Synthesis, Chemical Characterization and Biological Assessment of Amphiphilic Oligourethanes for Drug Delivery Coatings

by

Zachariah Grodzinski

A thesis submitted in conformity with the requirements for the degree of Masters of Applied Science
Institute of Biomaterials and Biomedical Engineering
University of Toronto

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Abstract

Medical devices may require drug delivery coatings that effectively transfer drug to a diseased site without causing undesired responses from the surrounding cells. This thesis focuses on the synthesis of Amphiphilic Oligourethanes (AOU) – consisting of polyethylene glycol, lysine and fluorinated segments. The molecules have been conceived as a platform on which to enable the generation of carriers that may bind a range of drug chemistries. Three families of AOUs were synthesised – anionic, non ionic hydrophilic, and non-ionic hydrophobic – in order to study their effect on smooth muscle cells and drug binding. Cell toxicity concentrations were defined for each oligourethane and were shown to be structure dependent, and possibly influenced by their surfactant character. The order of least toxic was anionic > non-ionic hydrophilic > non-ionic hydrophobic. Two titration techniques (Isothermal Calorimetry and Nuclear Magnetic Resonance) were investigated as methods to study intermolecular binding properties with a model drug, C6-Ceramide.
Dedication

On the evening of December 15th 2015, Dr. Mark Ernsting was murdered while enjoying an evening walk near his home in Toronto.

Mark received his PhD from Dr. Santerre’s Lab, and though I never had the chance to meet him, it is clear from the pages of this thesis that he laid the groundwork for many of the ideas worked on during my project.

Mark was a talented and passionate researcher who was not given the opportunity to complete his life’s goals.

This thesis is therefore presented in memory of Dr. Mark Ernsting to serve as a small testament to and continuation of his life’s work.
Acknowledgements

“In the name of God, the Lord of the World” (Genesis 21:33)

First and foremost I would like to thank my supervisor, Dr. Paul Santerre, for his constant guidance and support throughout my thesis. His strong technical background coupled with his unique ambition and imagination led to the formation of this interesting project and greatly helped me overcome its difficult obstacles along the way. I would also like to thank my committee members Dr. Eli Sone and Dr. Julie Audet for their support throughout the thesis.

Working in the Santerre Lab has really been an enjoyable experience. The strength of the lab however, is only as strong as its members. I’d therefore like to thank each one of them for all their time spent answering my questions and lightening the mood when things were not so light! In alphabetical order, thank you to; Dr. Kyle Battiston (all cell-related matters), Kate Brockman, Yasaman Delaviz, Dr. Soroor Sharifpoor, Ken Shiguetomi, Meghan Wright, Dr. Meilin Yang and Xiaoqing Zhang. I’d also like to thank Jennifer Logie (Shoichet Lab) for her help with the carbodiimide chemistry.

A special mention must be made to Dr. Maria Lopez-Donaire for all the time and support she gave to this project. From organic synthesis to biologic assays – and not to mention staying in the lab way into the night fixing broken equipment – this project would be incomplete without her.

I’d like to thank all the staff at the NMR Facility for all their help throughout this project. A special thank you goes to Dr. Sergiy Nokhrin for all his help with the NMR titrations and always managing to fit me in on the 700!

Nearing the end, I’d like to thank my parents for all the time and effort they have put into me allowing me to reach this point.

However – “the last is most cherished” (Rashi to Genesis 33:2) – I want to thank Leora [and Leeah] in the most simple, yet most complete way; Thank you for everything.
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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AOU</td>
<td>Amphiphilic Oligourethane</td>
</tr>
<tr>
<td>AOU6</td>
<td>Amphiphilic Oligourethane (coupled with 6 carbon alkyl chain)</td>
</tr>
<tr>
<td>AOU8</td>
<td>Amphiphilic Oligourethane (coupled with 8 carbon alkyl chain)</td>
</tr>
<tr>
<td>AOU12</td>
<td>Amphiphilic Oligourethane (coupled with 12 carbon alkyl chain)</td>
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<td>AOUH</td>
<td>Amphiphilic Oligourethane (with hydrolyzed methyl ester)</td>
</tr>
<tr>
<td>BMS</td>
<td>Bare Metal Stent</td>
</tr>
<tr>
<td>C-1-P</td>
<td>Ceramide-1-Phosphate</td>
</tr>
<tr>
<td>CMC</td>
<td>Critical Micelle Concentration</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic Obstructive Pulmonary Disease</td>
</tr>
<tr>
<td>COSY</td>
<td>Homonuclear Correlation Spectroscopy</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular Diseases</td>
</tr>
<tr>
<td>DBA</td>
<td>Dibutylamine</td>
</tr>
<tr>
<td>DBTDL</td>
<td>Dibutyltin dilaurate</td>
</tr>
<tr>
<td>DCB</td>
<td>Drug Coated Balloon</td>
</tr>
<tr>
<td>DCC</td>
<td>Dicyclohexyl Carbodiimide</td>
</tr>
<tr>
<td>DES</td>
<td>Drug Eluting Stent</td>
</tr>
<tr>
<td>DIC</td>
<td>Diisopropyl Carbodiimide</td>
</tr>
<tr>
<td>DMAc</td>
<td>Dimethylacetamide</td>
</tr>
<tr>
<td>DMAP</td>
<td>Dimethylaminopyridine</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DMEM-SP</td>
<td>Dulbecco’s Modified Eagle Medium with no Sodium Pyruvate</td>
</tr>
<tr>
<td>DMP</td>
<td>Dimorpholinophosphate</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>DOSY</td>
<td>Diffusion Ordered Spectroscopy</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial Cell</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>EDC</td>
<td>1-ethyl-3-(3 dimthylaminopropyl) carbodiimide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EPC</td>
<td>Endothelial Progenitor Cells</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FKBP</td>
<td>FK Binding Protein</td>
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<tr>
<td>GI</td>
<td>Gastrointestinal</td>
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<tr>
<td>GPC</td>
<td>Gel Permeation Chromatography</td>
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<td>HNMR</td>
<td>Proton Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>HOBt</td>
<td>Hydroxybenzotriazole</td>
</tr>
<tr>
<td>HSQC</td>
<td>Heteronuclear Single Quantum Correlation Spectroscopy</td>
</tr>
<tr>
<td>ICP-AES</td>
<td>Inductively Coupled Plasma Atomic Emission Spectroscopy</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>INF</td>
<td>Interferon</td>
</tr>
<tr>
<td>IPA</td>
<td>Isopropyl Alcohol</td>
</tr>
<tr>
<td>ISR</td>
<td>In-Stent Restenosis</td>
</tr>
<tr>
<td>ITC</td>
<td>Isothermal Titration Calorimetry</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate Dehydrogenase</td>
</tr>
<tr>
<td>LDI</td>
<td>Lysine Diisocyanate</td>
</tr>
<tr>
<td>MACE</td>
<td>Major Adverse Cardiac Event</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix Assisted Laser Desorption/Ionization Mass Spectrometry</td>
</tr>
<tr>
<td>MPEG</td>
<td>Methyoxy-Polyethylene-Glycol</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectroscopy</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>MTOR</td>
<td>Mammalian Target of Rapamycin</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular Weight</td>
</tr>
<tr>
<td>NCO</td>
<td>Isocyanate</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxysulfosuccinimide</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>NOE</td>
<td>Nuclear Overhauser Effect</td>
</tr>
<tr>
<td>oXLDL</td>
<td>Oxidized Low-Density Lipoproteins</td>
</tr>
<tr>
<td>PBMA</td>
<td>Poly n-Butyl Methacrylate</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCI</td>
<td>Percutaneous Cardiovascular Intervention</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet Derived Growth Factor</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene-glycol</td>
</tr>
<tr>
<td>PEVA</td>
<td>Polyethylene-co-vinyl Acetate</td>
</tr>
<tr>
<td>PFA</td>
<td>Perfluoro alcohol</td>
</tr>
<tr>
<td>PFG</td>
<td>Pulsed Field Gradient</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein Kinase B</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly(lactic-co-glycolide)</td>
</tr>
<tr>
<td>PTFE</td>
<td>Polytetrafluoroethylene</td>
</tr>
<tr>
<td>QRT PCR</td>
<td>Quantitative Real Time Polymerase Chain Reaction Analysis</td>
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<tr>
<td>SDF-1</td>
<td>Stromal Derived Factor-1</td>
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<tr>
<td>SIBS</td>
<td>Poly-Styrene-block-Isobutylene-block-Styrene</td>
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<tr>
<td>SMC</td>
<td>Smooth Muscle Cell</td>
</tr>
<tr>
<td>SMM</td>
<td>Surface Modifying Macromolecule</td>
</tr>
<tr>
<td>STD</td>
<td>Saturation Transfer Difference</td>
</tr>
<tr>
<td>TCB</td>
<td>Trichlorobenzene</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>TF</td>
<td>Tissue Factor</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic anhydride</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming Growth Factor</td>
</tr>
<tr>
<td>TMS</td>
<td>Tetramethylsilane</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour Necrosis Factor</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>VSMC</td>
<td>Vascular Smooth Muscle Cell</td>
</tr>
<tr>
<td>WST</td>
<td>Water Soluble Tetrazolium</td>
</tr>
</tbody>
</table>
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Figure 3.14  a) NCO titration, b) NMR and c) MALDI data for reactions performed using synthesis method 3. A relatively pure AOU is formed, with a slight bit of PEG-trimer still remaining. Large peak h represents all MPEG CH2 protons, while j represents the MPEG CH2 alpha to the urethane bond.

Figure 3.15  NMR spectra of AOU using the final stoichiometric method. * indicates solvent related impurity

Figure 3.16  MALDI spectra showing (from left to right), excess MPEG and pure AOU.

Figure 3.17  Section of NMR spectra before and after TFA addition, showing the formation of the proton signal from the MPEG CH2 alpha to the reacted TFA.

Chapter 4

Figure 4.1  1HNMR spectra of AOU. Integration with respect to peak a. Large peak h represents all MPEG CH2 protons, while j represents the MPEG CH2 alpha to the urethane bond. * indicates solvent related impurity.
Figure 4.2  $^1$HNMR spectra showing disappearance of CH$_2$ peak (i) on methyl ester after hydrolysis (*).

Figure 4.3 Top: NMR spectra characteristic of all AOU6,8,12. Bottom: Integrations of all relevant peaks. Peak u indicates complete successful coupling while peak $\alpha$ changes based on the number of repeated CH$_2$ units on the coupled molecule. Large peak h represent all MPEG CH$_2$ protons, while j represents the MPEG CH$_2$ alpha to the urethane bond. * indicates solvent related impurity, shifted ~1ppm from that in Figure 4.1, due to varied interactions in different systems.

Figure 4.4 WST-1 results for all materials. GM = growth media control. N = 9-12 for all samples (3 or 4 experiments performed in triplicates). Error bars represent standard error. * = statistically different that GM, # = statistically different that 0.1 mg/ml, ** = statistically different than next lowest concentration (indicating a dose response where applicable).

Figure 4.5 Hoechst DNA Results for all materials. GM = growth media control. N = 9-12 for all samples (3 or 4 experiments performed in triplicates). Error bars represent standard error. * = statistically different that GM, # = statistically different that 0.1 mg/ml, ** = statistically different than next lowest concentration (indicating a dose response where applicable).

Figure 4.6 LDH results for all samples. GM = growth media control. N = 9-12 for all samples (3 or 4 experiments performed in triplicates). Error bars represent standard error.
* = statistically different that GM, # = statistically different that 0.1 mg/ml, ** = statistically different than next lowest concentration (indicating a dose response where applicable).

Chapter 5

Figure 5.1 $^1$HNMR spectra of C6-Ceramide. Labeled peaks correspond to the peaks referred to in Table 5.1
Figure 5.2  $^1$HNMR spectra of interactions between AOU and C6-Ceramide in a) chloroform and b) ethanol. Notice the spectral shift over the varying concentrations in chloroform, and lack thereof in ethanol.

Figure 5.3  a) Isolated peak m from $^1$HNMR titrations with AOU and C6 Ceramide. b) peak m position data plotted against AOU concentration.

Figure 5.4  AOU structure with letters corresponding to NMR spectra. n=4 (AOU6), 6 (AOU8), 10 (AOU12).

Appendix A

Figure A.1  Titration data representing NCO conversion during synthesis method #1 reaction. After 1 hour, only 20% is reached as opposed to the expected 25%. Error bars represent standard deviation, n=3, except for time = 2 hours represents n=2.

Figure A.2  a) Molecular weight spectrum of raw MPEG, b) molecular structure of MPEG, and c) table describing calculation of MPEG molecular weight.

Figure A.3  a) MS and b) MALDI spectra of raw MPEG. N is normalized peak intensity, Mi is the molecular weight of each peak portion, and Mn is the number average molecular weight.

Figure A.4  Graph showing expected NCO conversion values based on different assumptions of MPEG molecular weight values. While 25% is shown if a MW of 618 is used, only 20% is expected if the MW is closer to 750.

Appendix B

Figure B.1  NCO titration data from large batch prepolymer reactions using 2 different sources of LDI, a) China and b) Germany. Approximately 50% NCO conversion is reached at t=50 mins.
Appendix C

Figure C.1 $^1$HNMR spectra of product after attempted direct coupling of K$_2$CO$_3$. a) Structure of hexylamine coupled to AOU, b) Structure of hexylamine.

Figure C.2 $^1$HNMR spectra of reaction after attempted coupling with EDC. Multiple unknown peaks are present.

Figure C.3 COSY NMR spectra of coupling reaction using EDC with no hexylamine.

Figure C.4 $^1$HNMR spectra from hexylamine coupling reaction using EDC-beads.

Figure C.5 HNMR spectra of hexylamine coupled to AOUH using DIC. * indicates residual water.

Appendix D

Figure D.1 Plot of absorbance vs. dilution factor in order to determine the optimal concentration for the LDH assay reagents whereby the sample falls within the linear portion and is significantly different from the growth media control.
Chapter 1
Introduction

1.1 Clinical Motivation

Cardiovascular disease (CVD) is the cause of approximately one third of Canadian and American mortalities each year (1, 2). It also places the largest economic burden of all diagnostic groups on the American health care system, costing approximately $315 billion per year, as compared to all cancer treatments for example, which expend approximately $200 billion (1). While CVD covers a wide range of diseases from high blood pressure to arrhythmia, the most common cause of CVD relates to diseased arteries. Arterial disease is often caused by atherosclerosis, the narrowing or weakening of a blood vessel due to accumulation of fat and cholesterol within the blood (1, 3). While mostly affecting the coronary artery (48% of all CVD) (1), atherosclerosis can occur in the peripheral system, affecting arteries such as the popliteal and carotid. In small arteries, such as the coronary, plaque build up significantly inhibits blood flow to the heart (stenosis), causing ischemia, which can lead to a myocardial infarction. In larger arteries, while the flow is not blocked, the vessel wall weakens causing either an aneurism or a dissection, both which can lead to vessel rupture. In all cases, the goal of the treatment is to restore blood flow to the heart – often through the use of minimal interventional therapies – by reopening (balloon), stabilizing (stents), or substituting (stent graft) the vessel (3).

The first balloon angioplasty was performed in 1977 (4), and since then the field of percutaneous interventions has evolved significantly. After elastic recoil was noticed following balloon-angioplasty, bare metal stents were introduced to support the reopened vessel. Endothelial denudation during stent placement however, led to a cascade of biological events resulting in neointimal hyperplasia – an event known as in-stent-restenosis – and after time re-occluded the vessel in 20-40% of the population treated (5-7). After systemic delivery of anti-proliferative drugs proved ineffective (8), bare metal stents were then coated with the drugs in order to deliver the required drugs directly to the injured site inhibiting the over-proliferation of smooth muscle cells (8, 9). While in-stent restenosis was significantly reduced with first generation drug eluting stents (DESs) (8, 10-12), late stage thrombosis (>30 days) became the next stent-related problem (13). Apart from patient related factors, the two main causes of late stage thrombosis were shown to be the extended exposure to prothrombogenic polymers, as well as delayed reendothelialization post stent-placement (14-17). While second generation DESs have managed
to help reduce the rates of late stage thrombosis (even below that experienced with bare metal stents) - through the combined use of thinner stent struts, more biocompatible polymer drug-carriers and more innovative anti-proliferative drugs (18) – thrombosis and restenosis are still problems requiring the development of non-thrombogenic biocompatible drug delivery coatings in combination with targeted drugs that don't prevent effective re-endothelialization post treatment.

More recently, drug-coated balloons (DCBs) have been presented as an additional method to treat stenosed vessels (19, 20). DCBs have two prime advantages over stent-based treatments; neointimal hyperplasia is prevented by the direct, homogenous delivery of anti-proliferative drugs, and late stage thrombosis is not as much a concern as the polymer device is removed immediately after delivery. While the lack of physical support has prevented its use in the treatment of de-novo lesions, DCBs are gaining more popularity in treating in-stent restenosis, lesions in more difficult locations with more complex geometries such as bifurcation lesions, and peripheral artery disease, which currently, when stented, experiences frequent stent fracture due to the higher stresses (21, 22). Being a newer technology however, DCBs inefficiently (23, 24), still elute first generation anti-proliferative drugs, from more primitive polymer matrices (21). Combinations of more developed biocompatible polymer matrices, in combination with effective anti-proliferative drugs however, may allow DCBs to become more effective in treating arterial diseases.

While the cardiovascular field has driven the development of percutaneous drug delivery devices, targeted delivery of drugs is a requirement in almost every field of medicine. Stent based drug delivery is used in gastrointestinal (GI) (25) and pulmonary (26) applications, drug coatings have been investigated to prevent fibrotic encapsulation on glaucoma-drainage shunts (27), and cochlear implants for example may be coated with dexamethasone to again prevent the fibrotic encapsulation of electrodes after insertion (28). Circumstances unique to each area however, require different properties from the drug excipient, from delivery of hydrophilic drugs in the case of GI stents, to extremely hydrophobic with the pulmonary stent. In order to treat a wide range of diseases in various biological environments, unique, customizable drug delivery coatings are required to efficiently deliver effective drugs to the required lesion sites without causing undesired biological responses from the surrounding environments.
1.1.1 Fluorinated Polyurethanes

Combining the thermal, stable and customizable properties of polyurethanes with the relative inertness and biocompatible properties of fluorinated polymers, the Santerre Lab has developed a family of fluorinated polymers specifically designed to provide unique interfacial characteristics (29-37). Due to their low interfacial energies, fluorinated segments rise to the material’s surface, effectively coating the underlying material with a unique highly fluorinated material, not subject to the effects of traditional additive coatings. Specifically regarding blood-contacting application, these surfaces have been shown to decrease platelet adhesion and reduce thrombosis-related events caused by protein adsorption (38).

1.1.2 Amphiphilic Oligourethane

Based on the above principles, work in the Santerre Lab in collaboration with industry partners has led towards the design of amphiphilic oligourethane (AOU) to be used as a platform on which to develop unique, tailored thin film drug delivery coatings. AOU (Figure 1.1) consists of 3 individual segments; a mono-functional polyethylene glycol unit to enhance solubility and drug release kinetics, lysine-diisocyanate center to serve as a point for additional functional groups to be added, and a perfluoroalcohol unit to provide the desired fluorinated properties.

![Figure 1.1: Structure of amphiphilic oligourethane. n~16, m~6](image-url)
Preliminary work on AOU synthesis yielded a product containing multiple unreacted and by-product species (non-AOU). Initial in-vitro cytotoxicity studies on a rat A-10 vascular smooth muscle cell (VSMC) line indicated that AOU affected smooth muscle cell function at defined concentrations. Three possible explanations were proposed to explain the observed result. 1) Impurities contained within the sample caused the cell effects; 2) AOU itself was a molecule that expressed a cellular response through extra- and or intra-cellular signaling processes; and 3) AOU’s amphiphilic structure caused a cell response which involved membrane disruption as seen with traditional surfactants (39). Hence, it became the objective of this current thesis to further understand the synthesis of AOU and expand upon its versatility in terms of chemical diversity, and to better understand the influence of its chemical structure on cell function.

1.1.3 Long Term Research Goal
Building on the biocompatibility of fluorinated polyurethanes, AOU will serve as a platform on which to develop unique and customized thin film drug delivery coatings to be used in conjunction with a range of devices specifically focused on treating arterial diseases. These coatings will protect the drug prior to deployment, release the drug at a desired rate upon deployment and should cause no undesired results to or cellular response from the surrounding biological environment.

1.2 Specific Research Hypotheses and Objectives

1.2.1 Hypothesis 1
It is hypothesized that pure AOU will elicit an in-vitro cell membrane dependent response on rat A-10 VSMCs which affects cell metabolism and DNA production.

1.2.1.1 Rationale
Non-ionic surfactants containing oxyethylene hydrophilic regions and hydrogenated or fluorinated hydrophobic regions (or both) have been shown to decrease cellular activity or cause complete cell death through membrane permeation (39-41).
1.2.1.2 Objective 1

Optimize AOU synthesis to obtain a pure product allowing for more accurate chemical and biological characterization to be performed.

1.2.1.3 Objective 2

Test overall AOU *in-vitro* toxicity on rat A-10 VSMCs using water-soluble tetrazolium-1 (WST-1) metabolic activity (42) and Hoescht DNA content assays (43), and assess the degree of membrane disruption using a lactate dehydrogenase (LDH) release assay.

1.2.2 Hypothesis 2

By modifying the pendant ester group of the AOU through changing its charge-state or by coupling additional hydrophobic alkyl chains, AOU’s amphiphilic structure will elicit an *in-vitro* cell membrane dependent response on rat A-10 VSMCs which affects cell metabolism and DNA production.

1.2.2.1 Rationale

Fluorinated surfactants have been shown to have less lytic potential than their equivalent hydrogenated counterparts (44, 45). Furthermore, toxicity of surfactants containing both fluorinated and hydrogenated regions has been shown to change depending on their respective ratios (40).

1.2.2.2 Objective 3

Synthesize AOU derivatives by changing charge-state through hydrolysis of methyl ester on the lysine group, generating an acidified analog AOU\textsubscript{H}; and coupling alkyl chains of different chain lengths (6, 8 and 12 carbons) to the lysine group creating AOU\textsubscript{6}, AOU\textsubscript{8} and AOU\textsubscript{12}.

1.2.2.3 Objective 4

Test overall *in-vitro* rat A-10 VSMC toxicity and degree of membrane disruption of AOU derivatives (WST-1, DNA, LDH assays).
1.2.2.4 Sub Hypothesis

Incorporating additional hydrophobic alkyl chains to AOU creating AOU-6,-8 and -12 will allow for increased drug binding with the hydrophobic anti-proliferative drug C6-Ceramide (which itself contains 2 long alkyl chains).

1.2.2.5 Rationale

C6-Ceramide was chosen as a model anti-proliferative drug with potential to be used to treat arterial diseases due to its ability to not only prevent smooth muscle proliferation, but also prevent endothelial cell death (46).

1.2.2.6 Objective #4

Use isothermal titration calorimetry (ITC) and nuclear magnetic resonance spectroscopy (NMR) (47-49) to measure the binding constants of AOUs with C6 Ceramide to determine if changing its structure alters its binding.

1.3 Thesis Outline

Chapter 2 provides a review of literature related to the clinical context surrounding drug delivery coatings with its primary focus on arterial devices, a summary of drugs currently used (in order to provide the context for new AOU chemistries), a description of their method of function, a background of the specific chemistries used for oligomeric fluoro-urethane synthesis, followed by an introduction into surfactant structure and their related cellular toxicity mechanisms.

Chapter 3 describes the optimization of AOU synthesis. Three main synthesis methods were investigated in order to – among other goals – prevent the generation of PEG-trimer (MPEG-LDI-MPEG) during synthesis, a common side reaction that takes place due to LDI’s symmetrical reactive groups. The presence of this undesired by-product presents a challenge due to its difficult separation from AOU due to their similar size and solubility characteristics.

Chapter 4 is the central chapter of the thesis and was written with the view of being submitted for publication to the journal Biomacromolecules. It begins with a concise review of the final
AOU synthesis method described in Chapter 3, followed by establishing synthesis methods used to generate new AOU derivatives – AOUH, AOU6, AOU8 and AOU12 (the details of AOU derivative synthesis optimization appears in Appendix C). Results from cell-interaction studies (using in-vitro rat A-10 VSMCs) are then presented for the full range of AOUs followed by an in-depth discussion regarding the results in the context of non-ionic, ionic and hybrid surfactants.

Chapter 5 describes preliminary work done to define the interactions of AOUs with C6-Ceramide. Background information is presented regarding isothermal titration calorimetry (ITC) and nuclear magnetic resonance (NMR) spectroscopy titrations as alternative methods to capillary electrophoresis methods for studying the interaction of drug and drug carriers. Results are presented only for the NMR experiments as a reliable protocol could not be established for ITC. These results serve as a demonstration of the potential for the use of NMR as a method to study AOU-drug interactions.

Chapter 6 presents the main conclusions from each chapter and Chapter 7 provides a list of future recommendations that should be considered as experiments to further the understanding and development of AOU-based molecules to be used as drug delivery coatings.

The series of appendices provide detail for some of the key studies which presented challenges in this thesis and were overcome with extensive experimentation.

Appendix A details the investigation into MPEG’s molecular weight in order to solve kinetic-related problems observed as a result of using the incorrect molecular weight.

Appendix B presents data from a small study reassessing the prepolymer reaction kinetics after increasing the reaction size.

Appendix C explains in detail the multiple reaction methods that were tested in order to optimize the synthesis of AOU derivatives.

Appendix D explains the process and results of LDH assay optimization.
1.4 References


Chapter 2
Review of Literature

2.1 Clinical Motivation – Atherosclerosis

Atherosclerosis is characterized by the slow formation of a lesion within an artery causing luminal narrowing, which can eventually inhibit blood flow to vital organs such as the heart, resulting in a myocardial infarction (1). While the pathology has been observed for decades, there has been a major development in the study of its cause and biomolecular mechanism. In the 1970’s Ross pointed towards the importance of smooth muscle cells in the plaque lesion formation (2). However it wasn't until the 1990’s when Hanson identified the process as a complex interaction between signaling factors and inflammation (3). At that time, Fuster began the work on mapping out a timeline of events that occur during the process (4). These pioneering studies laid the groundwork for the detailed descriptions of atherosclerosis that have recently appeared in review papers and complete books (5-7). Since the current thesis does not deal with the process of atherosclerosis per se, but rather novel drug carriers for drugs of interest in the management of atherosclerosis, it is the purpose of this section to simply outline the main events of the disease pathology and note the key biomolecules that play a role in the plaque development based on a number of comprehensive reviews (1, 8-10).

While it is clear that one of the main initiating players in the plaque formation is oxidized low-density lipoproteins (oxLDL), there is much controversy behind its specific risk factors and causes. Even though newer data and research has hypothesised factors such as genetic predisposition and infection, more traditional and significant factors point to age, hypertension and tobacco intake. The following outline will assume the presence of oxLDL.

oxLDL begins its damage by compromising the endothelium through enhancing permeability and increasing adhesion to monocytes (11, 12). The interaction between the endothelial cells (ECs) and oxLDL causes a release of monocyte activators, recruiting more monocytes and T-cells. As the site begins to take on the characteristics of an “injury”, platelets are recruited and the monocytes turn into macrophages, which subsequently begin to consume the oxLDL. These lipid containing foam cells begin to give form to the progressing lesion. By way of their consumption, the macrophages then begin to release more cytokines recruiting additional
molecules to the site. At this time, smooth muscle cells, which generally remain in the media layer of the vascular tissue, migrate to the intima and begin to proliferate, while those which have remained in the media begin to release additional pro-inflammatory signals. Activated macrophages begin to work externally, producing factors such as interleukin 2 (IL-2) and recruiting T-lymphocytes which themselves release classical inflammation factors such as interferon gamma (INFγ) and tumour necrosis factor alpha (TNFα). Once all of the mentioned constituents – and others – are joined together, the result is a mass of lipid-containing macrophages covered by a layer of smooth muscle cells incorporated in a connective tissue matrix. Now that the plaque is created, platelets from the flowing blood stream land on the activating surface and they too release multiple growth factors such as transforming growth factor α (TGFα), TGFβ and platelet derived growth factor (PDGF), further developing the injured lesion. A schematic pointing to the main processes described above is shown in Figure 2.1.

Figure 2.1: Schematic image of arterial wall cross section, indicating the main biomolecules involved in the atherosclerotic plaque progression. Reprinted from The International Journal of Pharmaceutics, 441, Puranik, A.S, Dawson, E.R, Peppas, N.A, Recent advances in drug eluting stents, 665-679, Copyright (2012), with permission from Elsevier.

While much research has gone into defining each stage of the plaque formation process, the above explanation is sufficient enough for the context of this thesis and provides a sense of how complex the process is, and how in the succinct words of Ross “What starts out as a protective response, in its excess becomes the disease entity atherosclerosis” (2). It is this plaque that serves
as the underlying cause of arterial diseases – such as stenosis, dissections, and aneurisms – which are most commonly treated through percutaneous interventions.
2.2 Current Treatments: Percutaneous Cardiovascular Interventions

2.2.1 Balloon Angioplasty and Bare Metal Stents

While atherosclerosis can affect all arteries, the following discussion regarding percutaneous interventions will use the coronary artery as a model, as it is the vessel most commonly treated percutaneously (approximately 1 million performed annually in the United States (13)), and therefore drove the research and development of percutaneous cardiovascular intervention (PCI) devices. While it has evolved over the past 30 years, the basis of this minimally invasive procedure is to use a thin wire, with a balloon at its end, which expands at the required site, reopening the blocked, or stenosed artery (14). First performed in Germany by Andreas Gruntzig in 1977, the initial results were quite promising (14), however it was soon evident that reclosure of the vessel, or restenosis was a serious problem with reports of restenosis in patients ranging between 30 and 60 percent (15, 16).

The next step in the development of PCIs resulted from a need to prevent restenosis, whereby a stent, an expandable metal scaffold, was placed at the plaque site following balloon expansion in order to support the vessel walls (17). First performed by Sigwart in 1987 (18), these stents, referred to as bare metal stents (BMSs), still resulted in restenosis in 32 percent of patients (19). While originally only offered as a suggestion (20), it was soon confirmed that this phenomenon of in-stent restenosis was a result of neointimal hyperplasia (21).

2.2.2 In Stent Restenosis

While early restenosis after balloon angioplasty was originally attributed to elastic recoil of the vessel, this was not the case for late stage restenosis following balloon angioplasty – and even more so following stent placement (where elastic recoil is not possible) (22). Rather, endothelial denudation caused during balloon expansion and stent placement initiates an inflammatory response consisting of a complex interplay of platelets, growth factors, cytokines and leukocytes. While much effort has been put into understanding the exact mechanisms, timelines and risk factors of this complex process, the following description will identify the key cellular and molecular constituents that play a role in order to better understand the rationale behind the proposed treatments.
Mechanical force exerted on the atherosclerotic plaque and the intima causes endothelial denudation releasing a large number of proteins and cytokines (22). Many of these proteins such as Tissue Factor (TF), activate platelets which, through P-selectin presented on their surface, begin to attract and bind leukocytes from the flowing blood (23). These activated platelets initiate a coagulation cascade where prothrombin is converted to thrombin and subsequently fibrinogen into fibrin. This isolated process causes a thrombotic cross-linked network to form (24). At the same time, due to the arterial injury as well as the activated platelets releasing mitogenic and chemotactic factors, PDGF and TGFβ, among others, are released and begin to recruit vascular smooth muscle cells (VSMCs) from the media. These VSMCs are shifted into the G1/S cycle increasing their migration and proliferation (22). Through IL-1 release, the leukocytes that were recruited during the thrombotic phase also add to the VSMC’s migration and proliferation potential (25) culminating in a large amount of neointimal hyperplasia over a few weeks post stent placement. Once the main response to injury has relaxed, the large number of VSMCs in the media continue to produce a complex extracellular matrix (ECM) covering the stent and inhibiting blood flow. This ECM-rich plaque differentiates itself from that of a typical atherosclerotic plaque which is much more cellular – an important point to take into account when choosing a mode of therapy such as a specific drug (22). A schematic pointing to the main processes described above is shown in Figure 2.2.

Figure 2.2: Schematic image of arterial wall cross section, indicating the main biomolecules involved in the in-stent restenosis process and subsequent neointimal hyperplasia. Reprinted from The International Journal of Pharmaceutics, 441, Puranik, A.S, Dawson, E.R, Peppas, N.A, Recent advances in drug eluting stents, 665-679, Copyright (2012), with permission from Elsevier.
2.2.3 Towards the Development of Drug Eluting Stents

In order to prevent restenosis post stent implantation, physicians began administering anti-restenosis drugs to patients. Since in-stent restenosis was most directly caused by neointimal hyperplasia, these drugs included smooth muscle inhibitors, platelet aggregation inhibitors, blood thinners, and combinations of these, however, none of the treatments produced any significant results (26). It was soon understood that while theoretically these drugs could inhibit neointimal hyperplasia, insufficient drug concentrations were being delivered to the required location to prove effective (27). The ability to administer therapeutically effective drug concentrations to the effected vessel however, became possible with the invention of the drug eluting stent (DES). In a DES, an anti proliferative drug is loaded onto the stent itself and is slowly released to the target tissue after implantation (26, 27).

When studying the evolution of DES development, one must focus on the interplay of 3 primary factors; the underlying stent platform, the specific anti-proliferative drug used, and the drug carrier (usually, but not always, a polymer matrix). However, since the majority of all investigated and federally approved DESs are developed by large medical device companies, not all of the material components, nor the logic behind their use, are disclosed in detail. Nevertheless, the following section will outline the material and clinical development behind the more famous and widely studied DESs. Five major groups can be traced; first and second generation permanent polymer-coating DESs, biodegradable polymer-coating DESs, polymer-free DESs, fully resorbable DESs, and bioconjugation stents.

2.2.4 First and Second Generation DES

The first generation of DESs (see Table 2.1) came on the market in the early 2000’s, and both were based on 316L stainless steel stent platforms. They included the Cypher (produced by Cordis) and TAXUS (produced by Boston Scientific) stents. Cypher used a polyethylene-co-vinyl acetate (PEVA) / poly n-butyl methacrylate (PBMA) coating with the drug Sirolimus, and TAXUS used a poly-styrene-block-isobutylene-block-styrene (SIBS) coating with Paclitaxel. While both systems contained very different coatings with drugs that functioned using different mechanisms (Section 2.4.1), both nevertheless produced very desirable initial results with significant reductions in target lesion revascularization (a metric of neointimal hyperplasia) (27-
After investigating the data in more depth however, it was realized that most trials were only able to prove non-inferiority of the DESs over BMSs (31). More troubling however, was the observation of late (> 30 days) and very late (> one year) stage thrombosis with patients treated with DESs, especially once anti-platelet therapy was terminated (32, 33). In order to settle the doubts regarding this effect, several meta-analyses were performed which showed no differences in MI and death occurrences between DESs and BMSs, but significant increases in late stage thrombosis in both first generation DESs (34, 35). Among others, the primary factors determined to cause this thrombosis were narrowed down to delayed reendothelialization and hypersensitivity reactions caused by prothrombogenic polymers (36-39).

Attempting to tackle the problems associated with the first generation DESs, a second generation of DESs appeared on the market in the late 2000’s (See Table 2.1). These stents are based on higher performance metallic platforms (allowing for thinner stent struts, and less material altogether), thinner drug carrier coatings and more developed anti proliferative drugs (40). While multiple clinical trials and meta-analyses have been performed testing the safety and efficacy of the second generation DESs, in general, as with the first generation DESs, only non-inferiority has been proven (31, 39). The Cobalt Chromium (Co-Cr) Everolimus eluting stent however, has been the most intensively studied second generation DES, thanks to it consistent reduction in stent thrombosis as compared to first generation DESs (41). Even more interesting though, is the data extracted from recent network meta analyses which indicate that the Co-Cr Everolimus eluting stent reduces stent thrombosis even more than with BMSs (42, 43). This represents what Palmerini has referred to as a “paradigm shift from the contention of an increased risk of stent thrombosis with DES compared with BMS to the converse” (31).

While multiple design factors affect the desirable outcome of the Co-Cr Everolimus stent, many have pointed to the extremely thin stent struts (81µm), the low polymer and drug load, and most importantly the thromboresistant fluorocopolymer (31). As will be discussed further in Section 2.5.3, the use of fluorinated materials in blood contacting devices has been shown to reduce thrombosis and platelet deposition, (44, 45) and therefore incorporating fluorinated chemistry into future designs of blood contacting drug delivery systems is a very attractive and important criteria to consider.
Table 2.1: List of the first and second generation DESs, including their base materials, the antiproliferative drug used, and a summary of the results from their main clinical trials. TLR = Target Lesions Revascularization, MACE = Major Adverse Cardiac Event, TVF = Target Vessel Failure, MI = Myocardial Infarction

<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>Name</th>
<th>Base Material</th>
<th>Polymer Coating</th>
<th>Drug</th>
<th>Main Clinical Trial Summaries</th>
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<tbody>
<tr>
<td><strong>First Generation</strong></td>
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<td>Cypher</td>
<td>Stainless Steel</td>
<td>PEVA/PBMA with PBMA top coat</td>
<td>Sirolimus</td>
<td>SIRIUS Clinical Trial (46)</td>
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<td>Study: Cypher vs. BMS, 5 years (n=1058)</td>
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<td>TLR (5 years): 9.4% vs. 24.2%</td>
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<td>MACE (5 years): 20.3% vs. 33.5%</td>
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<td>TVF (5 years): 22.5 vs. 33.5</td>
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<tr>
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<td>TAXUS</td>
<td>Stainless Steel</td>
<td>Poly-SIBS</td>
<td>Paclitaxel</td>
<td>TAXUS IV Clinilal Trial (30)</td>
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<td>MACE: 24.0% vs. 32.8%</td>
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<td>ENDEAVOR Clinical Trial (47)</td>
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<td>Study: Endeavor vs. 1st generation DES and BMS, 5 years (n=3616)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MACE: 16.1% vs 20.6% DES, 24.6% BMS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>TLR: 7.4% vs. 16.3% BMS, but similar to</td>
</tr>
<tr>
<td>Manufacturer</td>
<td>Model</td>
<td>Material</td>
<td>Polymer/Treatment</td>
<td>Study</td>
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<td>--------------------</td>
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</tbody>
</table>
| Medtronic          | Resolute    | Co-Cr Alloy        | Hydrophilic and hydrophobic elements | RESOLUTE Clinical Trial(48)  
Study: Resolute vs. Endeavor 2 years (n=1339)  
TLR: 12% Resolute vs. 16% Endeavor  
MI: 5.5% Resolute vs. 4.8% Endeavor  
Restenosis: All measures in favour of Resolute |
| Boston Scientific  | Promus Element | Pt-Cr Alloy       | Acrylic and fluorinated polymer | PLATINUM Clinical Trial (49)  
Study: Promus Element vs Resolute (n=1530)  
TLR: No significant difference  
MI: No significant difference |
| Abbott             | XIENCE V    | Co-Cr Alloy        | PVDF-HFP                  | XIENCE V Clinical Trial (50)  
Study: Meta Analysis of 4 SPIRIT trials of Xience V vs. 1st generation DES (n=4989)  
MACE: 9.4% Xience V vs. 13.0% DES  
TLR: 8.9% Xience V vs. 12.5% DES  
MI: 3.2% Xience V vs. 5.1% DES |
2.2.5 Biodegradable Polymer Coated DESs

While the DESs described above dominate the interventional cardiology market, the next most developed area of DES research is that of stents with biodegradable drug delivery coatings. These stents, while still based off the original metallic platforms, contain coatings of polylactides and similarly based copolymers (poly(lactic-co-glycolide)) (PLGA), incorporated with Paclitaxel or limus-based drugs. Over time, these coatings degrade and are metabolized by the Krebs-cycle to carbon dioxide and water (51). While non-inferiority as compared to previous generation DESs has been proven with stents such as Boston Scientific's SYNERGY (52) and Biosensor's BioMatrix Felix (53) – and the latter has even indicated a 5 year follow up decrease in major adverse cardiac event (MACE) and very late stage thrombosis (54) – this data however is still very preliminary, and is contested by data from other clinical trials testing the BioMatrix Felix against first generation DESs (55). Even more interesting however, is that results from meta-analyses comparing the newer biodegradable DESs are still not as attractive as those from the Co-Cr Everolimus stent (56), and in the words of a very recent reviewer “these data again suggest that [the Co-Cr Everolimus DES] may still have the best combination of efficacy and safety and that it seems hard to improve on currently approved DESs” (57). To summarize the point clearly, not one DES with a biodegradable polymer coating has been approved for commercial or investigational use in the United States (57). While not directly related to this project, the following section will make mention of three newer groups of stents for the purpose of completion.

2.2.6 Polymer Free, Bioconjugation and Bioresorbable Stents

As an alternative to modifying the drug carrier coating, research is currently underway investigating the use of polymer-free stents. This technology uses surface modifications to the metallic stent platform with the common taxus- and limus-based drugs incorporated therein. Biosensor’s BioFreedom stent showed no significant differences as compared to a first generation DES (58), and MIV Therapeutic’s VESTAsync with a nanothin-microporous hydroxyapatite surface coating, has been shown to successfully reduce key outcomes such as neointimal hyperplasia (59). Translumina’s Yukon polymer-free stent has the longest clinical follow up for a polymer-free stent, and its results place it within a similar efficacy range of
existing DESs (60). It should be noted that only one polymer-free stent has been approved (CE) (61), and it will still take many years of compiled data in order to approve its usage in the United States.

Since complete endothelialization is the ideal surface to allow for efficient hemodynamic properties and thrombosis prevention, much research has been done to use biomolecules to recruit endothelial cells onto the device surface (Bioconjugation Stents) (51). In this case endothelial progenitor cells (EPC) have been noted to be the most attractive source of endothelial cells to recruit (62). Many factors, including stromal cell derived factor 1 (SDF-1) and vascular endothelial growth factor (VEGF), have been investigated (51), however since EPCs only make up 0.002% of all peripheral blood mononuclear cells, and the inability of molecules to attract EPCs alone (63), the majority of this research area has remained on the bench. While Orbus’ Geneous R-stent – a stainless steel stent coated with monoclonal anti-human antibodies to attract CD34 – an EPC surface marker – received CE approval, further studies showed higher rates of thrombus formation as compared to BMSs (64) and questions again arise how well these novel stents will compare to the more developed generation of DESs.

Even with the development of newer drug eluting and bioconjugation stents, it is clear that ideally, after the vessel has fully remodelled and healed, the implanted foreign material should disappear or be removed. While still a very nascent area of research, fully resorbable stents have been developed and are currently undergoing preliminary clinical trials in areas outside the United States. A number of bioresorbable stents have been developed ranging in materials from lactide based polymers to magnesium (39, 40, 51, 57), but since these products are not directly related to the present research, mention will only be made of the most known bioresorbable stent, Abbot’s Bioabsorbable Everolimus Eluting Stent. This PLLA stent has shown no safety concerns after one year trials (65), however older European data seems to indicate a higher thrombus formation rate at earlier time points (66). Current studies will attempt to prove both short and long term safety and efficacy.

While some of the aforementioned stents have performed as well as more established DES, it is not clear what their long term results will be and how they will compare to those of the more effective second generation DESs.
2.2.7 Stent Grafts

As mentioned, atherosclerosis in small vessels causes a blockage of the blood flow and can be treated with stents and balloons, with the purpose of reopening the vessel. In larger vessels such as the thoracic and abdominal aorta, however, the build up of plaque can cause vessel swelling (aneurysm) or vessel dissection (creating an alternate blood flow path within the vessel wall), which can both lead to vessel destabilization and rupture. In these cases a more significant treatment is needed, and until 15 years ago, open surgical procedures replacing the affected vessel was the standard treatment. In 1999 however, stent grafts were introduced, allowing a replacement vessel portion to be placed at the diseased site by way of a catheter.

While the stent itself is simply a BMS, the stent graft is lined (either on the inside or outside of the stent) with a synthetic tube (for example expandable polytetrafluoroethylene or polyester). This new tube acts as a blood vessel removing the need to rely on the weak diseased vessel (67). So effective has the treatment been that one study, following 817 patients who received a stent graft to treat an aortic aneurysm, reported that 98% were free from aneurysm rupture post treatment (68). While many other associated complications exist with the device such as blood leakage in and around the graft, stent fracture and fabric erosion (67), the fact that a polymeric device is tracked through the vascular system leads to more traditional problems such as blood coagulation and thrombosis (69). The ability to deliver anticoagulant drugs to the blood-contacting surface without causing additional complications would be an effective way to improve the biocompatibility of these extremely important devices. While the drug carriers discussed in this project focus on carriers for antiproliferative drugs, they too could be used as non-thrombogenic drug delivery coatings on stent grafts.

2.2.8 Drug Coated Balloons

In many ways as a response to DESs, much attention has recently been paid to drug coated balloons (DCBs) as an effective method of delivering anti proliferative drugs to diseased arterial tissues. These balloons are tracked to the arterial target site as done with traditional PCIs, however upon balloon expansion and arterial reopening, drugs are delivered to the arterial wall, and the balloon is subsequently removed (17, 70, 71). DCBs therefore eliminate the problems associated with previous PCI methods; antiproliferative drugs are delivered directly to the lesions
removing the neointimal hyperplasia associated with BMSs, and since it is removed immediately after drug delivery, there is no risk of late stage thrombosis caused by extended drug delivery preventing reendothelialisation or extended exposure to a prothrombogenic polymer (72). Furthermore, balloons can provide a more homogeneous drug delivery to a larger surface area, and preserve the normal vessel anatomy, promoting a more natural healing post treatment (73).

The idea of delivering drugs on a balloon actually appeared in the early 1990’s, however unlike DESs, it is quite difficult to deliver enough drug to the vessel wall. In a pioneering study, Scheller et al. noticed that if delivered with iopromide (contrast agent), drug could remain on the vessel wall for a few seconds post delivery (74). This was the start of a long process in the development of tailored drug delivery coatings and excipients.

The most intuitive downside of DCBs is the lack of physical support it provides the vessel after removal. It is no surprise therefore that the initial studies looked at treating cases of in-stent restenosis (ISR) as opposed to de-novo lesions. PACCOCATH was the first study to investigate the efficacy of DCBs vs. non-coated balloons at treating BMS-in-stent restenosis. A 5 year follow up in 2012 demonstrated both significantly lower target vessel revascularization (9.3% vs. 38.9%) as well as major adverse cardiac events (75). A more realistic and relevant comparison however was that of DCBs vs. DESs in treating BMS-in stent restenosis. The PEPCAD II trial testing this combination showed comparable results between both the DCBs and DESs after 12 months, indicating the appropriateness of this treatment method (76).

While the DESs used in the previous studies delivered Paclitaxel to the lesion, based on the discussion above regarding DESs, a more challenging comparison would be a DCBs vs. Everolimus eluting stents. This was performed in the RIBS (77) and RIBS IV (78) trials. While multiple endpoints were evaluated, and different trials studied patients with DES- and BMS-in-stent restenosis, experts claim that the data suggests that the Everolimus eluting stents outperformed the DCBs in these cases (79). This clinical data, therefore has translated into recommendations from American and European associations, where the American Heart Association has ruled out the use of DCBs to treat coronary in stent restenosis in all cases (80), while the European Society of Cardiologists suggest the use of DCBs to treat BMS-in-stent
restenosis (81). These recommendations will likely change with the development of newer DCBs using more novel drugs such as Everolimus and Zotarolimus (79).

DCBs however, have been proposed for their use in a range of arterial diseases, not limited to main-stream coronary heart disease, two of which will be mentioned. Small vessel disease, effecting 20-30% of patients with coronary artery diseases, poses a challenge for cardiologists as patients usually experience higher restenosis rates. As before, two individual clinical trials (PICCOLETO (82) and BELLO (83)), showed conflicting results, and while explanations related to the different coatings used have been proposed, the American Heart Association still recommends the use of DESs to treat small vessel disease, due to DCBs seeming inability to prevent restenosis (80).

As with coronary arteries, restenosis occurs in peripheral arteries such as the femoral and popliteal, however very late stage thrombosis and the possibility of stent fracture in the peripheral vascular beds has driven the study of DCBs to serve as a treatment method. Many trials have proven DCB’s superiority over non-coated balloons (THUNDER (84), FemPac (85), PACIFIER (86)) with observations of reductions in late lumen loss and target lesion revascularization. Based on these data, on October 14, 2014, the FDA approved the use of a Lutonix ® DCB for treatment of de-novo and restenotic lesions in the superficial femoral-popliteal arteries (87). While DCBs have been approved in Europe for many years, this is the first approved DCB technology for clinical usage in the United States.

Even with the successful clinical trials underway, poor drug delivery is still an issue with DCBs, with the highest delivery dosage to date only reaching ~17% of the total drug loaded on the device, when performed in conjunction with a stent (72). When applied alone, these numbers fall to ~7%, with 90% of the drug being released into blood stream during tracking (72, 88). With increased research and development into the specific drugs used and their coating technologies, it is clear that more DCBs will be approved due to their transient usage as well as the ability to treat patients who can not undergo anti-platelet therapies.
2.3 Non-Vascular Applications of DESs

While the majority of research and literature of DES development focuses on arterial diseases, it should be noted that DESs play an important role in treating a wide range of non-vascular diseases from gastrointestinal tumours and sinusitis to urethral strictures and emphysema. The reader is directed to comprehensive reviews outlining the research areas and clinical trials in these cases (89, 90), while two examples will be summarized below to give the reader an appreciation of the wide range of applications that drug delivery coatings play. While DESs are used for many applications, coronary heart disease is still the driving force behind stent development and design, simply due to the fact that it affects the largest number of people.

2.3.1 Non Vascular Application #1: Gastrointestinal Stents

Gastrointestinal stents were developed originally as palliative devices to open obstructed tracts such as esophageal, biliary and colonic, generally caused by malignancies. Evolving from rigid plastic tubes, through uncoated expandable metal stents, and finally to fully polymer coated (polyethylene or polytetrafluoroethylene) stents (91), the gastrointestinal stent has its own design requirements, which cannot be overlooked during the design process. Most relevant however to this discussion is the newer research investigating the use of DESs for gastrointestinal applications. The rationale for delivering drugs to these locations is to prevent tumour ingrowth, which usually occurs once the simple polymer coating has degraded. While studies have been done with Paclitaxel coated stents and initial studies proved its anti-tumour effects (92), more recent comparative studies showed no significant difference between the Paclitaxel stent and the conventional polymer coated gastrointestinal stent (93). One reviewer sharply noted that an important step in the design process should be “selecting an adequate anti tumour agent depending on the nature of the cancer” and not relying on the previously studied systems (91).

Another factor in gastrointestinal drug eluting stents is the incorporation of Gemcitabine, a common bile-cancer anti tumour drug. Due to its hydrophilicity, a sustained drug release is required in order to be effective. Work has been made by Moon, whereby Gemcitabine is incorporated with a polysaccharide based coating and has shown a release profile lasting for 30 days (94). It has been noted however that increased drug delivery in the gastrointestinal tract could have damaging effects on normal digestive tract mucosa and non-target organs (91). While
a much smaller and less developed field than coronary stents, gastrointestinal DESs serve as an additional area where the design of customizable, biocompatible and safe drug carrier coatings is required.

2.3.2 Non Vascular Application #2: Airway Bypass Stents

Emphysema, just one of many chronic obstructive pulmonary diseases (COPD), is the weakening and eventual rupturing of the lungs’ parenchyma, most specifically the alveoli walls. This disease reduces the amount of oxygen that enters the blood, causes airway disruption and lung hyperinflation. In the absence of a lung transplant, the creation of a new airway passage between the pulmonary parenchyma and bronchial airways can serve as a bypass route for air to flow. The main problem however with the new opening is tissue ingrowth. While some non-practical solutions have been proposed, based on the literature described above it is no surprise that a Paclitaxel coated stent was investigated as a method to keep open the airway bypass route without occluding (89, 95).

Exhale®, a stainless steel stent with a Paclitaxel containing silicone rubber sleeve, has been investigated in both animal and human subjects (89, 95, 96). An 18 week initial trial, testing 25 dogs, with 157 transbrochial stents (50 with no Paclitaxel), showed that all control stents were completely occluded after 4 weeks while 95% of all the Paclitaxel stents were patent. Even after 18 weeks, 65% of the test stents still remained patent. Transferring this knowledge, in order to confirm safety and efficacy of these stents, the EASE trial studied 315 patients (208 people, 981 stents, vs. 107 people no bypass made), and monitored key indicators such as forced vital capacity, retention volume, and forced expiratory volume. After 6 months, while the key indicators remained the same, there were improvements in safety endpoints such as respiratory failure and death. The researchers claim the full benefit could not be realized due to stent occlusion when an insufficient dose of Paclitaxel was used. Regardless of this exact outcome, it is important to realize the wide-ranging applications of drug eluting stents and how important effective and safe drug delivery is.
2.4 Antiproliferative Drugs

2.4.1 Drugs Currently Used

While initial studies looked at coating arterial devices with anticoagulants such as heparin (97), it became clear (as described above in Section 2.2.2) that multiple factors lead to neointimal hyperplasia and restenosis, not simply thrombin formation (98). Taxus- and limus-based drugs, both with a long history in the medical field, have now become the only two groups of drugs used in approved arterial devices. Paclitaxel, having been used in cancer therapy since the 1970’s (99) was eluted from the Boston Scientific’s first generation Taxus stent, and Sirolium (or Rapamycin), widely used in organ transplantations (100) was eluted from Cordis’ first generation Cypher stent. All of the 14 clinically approved drug eluting stents (CE and or FDA) are loaded with newer limus-based drugs, with only 2 eluting Paclitaxel (101) while all 13 of the approved drug coated balloons (all CE, with 2 FDA(102) elute Paclitaxel (103)).

Paclitaxel binds to intracellular microtubules, stabilizing them and altering their assembly, inhibiting successful mitosis (104). Depending on the dosage, Paclitaxel can create a cytostatic or cytotoxic effect. Limus-based drugs, on the other hand bind to intracellular proteins (FK binding protein, FKBP), blocking the mammalian target of Rapamyacin (mTOR), inhibiting replication (105). While both drugs helped drastically reduce neointimal hyperplasia as compared to BMSs, high levels of late stage thrombosis was reported due to the use of prothrombogenic polymers as well as delayed reendothelialisation (36-39) – the latter which was caused due to both Paclitaxel ’s and Limus’ indiscriminate targeting of both smooth muscle and endothelial cells(106).

Even with delayed endothelialisation caused by these drugs, all of the drug eluting stents and balloons currently under clinical trials are still loaded with Paclitaxel or Limus-based drugs, simply due to the fact that these drugs have already been approved, removing the need to get separate FDA/CE approval on the drug itself, reducing the amount of time it takes for the device to make a profit. Given the non-specific attack towards the two cell types, however, there is still a lot of research looking into molecules to specifically target SMC proliferation without inhibiting endothelialisation, a necessary component in the prevention of thrombosis (107). A few examples of these novel molecules and their mechanism are given in Table 2.2.
Table 2.2: List of anti-proliferative drugs – currently used and proposed for use – to treat neointimal hyperplasia, including their main mechanisms.

<table>
<thead>
<tr>
<th>Family of Molecules</th>
<th>Specific Drug</th>
<th>Activating Mechanism</th>
<th>Cellular Response</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Limus Drugs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fermentation product of</td>
<td>Sirolimus (1st Generation)</td>
<td>Immunosuppressant drug which binds to FKBP12, subsequently blocking mammalian target</td>
<td>- Interrupts cell cycle at G1-S</td>
</tr>
<tr>
<td><em>Streptomyces hygroscopius</em></td>
<td>Zotarolimus (2nd Generation)</td>
<td>of Rapamycin (mTOR)</td>
<td>- Non discriminant between SMC and EC</td>
</tr>
<tr>
<td></td>
<td>Everolimus (2nd Generation)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Taxane Drugs</strong></td>
<td>Palitaxel (1st Generation)</td>
<td>Binds to cellular microtubules, stabilizing their structure, enhancing microtubule</td>
<td>- Inhibits cellular replication at G0-G1 and G2-M phase</td>
</tr>
<tr>
<td>Derived from bark of <em>taxus</em></td>
<td></td>
<td>assembly</td>
<td>- Non discriminant between SMC and EC</td>
</tr>
<tr>
<td><em>brevifolia</em> (39, 40)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Intracellular Protein Targets</strong></td>
<td>Cyclopentany Cytosine (108)</td>
<td>Inhibits CTP synthase, an important process during DNA and RNA synthesis</td>
<td>- Reduces SMC proliferation</td>
</tr>
<tr>
<td></td>
<td>Trimetazidine (109)</td>
<td>Inhibits mitochondrial enzyme which shifts energy from fatty</td>
<td>- Suppression of SMC proliferation</td>
</tr>
<tr>
<td>Extracellular Protein Targets</td>
<td>C-type natriuretic peptide (CNP) (110)</td>
<td>Activates ERK1/2 through Gi-coupled NPR3</td>
<td>- Increasing endothelial proliferation and reduced apoptosis</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>----------------------------------------</td>
<td>----------------------------------------</td>
<td>---------------------------------------------------------------</td>
</tr>
</tbody>
</table>
| Extracellular Protein Targets | Pitavastatin (111)                     | Inhibits HMG-CoA reductase (enzymes used to produce cholesterol) | - Promotes endothelial proliferation  
- Inhibits SCM proliferation and tissue factor expression |
| Non Coding RNA Targets        | miR-92a Inhibitor(112)                 | Increases phosphorylation of ERK1/2 and other protein kinases in ECs | - Enhances reendothelialization  
- No effect on SMC proliferation and migration in vitro |
| Cellular Messengers           | C₆ Ceramide (113, 114)                 | Stimulates c-jun N-terminal Kinases (JNKs), and supresses extracellular signal-related kinases (ERKs) | - In SMCs, acts as antiproliferative  
- In ECs, more is metabolized to the pro-mitogenic C-1-P, promoting cell survival. |
2.4.2 C6-Ceramide

Ceramides are a family of molecules composed of a sphingosine (18 carbon amino alcohol) and a carbon chain fatty acid. Apart from their structural role in the cell membrane, over the past twenty years these molecules have also been understood to serve as bioactive molecules and intra/extra cellular messengers, playing an important role in signal transduction, inflammation, angiogenesis and many other biomolecular processes (115). Most significant with regard to arterial devices is Ceramide’s ability to regulate cell responses, including growth arrest, senescence and apoptosis (113). A number of studies have pointed towards C6-Ceramide in particular (6 carbons in the fatty acid chain), as an ideal molecule to inhibit SMC proliferation, and consequently a number of mechanisms have been proposed (113, 116, 117). C6-Ceramide was shown to inhibit tyrosine kinase receptor and G-protein receptor-linked mitogenesis in aortic SMCs, and it was shown both in-vitro and in-vivo to inhibit SMC proliferation (causing growth arrest without apoptosis) after arterial stretch injury by down-regulating extracellular signal-related kinases (ERKs) and cell-survival-related protein kinase B (PKB).

Most interesting however, is C6-Ceramide’s ability to inhibit SMC growth while not inhibiting associated endothelial cell (EC) proliferation. This was shown based on data from a balloon angioplasty experiment delivering C6-Ceramide to porcine arteries, and an in-vitro study comparing the responses of human SMCs and ECs to C6-Ceramide. Among others, 2 primary factors were presented: Arterial ECs, by way of Ceramide kinase, metabolize C6-Ceramide (generally antiproliferative) into Ceramide-1-phosphate (C-1-P), a pro-mitogenic second messenger. Additionally, using quantitative real time polymerase chain reaction analysis (QRT-PCR), it was shown that the mRNA levels of Ceramide kinase and C-1-P were found to be almost double in ECs as compared to the SMCs. QRT-PCR additionally indicated much higher mRNA levels of galactosyl- and glucosyl-Ceramide synthase in SMCs, producing more than a three-fold increase in glycosylated Ceramide species, a less bioactive form of native Ceramide, as compared to the endothelial cells (118). While other factors have also been suggested for the different responses of smooth muscle and endothelial cells in the presence of C6-Ceramide, these findings make C6-Ceramide an attractive alternative to the anti-restenosis drugs currently used in drug eluting stents and balloons.
2.5 Chemistry

2.5.1 Polyurethanes

Introduced in the 1930’s by Otto Bayer in order to serve as competitor materials to DuPont’s polyamide-based Nylons, polyurethanes have become widely used in the biomedical field, from pacemaker leads to tissue engineering scaffolds (119). Traditional polyurethanes are synthesized in a two step process from three main components; a difunctional isocyanate, a diol, and a short chain simple diol or diamine know as a chain extender. The first step reacts the diol with excess diisocyanate to create a low molecular weight prepolymer, which is subsequently reacted with a chain extender. The resulting structure is a block-copolymer consisting of a glassy crystalline hard-segment (the region of the isocyanates and chain extenders), and rubbery soft segment (the region of the diols) (120). This unique structure creates thermoplastic elastomers, with non-crosslinked properties of thermoplastics, but high toughness and elasticity of thermosets. The polar urethane hard segments act as reinforcing microdomains within a more flexible matrix of the polyol, providing properties of both segments, which can be tailored depending on the exact application (119).

Since only the diisocyanates and alcohol components are used in this project, with no chain extender, the created molecules are termed oligourethanes. The exact same chemistry however is still at play whether synthesizing high molecular weight polyurethanes for artificial heart bladders or oligourethanes for drug delivery coatings.

The primary reaction used during the oligourethane synthesis can be classified in between a traditional addition and condensation polymerization. While no small molecule is released (as is in a condensation reaction), the kinetics and control over the reaction more closely resemble a condensation reaction. Some textbooks refer to it as a polyaddition reaction or rearrangement polymerization (120). The fundamental driving force of reaction between an alcohol and an isocyanate (N-C-O) is the latter’s resonance structure, where due to the electronegativity’s of both adjacent nitrogen and oxygen atoms, the carbon atom takes on a slightly positive charge making it more susceptible to nucleophilic attack (see Figure 2.3).
Due to the isocyanate’s unique structure, additional reactions can occur, making synthesis of urethanes quite a sensitive process. While a list of additional reactions are shown in Table 2.3, the two of most interest to this project are those of reaction with water and with additional isocyanates. The interference of the latter reaction is much less of an issue as it only occurs at temperatures that exceed 100ºC, however it does require the isocyanate to be distilled prior to use in order to remove dimer and trimer species. The problem of reaction with water however, is a much larger issue as it readily reacts with the isocyanate, and thus interferes with the desired reaction with the alcohol (121). Much care is therefore taken to remove all sources and residues of water through degassing reactants and performing the reactions under anhydrous nitrogen gas.

Figure 2.3: Top row shows the resonance structure of an isocyanate. Bottom row shows the nucleophilic attack of an alcohol on an isocyanate to form a urethane bond.
2.5.2 Chemical Characterization of Fluorinated Molecules

The unique properties of fluorinated molecules have been known in the biomedical field for the past 60 years (122). While Du Pont’s polytetrafluoroethylene (PTFE or Teflon) was originally used due to its high temperature and solvent resistance, it became apparent that its ‘inertness’ could be applied to medical applications as well, and once its manufacturability was improved it was used as an arterial bypass graft (123). The reason behind its unique properties is due to the effective overlapping of the C-F bond, creating the most stable bond in organic chemistry (124), as well as the atomic arrangement it forms around a carbon chain. Thanks to its large atomic size and dense electron cloud, the fluorine atoms on a CF₂-CF₂ chain essentially ‘mask’ the underlying carbon-carbon structure, and create a stiffer molecular chain than the typical hydrogen-saturated polyethylene. This, coupled with its high ionization energy and low polarizability gives fluorinated materials low interfacial energies and extreme hydrophobicity (123, 124).

2.5.3 Fluorinated Polyurethanes

While polyurethanes gained their popularity in the medical field for their physical properties as well as their biocompatibility (120, 121, 125), they do not match up as well in long-term blood contacting applications. As expected, on top of hydrolytic degradation, which occurs under the

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**Table 2.3: List of possible reactions with NCO**

<table>
<thead>
<tr>
<th>Reaction of NCO-&lt;br/&gt;with:</th>
<th>Creates:</th>
<th>Structure</th>
<th>Decomposes to:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amine</td>
<td>Urea</td>
<td><img src="structure1.png" alt="Urea structure" /></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>Secondary Amine</td>
<td><img src="structure2.png" alt="Secondary Amine structure" /></td>
<td>NH₂ + CO₂</td>
</tr>
<tr>
<td>Urethane</td>
<td>Allophanate</td>
<td><img src="structure3.png" alt="Allophanate structure" /></td>
<td></td>
</tr>
<tr>
<td>Urea</td>
<td>Biuret</td>
<td><img src="structure4.png" alt="Biuret structure" /></td>
<td></td>
</tr>
<tr>
<td>R'-NCO-</td>
<td>Dimer</td>
<td><img src="structure5.png" alt="Dimer structure" /></td>
<td></td>
</tr>
</tbody>
</table>
harsh conditions, they also experience protein adsorption, platelet activation and classical coagulation (126). Trying to incorporate the physical and thermal properties of polyurethanes with the unique blood contacting properties of fluorinated has led to the development of fluorinated polyurethanes. Originally the fluorinated group was used as one of the polyurethane blocks (soft segment (127), diisocyanate, or chain extender (128, 129)), however while increasing its blood compatibility, the lower molecular weight components compromised the underlying mechanical properties (130, 131).

Due to fluorine’s low surface energy, it was noticed that fluorinated groups, when placed in a matrix, naturally migrated and bloomed to the surface. Using this knowledge, the Santerre Group has developed a family of polyurethanes combined with fluorinated Surface Modifying Macromolecules (SMMs) for the use in biomedical applications (132-140). Appropriate biocompatible segments such as Polyethylene glycol (PEG) and lysine diisocyanate (LDI) can be chosen, which when degraded release non-toxic components (diols, amino-acids) that are removed via the renal system (141-143). These fluorinated SMMs significantly decrease platelet adhesion and fibrinogen activation (processes that lead to thrombosis), reduce protein denaturation when adsorbed onto the surface (a process that leads to blood coagulation) (132, 134, 135, 137, 138), and reduce hydrolytic degradation when compared to bare polyurethanes (132, 135, 137, 138).

Apart from the attractive blood contacting properties of fluorinated surfaces, its ability to protect the underlying polymer from the blood’s components has led researchers to study its ability to prevent premature drug release (a common problem with DCBs). Mathias using nuclear magnetic resonance (NMR) observed slower diffusion of cancer drugs through fluorinated regions of a hydrogel (144), while Tanaka prevented a burst release of drug from PLGA microcapsules by coating their surface with a fluorinated plasma treatment (145). Similar results have also been found when fluorine units were incorporated to other drug delivery systems such as fluorination of electrospun fibres (146) and the use of fluorocarbons in phospholipid micelles (147). Observation of such similar results over a range of different chemical structures is a testament to fluorine’s robust ability to inhibit premature drug release.

The combination of efficient chemical synthesis, ease of tailoring the individual components, good bio- and blood- compatibility, and the ability to prevent premature drug release is the basis
and rationale behind the development of the AOU backbone to be used to develop non-thrombogenic drug delivery coatings.

2.5.4 Carbodiimide Chemistry

Carbodiimides are part of a class of bioconjugation reagents known as zero-length crosslinkers which mediate the formation of amide linkages between carboxylates and amines or phosphoramidate linkages between phosphates and amines (148). The name zero length is given since the two original molecules (such as a peptide and protein) are covalently bonded together with no additional spacers (149). In general, the carbodiimide reacts with a carboxylic acid group to form an acylisourea active intermediate, which is quickly substituted by an amine-containing molecule, creating the desired conjugation and an isourea by-product. While multiple carbodiimides exist and their purposes are tailored to specific reaction conditions, the following discussion will highlight the two used in this project; 1-ethyl-3-(3 dimethylaminopropyl) carbodiimide) (EDC) and diisopropyl carbodiimide (DIC).

Thanks to its water solubility, EDC is the most commonly used bioconjugate agent. This removes the need to dissolve it in an organic solvent prior to the reaction, as well as makes purification easy with dialysis or gel filtration (149-151), both factors making it quite an attractive reagent to use when working with molecules that will used in biological environments. EDC reacts with a carboxyl group to form a highly reactive o-acylisourea, which is ideally attacked by an amine nucleophile (see Figure 2.4), however is susceptible to alternate attack by oxygen in water molecules and free hydroxyl groups. Through hydrolysis, water can cleave off the intermediate ester, creating an isourea and the original carboxylic acid. To prevent this problem, many add N-hydroxysulfosuccinimide (NHS), which forms a more soluble intermediate with a much higher reactivity towards amide formation (149).

DIC is a non water-soluble carbodiimide, which is easier to work with than its counterpart dicyclohexyl carbodiimides (DCC) due to its liquid state at room temperature and high solubility in organic solvents, and is used very often in organic synthesis (149). The general mechanism is similar with that described above with EDC and is shown in Figure 2.5. In order to prevent racemization of the intermediate from forming the unreactive enantiomer N-acylurea, very often a nucleophile that acts faster than the enantiomer-transfer is added to prevent the side reaction but still be active enough for an amine to couple. In EDC reactions, dimethylaminopyridine
(DMAP) is often used, while hydroxybenzotriazole (HOBr) is commonly used with DIC (152, 153).

Figure 2.4: Generic coupling reaction of COOH and NH using EDC.

Figure 2.5: Generic coupling reaction of COOH and NH using DIC.
2.6 Surfactants and their Toxicity

2.6.1 Non-Fluorinated Amphiphiles

Surface active agents, or surfactants, are a family of molecules consisting of a hydrophobic tale(s) and a hydrophilic head group, which can be positively, negatively or not-charged, corresponding to the three main surfactant groups; cationic, anionic, and non-ionic, respectively (154). When surfactants are placed in solutions in low concentrations, a portion will orient themselves at the water-air interface with others dispersed throughout the solution. At higher concentrations (the critical micelle concentration, CMC), the molecules will begin to arrange themselves in lower energy formations known as micelles, aligning their hydro-philic and -phobic components accordingly (155). While a surfactant’s similarity to phospholipids is harnessed in many biochemical assays to release intracellular components (SDS, Triton-X), it is also therefore a potentially toxic set of molecules when used in biomedical applications.

The two main mechanistic theories behind surfactant cell lysis are solubilisation and osmotic lysis (156) of the cell membrane. The solubilisation theory, pioneered in 1975 (157) has been divided into 3 main stages. In stage 1 individual surfactant molecules intercalate in between aqueous and lipid regions of the cell membrane which begin to lose shape and stability. Stage 2 is characterized by the saturation of surfactants in the cell’s lipid membrane and the formation of mixed lipid-surfactant micelles. As the surfactant concentration increases even more, stage 3 is reached with the fragmenting of surfactant-rich regions into individual mixed micelles and complete cell membrane solubilisation (158). In other cases, generally at lower concentrations, it is thought that surfactant intercalation simply causes increased membrane permeability, with subsequent water penetration and swelling causing membrane rupture and osmotic lysis (156, 159).

While the above explanation is useful in order to divide each step, Goni has shown that many of the steps such as surfactant flip-flop, lipid motion and membrane lysis/reassembly are not dependant on each other nor do they always occur successively (158). Furthermore, there are multiple factors that determine if, why and to what extent a molecule will permeate, disrupt and lyse a cell membrane. While surfactant related factors include length of hydro-philic and -phobic sections, its rigidity (160-162) and its induced surface pressure (163), the solvent in which the surfactant acts is of equal importance in determining the surfactant’s interactions with
cell membranes. Shalel studied a number of cationic, anionic, and non-ionic surfactants and their effects on erythrocytes in solutions of ranging ionic strength, osmolarity, and solute particle size (156). From a number of studies, it is relevant to point out that since cell membranes are generally negatively charged, surfactants with corresponding charge are less permeable than cationic surfactants. If the ionic strength of the solvent is increased however, the activity of the anionic surfactant is increased. Non-ionic surfactants are therefore not sensitive to changes in ionic strength. In closing, when studying surfactants to be used in biomedical applications, care should be taken to mimic the physiological conditions as well as possible.

2.6.2 Fluorinated Amphiphiles

As mentioned, fluorocarbons have been studied for use in biomedical applications due to their unique intra- and inter-molecular properties creating stable and inert structures. Fluorinated amphiphiles on the other, while used extensively in industrial applications, have been much less studied in the biomedical field, possibly due to difficult product purification (124). However, fluorocarbons’ robust properties directly affect their amphiphilic properties and can give rise to a unique family of biomedical surfactants (124, 164).

Fluorinated surfactants have CMCs equal to those of their hydrogenated counterparts with 50% longer chains, and each additional CF₂ group on a chain has a ~1.6 times the micellization potential compared to that of CH₂ (165). Another unique property of fluorinated amphiphiles is their tendency to form lamellar and threadlike micelle, less curved and spherical, due to the stiffness of fluorocarbon chains (164). These fluoro- surfactants however, give rise to quite non-typical surfactant properties. While increasing the chain length of hydrogenated surfactants typically increases cell hemolysis, the incorporation of fluorinated chains has been shown to reduce the effect, and even more, increasing the fluorinated chain length further decreases hemolysis (166, 167).

Courrier synthesized a range of surfactants with a dimorpholinophosphate (DMP) polar head group and semifluorinated hydrophobic tails (nCH₂-mCF₂) referred to as FnHmDMPs (n: 4-10, m: 2-11). Studies on mouse fibroblasts showed that those with hydrophobic chains of less than C12 were cytotoxic, while those above C12 were only non-cytotoxic when the fluorinated portion was above C4. To prove the protective shielding effect of the fluorinated chain, a fully hydrogenated version of identical length to a non-cytotoxic fluorinated analogue was cytotoxic.
Backed by additional data from assays with human lung epithelial cells, it was shown that the most important ratio (at least at surfactant concentrations below 1 wt. % in solution) was the fluorinated chain length/total chain length $F_n/(F_n+H_m)$ (168). While the complete story behind why and how the fluorinated chain shields the rest of the surfactant is not fully understood, it is clear that fluorinated surfactants should not be considered simply more hydrophobic versions of hydrogenated surfactants, rather they have their own unique cell compatibility properties not seen with other amphiphiles (124).
2.7 References


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Chapter 3
AOU Synthesis Optimization

3.1 Introduction

As described in section Chapter 1, AOU will serve as the primary backbone molecule, which will be used to test the project hypotheses. The full range of studies looking into the synthesis of AOU derivatives, their interaction with smooth muscle cells and drug binding properties will be discussed in the following chapters. However, before tests could be performed and derivatives synthesized, it was necessary to establish an AOU synthesis protocol that could yield a reproducible standard with a quality deemed acceptable in order to move on to the additional studies. This chapter therefore describes in detail the three primary steps that went into establishing the AOU synthesis.
3.2 Materials and Methods

3.2.1 Materials

The following materials were used as primary reactants during synthesis. The supplier and preparation information are listed in Table 3.1.

Table 3.1: List of primary reactants used during AOU synthesis.

<table>
<thead>
<tr>
<th>Material</th>
<th>Supplier</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine diisocyanate (LDI)</td>
<td>CHEMOS GmbH, Germany</td>
<td>Material from supplier was distilled under reduced pressure (0.05 mm Hg) at 120°C. Distilled product was stored under nitrogen at -20°C.</td>
</tr>
<tr>
<td>Polyethylene glycol methyl ether, 750. (MPEG)</td>
<td>Sigma Aldrich</td>
<td>Material from supplier was degassed under reduced pressure (0.1 mm Hg), while stirring, in an oil bath heated to 80°C for 24 hours prior to being used for a reaction.</td>
</tr>
<tr>
<td>Perfluoro-alcohol (PFA)</td>
<td>DuPont</td>
<td>Material from supplier was degassed under reduced pressure (0.1 mm Hg), while stirring, at room temperature for 24 hours. Degassed product was stored under nitrogen at room temperature.</td>
</tr>
</tbody>
</table>

The non-primary materials and reagents were used as delivered from supplier with no additional preparation and are listed in Table 3.2.

Table 3.2: List of non-primary reactants used during AOU synthesis.

<table>
<thead>
<tr>
<th>Material</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,2,4 Trichlorobenzene</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Acetone (reagent grade)</td>
<td>Caledon</td>
</tr>
<tr>
<td>AQUASTAR ® CombiCoulomat</td>
<td>EMD Chemicals Inc.</td>
</tr>
<tr>
<td>Bromophenol blue, 0.04 w% solution in water</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Compound</td>
<td>Supplier</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
<td>---------------------------------</td>
</tr>
<tr>
<td>Chloroform-d with 0.03% TMS</td>
<td>Cambridge Isotopes</td>
</tr>
<tr>
<td>Dibutylamine</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Dibutyltin dilaurate</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Drisolv ® Methanol</td>
<td>EMD Chemicals Inc.</td>
</tr>
<tr>
<td>Hexanes (ACS) (reagent grade)</td>
<td>Caledon</td>
</tr>
<tr>
<td>Hydrochloric Acid 0.1 N</td>
<td>BioShop ®</td>
</tr>
<tr>
<td>Hydrochloric Acid 1.0 N</td>
<td>BioShop ®</td>
</tr>
<tr>
<td>Isopropyl alcohol (ACS) (2-propanol)</td>
<td>EMD Chemicals Inc.</td>
</tr>
<tr>
<td>Methanol</td>
<td>Caledon</td>
</tr>
<tr>
<td>N,N-dimethylacetamide, anhydrous (99.8%)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>SiliaMetS® Cysteine Beads</td>
<td>SiliCylele</td>
</tr>
</tbody>
</table>
3.2.2 Synthesis Methods

3.2.2.1 Reaction Kinetics: Isocyanate Titrations

The kinetics of the AOU reaction were monitored following the procedure described by Sharifpour et al. (1). Briefly, approximately 1 ml of a dibutylamine (DBA) 1,2,4-trichlorobenzene (TCB) solution (1.3 M) was added into individual titration vials. 1 ml aliquots of the reaction mixture were added into the DBA/TCB solution to allow for the unreacted isocyanate groups to react with the DBA and produce a urea derivative (see Scheme 3.1). After at least one hour, 20 ml of methanol and three drops of bromophenol blue indicator were added to each vial after which the excess DBA was titrated using 0.1 N HCl. The relative isocyanate consumption was back-calculated using the following equation based off the amount of excess DBA remaining after the reaction.

\[
NCO \text{ Consumption} = \frac{[N_{HCl}]\left[M_{DBA,n} \frac{V_{HCl,C}}{M_{DBA,C}}\right] - V_{HCl,n}}{M_n[C_{NCO}]} \quad \text{Eq. 3.1}
\]

Where \(N_{HCl}\) is the normality of the HCl titrant (N), \(M_{DBA,n}\) is the mass of the DBA solution in the sample vial (g), \(V_{HCl,n}\) is the volume of the HCl used to reach equilibrium (L), \(M_n\) is the mass of the reaction aliquot (g), \(C_{NCO}\) is the isocyanate concentration of the aliquot (moles of NCO / total mass of reaction components), and \(V_{HCl,C}\) and \(M_{DBA,C}\) are the volume of HCl and mass of DBA solution respectively, used in the control titration vials with no reaction solution.

Scheme 3.1: The reaction of the partially reacted pre-polymer with excess DBA/TCB titration solution. Excess DBA is used to back calculate the free NCO concentration and the NCO conversion rate.
3.2.2.2  AOU Synthesis Method #1: 1 MPEG + 2 LDI + 3.3 PFA

In a nitrogen-purged glove box, distilled LDI was dissolved in dimethylacetamide (DMAc) in a round bottom flask to yield a 1 M solution, into which dibutyltin dilaurate (DBTDL) (0.5 wt. % relative to MPEG) was added. MPEG was dissolved in DMAc to yield a 0.1 M solution, analytically transferred into a separatory funnel and added dropwise to the LDI solution. This mixture was heated in a 40°C oil bath for one hour from the addition of DBTDL, then PFA was added directly by a syringe followed by a second addition of DBTDL (1 wt. % relative to PFA). The reaction vessel was passively cooled to room temperature and left to stir for an additional 22 hours. Reaction aliquots (~1 ml) were taken at various time points throughout the reaction to monitor NCO consumption (see Section 3.2.2.1)

3.2.2.3  AOU Purification Method #1a

Upon reaction completion, the DMAc was removed from the flask under reduced pressure using a rotary evaporator. The resulting product was dissolved in isopropyl-alcohol (IPA) to yield a 0.5 g/ml solution into which Hexane solvent (20x the volume of the IPA solution) was added. This mixture was stirred vigorously for four hours, after which the Hexane was removed and the resulting polymer rinsed with Hexane (12 ml/g product) three times. This process was repeated twice more (two hours stirring, three times rinse, one hour stirring, three times rinse). The final polymer was dissolved in distilled water at a concentration of 0.1 g/ml, sealed in a 1000 g/mol molecular weight cut off, regenerated cellulose dialysis membrane (Spectrum Labs, California), and dialyzed against distilled water (150 times volume of polymer-containing solution) for 24 hours. The dialysate was changed at two and four hours. The final solution was then lyophilized for 48 hours.

3.2.2.4  AOU Purification Method #1b

This method is the same as purification method #1, apart from the following changes which were made due to the results noticed after the first purification method. The IPA-AOU solution, described above, was placed in a separatory funnel and added dropwise into a beaker containing Hexane solvent (20x the volume of the IPA-AOU solution). The solution was vigorously stirred for four hours, after which the beaker was placed on ice and the Hexane supernatant solution was discarded. The remaining AOU was redissolved in IPA and the process was repeated with stirring cycles of two and one hours. The dialysis protocol followed that described in purification
method #1 however both the polymer and the dialysate solutions consisted of 50/50 (v/v) acetone and water.

3.2.2.5 AOU Synthesis Method #2: 1 MPEG + 1 LDI + n PFA

This method was first performed with n = 1, followed by n = 1.05, 1.1, 1.25.

MPEG was dissolved in DMAc to yield a 0.5 M solution and placed on ice (as will be explained in Section 3.3.3 MPEG was added from the beginning in order to help increase the reaction viscosity thereby promoting PFA-LDI formation as opposed to PFA-LDI-PFA). LDI was added directly to the flask followed by the dropwise addition of PFA (diluted three times by volume with DMAc). After PFA addition, DBTDL (0.7 wt. % LDI) was then added and the flask was placed in a 40°C oil bath and left to stir for two hours.

3.2.2.6 AOU Purification Method #2

Upon reaction completion, the DMAc was removed from the flask under reduced pressure using a rotary evaporator. Hexane solvent was then added to the AOU-containing flask (1.4 ml/g of AOU) and stirred vigorously for 30 minutes after which the Hexane solution was removed. This process was repeated 10 times. The resulting polymer was then dried under reduced pressure at 40°C overnight.

3.2.2.7 AOU Synthesis Method #3: Prepolymer Step 1 LDI + 1 PFA

In a nitrogen purged glove box, LDI was dissolved in DMAc in a round bottom flask to yield a 0.3 M solution (as explained in Section 3.3.3.3 a more dilute solution was chosen than for method #1 in order to better control the reaction and in order to obtain acceptable values for NCO titrations). PFA (diluted three times by volume with DMAc) was added dropwise to the LDI containing solution. Reaction aliquots were taken at various time points during the reaction in order to determine the point at which 50% of the NCO groups were consumed. After three hours the reaction was quenched with anhydrous methanol. Based on the results of this reaction, the identical reaction was performed however prior to the addition of PFA, DBTDL (0.7 wt. % relative to LDI) was added to the flask. The reaction was quenched with anhydrous methanol after three hours.
3.2.2.8 AOU Synthesis Method #3: 1 LDI + 1.25 PFA + 0.95 MPEG

The first step follows the exact process detailed above for the prepolymer step (Section 3.2.2.7). As determined from the prepolymer reaction, after one hour, MPEG dissolved in DMAc (0.6 M solution) was added directly to the flask. The reaction was placed in a 40ºC oil bath and left to stir overnight.

3.2.2.9 AOU Purification Method #3

This purification protocol is identical to that of purification method #1b, however the AOU-IPA solution was left to stir in the hexane solution for one hour. The hexane mixing cycle was repeated three times.

3.2.2.10 Residual Tin Catalyst Purification

AOU was dissolved in methanol at a concentration of 0.1 g/ml. 40 molar equivalents of SiliaMetS® Cysteine beads (SiliCycle, Quebec), relative to the measured elemental Tin content, were added into the solution. This number was calculated based off a measured Tin content (ICP – see Section 3.2.3.4) of 160 ppm post hexane purification. The solution was stirred vigorously for four hours and after four hours, the beads were centrifuged and removed. This process was repeated three times.

3.2.3 Characterization Methods

All characterization techniques were performed by the author unless specified otherwise.

3.2.3.1 Gel Permeation Chromatography

Samples were dissolved in dimethylformamide (DMF) at 2 mg/ml and 200 μl injection volumes were run on Styragel HR 3 DMF columns at a flow rate of 1 ml/minute and column temperature of 80ºC (Waters S10 HPLC Pump and Solvent Select Valve). Analytes were detected using a refractive index detector (Waters 2414), the spectra were generated using Empower Pro Software and processed on Microsoft Excel.

3.2.3.2 Nuclear Magnetic Resonance Spectroscopy (NMR)

Samples were dissolved in deuterated chloroform at 50 mg/ml, placed in 5mm Norelco NMR tubes and run on a Varian Mercury 400 MHz spectrometer (Department of Chemistry, University
of Toronto). The generated spectra were analyzed using MestReNova Version 8.1.4. Samples tested for Carbon-13 were run on a Agilent DD2-500 MHz spectrometer and analyzed using MestReNova Version 8.1.4.

3.2.3.3 Matrix Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI)

MALDI-MS was performed by Elna Luckham at The Biointerfaces Institute at McMaster University (Hamilton, Ontario). Samples were dried at 40ºC under reduced pressure (0.05 mm Hg) overnight prior to sending for analysis.

Saturated solutions of dithranol matrix and NaNO$_3$ were prepared in methanol. The polymers were prepared in methanol to a concentration of 10 mg/ml and the solutions were mixed in a ratio of 10:5:1 (matrix: polymer: salt). 0.5 µl were spotted on the MALDI target. Positive ion spectra were acquired in reflector mode on an Ultraflex MALDI-TOF mass spectrometer (Bruker, Bremen, Germany).

3.2.3.4 Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES)

ICP-AES was conducted using an Optima 7300 DV ICP-AES, and data was processed using ICP WinLab 32 (ANALEST Facility, University of Toronto). The following run parameters were used:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reading mode</td>
<td>Axial</td>
</tr>
<tr>
<td>Sample flow rate</td>
<td>1 ml/min</td>
</tr>
<tr>
<td>Sample flush time</td>
<td>30 seconds</td>
</tr>
<tr>
<td>Wash between samples (flow rate)</td>
<td>1.5 ml/min</td>
</tr>
<tr>
<td>Wash between samples (time)</td>
<td>60 seconds</td>
</tr>
<tr>
<td>Power</td>
<td>1500 W</td>
</tr>
<tr>
<td>Replicates</td>
<td>3</td>
</tr>
<tr>
<td>Calibration blank</td>
<td>1 N HCl</td>
</tr>
</tbody>
</table>

A Tin standard of 1000 µg/ml (in 1 N HCl) was obtained from the ANALEST facility (University of Toronto), from which diluted calibration standards were made (0.25, 0.5, 0.75, 1, 2 µg/ml.) A new calibration line was generated each time a group of samples were run. Samples, previously degassed under reduced pressure in a vacuum oven at 40°C overnight, were weighed out into a 15 ml falcon tube and diluted using 1 N HCl to obtain solutions of approximately 6 mg/ml (maintaining a minimum volume of 3 ml). Samples were sonicated for 45 minutes prior to analysis.

3.2.3.5 Water Content Analysis: Karl Fischer Titrations

Water content of reactants and reaction solvents was measured using a Mettler Toledo DL32 Karl Fischer Titrator. The sample beaker and electrodes were cleaned with methanol and AQUASTAR® CombiCoulomat solution, after which the sample beaker was filled with AQUASTAR® CombiCoulomat solution. Prior to each batch of sample runs, the titrator’s accuracy was tested using a 100 ppm known water standard (HYDRANAL® Water Standard, 0.1, Sigma Aldrich). Samples were added into the test-beaker using a syringe which was weighed before and after sample addition. The absolute value generated by the machine was converted to concentration (ppm) based on the known amount of sample added.
3.3 Results and Discussion

3.3.1 Background

As the AOU backbone molecule had been previously conceived in the Santerre Lab, a preliminary synthesis method had been developed. While this method did provide a strong basis for starting the current work, it also contained a number of issues which needed to be addressed. Most importantly, multiple by-products were generated during the reaction, making the purification difficult. Minimizing the side reactions, to generate the desired molecule, was the main focus behind varying the synthesis protocols. As mentioned, AOU consists of three groups; MPEG (one terminal CH$_3$, one terminal OH), LDI (two NCO groups), and PFA (one terminal OH, one terminal CF$_3$). Due to the similar reacting groups on many of the molecules, there are a number of combinations of molecules that can form:

a) AOU: MPEG-LDI-PFA (MW $\sim$1405 g/mol) (Figure 3.1)

\[
\begin{align*}
&\text{H}_3\text{C} - \text{O} - \text{CH}_3 - \text{O} - \text{CONH} - \text{N} - \text{CH}_2 - \text{O} - \text{CONH} - \text{N} - \text{CH}_2 - \text{O} - \text{CONH} - \text{N} - \text{CH}_2 - \text{O} - \text{CONH} - \text{N} - \text{CH}_2 - \text{O} - \text{CONH} - \text{N} - \text{CH}_2 - \text{O} - \text{CONH} - \text{N} - \text{CF}_3
\end{align*}
\]

**Figure 3.1:** Amphiphilic Oligourethane (AOU)

b) PEG-trimer: MPEG-LDI-MPEG (MW $\sim$1750 g/mol) (Figure 3.2)

\[
\begin{align*}
&\text{H}_3\text{C} - \text{O} - \text{CH}_3 - \text{O} - \text{CONH} - \text{N} - \text{CH}_2 - \text{O} - \text{CONH} - \text{N} - \text{CH}_2 - \text{O} - \text{CONH} - \text{N} - \text{CH}_2 - \text{O} - \text{CONH} - \text{N} - \text{CH}_2 - \text{O} - \text{CH}_3
\end{align*}
\]

**Figure 3.2:** PEG trimer – often formed during AOU synthesis
The formation of different trimeric species during syntheses with diisocyanates has been previously observed (2), however in the case of AOU, each of the three components is necessary in its correct location in order to provide the unique properties of fluorinated amphiphilic molecules, making non-AOU trimers completely unwanted. While the main goal was to only synthesize the desired product during the reaction, allowing the alternative products to form but removing them during purification was also considered as an option. This however was not easy since separation based on solubility only allowed the removal of PFA-trimer from the other two products (PFA-trimer is soluble in hexane solvent) while the molecular weights of AOU and PEG-trimer are too similar to allow for effective separation based on mass. Therefore, while the ideal scenario was to synthesize AOU exclusively, allowing for the presence of PFA-trimer could force a reduction in the amount of PEG trimer, and we would only have to deal with poor yields, as the PFA trimer itself could be readily separated from the desired product. While not efficient, this would achieve the desired product.

3.3.2 AOU Synthesis Method #1

This method was previously developed in the Santerre Lab (1 MPEG + 2 LDI + 3.3 PFA) (3). The MPEG was added dropwise into the excess LDI solution in order not to over-consume the NCO groups with MPEG, which would promote MPEG-trimer formation. After MPEG-LDI was formed, excess PFA was added in order to ideally cap each of the molecules, creating the final product. GPC profiles seemed to show a single product being formed, eluting in line with that synthesized by the previous student in the lab (Fig 3.4). $^1$H-NMR spectra, however did not indicate a single product, but rather multiple products. Furthermore, the results were not in line with those obtained by the previous student. Integration values of lysine protons were twice their
theoretical value and those corresponding to the MPEG protons were 20% above their theoretical values (Table 3.3 and Fig. 3.5).

Figure 3.4: GPC profile of product produced during synthesis method #1. As can be seen, there is a single distribution, and it lines up with that produced by a previous student

Table 3.3 Integration values for peaks in NMR spectra shown in Figure 3.5.

<table>
<thead>
<tr>
<th></th>
<th>k</th>
<th>j</th>
<th>h</th>
<th>g</th>
<th>f</th>
<th>e</th>
<th>d</th>
<th>c</th>
<th>b</th>
<th>a</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Theoretical</strong></td>
<td>3</td>
<td>1</td>
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In order to better understand the kinetics of the reaction and the rates of consumption, NCO titrations were performed on the reaction, both before and after PFA addition. Given the stoichiometry (1 MPEG : 2 LDI), at the end of the prepolymer stage when the desired product is MPEG-LDI-NCO, a 25% NCO consumption should be observed. The results however showed that 50% of the NCO groups were consumed even before the PFA was added (Figure 3.6). Since there were no additional reactants in the flask in order to consume the NCO, it was hypothesised that excess moisture (H₂O) was present in the flask, causing the extra consumption of NCO.

**Figure 3.5:** NMR Results of products formed during synthesis method #1. Table 3.3 above provides integration values for the peaks on the NMR spectra. a) Representative NMR spectra, and b) AOU structure with corresponding peaks labeled. Large peak h represent all MPEG CH₂ protons, while j represents the MPEG CH₂ alpha to the urethane bond.
Given that the LDI was distilled prior to use, and sealed anhydrous DMAc was being used, it was only possible that moisture was being introduced into the system via the MPEG. While the MPEG had been degassed under vacuum overnight prior to use, Karl Fischer titrations were performed on the MPEG in order to determine if the degassing process was effective. Results showed that even though the degassing process decreased the water content in the MPEG by 64% (8785 to 3138 ppm), the final value was by no means acceptable for a moisture sensitive reaction. Until this point the MPEG had been degassed at room temperature, therefore in order to increase the efficiency of the process, the MPEG was heated in a 40℃ oil bath, and resulted in the moisture content being reduced to 122 ppm. Using the updated degassing method, titrations were performed again, this time they showed that the theoretical 25% NCO consumption was achieved after the prepolymer stage (Figure 3.7).

Figure 3.6: Titration data representing NCO conversion. As can be seen, after one hour, 50% consumption is observed, twice the theoretical value. Error bars represent standard deviation, n=3.
In order to further improve the efficiency of the reaction, two changes were made to the purification protocol. Instead of adding the Hexane directly into the polymer-IPA solution, the polymer-IPA solution was added dropwise into the large hexane volume. This would increase the ability of the hexane to remove the excess PFA, PFA-trimer and residual DBTDL catalyst.

Furthermore, in order to prevent loss of product while removing the Hexane from the purified polymer, the entire beaker was placed on ice to solidify the precipitated polymer allowing for more effective removal of the impurity-containing Hexane solution.

Incorporating the updated degassing and purification protocols, a second batch of reactions were performed. While the $^1$H-NMR values were closer to the theoretical than before (Table 3.4), the integration values of the lysine proton peaks were still 1.5 times higher than theoretical. While these numbers matched those obtained by the previous graduate student project, they were not good enough to indicate that pure AOU had been formed. This conclusion was further validated after analyzing the samples with MALDI to observe the exact molecular weight distributions. As can be seen in Figure 3.8, three distinct distributions appear, corresponding to (from lowest to highest molecular weight) excess MPEG, AOU and PEG-trimer. This result shows that even

**Figure 3.7:** Titration data representing NCO conversion. As can be see after one hour the NCO consumption is in line with the 25% theoretical value. Error bars represent standard deviation, n=3, except for time = 4 hours represents n=2.
with excess LDI, given the reaction conditions, PEG-trimer is still formed resulting in a non-pure mixture of products.

At this point a side study, elaborated on in Appendix A, was undertaken to determine the molecular weight of MPEG. In summary the value previously used which was based off hydroxyl titrations (618 g/mol) was no longer used, rather a corrected value based off of NMR, MS and MALDI (750 g/mol) was used. This means that during synthesis method #1, the LDI:MPEG ratio was actually 2.5:1, further showing that excess LDI is not the solution to PEG-trimer prevention.

Table 3.4: NMR integration values showing LDI corresponding peaks (a,b,c,d) 1.5x their theoretical value

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**Figure 3.8**: MALDI data from product synthesized using method #1. 3 distinct distributions can be seen, from left to right: excess MPEG, AOU and PEG trimer. Peaks represent m/z data of single charged molecules with one sodium ion adduct. [M+Na]^+, where M is the molecule of interest.
3.3.3 AOU Synthesis Method #2

3.3.3.1 Initial Reaction Design

Due to PEG-trimer being formed in synthesis method #1, an alternative method was investigated. The reason PEG-trimer was being formed was due to the fact that the two reactants were added together in the same flask in the presence of heat and catalyst. There was no reason why an additional MPEG molecule would react with a free LDI as opposed to a reacted MPEG-LDI molecule. In order to overcome this problem it was hypothesised that the reaction could be performed in the opposite direction, that is to react PFA with LDI, and then add the MPEG. While the identical (but opposite) problem is evident since PFA-trimer would be formed, as explained earlier (Section 3.3.1) this alternative product is more easily removed thanks to its solubility in Hexane. As long as a sufficiently small amount of PFA-trimer is formed, the process could be considered a feasible approach at producing pure AOU.

Even though PFA-trimer can be removed, minimizing its formation from the start was still seen as a prime objective when designing the new reaction. When performing the reaction in solvent, as was shown in synthesis method #1, there was no preference given to bound verses unbound LDI molecules. It was hypothesised however, that by minimizing the amount of solvent being used, mass transfer would be controlled by diffusion as opposed to bulk flow. If diffusion would dominate, it would result in PFA-LDI, with its larger mass, having much lower diffusion rates compared to free LDI. Based on this logic, initial studies were done by adding pure PFA directly to pure LDI using various catalyst concentrations with no solvent. Regardless of the catalyst concentrations, all reactions became viscous gels within minutes, indicating the reactions had completed almost instantly. The following efforts were placed on slowing down the reaction while still allowing diffusion to dominate the molecular mass transfer.

3.3.3.2 Synthesis Results

In this method dilute PFA was added dropwise into a flask containing LDI and MPEG all in a 1:1:1 molar ratio (dissolved in a small amount of DMAc, 0.5 M) on ice. Since there was no catalyst and the flask was at ~0°C, it was assumed that only PFA and LDI would react (as it was known from synthesis method #1 that heat and catalyst were needed for MPEG and LDI to react). MPEG was placed in the flask from the start of the reaction, not only to allow for a fast
transfer from the first step (PFA + LDI) to the second step of the reaction (PFA-LDI + MPEG), but also to increase the viscosity of the entire reaction, further allowing diffusion to dominate.

\(^1\)H-NMR results from the new reaction method were very promising, with all peaks showing theoretical integration values (Figure 3.9). This however, was not surprising since each of the reactants had been added in equal ratios. MALDI spectra however, immediately showed that PEG-trimer was still being formed (Figure 3.10). This could only be explained by the fact that free LDI was being left over after the first step of the reaction, allowing both of LDI’s NCO groups to react with the MPEG in the second step. Excess PFA was added during the first step in order to overcome this problem, with the thought that less PEG-trimer would be formed, albeit at the expense of PFA-trimer. MALDI spectra of this reaction however again showed the presence of PEG-trimer (Figure 3.11).

![Figure 3.9: NMR spectra from reaction method #2 indicating close to theoretical integration values. Large peak h represents all MPEG CH\(_2\) protons, while j represents the MPEG CH\(_2\) alpha to the urethane bond.](image-url)
**Figure 3.10:** MALDI data from product synthesized using method #2. 2 distinct distributions can be seen, from left to right: AOU and PEG trimer. Peaks represent m/z data of [M+Na]+, where M is the molecule of interest.

**Figure 3.11:** MALDI data from product synthesized using method #2 with 25% excess PFA. 3 distinct distributions can be seen, from left to right: excess MPEG, AOU and PEG trimer. Peaks represent m/z data of [M+Na]+, where M is the molecule of interest.
3.3.3.3 PFA-LDI Investigation: NCO Titrations

As was done when problems arose during synthesis method #1, it was important to understand the kinetics of the reaction using NCO titrations. While the same reaction conditions used in synthesis method #2 could not be used due to the high LDI concentrations causing large errors during titration, a simplified dilute reaction – PFA (3x dilute in DMAc) added dropwise into a molar equivalent amount of dilute LDI on ice – was performed. In line with the initial PFA + LDI studies, it was assumed that 50% NCO conversion (consuming all the PFA) would be reached almost immediately, however as can be seen by the titration curves, even after one hour, only 7% of the NCOs were consumed (Figure 3.12).

![NCO Titrations: 1 LDI + 1 PFA [on ice]](image)

**Figure 3.12:** Titration data representing NCO conversion during a simplified LDI PFA reaction. After 1 hour, only 7% is reached as opposed to the expected 50%. Error bars represent standard deviation, n=2.

This observation provided a direct explanation as to why PEG-trimer was still being formed in synthesis method #2. PFA was not given enough time to react with LDI (since catalyst and MPEG were added immediately after PFA addition), resulting in a flask with unreacted PFA, LDI and MPEG in the presence of heat and catalyst. While there is not only one reason as to why the kinetics of PFA + LDI differed between the initial no-solvent studies and the actual full synthesis, it is likely that it was strongly due to the solvent that was used to dissolve the MPEG. Even though MPEG was used to increase the overall viscosity of the reaction, and only a small amount of solvent was used, this was enough to consider the reaction dilute and significantly slow down the rate. Overall, this showed that placing all the reactants together in a flask
produced a mixture of products not much different than that formed with the controlled steps used in synthesis method #1 (compare Figures 3.8 and 3.10, 3.11).

3.3.4 Synthesis Method #3

3.3.4.1 Initial Reaction Design

Building off the PFA-LDI titration data obtained above (Section 3.3.3.3), the next step was to increase the rate of NCO consumption, in order to determine the time that 50% NCO conversion was reached, and to add the MPEG. Titrations were performed on the same reaction as above, however this time at room temperature. As expected, the reaction proceeded much faster, however even after 2.5 hours, 50% conversion had not been reached (Figure 3.13a). While given the profile of the curve, the 50% mark would have been reached at approximately three hours, due to moisture constraints and the possibility of alternate LDI reactions, it was desired to increase the speed of this prepolymer reaction. DBTDL was added at the same concentration as used in the second step of method #2, and as can be seen in Figure 3.13b the reaction reached the 50% conversion rate at approximately one hour. As will be explained below (Section 3.3.4.2), an increased PFA:LDI ratio was used, which caused a 55% NCO conversion upon completion. Interestingly enough, this prepolymer reaction proceeded at the same rate as the initial prepolymer reaction performed in synthesis method #1. One hour was therefore decided as the time at which the concentrated MPEG would be added.

![Figure 3.13](image)

**Figure 3.13:** Titration data representing NCO conversion during prepolymer (PFA+LDI) studies. a) performed at room temperature with no catalyst and b) using the same conditions however with catalyst.
3.3.4.2 Synthesis Results

Based on the prepolymer titration data obtained in Section 3.3.4.1, a full reaction was performed using the following stoichiometry: 1 LDI + 1.1 PFA + 1.02 MPEG. Excess PFA was added in order to act as a buffer for the possible errors arising during MPEG addition. These could arise due to the incomplete addition of MPEG from the flask in which it was being dissolved, as well as due to the ambiguity that is associated with MPEG’s exact molecular weight (see Appendix A). The same logic explains the slight molar increase of MPEG. With this new stoichiometry, NCO titrations (Figure 3.14a) showed a ~50% NCO conversion at the 1 hour time point, as expected. NMR data also indicated a relatively pure AOU sample with all the peak integration values appearing within a few percentages of their theoretical values (Figure 3.14c). This result was further strengthened by the MALDI spectra, which indicated AOU with only a trace MPEG-trimer distribution (Figure 3.14b).

**Figure 3.14:** a) NCO titration, b) NMR and c) MALDI data for reactions performed using synthesis method 3. A relatively pure AOU is formed, with a slight bit of PEG-trimer still remaining. Large peak h represent all MPEG CH$_2$ protons, while j represents the MPEG CH$_2$ alpha to the urethane bond.
In order to be confident that further derivatization reactions could proceed uninhibited (see Chapter 4), it was the goal to completely remove PEG-trimer. In order to accomplish this, excess PFA was added and MPEG was slightly decreased. While this ratio would result in lower yields due to the sacrificial formation of PFA-trimer, it was still more desirable to form a pure product. The final ratio was set as 1.25 PFA, 1 LDI and 0.95 MPEG and all the characterization data was obtained from large batch reactions (12 g MPEG) in order to confirm the consistency (see Appendix B for an explanation regarding optimal MPEG addition time using a large scale reaction). While NMR data again indicated a relatively pure AOU (Figure 3.15), the most promising result was the demonstration of a MALDI spectra displaying AOU with no PEG-trimer (Figure 3.16), but rather only desired product and unreacted MPEG.

**Figure 3.15:** NMR spectra of AOU using the final stoichiometric method. Large peak h represent all MPEG CH$_2$ protons, while j represents the MPEG CH$_2$ alpha to the urethane bond. * indicates solvent related impurity.

**Figure 3.16:** MALDI spectra showing (from left to right), excess MPEG and pure AOU.
While it is clear that there was excess MPEG in the final product, due to the MALDI data’s qualitative nature, it is not possible to infer mass ratios based on peak intensities (which seem to indicate significantly more excess MPEG verses AOU). The difference in peak intensities can be affected by many factors such as sample homogeneity and differences in ionization efficiencies of the sample’s constituents (4).

To prove this point, a technique was used whereby trifluoroacetic anhydride (TFA) was added into the NMR tube containing the AOU, and allowed to react with the hydroxyl groups on the excess MPEG molecules. Due to TFA’s high electronegativity, the NMR proton signal on the α MPEG CH$_2$ would be shifted downfield. This provides a means to study the excess MPEG even while mixed with reacted MPEG (5). As shown in Figure 3.17, the integration value (~0.40) indicates approximately 20 mole % additional MPEG (which if calculated based on the total mixture is only 16 mole %). While it would be ideal to remove all excess molecules from AOU, this small amount of MPEG was deemed an acceptable amount in order to move onto the next steps of the project, especially since excess MPEG would not interfere with the determination of AOU’s potential cytotoxicity as PEG molecules have shown to be non-cytotoxic (6).

![Figure 3.17: Section of NMR spectra before and after TFA addition, showing the formation of the proton signal from the MPEG CH$_2$ alpha to the reacted TFA.](image)
Finally, in order to be deemed safe for cell work, according to studies performed by the previous graduate student, residual Tin levels had to be reduced to below 16 ppm (3). After Hexane solvent extractions, ICP-AES measured the Tin content in the sample to be ~160 ppm. Repeated runs of AOU on ICP-AES however, showed that the Cysteine scavenger bead protocol described above successfully removed residual Tin, with analysis consistently displaying tin contents below 10 ppm.

3.4 Conclusion

This chapter described in detail the process that went into optimizing the AOU synthesis. A preliminary synthesis method had previously been developed in the Santerre Lab, however it was observed that more than one product was being formed. From the GPC curves, through NMR analysis to MALDI where fine-tuned molecular weight observations could be made, it became clear that due to LDI’s ease of reacting via either NCO group and the similar reactivity’s of MPEG and PFA, non-AOU trimeric species were also being formed. The primary difficulty however, was the fact that the MPEG based trimer was too similar to AOU in molecular weight and solubility characteristics, making it essentially impossible to remove. An effort was therefore placed in trying to prevent its formation from the start.

The main design change came after realizing that if the reaction was performed in the opposite direction, the primary trimeric species would be PFA-trimer which, thanks to its solubility in hexane solvent, could be removed after the reaction. While a number of variations of this reaction were tested (varying temperature, catalyst concentrations, reaction times), the final method produced pure AOU, with no trimeric species being formed. While a slight amount of excess MPEG remained in the final product, this would not interfere with any further steps of the project. This AOU could now be used to complete the additional project objectives.
3.5 References


Chapter 4
Amphiphilic Oligourethane Synthesis and their Interactions with Smooth Muscle Cells

4.1 Forward

Understanding the interactions between drug delivery materials and their surrounding host environment is an important part in the overall development of tailored drug delivery coatings. Furthermore, understanding how a material’s molecular structure affects its associated cell response is important information that can be used to further develop and tailor the specific material depending on the given design criteria. Building off the synthesis of AOU presented in Chapter 3, this chapter outlines the synthesis of AOU (non ionic) and its derivatives; AOUH (anionic, hydrolyzed lysine center), AOU -6, -8 and -12 (non ionic, increasing hydrophobicity; 6, 8, and 12 carbon alkyl chains coupled to lysine center), followed by a detailed analysis into their interactions with in-vitro rat vascular smooth muscle cells. Apart from AOUH, all AOUs displayed some degree of toxicity, an effect that was hypothesized to be caused by their amphiphilic – and therefore surfactant like – properties. While at low and mid concentrations AOU was slightly toxic, at high concentrations, presumably above its critical micelle concentration (CMC), AOU was notably toxic, however even at the high concentration, AOU did not cause membrane solubilisation, an otherwise anticipated feature of non-fluorinated surfactants. Similar results were found with AOU6, however AOU8 and AOU12 at defined concentrations began to solubilize the membranes. AOU’s behaviour was explained by membrane intercalation reducing cellular metabolic activity, along with the low lytic potential of fluorinated surfactants, preventing membrane solubilisation. While the same explanation was given for AOU6, as the coupled hydrogenated chain increased in length – seemingly masking that of the fluorinated chain – AOU8 and AOU12 more closely resembled the behaviour of traditional surfactants, toxic and membrane-lytic. Competing anionic and hydrophilic segments seemed to reduce AOU’s propensity to aggregate in solution, yielding no detectable toxic effects, even at high concentrations. This is, to the author’s knowledge, the first report of cell interactions related to hybrid surfactants (surfactants that contain two hydrophobic tails – hydrogenated and fluorinated). The hypotheses presented in this study will be further analyzed with future experiments, and the results obtained will be used to further develop tailored non-toxic drug delivery coatings.
This chapter will be submitted for publication in the journal Biomacromolecules.

4.2 Introduction

The introduction of non-invasive medical devices has led to the development of biocompatible drug delivery coatings, which serve a dual role as a biocompatible interface, preventing undesired changes to the biological environment, as well as have the potential to deliver drug with controlled release rates (1). While the cardiovascular field has driven the research area of drug polymer conjugates as thin films on medical devices (2-4), tailored drug delivery coatings are required in numerous other fields of medicine (5), including gastrointestinal (6) and neural (7). Using cardiovascular restenosis as a model for design requirements, two primary challenges are noted with current coating formulations for thin film coatings on endovascular devices; premature drug release (8) and late-stent restenosis associated with polymer incompatibility with local tissues (9-12).

While the first-generation of polymers used in cardiovascular devices applied more traditional polymers (methacrylate based, poly-SIBS), a second-generation of devices have begun to incorporate fluorinated chemistries into their coatings (2, 12, 13) in order to resolve some of the blood compatibility challenges. Due to their unique intra- and inter-molecular characteristics, fluorinated molecules have extremely low interfacial energies (14) providing them with beneficial blood and protein interacting properties, reducing coagulation and protein denaturation, respectively, and protecting underlying structures (15). The introduction of fluorinated groups however can give a molecule complete or even partial surfactant-like character (14, 16, 17), warranting its biological safety to be tested.

In this study amphiphilic oligourethanes (AOU) were synthesized as a platform on which to develop unique thin film drug delivery coatings. Urethane segmented block chemistry provides an ideal chemical base from which to build such molecules since different segments can be incorporated in order to provide unique chemical features associated with blood compatibility, protein and drug interactions, solubility, physical character and degradability (15, 18). AOU chemistries consist of tri-segmented oligourethanes containing a mono-functional polyethylene glycol unit to enhance solubility in an aqueous base media and contribute to drug release kinetics, a diisocyanate (e.g. lysine diisocyanate (LDI)) central group to serve as a coupling site for additional functional groups, and a perfluoroalcohol (PFA) segment to generate a protein and
cell compatible fluorinated biological interface (19, 20). AOU was derivatized via the LDI moiety by hydrolysis of a pendant methyl ester (AOUH) and then subsequent reaction with a family of three hydrophobic segments with alkyl chains of different lengths (defined as AOU6, AOU8, and AOU12).

The interaction of the different AOU6s with in-vitro rat vascular smooth muscle cells – chosen as a cardiovascular model – was studied to determine if AOU structure-cell function differed across the different AOU6s, and to serve as a basis for further AOU development. The overall cellular response was measured using a metabolic activity assay and DNA analysis. Due to the inherent amphiphilicity of the molecules and their potential for interactions with cell membranes, the authors also carried out a lactate dehydrogenase (LDH) release assay – a known marker for cell membrane disruption (21, 22).
4.3 Experimental Section

4.3.1 Materials

Lysine Diisocyanate (LDI) (CHEMOS GmbH, Germany) was distilled under vacuum (0.05 mmHg) at 120°C prior to use to remove excess moisture and partially polymerized species from the monomers. Perfluoro-alcohol (PFA) (DuPont, USA) and Methoxypolyethylene glycol (MPEG) (avg. Mₙ ~750) (Sigma-Aldrich, Canada) were degassed under vacuum (0.05 mmHg) at 25°C and 80°C respectively, overnight prior to use to remove excess moisture. Molecular sieves, 4A 8-12 Mesh (Sigma-Aldrich, Canada) were activated at 300°C for 4 hours prior to use. Hexyl-, Octyl-, and Dodecyl-amine were purchased from Alfa Aesar and used as received. Acetone, Anhydrous 1,2,4 Trichlorobenzene (TCB), Anhydrous N,N-Dimethylacetamide (DMAc), Anhydrous N,N-Dimethylformamide (DMF), Dibutylamine (DBA), Bromophenol-blue 0.04 wt. % in H₂O, Dibutyltin Dilurate (DBTDL), Hexane, Methanol and Tert-Butanol were purchased from Sigma-Aldrich and used as received. Anhydrous methanol (EMD, Canada), Diisopropylcarbodiimide (DIC) (TCI Chemicals, USA), Hydrochloric Acid (1.0 N and 0.1 N) (BioShop, Canada), 1-Hyroxybenzotriazole (HOBt) (Anaspec, USA), Isopropyl-alcohol (IPA) (EMD, Canada), SiliaMet® Cysteine Beads (SiliCycle, Canada), Sodium Hydroxide (0.1 N) (NaOH) (BioShop, Canada) were used as received.

4.3.2 Non-Derivatized AOU Synthesis

AOU was synthesised using PFA, LDI and MPEG with molar ratios of 1.25, 1.00 and 0.95, respectively. In a controlled atmosphere glovebox under nitrogen gas, PFA (33 % v/v solution in DMAc) was added dropwise into LDI (0.3 M solution in DMAc) with DBTDL (0.7 wt. % LDI). The reaction flask was stirred continuously at 300 rpm for one hour at room temperature, at which time MPEG (0.6 M solution in DMAc) was added, the flask temperature was increased to 40°C and the reaction was left to progress overnight. Upon reaction completion and solvent removal using a rotary evaporator (Buchi, Switzerland) the raw product was dissolved in IPA (0.5 g/ml), dropped into a vigorously stirring solution of hexane for one hour to remove excess PFA and PFA-LDI-PFA trimer species. The precipitated product was recovered, redissolved in IPA and the process was repeated two more times. The final precipitated product was dissolved (0.1 g/ml in a 50% v/v solution of acetone), sealed in a 1000 g/mol molecular weight cut off, regenerated cellulose dialysis membrane (Spectrum Labs, California), and dialyzed against 50%
v/v solution of acetone (150 times volume of polymer-containing solution) for 24 hours to remove excess low-molecular weight impurities. The dialysate was changed at two and four hours. The acetone was removed and the resulting solution was lyophilized for 48 hours. In order to reduce the residual Tin content to below cytotoxic levels (23), 40 molar equivalents (to amount of elemental Tin in DBTDL added) of Cysteine beads were added to a solution of the final product (0.1 g/ml in Methanol) and stirred vigorously for four hours, at which time the saturated beads were replaced with fresh beads and the process was repeated twice more. The solvent was removed and the final product was dried under vacuum (0.05 mm Hg) at 40°C overnight.

4.3.3 AOUH Synthesis

AOUH was synthesized through hydrolysis of the methyl-ester moiety on the lysine group. 1.1 molar equivalents of 1 N NaOH (relative to AOU) was added to AOU (0.1 g/ml solution in 50% solution v/v Tert-butanol/water) and left to stir moderately for 18 hours at room temperature. Upon completion 1 N HCl (1.05 times amount of NaOH) was added to the flask and left to stir for five minutes to neutralize the solution. After removal of the solvent using a rotary evaporator (Buchi, Switzerland), the resulting product was dialyzed according to the protocol described above for the non-derivatized AOU, however both the polymer solution and dialysate were 100% distilled water. The final product was lyophilized for 48 hours.

4.3.4 AOUx Synthesis (x = 6,8,12)

AOU alkylated derivatives were synthesized by coupling one of either hexyl-, octyl-, dodecyl-amine to the carboxyl group on the lysine group of AOUH (optimization process detailed in Appendix C). For hexylamine coupling (AOU6); AOUH, DIC and HOBt with molar ratios of 1, 7.5 and 7.5 respectively were reacted. Molecular sieves (0.7g / g AOUH) were placed in the reaction flask containing the latter reagents, with anhydrous nitrogen air flowing through for five minutes. After five minutes, anhydrous DMF (30 ml/g AOUH) was added to the flask via a nitrogen-purged cannula and moderately stirred while purging with nitrogen in order to activate the carboxyl group. After 10 minutes, five molar equivalents of hexylamine were added via a nitrogen-purged syringe, the nitrogen flow was stopped and the reaction was sealed and left to stir for two days. Upon completion, the molecular sieves were removed through centrifugation and filter paper, the solvent was removed using a rotary evaporator and the resulting product was
dialyzed according to the protocol described for AOUH. The same process was performed for octyl- and dodecyl-amine, however the octlyamine reaction was dialyzed with a 50% v/v solution of acetone, and the dodecylamine was dialyzed with a 100% solution of acetone, due to their hydrophobic character.

4.3.5 Monitoring Reaction Kinetics

The reaction kinetics of the non-derivatized AOU synthesis was monitored using an isocyanate titration method to determine the required DBTDL concentration and the time at which 50% conversion was reached (in order to add the MPEG solution) (24). Briefly, 0.6 ml aliquots of the PFA-LDI reaction solution (with unreacted isocyanates) were mixed with 1 ml of a 1.3 M DBA/TCB solution, and left to react for one hour. Unreacted DBA (diluted with 20 ml of Methanol) was then titrated with 0.1 N HCl to determine the isocyanate consumption.

4.3.6 Chemical Characterization

4.3.6.1 Nuclear Magnetic Resonance (NMR) Spectroscopy

To confirm the structures of AOU and its derivatives (hydrolysis and amine-coupling), proton nuclear magnetic resonance spectroscopy (^1HNR), homonuclear correlation spectroscopy (COSY) and heteronuclear single quantum correlation spectroscopy (HSQC) experiments were performed on a Varian Mercury 400 MHz spectrometer (Department of Chemistry, University of Toronto). Samples were prepared in CDCl₃ at 50 mg/ml and run at room temperature. Peaks were characterized relative to a tetramethylsilane (TMS) reference.

4.3.6.2 Matrix Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS)

In order to characterize the molecular weight distribution of the starting non-derivatized AOU and confirm the removal of residual trimer by-products (MPEG-LDI-MPEG and PFA-LDI-PFA), samples were run on MALDI-MS (Biointerface Institute, McMaster University, Hamilton, Ontario). Saturated solutions of dithranol matrix and NaNO₃ were prepared in methanol. Samples were prepared in methanol (10 mg/ml) and the solutions were mixed in a ratio of 10:5:1 (matrix: polymer: salt). 0.5 µl was spotted on the MALDI target. Positive ion spectra were acquired in reflector mode on an Ultraflex MALDI-TOF mass spectrometer (Bruker, Bremen, Germany).
4.3.6.3 Induced Coupled Plasma Atomic Emission Spectrometry (ICP-AES)

Residual Tin content of AOU was measured using an (ICP-AES). Samples were prepared in 1 N HCl (6 mg/ml), sonicated for 45 minutes and run on an Optima 7300 DV ICP-AES (ANALEST, University of Toronto) detecting Sn (189.927 nm) with the following parameters; axial reading mode, 1 ml/min flow rate, 1500W power. Calibrations were performed using Tin standards (1000 µg/ml) (ANALEST, University of Toronto) diluted to 0.25, 0.5, 0.75, 1, 2, µg/ml). Triplicate data points were collected for all samples and calibrations.

4.3.7 Biological Characterization

4.3.7.1 Cell Culture and Sample Preparation

Cell culture experiments were performed using a rat thoracic artery-derived A10 vascular smooth muscle line (VSMC) (ATCC, CRL-1476). Cells were cultured in a 5% CO₂ environment at 37°C in Dulbecco’s Modified Eagle Medium (DMEM), supplemented with 10% and 2% (v/v) fetal bovine serum (FBS) and penicillin-streptomycin, respectively (Gibco Life Technologies, USA). Upon reaching approximately 80% confluence, cells were passaged using a 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) solution (v/v). Cells were always used between passage two and four to ensure consistency of cellular activity. 200 µl of cell suspension (125,000 cells/ml) were seeded in triplicate wells for each test and control group and incubated for 24 hours prior to exposure to samples.

Oligomer samples were dried under vacuum (0.05 mm Hg, 40°C) overnight prior to sample preparation. Samples were dissolved in supplemented DMEM at concentrations of 10, 1 and 0.1 mg/ml and sterile filtered through a 0.2 µm Acrodisc ® polyethersulfone syringe filter (Pall Life Sciences) prior to use for cell culture. Supplemented DMEM without sodium pyruvate (DMEM-SP) was used to prepare all samples for the LDH assay. All assays contained empty wells filled with respective media solution to measure background signals from growth media.

4.3.7.2 Metabolic Activity

Cellular metabolic activity was tested using a Water Soluble Tetrazolium-1 assay (WST-1) (Roche, Canada) (25). Dimethylsulfoxide (10 % solution (v/v) in supplemented media) (DMSO) (26) and supplemented media served as positive and negative controls respectively. After 24
hours of incubation with samples and controls, 110 µl of WST-1 (10% solution (v/v) in supplemented media) was added to the cells and incubated for one hour (colour change occurs). Plate was then read using a Bio-Rad Model 3550-UV absorbance reader at 450 nm and 690 nm for sample and background signals, respectively (Clokie Laboratory, Faculty of Dentistry, University of Toronto). Absorbance values from sample wells were normalized with respect to the negative control.

4.3.7.3 DNA Content

Total DNA content was measured using a cell lysis-DNA analysis technique (27). Controls were used as described for WST-1 assay. After 24 hours of incubation with samples and controls, cells were lysed with 100 µl of lysis buffer (0.05% Triton X-100, 50 mM EDTA in PBS) at 0ºC for 1 hour, followed by vigorous mechanical agitation (1.5 min/well) to ensure complete lysis. 100 µl of Hoescht dye (0.1% solution (v/v) in TNE buffer (10 mM Tris / 1 mM EDTA / 0.2 M NaCl in ddH2O) was added to the wells of a Black 96-well plate (MicroFluor-2, Thermo Scientific), followed by the addition of 10 µl of sample and control lysates per well. The plate was read against wells containing calf-thymus DNA standard (100-300 ng) into which 10 µl of lysis buffer was added in order to ensure consistent content between sample and calibration wells. All wells on the Black-96 well plate were performed in duplicates. The plate was then read using a fluorescence POLARstar Galaxy microplate reader (BMG Technologies, NC, USA) (Cvitkovitch Laboratory, Faculty of Dentistry, University of Toronto), using an excitation and emission wavelength of 360 and 460 nm respectively.

4.3.7.4 Membrane Lysis

In order to access the potential disruption of the cell membrane by the AOU molecules, intracellular lactate-dehydrogenase (LDH) release was measured (22) using a CytoTox ® Non Radioactive Cytotoxicity Assay Kit (Promega, Wisconsin). When seeding cells for the LDH assay, 6 sets of triplicate wells were seeded; 3 for low/medium/high concentrations of test sample, and 3 for the respective controls; positive, negative, and LDH natural release control. As above, growth media with no cells was added to one set of wells to measure the background growth media signal. Seeding protocol followed that described above followed by incubation in a 5% CO2 environment at 37ºC for 24 hours.
After 24 hours of cell culture, the media was discarded and wells were gently rinsed with supplemented DMEM-SP. Samples (200 µl solutions made in DMEM-SP as described above), were added to respective test wells, and 200 µl of DMEM-SP was added to the negative, positive, and background control wells. Triplicate wells of 200 µl of a 10% solution (v/v) ddH$_2$O in DMEM-SP (DMEM-H$_2$O) served as a natural-spontaneous LDH release control. The plate was incubated in a 5% CO$_2$ environment at 37ºC for 24 hours.

45 minutes prior to the 24-hour time point, 10 µl of lysis buffer (9% solution (v/v) Triton X-100 in PBS), 10 µl DMEM-H$_2$O and 10 µl of DMEM-SP was added to positive control (maximum LDH release), natural release control, and remaining sample and control wells, respectively. The plate was placed back in the incubator for a final 45 minutes after which the supernatant from each well was centrifuged (RCF 2100) for 10 minutes at 4ºC in a 500 µl eppendorf tube.

The following concentrations are based off an optimization study performed in order to obtain reliable absorption values, without oversaturating the assay (See Appendix D). In a new 96 well plate, 15 µl of solution from each eppendorf tube and 10 µl of DMEM-SP (with no FBS, as it affects the LDH release) was added into each well (each sample and control performed in duplicates). 50 µl of start solution (CytoTox ® 96 Reagent, Promega) was added into each well and left under dark conditions for 30 minutes, after which 50 µl of stop solution (1 M acetic acid) was added to each well. The plate was read on a Bio-Rad Model 3550-UV absorbance reader at 490 nm and 680 nm for sample and background signals, respectively (Clokie Laboratory, Faculty of Dentistry, University of Toronto). After correcting for background signals the percent cytotoxicity for each well was calculated as:

$$\% \text{Cytotoxicity} = \frac{\text{sample well average} - \text{natural release}}{\text{LDH maximum release} - \text{natural release}} \quad (Eq. 4.1)$$

where the values correspond to the absolute absorbance values.
4.3.8 Statistical Analysis

All cell characterization results were plotted using Microsoft Excel, and statistical difference between groups was measured using a one-way analysis of variance (ANOVA) and a post-hoc Student’s t-test using a significance level of $p < 0.05$ with a Bonferroni Correction (SPSS, IBM).
4.4 Results and Discussion

4.4.1 AOU Synthesis

Non-derivatized AOU (1) was synthesised as per Scheme 4.1 and its structure was confirmed by $^1$HNMR (Figure 4.1), COSY, HSQC and MALDI. Kinetic titrations confirmed 50% NCO consumption prior to MPEG addition and 100% consumption upon completion (see Figure 3.15a in Chapter 3). A combination of qualitative MALDI data (see Figure 3.16, in Chapter 3) (28) and a trifluoroacetic anhydride NMR technique (29) led to the confirmation that the final AOU contained ~15% unreacted MPEG (see Section 3.3.4.2 in Chapter 3). This residual MPEG resulted from the limitations in the dialysis purification but was deemed to have minimal influence on the subsequent derivatization reactions where amines would predominate the derivatization reaction over the residual hydroxyls from the MPEG molecules. As well, its presence would not be expected to have any downstream consequences on cell analysis as a result of the established biological safety of PEG molecules (30).

Scheme 4.1: Outline of AOU reaction; PFA + LDI + MPEG
4.4.2 AOU Hydrolysis

After optimizing reaction time and temperature, AOUH (2) was successfully synthesized through NaOH hydrolysis of the methyl ester to a carboxyl group on the lysine center group as per Scheme 4.2. Effectively complete hydrolysis was confirmed by $^1$HNMR with the disappearance of the CH$_3$ signal at 3.74ppm (Figure 4.2) (it is however difficult to calculate a hydrolysis efficiency value, as peak i is contained within the larger variable PEG signal).

Scheme 4.2: Outline of AOU hydrolysis to form AOUH

Figure 4.1: $^1$HNMR spectra of AOU. Integration with respect to peak a. Large peak h represent all MPEG CH$_2$ protons, while j represents the MPEG CH$_2$ alpha to the urethane bond. * indicates solvent related impurity.

Figure 4.2: $^1$HNMR spectra showing disappearance of CH$_3$ peak (i) on methyl ester after hydrolysis (*).
4.4.3 AOU Derivatives

Appendix C provides the full details of the different reactions undertaken for optimization of the coupling reaction. The use of DIC in conjunction with HOBt led to the successful coupling of hexyl-, octyl- and dodecyl-amine to AOUH (2) creating three new AOU-derivatives, AOU6 (3), AOU8 (4) and AOU12 (5), respectively as per Scheme 4.3. These structures were confirmed by $^1$HNMR by observing the downfield shift of the signal associated with the CH$_2$ alpha to the NH$_2$ on the unreacted coupling molecules from 2.68 to 3.14 ppm after amide-bond formation ($^1$H-NMR spectra shown in Figure 4.3). Monitoring peak z (CH$_3$ on coupled molecule) in Figure 4.3, determined a coupling efficiency of 86%.

Scheme 4.3: Outline of AOU coupling to create AOU6, 8 and 12
Figure 4.3: Top: NMR spectra characteristic of all AOU6,8,12. Bottom: Integrations of all relevant peaks. Peak $u$ indicates complete successful coupling while peak $\kappa$ changes based on the number of repeated CH$_2$ units on the coupled molecule. Large peak h represents all MPEG CH$_2$ protons, while j represents the MPEG CH$_2$ alpha to the urethane bond. * indicates solvent related impurity, shifted ~1ppm from that in Figure 4.1, due to varied interactions in different systems.
4.4.4 Interactions with VSMCs

The cytotoxicity of AOU, -H, -6, -8, and -12 was measured by exposing varying concentrations of dissolved samples to rat VSMCs for 24 hours after which the cells’ response was measured using 3 independent assays; WST-1 assay measuring metabolic activity, Hoescht DNA assay measuring total DNA content, and LDH measuring the release of intracellular LDH, indicating cell membrane disruption. The respective results are presented in Figures 4.4, 4.5 and 4.6.

Comparing Figures 4.4 and 4.5 show that in general the response of the cells was very consistent across metabolic activity and DNA content measurements with only two significant differences (AOU-10mg/ml and AOU6-0.1mg/ml). Discrepancies between these two methods have been noted by others (31), and while more accuracy is generally given to DNA measurements – due to the number of non-cytotoxic factors that can influence a cell’s metabolic activity – in this case equal veracity will be given to both due to their close agreement.

**Figure 4.4:** WST-1 results for all materials. GM = growth media control. N = 9-12 for all samples (3 or 4 experiments performed in triplicates). Error bars represent standard error. * = statistically different that GM, # = statistically different that 0.1 mg/ml, ** = statistically different than next lowest concentration (indicating a dose response where applicable).
Figure 4.5: Hoechst DNA Results for all materials. GM = growth media control. N = 9-12 for all samples (3 or 4 experiments performed in triplicates). Error bars represent standard error. * = statistically different that GM, # = statistically different that 0.1 mg/ml, ** = statistically different than next lowest concentration (indicating a dose response where applicable).

Figure 4.6: LDH results for all samples. GM = growth media control. N = 9-12 for all samples (3 or 4 experiments performed in triplicates). Error bars represent standard error. * = statistically different that GM, # = statistically different that 0.1 mg/ml, ** = statistically different than next lowest concentration (indicating a dose response where applicable).
4.4.4.1 AOU

Both WST and DNA measurements showed a marked decrease in VSMC metabolic activity and total DNA content, respectively, at concentrations at and above 1.0 mg/ml. As mentioned in the introduction, AOU is synthesized from three molecules – PEG, LDI, and PFA – all widely used non-toxic biocompatible components (15, 30, 32), and therefore while no individual component was anticipated to have an effect on cells at the established concentrations, their combination elicited a statistically significant cellular response. This combination effect becomes clearer after looking at AOU as a classical non-ionic surfactant composed of PEG and PFA – extremely hydrophilic and hydrophobic elements, respectively.

With possible exceptions (33), surfactants generally act most strongly on cell membranes above their critical micelle concentration (CMC) (34), a value dependant on the molecule’s structure (however can vary between different solutions (35)), and therefore the first step is to determine the possible range in which the AOU’s CMC occurs relative to each other. However, it must be pointed out that the LDH assay for AOU, AOUH and AOU6 all showed the absence of obvious cell membrane lysis, a result which will be discussed in detail below.

An underivatized AOU’s hydrophobic tail is a 6-carbon-long fluorinated chain, and its hydrophilic group is a repeated ethylene-oxide unit (~16 repeats) (with a lysine unit separating the two). Studying the effect of end-groups, Eastoe synthesised a non-ionic fluorinated surfactant with the structure F-(CF$_2$)$_6$-CH$_2$-O-(C$_2$H$_4$O)$_3$ (36) bearing the same fluorinated segment as AOU and the identical yet shorter hydrophilic chain. The CMC was calculated using tensiometry as 8x10$^{-5}$ M. Ravey, investigating the effect of fluorine and ethylene-oxide chain length on surfactant CMC, using the Wilhelmy Plate method found the CMCs of similar surfactants F-(CF$_2$)$_6$-(C$_2$H$_4$O)$_n$ (n=3,4,5,6) ranging between 10 and 25x10$^{-5}$ M (37), with the change in hydrophilicity causing only a small change in its CMC.

Comparing with data of non-fluorinated amphiphiles based on polymers containing MPEG, Shin synthesised MPEG-Polycaprolactone (PCL) surfactants with a constant MPEG portion (MW 5000) and a range of PCL units (ranging from n= 50-100); the CMCs ranged from 3.47 x 10$^{-7}$ M to 0.63 x10$^{-7}$ M respectively, decreasing with increasing hydrophobic chain length (38). Astafieva, also working with polymeric surfactants (polystyrene-poly-tert-butyl acrylate), changing the lengths of each portion, again found a decrease in CMC with increased
hydrophobic length on a large scale ($10^{-5} - 10^{-8}$ M), while the increase in the hydrophilic chain only caused small changes in the CMC (39). As conflicting reports have been given regarding the effect of the hydrophilic portion on the CMC (37, 39), some have noted that the hydrophilic portion does not significantly influence the onset point of micellization (38).

Therefore it is clear that choosing the correct model to compare CMC values depends almost completely on the hydrophobic portion of the surfactant. Additionally, fluorinated surfactants have CMCs that are on the same order of magnitude as those of their hydrogenated counterparts with 50% longer chains, and it has been shown that each additional CF$_2$ group on a chain has a ~1.6 times the micellization potential compared to that of CH$_2$ (40). Therefore, AOU (with (CF$_2$)$_6$) – at least for CMC purposes – could be compared to a hydrogenated counterpart consisting of (CH$_2$)$_{10}$. Prete reported the CMC of (CH$_2$)$_{10}$-(CH$_2$CH$_2$O)$_8$ as being on the order of 9.7x10$^{-4}$ M (41).

Although the CMC of an AOU may be expected to be slightly increased due to the methyl-ester side chain on the lysine which could inhibit effective molecular aggregation, it is conceived that the CMC of AOU is within the range described by Prete. Comparing those numbers with those found in Table 4.1 (AOU concentrations given in mol/L) and matching those against the WST-1 and DNA results in Figures 4.4 and 4.5, it is thought that at 0.1 mg/ml, AOU is most likely below its CMC and therefore has no significant effect on the cells. At 1.0 and 10 mg/ml, at or above its anticipated CMC, as suspected a significant effect is observed. The question then arises as to why no LDH was released – a classic marker for cell toxicity caused by membrane lysis (21, 22).

**Table 4.1:** Values of sample concentrations incubated with VSMC converted to mol/L to match reported CMC values in the literature.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>0.1 mg/ml (M)</th>
<th>1.0 mg/ml (M)</th>
<th>10 mg/ml (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AOU</td>
<td>7.35 x 10$^{-5}$</td>
<td>7.35 x 10$^{-4}$</td>
<td>7.35 x 10$^{-3}$</td>
</tr>
<tr>
<td>AOUH</td>
<td>7.43 x 10$^{-5}$</td>
<td>7.43 x 10$^{-4}$</td>
<td>7.43 x 10$^{-3}$</td>
</tr>
<tr>
<td>AOU6</td>
<td>6.91 x 10$^{-5}$</td>
<td>6.91 x 10$^{-4}$</td>
<td>6.91 x 10$^{-3}$</td>
</tr>
<tr>
<td>AOU8</td>
<td>6.78 x 10$^{-5}$</td>
<td>6.78 x 10$^{-4}$</td>
<td>6.78 x 10$^{-3}$</td>
</tr>
<tr>
<td>AOU12</td>
<td>6.53 x 10$^{-5}$</td>
<td>6.53 x 10$^{-4}$</td>
<td>6.53 x 10$^{-3}$</td>
</tr>
</tbody>
</table>
A two-part hypothesis is presented. Parteraroyo, studying the effects of Triton X-100 on melanoma cells found a similar trend as was observed with AOU (42). Cellular metabolic activity was reduced by 20-50% prior to the release of soluble-proteins, always at concentrations below the CMC of the surfactant under study. This was explained by the intercalation of the surfactant preventing effective membrane electrophoretic activity, a key component in the metabolic activity of a cell. Above the CMC however, standard membrane solubilisation occurred. This is in line with proposed theories of membrane lysis, where through osmotic lysis, surfactant monomers are the key players, while during solubilisation, surfactant aggregates creating mixed micelles with membrane proteins play the most significant role (43, 44). While this can explain the effect observed with AOU at low concentrations, it seems that at least at the highest concentration, 10 mg/ml – seemingly far above its proposed CMC – an additional factor is involved.

Due to their widespread usage in red-blood cell lysis for medical diagnostic purposes, surfactants are very often studied using erythrocytes. Comparing the haemolytic activity of hydrogenated and fluorinated surfactants, Riess observed a significantly lower (10 – 100 times) effect with fluorinated surfactants, and furthermore, haemolytic activity was shown to decrease as fluorine chain length increased (45, 46). It was proposed that the low affinity of the fluorocarbon chains for non-fluorinated materials (phospholipid membranes) is the cause behind this reduced activity (46, 47).

Therefore, it seems that the effect of AOU on VSMCs’ metabolic activity and DNA content is a result of two independent factors; its CMC and its fluorinated hydrophobic chain. Below its expected CMC, minimal effect on cellular activity is observed. At and above its expected CMC, while individual monomers or small aggregates may intercalate the cell membrane and possibly disrupt cross-membrane function reducing cellular activity, the low affinity of the potential fluorinated micelles for the cell membrane prevents membrane solubilisation and complete lysis, explaining the lack of LDH release shown in Figure 4.6.
4.4.4.2 AOU 6, 8, 12

WST-1 and DNA results both show a consistent decrease in metabolic activity and DNA content, respectively, as the coupled alkyl chain length increases (Figures 4.4 and 4.5). While AOU6 behaves in an almost identical manner to AOU, AOU8 and 12 are significantly more cytotoxic. This is further reflected by LDH results whereby no LDH is released by AOU6, yet significant amounts of LDH are released at the high concentrations of AOU8 and mid and high concentrations of AOU12, indicating not only reduced activity, but true membrane solubilisation and cell lysis. As was done with above with AOU, a chemical-structure verses cell-function relationship will be considered.

The coupling of a hydrogenated alkyl chain to the existing AOU oligomer has created what is now referred to as a hybrid surfactant, containing two individual hydrophobic chains (fluorinated and hydrocarbon), apart from a hydrophilic head group (16). While these surfactants have been studied due to their ability to co-solubilize hydrocarbon and fluorocarbon components – important in many industrial applications (17) – no studies have looked at their effects on cell toxicity or the stability of cell membranes.

The closest comparison to this dual fluorinated/hydrogenated surfactant (with toxicity data) is Courrier’s ‘semi-fluorinated surfactant’ composed of a dimorpholinophosphate (DMP) polar head group and semifluorinated hydrophobic tails (nCH₂-mCF₂) referred to as FnHmDMPs (n: 4-10, m: 2-11) (48). Testing their effect on mouse fibroblasts and human epithelial cells, it was found that below 0.1 wt. % (once corrected for MW, is within the range tested with AOUs), the surfactants were only non-cytotoxic when the fluorinated chain was above 4-carbons in length. The shielding effect of the fluorinated portion was proven by comparing it with a surfactant of identical carbon chain length – however fully hydrogenated – which was found to be highly toxic. The authors proposed that the decrease in toxicity was caused by the fluorocarbons’ ability to dissolve large amounts of gasses, thereby delivering oxygen to cells, improving their growth (48, 49), however the low lytic potential of fluorinated surfactants discussed above likely also played a role.

Translating the protective ability of fluorinated over hydrogenated chains to AOUs gives new insight into the cytotoxic behaviour of hybrid surfactants. Even though in general dual-tailed (not necessarily hybrid) surfactants are more likely to yield lower CMCs (17), with regard to their
interactions with the cell membranes, each tail acts independently. Recalling that one CF$_2$ is equivalent to ~1.6 CH$_2$ with regard to micellization, as well as fluorine’s extremely low intermolecular forces, it is clear that the fluorinated chain, (CF$_2$)$_6$, in AOU6 is more dominant than the coupled hydrogenated tail (CH$_2$)$_6$, thereby enabling AOU6 to behave in an almost identical manner to AOU. Even with AOU8 (whose (CH$_2$)$_8$ is equivalent to (CF$_2$)$_6$), at low and mid concentrations the oligomer still behaves similarly to AOU and AOU6. However, at high concentrations, and presumably above its CMC, especially due to its dual-tailed structure – the surfactant begins to solubilize the membrane (see LDH release in Figure 4.6), and its protective fluorine chain becomes less significant.

This point is made extremely clear when looking at AOU12, which showed significant toxicity even at low concentrations as well as substantial LDH release and membrane solubilisation at mid and high concentrations. Not only does the hydrogenated tail have a more significant micellization potential than the fluorinated segment (even after the CH$_2$:CF$_2$ correction), but due to its length it is likely to interact with a cell membrane before the shorter fluorinated segments. Compiling these factors, it is clear that the hydrogenated tail on AOU12 dictates its interactions with the cells and influences cytotoxicity, causing it to behave as a traditional surfactant with a long hydrocarbon tail – cytotoxic and membrane-lytic (44, 48, 50).

4.4.4.3 AOUH

Unlike all the other AOU derivatives, AOUH yielded a significantly different cellular response, inducing no change in metabolic activity (Figure 4.4), DNA content (Figure 4.5) and showing no sign of membrane disruption (Figure 4.6). In order to explain this peculiar result it is necessary to analyze AOUH as an anionic surfactant with a hydrophobic (specifically fluorinated) and hydrophilic tail. While typical anionic surfactants such as sodium dodecyl sulphate (SDS), form assemblies and micelles due to their anionic head groups and hydrophobic tails, and non-ionic surfactants such as Triton-X form assemblies due to their hydrophilic and hydrophobic regions, AOUH has characteristics of both; hydrophilic and anionic. Determining the surfactant’s assembly structure – and associated function – is therefore not straightforward.

A number of studies have looked at anionic surfactants with oxyethylene units inserted in between the anionic and hydrophobic regions – (anionic)-(oxyethylene)-(hydrophobic) (51-54). In general, an increase in the oxyethylene region leads to a decrease in CMC, and inserting more
than 3 oxyethylene units has been shown to dictate the surfactant’s properties, causing it to behave more like a non-ionic surfactant (52). While it is possible that the ~16 oxyethylene units in AOUH do play a significant role in its overall behaviour, it is important to note that unlike the aforementioned anionic surfactants, the ionic group in AOUH is placed in between the hydrophobic and hydrophilic regions.

Lijima synthesised a similar surfactant to AOUH, containing an anionic headgroup (OP(OH)\(_2\)O) with a 12 unit PEG hydrophilic chain and a 12 carbon alkyl hydrophobic chain, however since its purpose was to stabilize TiO\(_2\) nanoparticles, no cell data was presented with respect to the pure surfactant (55). Sagisaka however, also created an anionic hybrid surfactant, containing an anionic headgroup (sulfonate and carbonyl moieties) with 2 tails; a fluorinated hydrophobic tail (\(\text{CH}_2\)\(_2\)(\(\text{CF}_2\))\(_2\)CF\(_3\)), and an alkyl chain with inserted oxyethylene units (\(\text{CH}_2\text{CH(OH)O}(\text{CH}_2\text{CH}_3\)\(_3\)). Since the surfactant was designed to form a quasi-ion channel bilayer (as drug delivery capsules), they were only tested at very high concentrations, 10-30 wt.%, far above that studied for typical micelles. However, even at those extreme concentrations, the authors noticed two key points. At temperatures below 40ºC, the oxyethylene units became hydrated, loosening their packing and distorting the aggregates. Furthermore, since two isomers were present, once hydrated, the oxyethylene chain would be able to orient away from the fluorinated tail, ridding the molecule of its surfactant-micelle like character. In a very recent study, Sagisaka confirmed that this surfactant only began to form aggregates in water above 10 wt. % (57), 10 times above the highest concentration studied with AOUH.

It is therefore clear that due to the presence of a long hydrophilic tail, coupled with the competing anionic headgroup, AOUH does not aggregate in solution. AOUH must therefore be treated as a monomer with 3 individual units (extremely hydrophilic, anionic and fluorinated), each of which is non-compatible with negatively charged cellular lipid membranes. While the fluorinated section is hydrophobic, in general – as explained above – fluorinated surfactants (even in aggregated form) have a much lower lytic potential than hydrogenated counterparts due to its dissimilarity with a standard phospholipid membranes, a key requirement for membrane permeability (58).
4.5 Conclusion

This work introduced AOU as a platform of carrier oligomers on which to further develop tailored conjugates for drugs, proteins and other biologically active molecules to coat the surfaces of medical devices. In total, 3 groups of AOUs were synthesised, non-ionic (AOU), anionic (AOUH), and non-ionic-hydrophobic (AOU6,8,12). Interactions with VSMCs were monitored using metabolic activity, DNA content and membrane stability assays. Understanding AOUs’ surfactant-like properties allowed for a strong correlation between the individual AOU’s structure and its associated affect on VSMCs to be made. AOU alone was shown to reduce cellular activity at high concentrations, however even above its expected CMC (based on existing models), it did not solubilize the cell membranes – a result consistent with the low lytic potential of fluorinated surfactants. Increasing the length of the coupled alkyl chain led to increased cytotoxic effects and ultimately, cell lysis with AOU 8 and 12. This is the first study to report on the effect of hybrid surfactants with cell membranes, and it showed that the length of the hydrogenated tail dictated the surfactant’s properties, and at a point removed the protective ability (with respect to cell interactions) of the fluorinated tail. The introduction of an anionic group in between the hydrophilic and hydrophobic segments significantly altered the cell response relative to the non-anionic oligomers, causing no decrease in cell activity, even at elevated concentrations. This was explained by the competing hydrophilic and ionic portions potentially inhibiting micellization or even aggregation in solution at the concentrations studied.

This study indicates the ability for atomic-level changes on an oligomeric molecule to elicit a significantly different cell response. Future work will look at developing additional AOU formulations as well as studying AOU-drug interactions, in order to tailor the AOU to enhance binding to specific drugs depending on the needed application.
4.6 References


Chapter 5
AOU-Drug Binding Studies

5.1 Introduction

5.1.1 Drug Incorporation

Drugs can be incorporated into polymer matrices in a number of ways. The most basic, yet widely used approach is to mix the polymer and drug together in a specific ratio and coat the mixture onto a surface. Drug release from these coatings or reservoirs is controlled by diffusion of the drug through the polymer (1). Due to its simplicity it is no surprise that this method was used in the first generation drug loaded stents (e.g. Taxus ® stent from Boston Scientific). Adding a polymer top-coat (or membrane) adds an additional level of control, especially preventing a burst release. This method was incorporated in the Cypher ® stent (2, 3). Drugs can also be covalently bound to polymer matrices requiring chemical degradation – through hydrolysis or enzymes – to release the drug. Biodegradable polymers as drug carriers bear properties of both aforementioned systems. Though drugs are non-covalently incorporated, drug release is largely controlled by the degradation of the carrier matrix (4).

All drug coated arterial devices reported on in Chapter 2, incorporated specific drugs non-covalently. The release of drug from a polymer matrix is influenced by multiple physical parameters including solubility of the polymer in the delivery environment, the polymer’s glass transition temperature (T_g) (lower T_g, faster release), molecular weight (MW) (lower MW, faster release) and crystallinity (lower crystallinity, faster release) (5), as well as the polymer-drug interactions on a molecular level (6). The latter is particularly important for AOUs given that solubility in an aqueous media is a design feature and hence, understanding the potential for the drug to remain complexed or disassociated once the carrier complex is dissolved in media is of value in terms of knowing if the drug is available for interaction with its physiological substrate. The following section describes two methods – Isothermal Titration Calorimetry (ITC) and NMR Titrations – that were considered for use in studying the molecular interactions between AOU and a model drug, C6-Ceramide, with the view of determining a structure-function relationship between the specific AOU and its associated binding with C6-Ceramide. This particular drug has polar group and long aliphatic chains that could associate with either of the polar urethane or alkyl chain binding motifs that were incorporated into the AOU.
5.1.2 ITC for Monitoring Drug Binding

Molecular interactions can be defined by the thermodynamics of the association/disassociation process. The change in enthalpy that occurs during an interaction is reflective of the type and strength of the interaction. Isothermal titration calorimetry (ITC) is a simple, yet powerful method to measure interaction enthalpies, molar ratios of interacting species, binding constants (k) and reaction rates for a range of molecular interactions. Modern ITCs are referred to as micro- and nano-calorimeters in that they can detect heat changes in the micro- and nano-watt ranges, and therefore have the ability of detecting dissociation constants down to the $10^{-8}$ to $10^{-9}$ M level (7).

An ITC consists of two identical cells, a reaction or calorimetric cell in which one of the binding molecules is dissolved, and a reference cell, which contains water or another solvent. Molecule A, in solution, is titrated into the reaction cell (containing molecule B), and depending on the nature of the interaction – endothermic or exothermic – external power is applied or removed from the cell in order to keep the temperature exactly the same as that in the reference cell. During the first injection, molecule A fully interacts with the free molecule B releasing or absorbing a certain amount of heat. During subsequent injections, less of molecule B is available for interaction, therefore resulting in constantly lower heats of interaction (exo- or endo-thermic). Once molecule B is fully saturated with A, no further thermodynamic changes occur. The measured heat values can be integrated with respect to time and plotted against molar ratios. If one converts standard equilibrium values for two molecules A and B, such as defined below:

$$[A] + [B] \xrightleftharpoons{}^{k_d} [AB]$$

into thermodynamic parameters ($G =$ Gibbs Free Energy, $R =$ Gas Constant) using the following relationship

$$\Delta G = \Delta G^o + RT ln \left( \frac{[A \cdot B]}{[AB]} \right) = RTln(K_d)$$

it is possible to establish models to yield binding and dissociation constants, and in certain cases even the free energy ($\Delta G$) and entropy ($\Delta S$) can be calculated (7-9). While ITC is traditionally employed to study biological interactions such as proteins, enzymes and drugs (7) it has more
recently been used to study interactions of synthetic-biological molecules (10, 11), including polymer-drug interactions for the development of drug carriers (12-15). It is the latter scenario, which is used in this study to measure the binding properties between the lipid drug C6-Ceramide and AOU-based drug carriers.

5.1.3 NMR for Monitoring Drug Binding

Chemical shift, relaxation times and line widths obtained during an NMR experiment are precise measurements that reflect an atom’s chemical environment. Therefore, during the interaction of a protein and ligand, drug and ligand, drug and carrier, or any two molecules, the chemical environment surrounding an atom on one of the molecules will be affected, thereby changing the measured values. Using sensitive probes, NMR can monitor interactions with a large range of affinities ($10^{-9}$ - $10^{-3}$ M), and yield information about which part of the molecules are interacting. NMR is therefore widely used to measure intermolecular interactions to understand the regulation of biological processes (protein-ligand), and is now being used as a screening technique for drug development (drug-protein) (16-18). While numerous protein-binding-NMR-techniques exist, from Magnetization Transfer Rates which yield information about the kinetics of the interaction, to the Nuclear Overhauser Effect (NOE) which provides precise information regarding intermolecular distances, the most fundamental and widely used approach is chemical shift mapping or titrations which exploit the change in a chemical shift between bound and unbound species in order to measure binding strength (16). The basis of this method is provided below using the interaction between a drug (D) and a macromolecule or drug carrier (M) as an example, based off the works of Craik, Wilce (19) and Lian (20).

The first step when studying molecular interactions using NMR is to determine the time scale of the specific parameters. When a drug interacts with another molecule, an exchange equilibrium is created

$$[D] + [M] \rightleftharpoons [DM] \quad (Eq. 5.1)$$

where $k_d$ is defined as

$$K_d = \frac{[D][M]}{[DM]} = \frac{k_{off}}{k_{on}} \quad (Eq. 5.2)$$
with \( k_{on} \) being the rate of association of drug and molecule and \( k_{off} \) being the rate of disassociation of drug from the drug-molecule complex. From these values, a time constant \( 1/\tau \), is calculated (where \( \tau \) is a single exchange lifetime) to represent the lifetime of an interaction state. When \( 1/\tau \) is much slower than the chemical shift separation, the bound event is relatively long (on an NMR time-scale) and slow-exchange is occurring. These interactions correspond to tight binding with \( k_d \) values in the region of \( 10^{-9} \) M. As the event is slower than the NMR chemical shift separation and relaxation rates, two distinct peaks appear on the spectra corresponding to the bound and unbound state. In the opposite extreme when \( 1/\tau \) is much faster than the chemical shift separation and relaxation time, the system is in fast exchange representing relatively weaker binding states, on the order on \( 10^{-3} \) M. In this case, a single resonance is observed and represents a weighted average of the bound and unbound states. A drug-related signal for example, is therefore defined as

\[
\delta_{obs} = \delta_D \rho_D + \delta_{DM} \rho_{DM} \quad (Eq. 5.3)
\]

where \( \delta_{obs} \) is the observed chemical shift and \( \rho_D \) and \( \rho_{DM} \) are the molar ratios of the unbound and bound drug respectively, with their theoretical unbound and bound chemical shifts \( \delta_D \) and \( \delta_{DM} \), respectively. With intermediate exchange, the rate of exchange between bound and unbound molecules is similar to the NMR exchange rates. In these cases the spectral lines become very broad with no simple method of analysis. The drug-molecule system used in this study relates to a fast-exchange interaction, which will be assumed from here on.

Chemical shift mapping or titration consists of testing a series of solutions in which the concentration of one molecule is held constant while the concentration of the second is gradually increased. When the ratio of D:M is 1:0, the signal represents D in its unbound state. As M is added in, the signal shifts to represent an average resonance of D and DM. Eventually the signal begins to remain constant after binding has reached saturation. From this data it is possible to obtain the dissociation constant for the interaction. Combining Equations 5.2 and 5.3 above leads to the following derivation.

\[
K_d = \frac{[D][M]}{[DM]} = \frac{([D] - [DM])([M] - [DM])}{[DM]} \quad (Eq. 5.4)
\]
\[ K_d[DM] = [D][M] - [D][DM] - [M][DM] + [DM]^2 \quad (Eq. 5.5) \]

\[ \delta_{obs} \propto [DM] = \frac{(K_d + [D] + [M]) - \sqrt{(K_d + [D] + [M])^2 - 4[D][M]}}{2[D]} \quad (Eq. 5.6) \]

As mentioned, this technique and others have been used in studying protein-ligand and more recently in the study of drug-protein interactions. While fewer in number, some studies have used these techniques to investigate drug-polymer interaction for the development of customized drug carriers, for example poly(amidoamine) dendrimers as heparin delivery vehicles (21) and corn-derived zein proteins to deliver amoxicillin (22). The current study employs a \(^1\)HNMR titration technique to measure the binding affinity of the lipid drug C6-Ceramide to AOU and AOU-derived drug carriers.

### 5.2 Materials and Method

#### 5.2.1 ITC

ITC experiments were run on a TAM III multi channel calorimeter (TA Instruments) and the data was processed using TAM Assistant software (BioZone, University of Toronto).

After rigorously cleaning the ITC using ethanol and distilled water and sealing the ampules with vacuum grease to prevent solvent evaporation, the AOUx sample was dissolved in anhydrous ethanol to yield a 40 mM solution, and 800 µl was placed in the sample ampule equipped with a gold stirrer (set at 50 rpm). C6-Ceramide was dissolved in anhydrous ethanol to yield a 12.5 mM solution and transferred into a 250 µl cannula glass syringe, which was placed in a 3810 automatic syringe pump (TA Instruments). 26 injections of C6-Ceramide, each with a volume of 10 µl, were injected directly into the AOU-containing ampule (1 µl/second, eight minute recovery time after each injection). The data was processed using the TAM Assistant Software.

#### 5.2.2 C6-Ceramide NMR Characterization

Before monitoring drug binding using NMR, it was necessary to fully characterize the C6-Ceramide spectra in order to understand which specific peaks were being monitored. 1D \(^1\)H and
$^{13}$C and 2D HSQC ($^{13}$C-$^1$H) and COSY ($^1$H-$^1$H) NMR experiments were run on a Varian Mercury 400 MHz spectrometer (Department of Chemistry, University of Toronto). The generated spectra were analyzed using MestReNova Version 8.1.4.

5.2.3 $^1$HNMR Titrations

Sample solutions were made by first preparing concentrated solutions (approximately 500 mM) of respective AOU sample in deuterated chloroform. Dilute solutions were made from the one concentrated sample in order not to propagate the error due to the volume of the sample itself. Depending on sample viscosity, in order to prevent error, sometimes a more dilute stock sample was used to generate the very dilute solutions. In full experiments, a complete set of AOU samples consisting of 500, 400, 300, 200, 100, 75, 50, 25, 10, 1, 0.1 mM solutions were used. A 10 mM solution of C6-Ceramide was also prepared in deuterated chloroform.

150 µl of each solution (10 mM C6-Ceramide, and respective concentration of sample solution) were added into a 4 ml glass vial and vortexed for 10 seconds. The mixed solution was transferred into a 3 mm Norelco NMR tube using a syringe. Samples were run on an Agilent DD2-700 MHz NMR spectrometer (Department of Chemistry, University of Toronto) and data was analyzed using MestReNova Version 8.1.4.

All spectra were normalized to the characteristic chloroform singlet at 7.260 ppm. Individual peaks were monitored for the change in chemical shift and were normalized to range between 0 and 1. These values were plotted against the concentration of polymer sample in the respective tube. A 1:1 binding curve was fitted to the plotted points with a non-linear regression using CurveExpert Professional Version 2.20 and Equation 5.6.

$$
\delta_{obs} \propto [DM] = \frac{(K_d + [D] + [M]) - \sqrt{(K_d + [D] + [M])^2 - 4[D][M]}}{2[D]} \quad (Eq. 5.6)
$$

Where $K_d$ is the dissociation constant in mM, [D] is the constant concentration of C6-Ceramide (mM) and [M] is the concentration of polymer sample (mM).
The same steps were repeated for all AOUs and C6-Ceramide samples made up in deuterated ethanol. Spectra were normalized to the characteristic ethanol multiplet at 1.110 ppm.
5.3 Results and Discussion

5.3.1 ITC

3 attempts were made to measure the association/disassociation constants for C6-Ceramide and the non-derivatized AOU using the ITC system. Each run yielded a different curve; endothermic, exothermic and a non-significant curve. A number of possibilities could explain the non-significant responses. Insufficient heat-flow stabilization within the ampule chamber could have masked the actual measured heat of interactions. As well, it is possible that the fast-evaporating ethanol could have evaporated slightly, causing incorrect concentrations to be measured. Moreover, as will be presented in the NMR section below (Section 5.3.3), the two molecules (AOU and C6-Ceramide) displayed little or no interaction in ethanol. While the results from ITC do not conclusively show a non-interacting system, it is possible that the low-level interaction coupled with the background interferences yielded non-significant results. Due to time constraints and the fact that an additional technique was still available (NMR), no further troubleshooting was attempted with the ITC method.

5.3.2 NMR: C6 Ceramide Characterization

The complete characterization of C6-Ceramide is presented in Figure 5.1.

![Figure 5.1: $^1$H NMR spectra of C6-Ceramide. Labeled peaks correspond to the peaks referred to in Table 5.1](image-url)
5.3.3 NMR Titrations

5.3.3.1 Solvent Related Factors

Due to solubility constraints of the drug in physiological solvents such as water, in order to measure the molecular interactions in polar and non-polar solvents, NMR titrations were performed in chloroform (non-polar) and ethanol (polar). Characteristic series of $^1$HNMR titrations in chloroform and ethanol are presented for AOU-C6-Ceramide binding in Figure 5.2 a and b respectively.

![HNMR spectra](image_url)

**Figure 5.2:** $^1$HNMR spectra of interactions between AOU and C6-Ceramide in a) chloroform and b) ethanol. Notice the spectral shift over the varying concentrations in chloroform, and lack thereof in ethanol.

As can be seen, in chloroform (Figure 5.2a) the spectra shifted as the ratio of AOU to C6-Ceramide was increased (from top to bottom), indicating a change in the chemical environment due to a molecular interaction. In ethanol however (Figure 5.2b), no change in chemical shift was observed for any peak across the full range of AOU:C6-Ceramide ratios (0.01-50). This effect was observed with all AOUs (AOU, H, 6, 8, 12) in ethanol solutions with C6-Ceramide. This was likely caused by the individual molecules (AOU and C6-Ceramide) preferentially interacting with the solvent over each other, an effect that would remain constant over the full range of concentrations, thereby causing no change in the NMR spectra.
It has been reported that intra- (23) and inter-molecular (24) equilibrium states change depending on the solvent in which it is tested, due to secondary interactions such as hydrogen bonds and van der Walls force forming between the solvent and the molecules under study (25, 26). Hydrogen bonding donors and acceptors become solvated in more polar solvents, decreasing the binding strength between the molecules in solution being tested (27). While the following analysis therefore represents equilibrium data obtained from interactions measured in chloroform, it is important to note that this data reflect findings in a hydrophobic solvent rather than an aqueous base medium. In practice however, the interaction between drug and polymer will likely take place in a blood-like environment, significantly more polar than chloroform. The actual binding measurement is therefore somewhere in between that measured in chloroform and ethanol. However, for the purpose of comparing the effect of different AOU chemistry with the drug, the current system provides us with some insight of the interactions relative to each AOU, rather than providing absolute values of a given AOU in a physiological application.

5.3.4 Sample Calculation

The following presents a sample calculation process for extracting $K_d$ (dissociation constant) values from a series of $^1$HNMR spectra using AOU as the example. After performing the $^1$HNMR series, the C6-Ceramide peak m (Figure 5.1) was chosen as one of the peaks to monitor due to its clear and unique (not overlapping) signal across the entire range of concentrations (Figure 5.3a). The peak position (ppm) was then plotted against the total AOU concentration (Figure 5.3b) and a curve was fit to the data points using Equation 5.6.
This data yielded a dissociation constant of 20 mM (the significance of this number will be discussed below). The process described was performed for each AOUx-C6-Ceramide interaction, monitoring C6-Ceramide peaks m, e and a (Figure 5.1), and AOU peaks e, f, and x (where applicable) (Figure 5.4). The complete data is presented in Table 5.1.

Figure 5.3: a) Isolated peak m from $^1$HNMR titrations with AOU and C6 Ceramide. b) peak m position data plotted against AOU concentration.

Figure 5.4: AOU structure with letters corresponding to NMR spectra. n=4 (AOU6), 6 (AOU8), 10 (AOU12).
**Table 5.1:** Dissociation constant data for AOUx-C6-Ceramide interactions using different NMR peak shifts. \( n \) = number of points included in curve fitting, (AOUx-C6-Ceramide interaction was performed once).

<table>
<thead>
<tr>
<th>Drug (M)</th>
<th>AOU</th>
<th>AOUH</th>
<th>AOU6</th>
<th>AOU8</th>
<th>AOU12</th>
</tr>
</thead>
<tbody>
<tr>
<td>( K_d ) [mM]</td>
<td>20</td>
<td>38</td>
<td>39</td>
<td>54</td>
<td>15</td>
</tr>
<tr>
<td>Std. Err.</td>
<td>3</td>
<td>3</td>
<td>11</td>
<td>12</td>
<td>7</td>
</tr>
<tr>
<td>95% Interval</td>
<td>13-27</td>
<td>30-45</td>
<td>9-69</td>
<td>23-82</td>
<td>0-33</td>
</tr>
<tr>
<td>( n )</td>
<td>9</td>
<td>9</td>
<td>5</td>
<td>8</td>
<td>5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Drug (E)</th>
<th>AOU</th>
<th>AOUH</th>
<th>AOU6</th>
<th>AOU8</th>
<th>AOU12</th>
</tr>
</thead>
<tbody>
<tr>
<td>( K_d ) [mM]</td>
<td>34</td>
<td>127</td>
<td>65</td>
<td>81</td>
<td>79</td>
</tr>
<tr>
<td>Std. Err.</td>
<td>8</td>
<td>29</td>
<td>23</td>
<td>23</td>
<td>21</td>
</tr>
<tr>
<td>95% Interval</td>
<td>14-53</td>
<td>62-191</td>
<td>0-129</td>
<td>26-135</td>
<td>27-130</td>
</tr>
<tr>
<td>( n )</td>
<td>8</td>
<td>9</td>
<td>5</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Drug (A)</th>
<th>AOU</th>
<th>AOUH</th>
<th>AOU6</th>
<th>AOU8</th>
<th>AOU12</th>
</tr>
</thead>
<tbody>
<tr>
<td>( K_d ) [mM]</td>
<td>79</td>
<td>128</td>
<td>63</td>
<td>81</td>
<td>80</td>
</tr>
<tr>
<td>Std. Err.</td>
<td>13</td>
<td>29</td>
<td>22</td>
<td>23</td>
<td>19</td>
</tr>
<tr>
<td>95% Interval</td>
<td>47-110</td>
<td>62-194</td>
<td>0-129</td>
<td>26-134</td>
<td>34-125</td>
</tr>
<tr>
<td>( n )</td>
<td>8</td>
<td>9</td>
<td>5</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>AOU (E)</th>
<th>AOU</th>
<th>AOUH</th>
<th>AOU6</th>
<th>AOU8</th>
<th>AOU12</th>
</tr>
</thead>
<tbody>
<tr>
<td>( K_d ) [mM]</td>
<td>83</td>
<td>32</td>
<td>182</td>
<td>181</td>
<td>93</td>
</tr>
<tr>
<td>Std. Err.</td>
<td>16</td>
<td>7</td>
<td>64</td>
<td>9</td>
<td>25</td>
</tr>
<tr>
<td>95% Interval</td>
<td>44-121</td>
<td>15-49</td>
<td>17-348</td>
<td>156-206</td>
<td>32-152</td>
</tr>
<tr>
<td>( n )</td>
<td>7</td>
<td>9</td>
<td>6</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>AOU (F)</th>
<th>AOU</th>
<th>AOUH</th>
<th>AOU6</th>
<th>AOU8</th>
<th>AOU12</th>
</tr>
</thead>
<tbody>
<tr>
<td>( K_d ) [mM]</td>
<td>81</td>
<td>41</td>
<td>187</td>
<td>88</td>
<td>110</td>
</tr>
<tr>
<td>Std. Err.</td>
<td>15</td>
<td>10</td>
<td>67</td>
<td>26</td>
<td>35</td>
</tr>
<tr>
<td>95% Interval</td>
<td>44-119</td>
<td>18-64</td>
<td>14-361</td>
<td>26-151</td>
<td>25-193</td>
</tr>
<tr>
<td>( n )</td>
<td>7</td>
<td>8</td>
<td>6</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>AOU (X)</th>
<th>AOU</th>
<th>AOUH</th>
<th>AOU6</th>
<th>AOU8</th>
<th>AOU12</th>
</tr>
</thead>
<tbody>
<tr>
<td>( K_d ) [mM]</td>
<td>-</td>
<td>-</td>
<td>173</td>
<td>87</td>
<td>92</td>
</tr>
<tr>
<td>Std. Err.</td>
<td>-</td>
<td>-</td>
<td>53</td>
<td>26</td>
<td>25</td>
</tr>
<tr>
<td>95% Interval</td>
<td>-</td>
<td>-</td>
<td>44-302</td>
<td>25-149</td>
<td>33-150</td>
</tr>
<tr>
<td>( n )</td>
<td>-</td>
<td>-</td>
<td>7</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>
All titrations contained at least 9 individual data points, however for some of the samples (especially AOU6 and AOU12) the first 5-7 points followed a curve, while the points containing very high amounts of AOU (~250 mM and more) deviated from the original curve and followed a straight sloped line. These points were therefore not included in the curve modeling (See Table 5.1) decreasing its statistical significance. At very high AOU concentrations, especially due to its high viscosity, when in solution with a very small amount of C6-Ceramide, the detected signal was dominated by the AOU alone. As the concentrations increased the signal followed a sloped line (not a curve), due to solvent and other background conditions causing proportional changes in the spectra. This linear shift however, does not reflect the intermolecular behavior of AOU and C6-Ceramide.

5.3.4.1 Data Analysis

Binding constants were calculated by monitoring different peaks on the AOU molecule and the drug (See Table 5.1). These peaks were chosen to provide the strength and nature of the interaction across different bonds and different segments of the molecules (polar (drug peak m, NH, AOU peak a CH\textsubscript{2} alpha to urethane bond), hydrophobic (drug peak e CH\textsubscript{2}, drug peak a CH\textsubscript{3}, AOU peak x CH\textsubscript{2}), fluorinated (AOU peak e CH\textsubscript{2} alpha to CF\textsubscript{2}), a technique commonly used in NMR binding experiments (6, 12). However, after looking at the standard-error values, the 95% confidence intervals and sample sizes for each $K_d$ value (Table 5.1), it is clear that the data is not powerful enough to extract strong conclusions. The following points however can be made.

1. **Range of Binding Strength:** Regardless of the exact value, all of the reported binding strengths fell within the mM range. While these numbers do not compare to traditional ligand protein binding strengths (nM-µM range) (17), and are also weaker than synthetic receptors (11, 14), they do fall in line with binding strengths of other drug-polymer carrier interactions (12, 22, 27).

2. **Effect of Hydrogen Bonding:** While no formal investigation was carried out to study the effect of hydrogen bonding in the AOU-C6-Ceramide interaction, it is clear from Table 5.1 that the binding strength measured from peak m (NH, a hydrogen bonding donor and or acceptor) with all AOUs is consistently stronger than that measured from all other drug or polymer peaks. The effect of hydrogen bonding during molecular interactions is an
important factor that is considered during drug-carrier design and has been shown to significantly affect the binding strength (6, 27).

3. **Effect of Side Chain:** Observing the binding values for AOU6, 8, and 12 in Table 5.1, it is possible that by incorporating the alkyl side chain, instead of increasing binding - as had been originally hypothesized - the large chain actually interfered with the more significant polar/hydrogen bonding groups, decreasing its binding strength as compared with AOU. Comparing k values (and their 95% CI ranges) from AOU peaks f, e, and drug peak e, on AOU6, 8 and 12 as compared to those on AOU, it is noted that k values increase for all alkyl chain systems relative to the AOU implying a decrease in the binding affinity possibly due to steric hindrances introduced with the incorporation of the hydrocarbon side chains. The values from drug-related peaks m and k which do not follow the exact trend described, could be testament to the false-nature of the assumption, or could be due to the distant location of proton a and its stable CH$_3$ signal as well as the polar nature of peak m masking the effects of hydrophobic binding.

Due to the lack of statistical power of the current data, no significant claims can be made regarding the effect of AOU molecular structure changes on intermolecular binding strength. This is mainly due to the insufficient number of data points used to generate the binding curves of the derivatized AOUs. This occurred due to the quantity of material needed to generate the full range of concentrations and the fact that the derivatized AOU synthesis was only optimized close to the end of the project. Additional techniques that can be used to obtain more significant binding data is provided in the Future Recommendations Chapter, #6 and 7. This data does however serve as a proof of concept for the use of NMR as a powerful technique to study binding properties between AOU molecules and anti-proliferative drugs such as C6 Ceramide.

### 5.4 Conclusion

ITC and NMR were used as methods to study molecular interactions between AOU based carriers and C6-Ceramide. Due to solvent and equipment related factors, no binding data could be extracted from ITC experiments. $^1$HNMR titrations performed in ethanol indicated no binding likely due to solvent interactions competing with the hydrogen bond donors and acceptors on the AOUs and the drug. Experiments in chloroform however yielded characteristic binding curves that were fit to a binding model to obtain associated K$_d$. Due to the lack of statistical
significance, no conclusion on structure-function relationship could be made between different AOUs, however it seemed that the hydrogen bonding groups played a significant role in the binding. This study has shown that NMR is a powerful method that can be used to study interactions between polymer carriers and anti-proliferative drugs. Additional $^1$HNMR titrations using more data points as well as additional NMR techniques (NOESY determining interaction sites, and DOSY calculating bound diffusion constants), may possibly lead to a better understanding of the AOU drug-binding structure-function relationship to allow for the development of AOUs tailored for specific drug binding properties.

5.5 Acknowledgements

The author wishes to acknowledge the Canadian Foundation for Innovation, project number 19119, and the Ontario Research Fund for funding of the Centre for Spectroscopic Investigation of Complex Organic Molecules and Polymers.
5.6 References


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Chapter 6
Conclusions

The use of stent and balloon related devices to treat de-novo and iatrogenic diseases requires the development of drug delivery carriers that effectively deliver drugs to the injured site without causing any undesired responses from the surrounding environments. Coupling the tailorability of polyurethanes with the unique interfacial properties of fluoropolymers has led to the development of amphiphilic oligourethane (AOU) to serve as a platform on which to synthesise variable drug delivery carriers. It was hypothesized that due to its amphiphilic character, AOU would cause a response from in-vitro rat smooth muscle cells, and that by altering AOU’s structure (charge state and hydrophobicity) a change in the cell response would be observed. The two primary objectives of this thesis therefore were the synthesis of AOU and its derivatives and biological characterization of all AOUs, testing cellular toxicity and membrane stability. As a side-hypothesis, it was anticipated that the incorporation of hydrophobic motifs to AOUs would increase its drug binding with a hydrophobic drug.

Chapter 3 introduced AOU and detailed the optimization process that went into synthesizing a relatively pure AOU. The preliminary AOU synthesis method that had been developed in the Santerre Lab yielded a non-pure AOU containing trimeric by-product species including PEG-trimer, synthesized due to the dual functional groups on LDI. Due to this species’ similar size and solubility characteristics to AOU, it was not easily separated from AOU (observed using NMR and MALDI). Two approaches were undertaken in order to address this issue.

1) The reaction, originally performed reacting MPEG with LDI followed by capping with PFA, was reversed to form a PFA-LDI prepolymer followed by capping with MPEG. This would allow PFA-trimer to form (which is easily removed in Hexane) instead of the more difficult PEG-trimer. While the reverse synthesis was initially performed on ice (due to the assumption of a fast prepolymer reaction step), this effectively formed a one-pot reaction, yielding results not different than those obtained from the first synthesis method (observed using NMR and MALDI).

2) The next reaction method was again performed using the reverse order, however at room temperature with catalyst. Optimizing the individual parameters (time, temperature and catalyst concentration), led to the synthesis of AOU with no trimeric species, and ~15
molar % of residual unreacted MPEG which would not cause any problems in subsequent reaction or characterization steps. This synthesis completed the first objective and served as the basis for all further experiments testing the additional project hypotheses.

Using the optimized synthesis method developed in Chapter 3, Chapter 4 introduced AOU derivatives and presented their optimized synthesis methods (optimization process detailed in Appendix C).

3) AOUH was synthesized by hydrolyzing the methyl ester on AOU’s lysine pendant chain with NaOH, forming an anionic carboxyl moiety. This structure was confirmed with NMR by the disappearance of the CH$_3$ peak from the methyl ester.

4) AOU6, 8, and 12 were synthesized by coupling amine functionalized alkyl chains of 6, 8 and 12 carbons, respectively, to the carboxyl group on AOUH using carbodiimide chemistry. These structures were confirmed with NMR by monitoring the chemical shift change on coupled molecules before and following the reaction.

After having fulfilled all synthesis-related objectives, Chapter 4 continued onto $in$-$vitro$ biological characterization of AOU and its derivatives with rat vascular smooth muscle cells, measuring cellular metabolic activity (WST-1), total DNA content (Hoescht DNA) and membrane solubilization (LDH release), followed by a discussion of the results in the context of traditional, fluorinated and hybrid surfactants.

5) AOU, a fluorinated amphiphile, caused a decrease in cellular activity and DNA content by ~50% at the highest concentration measured, without however any observed membrane solubilization that could be detected. This was explained by individual AOU molecules possibly intercalating the membrane causing a decrease in cellular activity, however not causing any detectable membrane solubilisation due to fluorine’s low adhesive interactions and lytic potential.

6) AOU6, with the structure of a hybrid surfactant (two terminal hydrophobic tails – fluorinated and hydrogenated – and one hydrophilic tail), behaved similarly to AOU. Normalizing the effective lengths of both the fluorinated and hydrogenated chains due to the increased micellization potential of fluorinated chains, it was concluded that the coupled chain (CH$_2$)$_6$ likely played no significant role in determining its effect on the cells.
7) AOU8, with its longer coupled chain, reduced cellular metabolic activity and DNA content in comparison to that of AOU and AOU6, and appears to have caused a small amount of membrane solubilisation at mid and high concentrations tested, supported by the LDH data. This indicated that the coupled chain (with its similar effective chain length to PFA) began to influence the properties of the molecule, causing it to behave more like a traditional hydrogenated surfactant, toxic and membrane lytic.

8) This trend related to the effect of hydrocarbon chain length became clearer when looking at the results from AOU12. The coupled chain significantly masked the protective effects of the fluorinated chain as seen with AOU, and not only caused high levels of toxicity as measured using the WST and DNA assays, but resulted in significant membrane lysis as displayed by the LDH release data.

9) The introduction of an anionic group in between the hydrophilic and hydrophobic segments on AOUH seemingly disrupted the molecular arrangement of the AOU in solution, resulting in non-aggregated molecules containing individual segments (hydrophilic, anionic and fluorinated) each with a low affinity for biological membranes. While the precise mechanistic action of the latter is not known at this time, the end point is supported by the observation that there was no sign of toxicity or membrane solubilization at all measured concentrations.

The above study strongly confirmed the two main thesis hypotheses by showing that AOU elicited a response on smooth muscle cells and that a differential response was observed after AOU modification.

Chapter 5 introduced ITC and NMR as methods to be used to study intermolecular binding properties, as a basis for future work to study the effects of structural changes to AOU on drug binding. Methods were described for AOUs in combination with a model antiproliferative drug C6-Ceramide.

10) Due to possible errors stemming from the instrument and the solvent, as well as the reduced binding in ethanol (see #11 below), ITC did not yield any valuable results.

11) NMR titrations, mapping the changes in chemical shifts, were performed with all AOUs in chloroform and ethanol. Binding was observed in chloroform, however no binding (no change in chemical shift) was observed in ethanol. It was hypothesized that this was as a
result of the individual molecules preferentially interacting with the polar solvent as opposed to the other molecule.

12) The binding constants between the AOU6 and C6-Ceramide fell within the mM range, similar to those reported in the literature for similar polymer-drug binding systems.

13) While low statistical significance of the values didn't allow statistical comparisons to be made across the different AOU6 and different monitored peaks, it is possible that hydrogen-bonding played a significant role in the binding, due to the consistent increase in binding constant when calculated from monitoring the NH-related peak.

14) Additionally, it is anticipated that the incorporation of the alkyl side chains on AOU6, 8 and 12 could possibly decrease the binding strength as opposed to increase it by introducing more hydrophobic binding sites. This could be caused by the hydrocarbon chains forming steric hindrances blocking the more significant polar binding sites.

This project successfully developed AOU as a variable platform for drug delivery coatings. Comprehensive synthesis studies were performed furthering the understanding of isocyanate chemistry and its effect on oligourethane synthesis, as well as developing efficient conjugation protocols that can be used in further AOU development. Through the study of AOU and its derivatives, it was shown that amphiphilic structures can elicit a toxic cell response without any membrane solubilisation. AOU6s in the form of hybrid-surfactants were shown to elicit a varied cell response as a function of hydrocarbon chain length with this being the first study in the literature reporting on the effect of hybrid surfactants on cells. Furthermore, it was shown that a simple anionic modification on an amphiphilic structure can significantly alter its behavior with respect to cell membrane interactions. The project concluded with a preliminary study developing a protocol for the use of ITC and NMR as methods to study AOU-drug interactions which serves as an additional component in the overall development of tailored drug delivery coatings.
Chapter 7
Future Recommendations

7.1 Recommendations Directly Related to this Project

1) As described in Chapter 3, AOU, while free of PEG-trimer still contained ~16% excess free MPEG. While this didn't prevent further AOU characterization and development, being able to fully remove it would allow for more efficient use of AOU-based materials and more accurate characterization. 3 possible methods could be tried to generate a more pure AOU.

   a. To try to prevent its presence following the reaction, an optimization study could be performed, where all reaction conditions are held constant however, the MPEG ratio is constantly decreased. The optimal amount of RPEG used in the reaction would be the amount that produces the smallest ratio of MPEG:AOU following the reaction.

   b. MPEG’s aggregation in water (1) could prevent its removal simply using dialysis with water. Dialysis in different solvents could be attempted to enhance MPEG removal.

   c. Excess MPEG, and even minute traces of other side products could be removed by separation using size exclusion and liquid chromatography methods as described with a similar amphiphilic system (2).

   The latter two methods apply to the small amount of excess PFA, which was shown to be present (see Appendix C, where it was shown that during the EDC coupling reactions, small amounts of PFA coupled to the hydrolyzed lysine center).

2) As most of the theories behind the cytotoxicity of AOUs relates to its aggregation or micellization, the critical micelle concentration (CMC) must be determined. This can be done with one of the most standard and precise methods for CMC determination, pyrene fluorescence (3). The calculated values can be compared against the toxicity results and hypotheses discussed in Chapter 4.

3) While LDH release is a standard method for measuring cell membrane disruption and membrane lysis, it is also possible to use transmission electron microscopy (TEM) to
visually observe the possible disruption of the membrane (4, 5). Adding this level of information could possibly confirm the osmotic lysis hypothesis presented in Chapter 4, whereby low sample concentrations caused a decrease in cellular activity through membrane intercalation, without however any membrane solubilisation.

4) The identical cell experiments should be performed using endothelial cells. This would provide more robust data regarding the use of AOUs as arterial drug delivery carriers, especially since effective reendothelialization is such a key factor following percutaneous interventions (See Chapter 2).

5) WST assays should be repeated for AOUH with increased concentration ranges in order to determine if an increase in AOUH concentration results in increased cellular metabolic activity, an observation already made with regard to COOH-functionalized carbon nanotubes (6). Since it was proposed that the negative charge on the COOH groups mimicked extracellular components enhancing the chemoelectrophysical regulation and streaming potentials of the cells, additional WST assays could be performed on AOUs with more COOH groups (as proposed in 8b below) to aid in confirming this observation.

6) The exact same $^1$HNMR titrations should be performed as described in Chapter 5, however with more titration points, especially at higher AOU concentrations. This will allow for
   a. Better resolution at the curve and plateau regions, increasing the significance of the modeled curve and extracted dissociation constants.
   b. More accurate comparisons to be made between the degree of chemical-shift-change for different peaks in the structure, allowing for a more precise determination of the key binding sites (7, 8).

7) Apart from $^1$HNMR titrations, the following three NMR experiments have become standard NMR-binding experiments that can provide additional data regarding the AOU-C6-Ceramide interaction.
a. **Pulsed Field-Gradient (PFG) NMR or Diffusion-Ordered Spectroscopy (DOSY):** This method studies intermolecular aggregation by monitoring self-diffusion coefficients of the unbound and bound molecules (8). This data can further be used to determine the aggregate’s hydrodynamic radius using the Stokes-Einstein equation (7).

b. **Nuclear Overhauser Effect Spectroscopy (NOE):** This powerful technique provides binding site information by determining the spatial distance between two nuclei through cross-peak correlation (8, 9).

c. **Saturation Transfer Difference (STD):** Measuring the difference between the saturation-on signal (drug signal affected by binding with macromolecule) and saturation-off spectra (drug signal not affected by binding with the macromolecule), one can identify which protons interact with the macromolecule (10). An STD titration can be used to calculate the binding constants (11).

### 7.2 Recommendations for Project Extensions

8) Additional derivatives of AOU should be designed based on 1 or 2 of the following criteria; a) to further understand how fluorinated hybrid amphiphiles in general and AOU specifically, react with VSMCs, and b) to determine what functional groups could be added to alter drug binding. A few examples of coupling molecules are presented:

a. **Methoxy-Polyethylene Glycol** (4, 6, 12 units; Sigma JKP 1200). This would increase the strength of the hydrophilic portion of AOU, possibly promoting aggregation or micellization, however the extra chain could also interfere with molecular arrangement. Testing its toxicity could determine which of the two factors is more significant. Furthermore, the increased hydrophilicity could possibly further decrease the drug binding properties, or allow for more polar binding sites – possibly increasing the binding.

b. **ButaneTetraCarboxylic Acid** (Sigma 257303): This would significantly increase the ionic character of the central group, possibly amplifying the behaviour of AOUH (which was assumed not to form aggregates). Furthermore, the increased number of hydrogen-bonding sites could enhance the binding properties with C6-Ceramide, which, as described in Chapter 5, could significantly increase the binding potential.
c. **Perfluoro Amines** (NH$_2$(CF$_2$)$_6$CF$_3$; Sigma CDS002883). This would increase AOU’s hydrophobic portion as was done with AOU6,8 and 12, however the use of a fluorinated chain – creating a two-tailed-fluorinated surfactant – could significantly alter the interaction with cell membranes. If its toxic effect were equal to or less than that of AOU, it would confirm the hypothesis regarding the decreased lytic potential of fluorinated chains (See Chapter 4), as well as allow it to be applied to two-tailed surfactants.

9) As discussed in Chapter 4, both the hydrophobic and hydrophilic (though less so) portions affect an amphiphile’s CMC and associated cell toxicity. As the fundamental synthesis method for AOU has already been optimized, AOUs with both shorter and longer hydrophobic and hydrophilic segments could be synthesized, requiring only NCO titrations to monitor the reaction kinetics and calculate at which time to add the PEG. These new AOU combinations could for example significantly effect toxicity (increasing fluorinated segment), and increase drug release kinetics (increasing hydrophilic portion).

10) While the NMR and ITC experiments can provide valuable binding information on the molecular level, in practice however, the clinically relevant value is actually how much drug is released from an AOU film over a period of time. AOU-C6-Ceramide films can be cast and placed in biological solutions such as PBS. At certain time points, aliquots are taken and the drug quantified using ultraviolet spectroscopy or high-performance liquid chromatography, two methods commonly use to study drug release (12, 13). Furthermore, comparisons could be made between actual drug release kinetics and the binding constants calculated using the NMR methods.

11) Layered films of different AOUs can be made to serve unique functions. For example a drug eluting balloon model requiring rapid release could consist of the drug loaded in an extremely hydrophilic AOU, with a thin hydrophobic AOU top-coat to prevent premature release. A more long term delivery device could contain have the drug loaded in alternating layers of hydrophobic AOUs, placed in between layers of hydrophobic AOUs with no loaded drugs. The efficacy of these kinds of combinations can be tested using the methods described in #10.
Appendices

Appendix A: MPEG Molecular Weight Analysis

Before performing AOU synthesis method #2 an investigation was made into determining the molecular weight of the MPEG used in the AOU reactions. The following section describes the study in detail.

After performing AOU synthesis method #1 a number of times, an interesting phenomenon was observed. Instead of reaching 25% NCO conversion after one hour (as had been expected based on the 4:1 NCO:OH ratio, and as had been observed earlier (see Figure 3.7), the titrations began to consistently display 20% NCO conversion after one hour (Figure A.1). While observing this once could be explained by the margin of error, but due to its multiple occurrence (n=4) an alternate explanation was needed. Since this titration method had been used by others and provided reliable results (1), other possible errors could only be attributed to incorrect molecular weight data. Since LDI and PFA are relatively small molecules, the molecular weight of MPEG was left as a possible source of error.

![NCO Conversion Graph](image)

**Figure A.1:** Titration data representing NCO conversion during synthesis method #1 reaction. After one hour, only 20% is reached as opposed to the expected 25%. Error bars represent standard deviation, n=3, except for time = 2 hours represents n=2.
As was already observed by Liu (2) and Dust (3), determining the correct molecular weight of a long chain PEG molecule is not trivial and can vary depending on the method chosen. Following the protocol of the previous student, a molecular weight of 618 g/mol had been used as the molecular weight of MPEG, a number obtained based on hydroxyl titrations. Due to the hygroscopicity of the MPEG, obtaining an accurate result using this method is actually quite difficult. Hence, other methods were explored and NMR, MS and MALDI all indicated significantly higher molecular weight values; 781 (Figure A.2), 750 (Figure A.3a) and 810 g/mol (Figure A.3b), respectively.

**Figure A.2:** a) Molecular weight spectrum of raw MPEG, b) molecular structure of MPEG, and c) table describing calculation of MPEG molecular weight.
Using this observation, old titration data was recalculated based on updated molecular weight values of MPEG. As can be seen in Figure (A.4), assuming the same stoichiometric ratio, and assuming that all the MPEG molecules are consumed by NCO groups – using a molecular weight of 618 results in 25% NCO conversion. However, if a larger value, approximately 750 is used – as expected – a 20% NCO conversion ratio is observed. Practically this means that during these reactions, instead of having a 2 times LDI:MPEG ratio, in actual fact less MPEG was being added, resulting in a ~2.5 times LDI:MPEG ratio. Not only was this wasting an expensive reagent, but it further demonstrated that adding excess LDI did not prevent MPEG-trimer formation.

**Figure A.3.** a) MS and b) MALDI spectra of raw MPEG. N is normalized peak intensity, $M_i$ is the molecular weight of each peak portion, and $M_n$ is the number average molecular weight.

\[
M_n = \frac{\sum N_i M_i}{\sum N_i}
\]

<table>
<thead>
<tr>
<th>Max Intensity</th>
<th>14008.84</th>
<th>13362.71</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sum of N</td>
<td>30.88</td>
<td>10.62</td>
</tr>
<tr>
<td>Sum of MW*N</td>
<td>23195.45</td>
<td>8595.51</td>
</tr>
<tr>
<td>Sum / Sum N</td>
<td>751.12</td>
<td>809.29</td>
</tr>
</tbody>
</table>

Using this observation, old titration data was recalculated based on updated molecular weight values of MPEG. As can be seen in Figure (A.4), assuming the same stoichiometric ratio, and assuming that all the MPEG molecules are consumed by NCO groups – using a molecular weight of 618 results in 25% NCO conversion. However, if a larger value, approximately 750 is used – as expected – a 20% NCO conversion ratio is observed. Practically this means that during these reactions, instead of having a 2 times LDI:MPEG ratio, in actual fact less MPEG was being added, resulting in a ~2.5 times LDI:MPEG ratio. Not only was this wasting an expensive reagent, but it further demonstrated that adding excess LDI did not prevent MPEG-trimer formation.
References


Figure A.4: Graph showing expected NCO conversion values based on different assumptions of MPEG molecular weight values. While 25% is shown if a MW of 618 is used, only 20% is expected if the MW is closer to 750.
Appendix B: NCO Titration Data for an increase in Reaction Size

During AOU reaction optimization, each reaction contained approximately 3 g of MPEG. It was on this reaction size that the one hour prepolymer reaction time in Method #3 was determined. This was shown in Figure 3.13 and 3.14. In order to prevent large amounts of excess MPEG being left in the AOU mixture, the ratios of PFA and MPEG to LDI were increased and decreased, respectfully. Additionally, in order to supply enough material for the required characterization studies, the reaction sizes were increased four-fold.

Two new factors were introduced with these changes. The increased PFA:LDI ratio had an effect on prepolymer reaction time and therefore required an updated kinetic study, but less noticeable was the fact that since the reaction sizes were increased, the time it took to drop the PFA into the LDI also increased. There was now a significant difference between timing the reaction from catalyst addition verses the complete addition of PFA. NCO titrations were therefore performed and the results from the reactions using different batches of LDI are presented in Figures B.1a and B.1b. Based on the results, it was decided that the MPEG should be added 50 minutes after catalyst addition.

![Figure B.1](image)

**Figure B.1:** NCO titration data from large batch prepolymer reactions using 2 different sources of LDI, a) China and b) Germany. Approximately 50% NCO conversion is reached at t=50 mins.
Appendix C: Coupling Reaction Optimization

C.1 Introduction

The purpose of this reaction step was to couple a short hydrophobic chain (hexyl-, octyl, dodecyl-amine) through its NH₂ terminal-functionality to the carboxyl group on the lysine moiety of AOU. Work in the literature has shown successful coupling of amine-functional groups to methyl-ester moieties using a K₂CO₃ direct coupling approach (1, 2). This was therefore the first approach taken. Work in the Santerre Lab used carbodiimide chemistry to couple amines and hydroxyl groups to carboxyl groups and therefore was the second method chosen (3, 4).

C.2 Methods

During the optimization process, reactions were performed first – and sometimes only – with hexylamine, as it was the most soluble of the three coupling molecules. Unless otherwise mentioned, the reaction conditions were the same if performed with octyl- or dodecyl-amine.

C.2.1 Coupling Reaction #1: K₂CO₃ Direct Coupling

Degassed AOU, K₂CO₃ (2 molar equivalents to AOUH), and molecular sieves (approximately 0.7 g/g AOU) were added into a round bottom flask and purged with nitrogen for five minutes. Anhydrous methanol (EMD, Canada) was added via cannula (approximately 30 ml/g AOU) and hexylamine (1.05 molar equivalents of AOU) was added via syringe. The flask was placed in a 45°C oil bath and equipped with a condenser. The entire system was bubbled with nitrogen for 30 minutes after which the flask was sealed under a nitrogen balloon and left to stir for 10 days. 3 ml aliquots were taken via a syringe on days one, four and seven to monitor reaction kinetics.

C.2.2 Purification Method #1

Aliquot samples were passed through a cotton filter in a Pasteur pipette to remove the molecular sieves, the solvent was removed using a rotary evaporator under reduced pressure (0.05 mm Hg) and the resulting sample was analyzed using ¹HNMR.

The final reaction solution was centrifuged at 4000 rpm for five minutes to remove the molecular sieves after which the solvent was removed using a rotary evaporator under reduced pressure,
redissolved in acetone and recentrifuged. This process was repeated three times in order to precipitate and remove the potassium carbonate. In order to remove excess hexylamine, hexane solvent was added to the product (0.1 g/ml), vortexed (one minute) and centrifuged (4000 rpm, five minutes) after which the supernatant was discarded. This process was repeated five times. The final precipitated product was dried under reduced pressure at 40ºC and analyzed using $^1$HNMR.

C.2.3 Coupling Reaction #2: Carbodiimide Chemistry

Degassed AOUH, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (eight molar equivalents to AOUH), dimethylaminopyridine (DMAP) (Sigma-Aldrich, Canada) (0.5 molar equivalents to AOUH) molecular sieves (approximately 0.7 g/g AOUH) were added into a round bottom flask and purged with nitrogen for five minutes. Anhydrous dimethylformamide (DMF) was added via cannula (approximately 30 ml/g AOUH) and hexylamine (1.1 molar equivalents of AOUH) was added via syringe. The flask was repurged with nitrogen for five minutes after which it was sealed under a nitrogen balloon and left to stir for seven days.

C.2.4 Purification Method #2

Upon reaction completion, the solution was centrifuged at 4000 rpm for five minutes to remove molecular sieves. The solvent was removed using a rotary evaporator under reduced pressure (0.05 mm Hg). In order to remove residual EDC, EDC byproducts, DMAP and excess hexylamine, the resulting product was dissolved in water at a concentration of 0.1 g/ml, sealed in a 1000 g/mol molecular weight cut off, regenerated cellulose dialysis membrane (Spectrum Labs, California), and dialyzed against distilled water (150 times volume of polymer-containing solution) for 24 hours. The dialysate was changed at two and four hours. The final solution was frozen and lyophilized for 48 hours.

C.2.5 Coupling Reactions 3-8

The following reactions were all performed using the same procedure as described in Coupling Reaction #2, however changes were made to the molar ratios of the reactants, the type of carbodiimides and nucleophilic catalyst (Dimethylaminopyridine, DMAP or Hydroxybenzotriazole, HOBr) used and reaction time. The exact conditions are summarized in Table C.1.
Table C.1: The table below summarizes the various reaction conditions tested during the carbodiimide coupling optimization. CD = Carbodiimide, NC = Nucleophilic Catalyst. EDC Beads = SiliaBond ® EDC (Silicycle, Quebec).

<table>
<thead>
<tr>
<th>Reaction Method</th>
<th>Carbodiimide (CD)</th>
<th>Nucleophilic Catalyst (NC)</th>
<th>AOUH:Amine:CD:NC</th>
<th>Time (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>EDC</td>
<td>DMAP</td>
<td>1:1:1:8:0.5</td>
<td>7</td>
</tr>
<tr>
<td>3 (Investigative)</td>
<td>EDC</td>
<td>DMAP</td>
<td>1:0:8:0.5</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>EDC</td>
<td>DMAP</td>
<td>1:3:3:0.5</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>EDC Beads</td>
<td>-</td>
<td>1:5:3:0</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>EDC Beads</td>
<td>-</td>
<td>1:5:6:0</td>
<td>3</td>
</tr>
<tr>
<td>7</td>
<td>EDC Beads</td>
<td>HOBt</td>
<td>1:5:6:3</td>
<td>3</td>
</tr>
<tr>
<td>8</td>
<td>DIC</td>
<td>HOBt</td>
<td>1:5:7.5:7.5</td>
<td>3</td>
</tr>
</tbody>
</table>

C.2.6 Purification Methods 3-8

Reaction methods 3-8 all used the identical purification method as described in purification method #2. The only difference occurred in reaction method 8, during which dialysis was performed for 48 hours and the dialysate was changed at two, four, and 24 hours.
C.3 Results and Discussion

C.3.1 Reaction Method #1 (Direct Coupling)

The direct coupling approach using K$_2$CO$_3$ did not succeed in coupling any of the hexylamine to the carboxyl group. This was proven by $^1$HNMR using the following observations. The proton signal from the CH$_2$ alpha to the unreacted amine appears at 2.68 ppm (triplet). After amide-bond formation, that signal should move downfield to approximately 3.1 ppm. As can be seen in the $^1$HNMR spectra after purification (Figure C.1), a signal appears at ~2.6 (*, indicating unreacted hexylamine), with no increase in the signal at 3.1 (f, indicating no reacted hexylamine). Furthermore, after mixing with hexanes, the signal at 2.6 disappeared, as all the free hexylamine was washed away.

![Diagram](image)

**Figure C.1:** $^1$HNMR spectra of product after attempted direct coupling of K$_2$CO$_3$. a) Structure of hexylamine coupled to AOU, b) Structure of hexylamine.

It should be noted that while this method was not able to couple the amine to the carboxyl group, it did manage to hydrolyze the methyl-ester, as can be seen by the disappearance of the CH$_3$ peak at 3.7 ppm indicated by $\times$ in Figure C.1. This however was not sufficient in terms of coupling.
hexylamine, and therefore since other methods had been previously used in the Santerre Lab, they were tested before attempting to troubleshoot the direct coupling reaction.

C.3.2 Reaction Method #2 (EDC Chemistry)

In order to perform hexylamine coupling with EDC, AOU had to first be hydrolyzed. This method is detailed in Section 4.3.3 of Chapter 4, using a NaOH hydrolysis method. $^1$HNMR spectra from reactions using EDC to couple hexylamine to AOUH were associated with a number of peaks which were not immediately identifiable. As can be seen in Figure C.2, there is no unreacted hexylamine (no triplet at 2.6 ppm), however this was not because it was all removed during dialysis, since a new peak (u) has appeared indicating that at least some hexylamine has coupled to the carboxyl group (~25%, compare integration values of peak z (0.82) to theoretical (3)).

Furthermore, since some of the newly formed peaks could not easily be attributed to EDC or EDC byproducts (U$_4$,5,6), it was clear that additional reactions were taking place during the coupling process. The next investigative reaction attempted to solve some of these questions.

**Figure C.2:** $^1$HNMR spectra of reaction after attempted coupling with EDC. Multiple unknown peaks are present.
C.3.3 Reaction Method #3 (Investigative)

In order to isolate the additional reactions that were taking place in method #2, the next step was undertaken whereby the exact reaction in method #2 was run, however with no hexylamine. Therefore, all reactions that were taking place due to the reaction conditions (EDC, DMAP, time, temperature) could be observed, however artefacts of hexylamine coupling would not cloud the spectra.

Figure C.3 presents a 2D Correlation Spectroscopy (COSY) spectra ($^1$H-$^1$H) with the associated 1D $^1$HNMR labelled on the top of the coupling reaction with no hexylamine present. After analyzing the spectra alongside Heteronuclear Signle Quantum Coherence (HSQC) data ($^{13}$C-$^1$H) from the same reaction, many of the unknown peaks were identified. $e'$ and $l'$ were associated with some residual unreacted PFA coupling to the carboxyl (as can be seen since they are neighbouring species, connected by the e-l horizontal line – simply shifted downfield). Likewise $j$ was associated with the same phenomenon with residual unreacted MPEG coupling to the carboxyl group. While the presence of these unreacted species wouldn't have been expect to cause a problem with respect to their biological effects, their competitive binding reactions however, did interfere with additional reaction steps, creating small amounts of impurities.
Additionally, after further analysis it became clear that peaks U_{1,2,3} were an indication of residual EDC, or impurities contained within the EDC and not removed during purification. Again, based on these observations the next methods attempted to attenuate or remove some of these products.

**C.3.4 Reaction Method #4**

Based on the data obtained from the investigative reaction above, two modifications were made to prevent the side reactions and residual components; the hexylamine ratio was increased three fold in order to prevent residual PFA or MPEG from competing with the binding reaction, and the amount of EDC was decreased in order to facilitate the removal of its residuals during purification. $^1$HNMR spectra from this reaction (not shown) did not look significantly different than that from method #2. While the strength of the coupled-PFA and coupled MPEG were decreased (due to the lower amounts of EDC), the amount of residual EDC was not changed after purification. Furthermore, due to the decreased amount of EDC, the hexylamine coupling efficiency was reduced to $\sim15\%$. This showed that method #2 was more effective at coupling hexylamine than method #4.

If method #2 was going to be chosen however, it was clear that the excess EDC / impurities in the EDC should be removed. Increased dialysis purifications did not help remove these products, and it was assumed that they were forming secondary interactions with the AOUH not enabling its removal. Extractions using dichloromethane and water (to further separate the AOU and residual EDC products) as well as dialysis using an ionic solution (dissolved NaCl) to break the secondary interactions were both tried, yet the residual EDC-impurity peaks still remained. It became clear that another batch of EDC or another carbodiimide altogether should be tried.

**C.3.5 Reaction Method #5 and 6 (EDC Beads)**

EDC, bonded onto a non-reactive silica bead (Silicycle, Quebec) was chosen as a suitable carbodiimide for this reaction, as it could be simply removed with centrifugation and filtration post-reaction. Excess hexylamine (5 times AOUH) was used in order to prevent the competitive reactions (residual MPEG and PFA), and the rest of the reaction parameters were held constant. While no residual EDC-related peaks are present on the $^1$NMR spectra from the purified sample (Figure C.4), the integration value of peak $z$ (CH$_3$, 0.89 ppm) indicates only $\sim13\%$ coupling efficiency of the alkyl amine was achieved.
In reaction method #6, an attempt was made to increase coupling efficiency by increasing the ratio of the EDC beads (which were now proven to be easily removed during purification, eliminating the problem caused when increased EDC concentrations were used in methods 2-4). Similar efficiencies however, were still observed after increasing EDC bead concentrations.

C.3.6 Reaction Method #7 (EDC Beads + HOBt)

As described in Chapter 2, incorporating an N-hydroxy-derivative (such as hydroxybezotriazole (HOBt) assists in a more efficient amine-coupling, by preventing molecular reactions from occurring and pushing the reaction towards forming an active ester available for attack by a free amine (5). HOBt was therefore added to the reaction with EDC beads, and while the efficiency of the reaction increased to 23% (back to that originally reached using method 2, however this time with no residual EDC-species), it was clear that based on the reaction conditions, EDC was not the correct coupling agent to be using in this reaction.

C.3.7 Reaction Method #8 (DIC)

EDC is known for its water solubility, allowing biological reactants to be used without first dissolving in a solvent (see Section 2.5.4 in Chapter 2). While EDC was chosen as the first carbodiimide to be tested – as many people in the Santerre Lab had already used it – after its unsuccessful performance in these reaction conditions, another carbodiimide was chosen. Dicyclohexylcarbodiimide (DCC) is traditionally the most commonly used peptide coupling agent, its more updated analogue with easier purification characteristics –

**Figure C.4**: $^1$H NMR spectra from hexylamine coupling reaction using EDC-beads.
diisopropylcarbodiimide (DIC) – was therefore the next coupling agent chosen. A reaction with both DIC and HOBr, with ratios similar to that of method 2, successfully coupled hexylamine to AOUH with ~85% efficiency, with no competitive reactions nor any presence of residual carbodiimide-related species. The $^1$H-NMR spectra of AOUH coupled hexylamine, or AOU6 is shown in Figure C.5. The new positions of peaks u and k were confirmed with COSY and HSQC. This method was therefore chosen in order to couple the additional molecules (octyl- and dodecyl-amine) as described in Chapter 4.

**Figure C.5:** HNMR spectra of hexylamine coupled to AOUH using DIC. * indicates residual water.
C.4 References


Appendix D: LDH Assay Optimization

In order to confirm the optimal amount of LDH-containing supernatant to measure using the start and stop solutions of the LDH assay, it was necessary to perform an optimization study whereby a range of concentrations of lysed cells were reacted with the start and stop solutions. The ideal concentration was chosen whereby the point falls on the linear portion of the concentration-absorbance graph, and also is significantly different than the natural LDH release control.

3 groups of triplicate wells of A-10 VSMCs were seeded in a 96-well plate (200 µl / well, 25,000 cells / well) and left to incubate in a 5% CO₂ environment at 37°C for 24 hours. One set of triplicate wells contained growth media with no cells to serve as a background control. After 24 hours, the wells were rinsed and the media was changed in all the wells with 200 µl of DMEM-SP (DMEM with no sodium pyruvate), except for one set to which 200 µl of water media (10% solution v/v of H₂O in DMEM-SP) was added. The plate was returned to the incubator for another 24 hours. 45 minutes prior to the 24 hour time point, 10 µl of white media, water media and lysis solution (Triton-X) was added to the growth media control, natural release control and complete lysis control wells, respectively. After 45 minutes, the supernatant was removed, centrifuged and placed on ice.

In a new 96-well plate, increasing volumes of the test solutions were added to five wells (10, 20, 30, 40, 50 µl) to which DMEM-SP (with no FBS, as it can affect the LDH release) was also added to reach a constant volume of 50 µl in each well. 100 µl of start solution was added to each well, left in a dark environment for 30 minutes followed by the addition of 100 µl of stop solution. The plate was read on a Bio-Rad Model 3550-UV absorbance reader at 490 nm and 680 nm for sample and background signals, respectively (Clokie Laboratory, Faculty of Dentistry, University of Toronto). The absorbance vs. sample dilution factor was plotted on a graph and is shown in Figure D.1.

As can be seen in Figure D.1, the linear portion of the maximum LDH release curve is in the 40–80 % dilution factor range. In order to remain within that range and to maintain a significant difference from the growth media control, a 60% solution of test sample was chosen for further experiments. However, in order to reduce the amount of start and stop solutions used, the total volumes were divided in half. This therefore led to the final protocol used above (Section
4.3.7.4) whereby 15 and 10 µl of sample and DMEM-SP (with no FBS) respectively, were used for the LDH assay.

Figure D.1: Plot of absorbance vs. dilution factor in order to determine the optimal concentration for the LDH assay reagents whereby the sample falls within the linear portion and is significantly different from the growth media control.