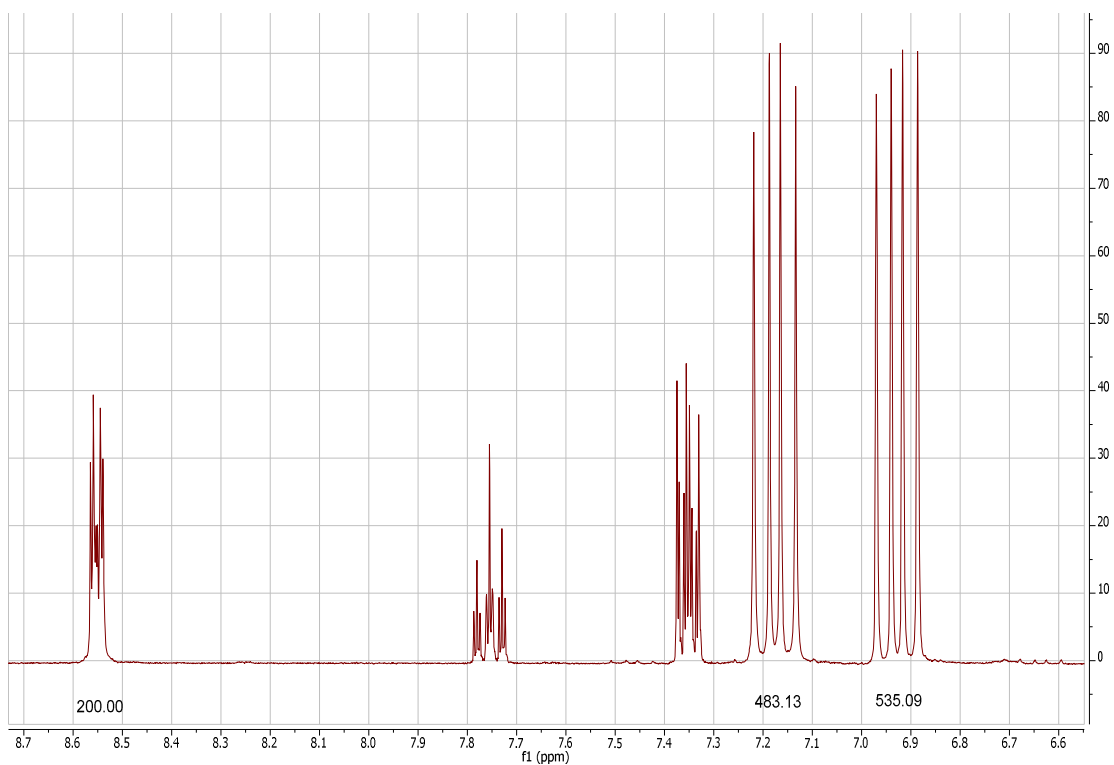


Figure 62. NMR spectra of TBVCL and VP at molar feeding ratio of 1, after polymerization at 65 °C for 20 minutes.

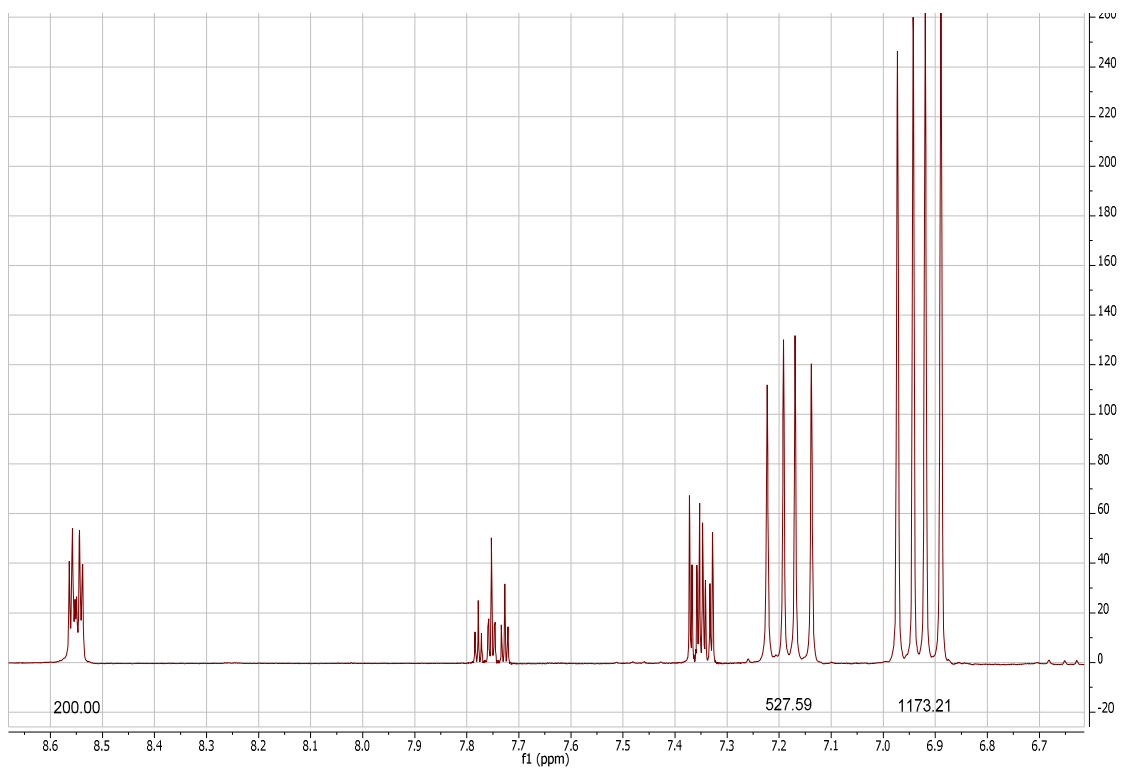


Figure 63. NMR spectra of TBVCL and VP at molar feeding ratio of 2, before polymerization

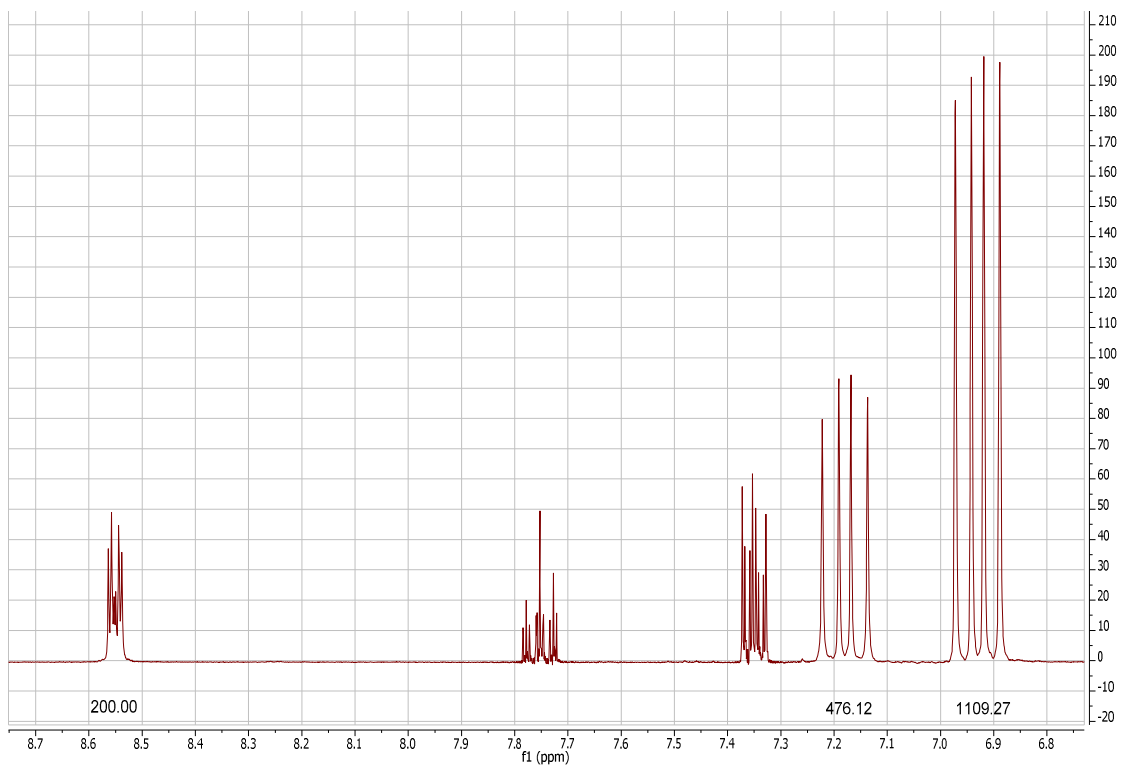


Figure 64. NMR spectra of TBVCL and VP at molar feeding ratio of 2, after polymerization at 65 °C for 20 minutes.

VITA

Yu Cao was born and raised in Zhongxiang, HuBei, China. He obtained his Bachelor's degree in 2005, majoring in Materials Science and Engineering, from Tongji University, Shanghai, China. Then he continued his graduate study in Tsinghua University, Beijing, China and received his Master's degree in 2008. In August of 2008, he enrolled in the doctoral program of Polymer Engineering in the University of Tennessee, Knoxville.