Dissipative Assembly of an Ion Transport System

by

Paul Vu
Bachelor of Science, The University of Calgary, 2012

A Thesis Submitted in Partial Fulfillment
of the Requirements for the Degree of

MASTER OF SCIENCE

in the Department of Chemistry

© Paul Vu, 2013
University of Victoria
All rights reserved. This Thesis may not be reproduced in whole or in part, by
photocopy or other means, without the permission of the author.
Dissipative Assembly of an Ion Transport System

by

Paul Vu
Bachelor of Science, The University of Calgary, 2012

Supervisory Committee

Dr. Thomas Fyles, Department of Chemistry
Supervisor

Dr. Robin Hicks, Department of Chemistry
Departmental Member
Abstract

This thesis describes the development of an ion channel system exhibiting dissipative assembly characteristics. In this system an active transporter based on an oligoester fragment terminated in a thioester of 6-aminohexanoic acid (HO₂C-Hex-Adip-OctS-Hex-NH₂) undergoes thioester cleavage to form a thiol terminated oligoester (HO₂C-Hex-ADip-Oct-SH). This fragment was expected to be inactive for ion transport but previous work showed high activity in planar bilayer experiments. In this work, the high activity was shown to be due to the oxidized form of the thiol, the disulfide HO₂C-ADip-Oct-SS-Oct-ADip-Hex-CO₂H. Air oxidation was found to be quite rapid for the thiol based on ESI-MS (negative ion) and HPLC analysis. Under anaerobic conditions, the thiol fragment was an inactive species for ion transport. In situ air oxidation initiated transport activity associated with the disulfide.

The transporter HO₂C-Hex-Adip-Oct-Hex-NH₂ was active in planar bilayer experiments and was compared to the disulfide via activity grids. The activity of these two compounds was shown to be distinct from each other by conductance and channel duration differences. The activity of HO₂C-Hex-Adip-Oct-Hex-NH₂ was shown to die off in a period of 30 minutes at pH 8.2. Techniques were developed to stimulate and monitor activity and bilayer quality so that an inactive condition could be confirmed. The addition of Pr-S-Hex-NH₂⁺Cl as a fuel was shown to extend the activity of HO₂C-Hex-Adip-Oct-Hex-NH₂ by eight-fold in 1M CsCl electrolyte. Previous work had established the capability of thioester exchange reactions by a reaction between Pr-S-Hex-NH₂⁺Cl and benzyl thiol in a homogenous solution. The extended activity indicated that the same process may occur in a heterogeneous bilayer system. An inactive system created by the die-off in activity of HO₂C-Hex-Adip-Oct-S-Hex-NH₂ was treated with Pr-S-Hex-NH₂⁺Cl to regenerate activity. This cycle could be repeated once the activity died off again. All these findings are consistent with the dissipative assembly of a membrane transport system.
# Table of Contents

Supervisory Committee .................................................................................................................. ii  
Abstract........................................................................................................................................ iii 
Table of Contents .......................................................................................................................... iv  
List of Figures .................................................................................................................................. v  
List of Schemes .............................................................................................................................. viii  
Acknowledgements ....................................................................................................................... ix  

1 Introduction .................................................................................................................................... 1 
  1.1 Dissipative assembly .................................................................................................................. 1  
  1.2 Synthetic ion transport ............................................................................................................. 3  
  1.3 Planar bilayer experiment ......................................................................................................... 6  
  1.4 Prior work ............................................................................................................................... 10  
  1.5 Thesis goals ............................................................................................................................. 12  

2 Distinguishing HO₂C-Hex-ADip-Oct-SH from its dimer ............................................................. 13  
  2.1 Synthesis of HO₂C-Hex-ADip-Oct-SH .................................................................................... 13  
  2.2 Identifying the disulfide .......................................................................................................... 15  
  2.3 Modified synthesis of HO₂C-Hex-ADip-Oct-SH and converting it to disulfide ......... 18  
  2.4 Establishing the activity of the disulfide and the thiol ......................................................... 20  
  2.5 Conclusions and future work ................................................................................................. 25  

3 Dissipative Assembly of an Ion Transport System ....................................................................... 26  
  3.1 Establishing the activity of HO₂C-Hex-ADip-Oct-S(CO)-Hex-NH₂ ........................................ 27  
  3.2 Developing a protocol to maintain activity ............................................................................. 31  
  3.3 Testing the effectiveness of Pr-S-Hex-NH₃⁺Cl as a fuel in the bilayer ............................... 34  
  3.4 Dissipative assembly of an ion transport system .................................................................. 36  
  3.5 Conclusions and future work ................................................................................................. 40  

4 Experimental ............................................................................................................................... 41  

References ......................................................................................................................................... 43
List of Figures

Figure 1-1. A simple illustration of a conventional ion transport system (left) versus a dissipative assembly system (right) .................................................................................................................................................. 1

Figure 1-2. Some examples of synthetic ion channels that have been developed; A) Tabushi’s β-cyclodextrin, B) Matile’s octi-phenyl backbone, C) Ghadiri’s ring shaped peptide chains, D) Moszynski’s oligoesters. ........................................................................................................................................... 4

Figure 1-3. The set-up of a bilayer experiment and an example of a system with active ion channels opening and closing on a current (pA) versus time (s) recording. A polystyrene cup (a) with an aperture is immersed into an electrolyte chamber (b), lipid is applied to the aperture to form a bilayer. Electrical contact is made when agar salt-bridges (c) are used to connect the reference electrolyte (d) and Ag/AgCl reference electrodes (e) to the chamber ......................... 6

Figure 1-4. The different types of current patterns that have been identified; Square-top, Multi-level, Flicker, Spike, Erratic, and parameters beyond the detection limits ......................................................... 9

Figure 1-5. The mapping grid for the activity of active compounds. An empty grid is shown on the left and an example of an active compound (from Figure 1-2D, R = Hex-OH) is shown on the right.................................................................................................................................................. 9

Figure 2-1. Negative ion electrospray ionization mass spectrometry of HO$_2$C-ADip-Oct-SS-Oct-ADip-Hex-CO$_2$H (in methanol with 0.1% N,N-diisopropylethylamine) .............................................................. 16

Figure 2-2. $^1$H NMR analysis of HO$_2$C-ADip-Oct-SS-Oct-ADip-Hex-CO$_2$H and identification of the key proton .................................................................................................................................................. 17

Figure 2-3. HPLC and ESI-MS studies of thiol prior to oxidation to disulfide. Compound elution time: 8.85min.................................................................................................................................................. 19
Figure 2-4. HPLC and ESI-MS studies of disulfide after oxidation with thiol. Compound elution time: 11.37 min.

Figure 2-5. A representative trace of erratic type activity of HO$_2$C-Hex-ADip-Oct-S-S-Oct-Adip-Hex-COOH recorded in three experiments. Conditions: diPhyPC bilayer, 250µm aperture, Ag/AgCl electrodes, KCl junction solution with agar salt bridges, in 1M CsCl at pH 8.2 and an applied potential of +20 mV.

Figure 2-6. A representative trace of the “thiol” activity reported previously.

Figure 2-7. A comparison of HO$_2$C-ADip-Oct-SS-Oct-ADip-Hex-CO$_2$H (left) and the “thiol” as reported previously (right) via activity grids.

Figure 2-8. A representative trace of HO$_2$C-Hex-ADip-Oct-SH inactivity in the bilayer clamp. Conditions: diPhyPC bilayer, 250µm aperture, Ag/AgCl electrodes, KCl junction solution with agar salt bridges, in 1M CsCl at pH 8.2 and an applied potential of +100 mV.

Figure 2-9. A diagram of current, voltage, capacitance, and average conductance over 5 minute intervals plotted against experiment time. Showing the in situ thiol oxidation to disulfide in the bilayer clamp with detection by membrane activity. diPhyPC bilayer, 250µm aperture, Ag/AgCl electrodes, KCl junction solution with agar salt bridges, in 1M CsCl at pH 8.2.

Figure 3-1. a) Representative traces of erratic type activity for HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$. Conditions: diPhyPC bilayer, 250µm aperture, Ag/AgCl electrodes, KCl junction solution with agar salt bridges, in 1M CsCl at pH 8.2 and a varied applied potential of +100-200 mV. b) A comparison of activity grids of HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$ (left) and the same compound reported prior to this work (right).

Figure 3-2. The activity grids of the HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$ (left) and HO$_2$C-ADip-Oct-SS-Oct-ADip-Hex-CO$_2$H (right) for comparison.
Figure 3-3. A diagram of current, voltage, capacitance, and average conductance over 5 minute intervals plotted against experiment time. Showing HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$ activity until disappearance in the bilayer clamp. Conditions: diPhyPC bilayer, 250µm aperture, Ag/AgCl electrodes, KCl junction solution with agar salt bridges, in 1M CsCl at pH 8.2 and a varied applied potential of +120-200mV ................................................................. 30

Figure 3-4. Voltage-dependent activity of HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$. a) and b): a single cycle of an episodic protocol showing conductance as a function of time and the applied potential changes. c) and d): averaged conductance as a function of applied potential; c) is the data from panel a) while d) is the data from panel b)................................................................. 33

Figure 3-5. A diagram of current, voltage, capacitance, and average conductance over 30 minute intervals plotted against experiment time for HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$ premixed with Pr-S-Hex-NH$_3^+$Cl. Conditions: diPhyPC bilayer, 250µm aperture, Ag/AgCl electrodes, KCl junction solution with agar salt bridges, 1.6mM Cl$^-$NH$_3^-$Hex-(CO)S-Pr in 1M CsCl at pH 8.2, with varying potential ±80-120mV .............................................................................. 35

Figure 3-6. A diagram of current, voltage, capacitance, and average conductance over 5 minute intervals plotted against experiment time. Showing one cycle of the dissipative assembly system. 22µmol of fuel was added. Conditions: diPhyPC bilayer, 250µm aperture, Ag/AgCl electrodes, KCl junction solution with agar salt bridges, in 1M CsCl at pH 8.2 ......................................................... 37

Figure 3-7. Activity grids summarizing 100 minutes of the dissipative assembly system with one cycle (left). Compared to the activity grid of HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$ (middle) and HO$_2$C-ADip-Oct-SS-Oct-ADip-Hex-CO$_2$H (right) ........................................................................................................ 38

Figure 3-8. A diagram of current, voltage, capacitance, and average conductance over 5 minute intervals plotted against experiment time. Showing three cycles of the dissipative assembly system. Three injections of fuel were added in the order: 22µmol, 27µmol, and 36µmol. diPhyPC bilayer, 250µm aperture, Ag/AgCl electrodes, KCl junction solution with agar salt bridges, in 1M CsCl at pH 8.2 .......................................................................................... 39

Figure 3-9. Activity grids summarizing 120 minutes of the dissipative assembly system with three cycles (left) compared to the activity grid of HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$ (middle) and HO$_2$C-ADip-Oct-SS-Oct-ADip-Hex-CO$_2$H (right) ........................................................................................................ 40
List of Schemes

Scheme 1-1. Chemical interconversions of the proposed dissipative assembly system ............... 11


Scheme 2-2. Formation of side products through air-oxidation of HO₂C-Hex-Adip-Oct-SH ....... 15

Scheme 3-1. Reaction cycle of the proposed dissipative assembly system at pH 8.2 ............... 26
Acknowledgements

I would like to thank my supervisor Tom Fyles for all his advice and support; you have been a great mentor and definitely one of the best. To Joanne Moszynski and Jon Chui for all their work in this field, you have paved the way for me. To Andrew Dambenieks, thank you for having all of the samples prepared for me, you made completing this system a hell of a lot easier. I would like to thank the faculty and support staff in the University of Victoria chemistry department for making my work smooth and seamless. To my lab mates Paria, Gavin, and Ye, we had some good times and it wouldn’t have been as enjoyable with you guys. To all my family and friends who have support me since the very beginning, I love you all. To my grandfather who always saw the best in me and always reminded me of it, this one’s for you!
1 Introduction

1.1 Dissipative assembly

Dissipative assembly refers to a system that functions continuously in a state far from equilibrium and is maintained in the non-equilibrium state by a continuous supply of energy. The input energy is dissipated over time by irreversible processes and it is the dissipation of energy which maintains the structural and functional characteristics of the non-equilibrium system. The irreversible processes attempt to bring the higher energy non-equilibrium system to a thermodynamically favored state (global minimum on a potential energy surface). If the input energy is cut off or exhausted, the system collapses to equilibrium and ceases to function.¹

This thesis discusses the development of an ion transport system that displays dissipative assembly characteristics. Dissipative assembly in this case is the maintaining of an active structure (and hence ion transport activity) at a state away from equilibrium. The ion transport activity is maintained by a chemical fuel that is consumed (dissipates energy) over time as the system moves back to equilibrium. At equilibrium the system does not function as an ion transporter. This is similar to living cells that function away from equilibrium and require the use of fuels to maintain cellular functions.

![Figure 1-1. A simple illustration of a self-assembled ion transport system (left) versus a dissipative assembly system (right).](image-url)
The difference between a dissipative assembly system and a conventional ion transport system is that the latter operates (has membrane transport activity) at an equilibrium state. The active structures of membrane transporters typically involve the assembly of a number of molecules into an active structure which may viewed as a type of equilibrium “self-assembly” process (see examples discussed in section 1.2). Ion transport can be driven through the self-assembled transporting structure via concentration and potential gradients that involve flow of energy, but the transport system itself is at a thermodynamic minimum. The difference between the equilibrium assembly and dissipative assembly is illustrated in Figure 1-1. In a conventional ion transport system (Figure 1-1, left), self-assembly occurs as the system reaches lower potential energy states; at the global minimum potential energy state a fully functioning ion channel exists. For dissipative assembly (Figure 1-1, right), a fully functional transport system exists at high potential energy states (non-equilibrium) and over time as the system reaches lower potential energy states (thermodynamic equilibrium), the function of the transport system disappears. Although many types of self-assembly systems have been developed more extensively than dissipative assembly systems, their potential as a functioning system is limited by their static nature. The constant influx of energy to maintain a dissipative assembly system allows such systems to be unique, for example they could be directly controlled (via an input of energy), switched between states, and they allow for the possibility to couple together unrelated functions that use the same source of input energy; these are signs that are seen in living cells.

In a general context, dissipative assembly has been achieved in a number of macroscopic examples. The essential characteristic for dissipative assembly systems is that an energy input is required to form some kind of structure that puts the system at a higher energy level. This higher energy level system can then be detected while it is in a state far from equilibrium. For example, Grzybowski and coworkers synthesized a noble metal nanoparticle covered with a self-assembled monolayer mixture of azobenzene containing alkane thiol. When a solution of this noble metal nanoparticle was exposed to UV light (the external energy in this case), photoisomerization allowed cis-azobenzene to form. Dipole-dipole interactions between cis-azobenzene caused the formation of macroscopic crystals as the nanoparticles would organize together. In the absence of UV light, these crystals just simply broke apart. Obviously the higher energy states of these structures/systems do not have any function, but a structure was created and maintained by a constant influx of energy. Other examples reviewed are...
similar; a continuous energy flux maintains a macroscopic organized structure. The structures are certainly organized and maintained by the input energy but they do not do any chemical function.

A notable exception, conceptually related to the dissipative assembly system from this thesis, has been reported by van Esch. The system is based on a synthetic gelator designed to operate away from equilibrium. Synthetic gelators are self-assembled fibrous networks that encapsulate solvent molecules between their strands. The synthetic gelators in the van Esch system contain diesters (active state) that are prone to hydrolysis. Under the conditions of the experiment, these diesters hydrolyze to their diacid (inactive state) form. A chemical fuel (in the form of methyl iodide) is then used to doubly alkylate the diacid to bring them back to the active state, the diester. Gelation is detected using a macroscopic assay (invert the vial) so is similar to other macroscopic dissipative assembly systems reported. However this system does show how to link system behaviors to chemical transformations. The total experimental time for this system to display dissipative assembly is an issue; experiments took upwards of 900 hours per cycle.

This thesis reports a dissipative ion transport system. Unlike all previous dissipative assembly systems the outcome of energy dissipation is a function rather than a structure. Structures certainly form, but it is the functional characteristics that matter.

1.2 Synthetic ion transport

Over the decades, synthetic ion transport molecules have been extensively worked on to replicate the functionality of their natural counterparts all the while having a simpler chemical structure. In the present day, a variety of synthetic ion transport molecules have been synthesized ranging from the first ion transport molecule as a β-cyclodextrin derivative to the compounds synthesized in our lab. There are plenty of reviews on all the different types of synthetic ion transport molecules and some examples are shown below in Figure 1-2 selected to focus on the role self-assembly plays in the transport function.
Figure 1-2. Some examples of synthetic ion channels that have been developed; A) Tabushi’s β-cyclodextrin⁶, B) Matile’s octi-phenyl backbone¹⁰, C) Ghadiri’s cyclic peptide chains¹¹, D) Moszynski’s oligoesters¹².

All the ion transport systems shown in Figure 1-2 involve self-assembly. The Tabushi channel (Figure 1-2 A) is presumed to involve an end-to-end dimer to allow passage of ions through the membrane. A clearer example is the system developed by Ghadiri.¹¹ Cyclic peptides (Figure 1-2 C) stack on top of each other in bilayer membranes to form a cylindrical β-sheet peptide structure stabilized by hydrogen bonding. When stacked on top of each other, these peptide nanotube structures displayed ion transport activity. This is an example of a self-assembled ion transport system with the energy profile which was described in Figure 1-1. At higher potential energy states (when the cyclic peptides are monomers) no transport activity was detected. But over the course of the experiment as peptide rings began to stack on top of
each other (self-assemble), activity was detected. Once formed, the stable peptide nanotubes continue to transport. Similar examples have been reported by Matile (Figure 1-2 B). In these cases, peptides on an octi-phenyl backbone are self-assembled into a tubular β-barrel that displays activity. As monomers, these octi-phenyl backbones do not display transport activity and according to Figure 1-1, interactions between the peptide octi-phenyl backbones result in the self-assembly of a more stable aggregate that has ion transport capability.

The class of compounds discussed in this thesis are related to those synthesized by Joanne Moszynski;¹² these are shown in Figure 1-2 D. These are the most recent examples of a series of oligoester transporters developed at the University of Victoria.⁹,¹³,¹⁵ The self-assembly that occurs in this system is the formation of small aggregates in the bilayer membrane. The active structures themselves are less organized than in the previous examples but nonetheless are active and follow the energetic scheme of Figure 1-1. Ion transport activities from these compounds were detected in both vesicle and planar bilayer experiments. The data reported for these compounds are consistent with U-shaped inserts that cluster to make an active transporter. The dissipative assembly design based on this class of compound is discussed below in Section 1-4.

These synthetic molecules have advantages over their natural counterparts because the complexity of these natural counterparts are often more difficult to synthesize (via genetic manipulation or some other method) whereas the methods to obtain synthetic molecules are easier due to the simplicity of these synthetic molecules. Due to their simpler structure, characterization of synthetic ion transport molecules (in terms of their activity in bilayer membranes) as a result of certain differences in their structure can be made. Being able to synthesize ion transport molecules allows for the possibility to build upon the ideas seen in nature to develop channels with larger pores and/or channels that simply stay active for a longer period of time. Synthetic ion transport systems have come a long way since their birth and being able to mimic the abilities of those seen in nature could prove to be advantageous; especially in drug development since the control of channel size/durations could help with drug delivery systems.¹⁷
1.3 Planar bilayer experiments

One of the two common experiments when dealing with activities of synthetic ion channels is a planar bilayer experiment. In bilayer experiments, planar lipid bilayers are created using a bilayer clamp apparatus (shown in Figure 1-3 below). The apparatus is set up with a polystyrene cup (with a small aperture) immersed into an electrolyte chamber. Electrical contact is made with Ag/AgCl reference electrodes that are connected to the chamber via agar salt-bridges. The other common experiment is vesicle studies, where a spherical lipid bilayer is utilized instead of a planar bilayer.

Figure 1-3. The set-up of a bilayer experiment and an example of a system with active ion channels opening and closing on a current (pA) versus time (s) recording. A polystyrene cup (a) with an aperture is immersed into an electrolyte chamber (b), lipid is applied to the aperture to form a bilayer. Electrical contact is made when agar salt-bridges (c) are used to connect the reference electrolyte (d) and Ag/AgCl reference electrodes (e) to the chamber.

In the set-up of a bilayer experiment, a voltage is applied over a planar lipid bilayer as part of a circuit. This lipid bilayer has been formed over a small aperture that is typically about 250µm in diameter. This aperture intersects two chambers filled with an electrolyte; hence the lipid is in contact with the electrolyte in the two chambers. One of the characteristics of the bilayer is that it acts as a resistor; a fully established bilayer will break the circuit and a current will not be established. The thickness and integrity of the bilayer generally varies by quite a bit so the bilayer is judged by a capacitance test that determines the quality of the bilayer. A capacitance of 160pF or higher indicates the formation of a single bilayer over the aperture;
though a capacitance greater than 200pF indicates that the bilayer is quite thin and any value higher than this does risk bilayer breakage. Once a bilayer has been fully established and is shown to be stable (will not break or leak under various applied potentials for the span of several minutes), the compound to be investigated is introduced into the electrolyte. The voltage applied to the circuit is recorded in millivolts (mV) and the detected currents are recorded in picoAmperes (pA); modern instruments are capable of detecting single channels, especially for active compounds that have a square-top type activity (indicated in Figure 1-3 above). If the compound is active as an ion channel, ions will flow across the bilayer to reconnect the circuit and the current that is produced will be detected; larger currents will indicate a larger channel and/or multiple ion channels.

When channels are detected, an increase in current is also detected; the magnitude of the current increase depends on the size and number of channels formed. As a channel collapses the bilayer goes back to being a good resistor and prevents current flow; the current value then drops back down to 0pA (baseline). The duration and magnitude of these currents give insight about the characteristics of the compound as an ion channel. The current is shown on the y-axis and is recorded over time (on the x-axis). The duration of the currents give insight on the stability of the active structure relative to the inactive state. Once obtained, these current values can then be converted to conductance (measured in picoSiemens, pS) using Ohm’s law (Equation 1-1) where I = current, g = conductance, and V = the applied potential.

\[ I = gV \] 

Equation 1-1

From conductance values, the diameter of the channels studied can be determined alongside other characteristics such as the amount of charge that has flowed through the channel. The Hille equation\textsuperscript{14} (Equation 1-2) is used to calculate the apparent diameter of a channel where g is conductance, l is the channel length, d is the channel diameter, and \( \rho \) is the resistivity of the electrolyte; this equation assumes the channels take the shape of a cylinder with a domed end.

\[ \frac{1}{g} = l\rho[\pi(d/2)^2] + \frac{\rho}{d} \] 

Equation 1-2

Although these methods are well developed to look at ion transport systems, there are several problems that routinely arise. Firstly, the bilayer clamp is very sensitive to sound and vibrations in the surrounding environments so artifacts in the recorded data are common.
Secondly, a leaking bilayer could potentially produce artificial activity as this would allow the flow of ions and inadvertently cause a signal to be present. Impurities from other compounds could also produce an inaccurate result of the key compound being examined.

A repetition of experiments on a certain compound is strongly recommended to achieve a reliable result. This is especially true for compounds that are believed to be inactive in bilayers, since the absence of activity is not a decisive indicator that a compound is inactive. Three successful experiments was usually a good indicator of the transport activity of the compound. Failed experiments were experiments that contained an unstable bilayers leading to inaccurate recordings of transport activity.

The square-top openings shown in Figure 1-3 are the ideal on-off behavior. Synthetic ion channels can show this, but frequently show other types of openings due to their structure and/or interactions with other ion channel molecules. Aggregates of ion channel molecules could produce different transport activity then that of a single ion channel molecule. The types of patterns that have been identified in previous work are shown below in Figure 1-4. The activity color indicates that a pattern similar to the ideal case has been observed: square top is green, multi-level is blue, flicker is yellow, spike is red, erratic is purple, and grey is for the parameters beyond the detection limits. “Flicker” is a transition between two open states while a “multi-level” pattern indicates multiple related open states. “Spikes” are short duration; they may be square-top or other activities but their duration is below the measurement time of the instrument. “Erratic” encompasses a wide range of activities that have no discernible pattern. This indicates that many closely related conducting pathways are present either sequentially or simultaneously. Erratic activity is the most commonly observed condition for many synthetic ion channels. In order to summarize and compare all types of openings, a gridding method has been developed by Jon Chui to describe the characteristics of active compounds. These grids shown in Figure 1-5 show the conductance and the duration of each opening on a coarse scale. The cells are filled in with a specific color representing the pattern of activity for that compound. The detected current-time profiles represent a pattern that indicates the characteristics of the active compound as an ion transporter.
Figure 1-4. The different types of current patterns that have been identified; Square-top, Multi-level, Flicker, Spike, Erratic, and parameters beyond the detection limits.¹⁶

Figure 1-5. The mapping grid for the activity of active compounds. An empty grid is shown on the left and an example of an active compound (from Figure 1-2D, R = Hex-OH) is shown on the right.
Once a color is chosen, the information from the raw data of the compounds is interpreted and filled into the grid in terms of conductance and opened duration of channels. For a compound with multiple patterns, more than one color can be used to illustrate this characteristic. In Figure 1-5 (right), the active compound example was shown to have square-top, multi-level, spike, and erratic type activities. These different types of activities could arise from the interaction of the ion transport molecule with others, resulting in aggregation of ion transport molecules. These grids help distinguish active compounds from each other and essentially act as a fingerprinting method for every compound.

Having control of the applied potential, the pH of the electrolyte, and the thickness of a bilayer, the development of a dissipative assembly system involving an ion transport system could be carried out. The detection of current changes in this bilayer clamp assay allows for the investigation of this dissipative assembly system at a molecular level, potentially down to a single molecule level. This includes the ability to detect when the system switches from being in an active state to an inactive state and vice versa.

1.4 Prior work

The dissipative assembly system developed and discussed in this thesis (shown in Scheme 1-1) was first conceived by Andrew Dambenieks\textsuperscript{17}. The names of the compounds in this thesis follow an oligoester naming convention.\textsuperscript{17} Naming is as follows starting from the carboxylic acid end, all the ester sections of a compound are named until the other end of the molecule is reached and the name is terminated by the functional group on other end. For HO\textsubscript{2}C-Hex-Adip-Oct-S-Hex\textsubscript{2}, HO\textsubscript{2}C represents the carboxylic end, Hex is a six carbon ester, Adip is an abbreviation for a modified diphenylacetylene ester, Oct is an eight carbon ester with S indicating a thioester, Hex is another six carbon ester, and NH\textsubscript{2} is the amine end. Naming is similar for all other compounds named in this thesis.

The proposed dissipative assembly system, shown in Scheme 1-1, is based on an active transporter, HO\textsubscript{2}C-Hex-Adip-Oct-S-Hex-NH\textsubscript{2}, which was expected to show activity. It would also undergo intramolecular cyclization and produce a truncated product, HO\textsubscript{2}C-Hex-Adip-Oct-SH which was assumed to be inactive in planar bilayer experiments, along with a lactam by-product. In order to restore activity and restart the cycle, the truncated inactive product would react with an external fuel, Pr-S-Hex-NH\textsubscript{3}\textsuperscript{+}Cl, by a thioester exchange reaction that would produce the active compound once again. The thioester exchange reaction is based on literature precedents.
with small molecule examples.\textsuperscript{18} It was expected that the full length compound, HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$, would be active based on prior work by Joanne Moszynski\textsuperscript{12} discussed above in Figure 1-2. In her work, a twenty-fold decrease in activity was observed when the full length compound (Figure 1-2 D, R = Hex-OH) was truncated to a hydroxyl terminal group (Figure 1-2 D, R = OH). This result gave way to an assumption that an analogue of the compound in Figure 1-2 D would see a similar decrease in activity following intramolecular cyclization. Some of the internal segments in HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$ (for the dissipative assembly system) were also rearranged from previous oligoester analogues (Figure 1-2 D) to prevent inadvertent ester thiolysis.

Scheme 1-1. Chemical interconversions of the proposed dissipative assembly system.

Previous studies showed that vesicle experiments could not be used because the activity of HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$ was inactive in vesicle assays but active in planar bilayer assays.\textsuperscript{17} Since this compound is an integral part in the proposed dissipative assembly system, it was decided that planar bilayer experiments were the only way to investigate this system. Although HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$ was shown to be active in bilayer clamp experiments, the main focus in previous work was on the more stable compound HO$_2$C-Hex-Adip-Oct-S-Hex-OH.\textsuperscript{17} More experiments are needed to fully characterize the activity of HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$.

In his work, Andrew Dambenieks synthesized and characterized all the needed compounds in Scheme 1-1 along with a number of related compounds terminated in alcohol
groups. He showed activity of the full-length compounds in bilayers and demonstrated that HO₂C-Hex-Adip-Oct-S-Hex-NH₂ underwent intramolecular shortening via HPLC studies conducted in a homogeneous solution. Thioester exchange reactions were evaluated with the fuel and model compounds in proton NMR studies. The disappearance of a key peak and the appearance of another peak showed that thioester exchange reactions were possible for the fuel.

The design appeared to be flawed when very high activity in bilayers was also detected for the supposed inactive compound (HO₂C-Hex-Adip-Oct-SH). The detected activity was unusually high and long duration and was readily detected. This posed as a fatal flaw in the dissipative assembly system design since an inactive state could not be reached and the system would just cycle between two active states. At this point the design was dropped. The puzzling very high activity of the thiol was not further investigated.

1.5 Thesis goals

Looking at the previous work, one area that appeared to be a good starting point for investigation was the very high activity seen for the thiol (HO₂C-Hex-Adip-Oct-SH). Why is it so active? Why are the channels so big and open for so long? This is unexpected as this compound is quite similar to earlier compounds (Figure 1-2 D, R = Hex-OH) in terms of length and functionality, yet its activity is unusually high. Further investigations of HO₂C-Hex-Adip-Oct-SH in ESI-MS, NMR, and planar bilayer experiments are required. Chapter 2 will show that the thiol oxidizes quite readily to a disulfide and that the high activity is associated with the disulfide, not the thiol.

Since the thiol under anaerobic conditions is in fact quite inactive as a transporter, Chapter 3 explores the modifications made towards the development of a dissipative assembly system based on the design of Scheme 1-1. All the pieces of the dissipative system would have to be accounted for before carrying out the system itself. These pieces include: a compound active as an ion transporter, an inactive compound, an external fuel to regenerate activity, and reactions to switch between the two compounds. These reactions are the intramolecular reaction and the thioester exchange reaction. Next, a protocol will need to be developed to monitor the transport activity of the dissipative assembly system over a long enough period of time. After that, the dissipative assembly design can be tested by regenerating activity with the external fuel after activity has died off, this cycle will then be repeated.
2 Synthesis and membrane activity of HO$_2$C-Hex-Adip-Oct-SH and its disulfide

2.1 Synthesis of HO$_2$C-Hex-Adip-Oct-SH

The synthesis of this compound was reported previously by Andrew Dambenieks. In summary, HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$ was treated with a solution of 1M sodium bicarbonate to deprotonate the amine and initiate intramolecular cyclization of the compound producing a terminal thiolate anion and a 7-membered lactam. This reaction was then followed up with an acid work-up to protonate the thiolate anion. Following these reactions shown in Scheme 2-1, a white precipitate was collected and this was confirmed to be the thiol via NMR studies ($^1$H and $^{13}$C).

It is known that thiol functional groups oxidize in the presence of oxygen to form disulfide bonds; and on occasion oxygen could be a strong enough oxidizing agent to produce sulfonic acids from thiols. The investigation into thiol air-oxidation by Liu showed that these oxidation processes generally took four to six hours to oxidize thiol to disulfide. In the reaction above, no precautions were taken to prevent the formation of disulfide or sulfonic acids so it was quite possible that these could have existed as impurities. Scheme 2-2 below shows the possible air-oxidation products of HO₂C-Hex-ADip-Oct-SH, these are predicted to be the disulfide, HO₂C-ADip-Oct-SS-Oct-ADip-Hex-CO₂H, and sulfonic acid, HO₂C-Hex-ADip-Oct-SO₃H. Other potential products include a sulfoxide and a sulfone in the process of sulfonic acid synthesis. The magnitude of this issue cannot be assessed until the thiol product mixture is characterized; in this case electrospray ionization mass spectrometry set to detect anions (negative mode) would be the best method to identify impurities. A signal at 523m/z in negative ion mode was needed in order to confirm the presence of the thiol (deprotonation at the carboxylic acid end leaving the thiol end intact). Another peak at 261m/z could also indicate the dianion form of the thiol. A signal at 1045m/z will be consistent with that of the disulfide molecule minus one proton at one carboxylic acid end (with the other end still protonated). Another impurity at 522m/z would indicate the dianion of the disulfide.
2.2 Identifying the disulfide

Identification and characterization of HO₂C-Hex-ADip-Oct-SH and possible oxidation products was carried out with electrospray ionization mass spectrometry in negative mode. When a methanol solution of the thiol (with 0.1% N,N-diisopropylethylamine) was observed by ESI-MS in negative mode, only one signal was observed at 1045m/z, shown in Figure 2-1. This signal was determined to be the disulfide minus one hydrogen atom at the carboxylic end. The sulfonic acid derivative of the thiol was not confirmed as its peak was expected to be at 571m/z for the deprotonation of a carboxylic proton.
Figure 2-1. Negative ion electrospray ionization mass spectrometry of HO₂C-ADip-Oct-SS-Oct-ADip-Hex-CO₂H (in methanol with 0.1% N,N-diisopropylethylamine).

¹H NMR studies were also utilized to confirm that this sample did contain HO₂C-ADip-Oct-SS-Oct-ADip-Hex-CO₂H and as such this is established in Figure 2-2 below. The identification of a key triplet peak at a chemical shift of 2.69ppm confirmed the presence of the disulfide molecule. This peak had an integration of four, a multiplicity of a triplet, and was determined to belong to the -CH₂ group adjacent to the sulfur atom.
Figure 2-2. $^1$H NMR analysis of HO$_2$C-ADip-Oct-SS-Oct-ADip-Hex-CO$_2$H and identification of the key proton.

Although the reaction to reduce disulfide molecules to free thiols has been well established in the literature$^{21}$, these methods were unable to reduce this disulfide to the thiol, HO$_2$C-Hex-ADip-Oct-SH. Tris(2-carboxyethyl)phosphine (TCEP) was chosen over other reducing agents such as dithioerythritol and dithiothreitol because its efficiency at disulfide reduction and ease of purification of the products. When TCEP was reacted with HO$_2$C-ADip-Oct-SS-Oct-ADip-Hex-CO$_2$H in water, no thiol was identified (through ESI-MS) but only the by-product of the reaction, the phosphine oxide form of TCEP. Reduction under any conditions proved to be quite difficult so reduction reactions with dithioerythritol and dithiothreitol were not attempted. Preventing the oxidation of thiol seemed to be the best route to take in terms of synthesizing the required thiol.
2.3 Modified synthesis of HO₂C-Hex-ADip-Oct-SH and converting it to disulfide

After identifying the problem that prevented the synthesis of the thiol, modifications to the synthesis could be made to prevent any oxidation of the thiol with oxygen. In order to prevent the oxidation of the thiol to disulfide, air was to be simply excluded in the reaction described above in Scheme 2-1. The absence of oxygen in the reaction was predicted to prevent the formation of any thiol. A Schlenk line was utilized to introduce argon gas and carry out the reaction.

The modification made to the reaction was indeed successful and HO₂C-Hex-Adip-Oct-SH was correctly synthesized. A combination of high pressure liquid chromatography (HPLC) and ESI-MS analysis was used to properly observe the oxidation of the thiol to the disulfide; this is shown in Figure 2-3. ESI-MS analysis in negative mode showed a 523m/z peak, this was identified as the thiol since 523m/z corresponds with the thiol minus one hydrogen atom (as predicted earlier) but additional peaks were also present. A peak at 1047m/z was present, most likely correlating with the aggregation of two thiol molecules, 2M-H. The peaks at 409m/z and 571m/z were impurities within the ESI-MS instrument.

A purified sample of the thiol in methanol was run through the HPLC to confirm the presence of only the thiol. After that, the collected sample containing thiol from the HPLC was evaporated in a stream of nitrogen and was brought back up in 1mL of methanol. This sample was simply left in air overnight to allow oxidation of the thiol. Figure 2-3 below shows that the thiol eluted from the HPLC at 8.85min, different from the disulfide which eluted at 11.37min and is shown in Figure 2-4. The conclusion from these experiments is that the thiol was synthesized previously but due to handling in normal conditions, the thiol was easily air oxidized to the disulfide. The NMR of the thiol was not obtained in this work because it was not possible to remove all the HPLC solvent without oxidation to form the disulfide.
Figure 2-3. HPLC and ESI-MS studies of HO₂C-Hex-ADip-Oct-SH prior to oxidation to disulfide.

Compound elution time: 8.85 min.

Figure 2-4. HPLC and ESI-MS studies of HO₂C-ADip-Oct-SS-Oct-ADip-Hex-CO₂H after oxidation with thiol. Compound elution time: 11.37 min.
2.4 Establishing the transport activity of the disulfide and the thiol

The transport activity of the thiol reported previously\(^\text{17}\) was most likely the disulfide because after confirmation that HO\(_2\)C-ADip-Oct-SS-Oct-ADip-Hex-CO\(_2\)H was indeed synthesized, its activity in the bilayer clamp was evaluated. Shown in Figure 2-5 below is a representative trace of the disulfide. A representative trace is a snippet of a recording of the activity that best illustrates the activity pattern of the compound. The disulfide was shown to be quite active and this activity was maintained for the duration of many experiments with durations upwards of 4 hours each. HO\(_2\)C-ADip-Oct-SS-Oct-ADip-Hex-CO\(_2\)H also had erratic type activity which is the same activity pattern that was reported previously for the thiol.\(^\text{17}\) Both the activity from the disulfide and the reported “thiol” were shown to have high conductance and open channel durations for hours at a time; this comparison is shown in Figure 2-6. In order to see a clearer comparison between these two activity traces, Figure 2-7 shows the activity grid constructed for the disulfide (prepared as described above) and the “thiol” as reported previously.\(^\text{17}\) When comparing these two grids, one can notice that the activity characteristics of both these compounds are similar because they display the same grid coordinate and color\(^\text{16}\), an unusually high conductance with long durations.

![Figure 2-5. A representative trace of erratic type activity of HO\(_2\)C-Hex-ADip-Oct-S-S-Oct-Adip-Hex-COOH recorded in three experiments. Conditions: diPhyPC bilayer, 250\(\mu\)m aperture, Ag/AgCl electrodes, KCl junction solution with agar salt bridges, in 1M CsCl at pH 8.2 and an applied potential of +20 mV.](image-url)
Figure 2-6. A representative trace of the “thiol” activity reported previously.\textsuperscript{17}

Figure 2-7. A comparison of HO\textsubscript{2}C-ADip-Oct-SS-Oct-ADip-Hex-CO\textsubscript{2}H (left) and the “thiol” as reported previously (right) via activity grids.

The similarity in activity between the synthesized disulfide and the “thiol” in previous literature gives further evidence that “thiol” reported previously had oxidized in air to form the disulfide. That being the case, the activity of the thiol needed to be established and its activity in bilayer experiments will dictate how the dissipative assembly system will be developed. After confirmation of the thiol with ESI-MS analysis, the activity of this compound was tested in the bilayer clamp. Due to the risk of oxidation, the bilayer cell was placed into a box where a small stream of argon gas was constantly blown over the electrolyte chamber to ensure a blanket of argon over the system. The electrolyte was also bubbled with argon before use to remove the
majority of any oxygen dissolved into the electrolyte. When the thiol was tested in the bilayer clamp, no activity was detected and this is shown in Figure 2-8.

![Figure 2-8](image)

Figure 2-8. A representative trace of HO₂C-Hex-ADip-Oct-SH inactivity in the bilayer clamp. Conditions: diPhyPC bilayer, 250µm aperture, Ag/AgCl electrodes, KCl junction solution with agar salt bridges, in 1M CsCl at pH 8.2 and an applied potential of +100 mV.

Although there is an absence of activity, this does not rule out that the thiol could be active. Perhaps the thiol would be active when premixed into the lipid; this was not attempted as it is impossible to establish that the thiol would survive the usual lipid handling without oxidation. It is believed that Figure 2-8 shows a bilayer with thiol in it because of the billions of thiol molecules injected near the bilayer surface, only a few are required to diffuse into the bilayer to produce transport activity (if the compound was active). If this is the case then oxidation of this system will generate some disulfide and activity will start. Figure 2-9 shows the conversion of the thiol to disulfide in a bilayer experiment; this experiment was successful once in three attempts. In this experiment, HO₂C-Hex-ADip-Oct-SH was gently injected into the system via a 10µL syringe (1cm away from the aperture) and the applied potential was slowly increased to see if there was any activity. After 60 minutes of inactivity, 2x10µL injections of air were injected into the bilayer (1cm from the aperture). This small bubble broke the bilayer and after reforming the bilayer and applying a small applied potential spike, activity was present. There was a twenty minute duration of little activity between the 90 minute mark to 110 minutes. This was due to the bilayer breaking at the 90 minute mark and after reforming it, it took some time for the disulfide molecules that had formed to diffuse back into the bilayer membrane to produce activity again.
In terms of processing the data (like that of Figure 2-9), a graph showing the average current every five minutes to represent the data was considered but ultimately discarded as there was too much current fluctuation in this type of erratic opening. As a result some periods where there was obvious activity appeared to be relatively inactive. Another graph outlining the total time of channel activity above a threshold value averaged over a four minute interval was also explored but was not accepted because it gave values mostly at the extremes of fully open and fully closed which is not what the raw data look like. The final decision was to use a four-panel display as shown in Figure 2-9. The top panel is data from the current channel (in pA) as a function of the total experiment time. The raw data was removed of any signals reaching the digitizer limit, any signals from re-establishing the bilayer, and signals from capacitance tests all of which are clearly unrelated to channel activity. The protocol to remove these signals was carried out by converting the signals to data values and filtering out the values of the signals listed above. The second panel is a trace of the applied potential; the potential changes at points throughout the experiment as required, and some of the changes in the current channel (top panel) are only due to potential changes. The applied potential seems erratic because there was no protocol in the applied potential, the value was constantly changed to prevent bilayer breakage and induce/maintain activity. Protocols in applied potential were attempted but were shown to be difficult to maintain due to the bilayer breaking on occasion which would result in the required shift of the applied potential to 0mV to re-establish the bilayer. The third panel is a graph of capacitance to show the state of the bilayer through the course of the experiment. This panel in Figure 2-9 has only a few values related to bilayer breakage; it carries more data in subsequent experiments. The changes in the applied potential needed to measure capacitance are automatically excluded from the applied potential channel in the bilayer clamp. The fourth panel shows the average conductance over a five minute interval, the average conductance is obtained by taking a histogram of all the data values in the five minute interval and running a Gaussian curve through the resulting peak to determine the average value and standard deviation. This representation is the most direct measure of the activity presented in a way that can be interpreted based on the experimental data given in the other parts of the figure.
Figure 2-9. A diagram of current, voltage, capacitance, and average conductance over 5 minute intervals plotted against experiment time. Showing the in situ thiol oxidation to disulfide in the bilayer clamp with detection by membrane activity. diPhyPC bilayer, 250µm aperture, Ag/AgCl electrodes, KCl junction solution with agar salt bridges, in 1M CsCl at pH 8.2.
2.5 Conclusions

This chapter explored the activity of HO₂C-Hex-ADip-Oct-SH, a compound that was previously described to be highly active in planar bilayer experiments. Further analysis of this compound showed that the high activity was in fact due to HO₂C-ADip-Oct-SS-Oct-ADip-Hex-CO₂H formed by air oxidation. Upon procedural corrections to obtain HO₂C-Hex-ADip-Oct-SH, HPLC and ESI-MS studies showed that air oxidation was the cause of HO₂C-Hex-ADip-Oct-SH converting to its disulfide form. Planar bilayer experiments did not show any activity for HO₂C-Hex-ADip-Oct-SH and a method was developed to show HO₂C-ADip-Oct-SS-Oct-ADip-Hex-CO₂H channel activity could be turned on by air oxidation.

The inactivity of the thiol confirms that no major changes to the original proposed system of dissipative assembly (Scheme 1-1) are needed. The only change needed would be the use of an inert gas (such as argon) to prevent any oxidation of thiol in the time of the experiment. Conversely, it will be important to establish that generated activity is not due to introduction of oxygen and inadvertent formation of the disulfide.

Future work in areas of this chapter should look into the stability and characteristics of the disulfide; why were the reduction reactions using TCEP not successful in recovering any thiol? The high activity of the disulfide in planar bilayer experiments should also be of interest in future synthetic ion channel research. The aspects of the disulfide that produce this high activity could potentially lead to a new class of highly active compounds.
3 Dissipative Assembly of an Ion Transport System

In this proposed dissipative assembly system (shown in Scheme 3-1 for the species expected at pH 8.2), HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$ would undergo intramolecular reaction to form the inactive thiol, HO$_2$C-Adip-Oct-SH, and a by-product, probably the 7-membered lactam. A fuel, Pr-S-Hex-NH$_3^+$Cl, would then be introduced into the system to undergo a thioester exchange reaction with HO$_2$C-Adip-Oct-SH and reproduce HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$ with a propanethiol by-product; this exchange reaction was established previously.$^{17}$

Upon resolving the activity of the thiol, it was possible to move forward with experiments to probe the dissipative assembly design. All planar bilayer experiments were carried out under argon; this includes bubbling the electrolyte with argon to remove any oxygen dissolved in the liquid. This would prevent the formation of disulfide so that the state of the system can be observed by the bilayer activity. HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$ was chosen as the lead active compound instead of HO$_2$C-Hex-Adip-Oct-S-Hex-OH due to a quicker decomposition rate in HPLC studies.$^{17}$ HO$_2$C-Hex-Adip-Oct-S-Hex-OH was shown to be more stable in basic conditions than HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$; in two equivalents of base (diisopropylethylamine – DIEPA), the nearly all the compound still existed after 24 hours. In zero equivalents of base, HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$ nearly disappeared after 24 hours and in one
equivalent of base, \( \text{HO}_2\text{C-Hex-Adip-Oct-S-Hex-NH}_2 \) had completely disappeared. These compounds were measured in HPLC by taking an integration of the signals. A quicker decomposition was desired simply to ensure the duration of the dissipative assembly system was conducted within a reasonable experiment time (hours instead of days). The decay of these compounds were measured by The pKa for the membrane-associated species is not known, but a reasonable expectation is made in that increasing the pH from 8.2 will progressively increase the proportion of the amine form and thus accelerate the degradation. A reasonable pH was expected to allow the experiment to be conducted in a convenient time range. Ideally the experiment will be able to cycle the system a few times within the working lifetime of a bilayer of a couple hours.

### 3.1 Establishing the activity of \( \text{HO}_2\text{C-Hex-ADip-Oct-S-Hex-NH}_2 \)

In order to proceed with the dissipative assembly system, the activity of \( \text{HO}_2\text{C-Hex-Adip-Oct-S-Hex-NH}_2 \) had to be characterized within planar bilayer experiments and distinguished from impurities that may also produce their own activity such as \( \text{HO}_2\text{C-ADip-Oct-SS-Oct-ADip-Hex-CO}_2\text{H} \). Taking into account the results obtained in the previous chapter, the bilayer clamp experiments in this chapter were all performed in a chamber filled with argon gas to prevent the oxidation of any thiol to disulfide. The activity observed for \( \text{HO}_2\text{C-Hex-Adip-Oct-S-Hex-NH}_2 \) is shown below in Figure 3-1a as a representative trace. The activity of this compound (Figure 3-1b, left) is also compared to previous analyses of this compound\(^7\) (Figure 3-1b, right) via activity grids. The two activity grids of the compound are quite similar as both have erratic (purple) activity and similar duration conductance ranges. It is not known how much care was taken in previous experiments to collect early data (before decomposition) and to prevent disulfide activity, so the purple activity in the upper right hand corner might be due to disulfide that has formed. The synthesis in Chapter 2 showed that \( \text{HO}_2\text{C-Hex-Adip-Oct-S-Hex-NH}_2 \) broke down in basic environments but the rate that this reaction occurred was not explored. Taking this into account for a system at pH 8.2, activity had to be established right away but generally took about 5 minutes for channels to be formed once the compound had been introduced. Data was taken in early recordings to ensure that the activity that was looked at was in fact \( \text{HO}_2\text{C-Hex-Adip-Oct-S-Hex-NH}_2 \).
Figure 3-1. a) Representative traces of erratic type activity for HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$.

Conditions: diPhyPC bilayer, 250µm aperture, Ag/AgCl electrodes, KCl junction solution with agar salt bridges, in 1M CsCl at pH 8.2 and a varied applied potential of +100-200 mV. b) A comparison of activity grids of HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$ (left) and the same compound reported prior to this work (right).

From this trace and activity grid comparisons, this compound was shown to have an erratic type activity. The duration of these channels that were open lasted as long as several minutes (upwards of 10minutes at the beginning when HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$ was still abundant) to as short as 100ms (occurred when the compound was about to die off). The conductance of these channels varied quite a bit but all within the range of 400-1200pS when fully active and 100-400pS when about to die off. Comparing this activity to that of the disulfide, the conductance of HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$ is significantly lower. The conductance of the disulfide generally exceeded 2000pS quite easily and rarely had a conductance lower than that. On top of that, at applied potentials around 150mV, the activity of HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$ almost always disappeared within 30 minutes meanwhile the activity of the disulfide would
continue apparently indefinitely. And lastly, distinguishing between the two compounds, the channels of the disulfide are open much longer than HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$, lasting from minutes to hours at a time instead of milliseconds to minutes; this is illustrated in Figure 3-2. The grids of the HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$ (left) and the disulfide (right) are visually compared to each other and do not match. At no time was an activity of HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$ surpassing 3000pS observed.

Figure 3-2. The activity grids of the HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$ (left) and HO$_2$C-ADip-Oct-SS-Oct-ADip-Hex-CO$_2$H (right) for comparison.

Figure 3-3 shows a trace of the lifetime of HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$ as a representative trace of activity. When the applied potential was first held at 200mV it took a few minutes for activity to arise. A pH of 8.20 in the CsCl solution was enough to cause the activity to disappear within 30-45 minutes. The capacitance is degrading as the disappearance occurs, but the best evidence is indicated in the conductance versus time panel. The initial activity (conductance > 1000pS) does decrease to a low level as there was still activity near the end of the plot around 200pS, but it is possible to use this in the dissipative assembly system. Two conductance values at 25min indicated that two levels of activity were present. After activity had died off attempts were made to re-establish the activity. These attempts included shifting the applied potential to an extreme (generally 200mV, any higher usually broke the bilayer) and lifting the cup to reorganize the bilayer, but these were uniformly unsuccessful. One can assume that this is the result of an absence of HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$ and the presence of HO$_2$C-Hex-ADip-Oct-SH in the bilayer. It is pretty clear that there is neither “pure” HO$_2$C-Hex-ADip-Oct-S-Hex-NH$_2$ nor any HO$_2$C-ADip-Oct-SS-Oct-ADip-Hex-CO$_2$H in the bilayer.
Figure 3. A diagram of current, voltage, capacitance, and average conductance over 5 minute intervals plotted against experiment time. Showing HO₂C-Hex-Adip-Oct-S-Hex-NH₂ activity until disappearance in the bilayer clamp. Conditions: diPhyPC bilayer, 250µm aperture, Ag/AgCl electrodes, KCl junction solution with agar salt bridges, in 1M CsCl at pH 8.2 and a varied applied potential of +120-200mV.
3.2 Developing a protocol to maintain activity

The development of a protocol arose due to the idea of wanting to perform the experiment with limited interference from the operator. Running a fixed potential over a long period of time adds stress on the bilayer. Therefore, longer experiments require a varying applied potential; this results in a constant thinning and thickening of the membrane in response to the potential change and the net result is that a sealed bilayer is maintained. An episodic-type configuration is a set-up in the recording process where the detection signal is recorded in episodes instead of continuously (which is known as a gap-free configuration). The episodes are set-up so that certain parameters are automated, such as the applied potential of the system. In this experiment, a 90 second cycle from a positive value to a negative value was used to have ions flow in opposite directions to maintain overall balance between both sides of the bilayer. This method was able to somewhat maintain activity but removed the ability to control the voltage and any issue that would arise would almost always result in the premature stopping of a recording. These issues pertain to bilayer integrity (since over time the bilayer would begin to leak and/or just simply break) and keeping the activity signal within a visible range. Sometimes the activity would exceed the digitizer range of the bilayer; this is because a gain telegraph was not implemented into the system. Under this set-up a lower voltage was required to bring the signal into the digitizer range.

Failing to truly maintain activity and/or bilayer stability, an episodic system was considered to be not the best method although it would make running the dissipative assembly system much easier. In order to maintain activity and bilayer stability, full control of the bilayer clamp was the necessary route to proceed; a gap-free configuration was then taken up to do this. A gap-free configuration is a continuous recording that allowed the ability to specifically control the applied potential running through the bilayer clamp at any given time during the recording. A higher applied potential was required to activate the channels but this risked the integrity of the bilayer and a slightly lower potential was required to maintain bilayer stability. During these experiments, the applied potential was set at 200mV to initiate activity and the moment activity was observed the potential was lowered, usually within the range of 120-150mV. This lower range was generally high enough to maintain activity for a period of time and low enough so that bilayer breakage requiring repair only occurred every couple of hours.
Repairing would include raising/lowering the cup to reform the bilayer from available lipids and brushing additional lipid onto the aperture if needed.

Both the episodic and gap-free configurations can have a math channel that runs parallel with the current detection signal. The math channel takes the active current signal and directly converts it to conductance by dividing by the applied potential. This provides a real-time conductance analysis of active compounds as they are forming channels in the bilayer clamp.

What is the basis for adjusting the applied potential to maintain activity? What property of the channels formed by HO₂C-Hex-Adip-Oct-S-Hex-NH₂ is responsible? As this system was explored it appeared that the compound activity depended on the applied potential, with much higher activity at higher potentials. Although this was not explored in a detailed fashion, there is some evidence that this is the case; the experimental data is shown in Figure 3-4 a) and b). Initially, 90nmol of HO₂C-Hex-Adip-Oct-S-Hex-NH₂ was injected into the system. These experiments were conducted using an episodic protocol in which a brief period in each cycle was placed at a low potential (30 mV). Panels a) and b) show that this low potential period has little to no conductance relative to the signals at higher applied potentials (either positive or negative). Averaged data presented in panels c) and d) show that this activity is clearly not a steady function of applied potential. A channel that follows Ohm’s law would have a constant conductance no matter what the applied potential. This would appear as a horizontal line in a plot like panel c) or d).

This is evidence that the channels formed by HO₂C-Hex-Adip-Oct-S-Hex-NH₂ are voltage dependent. Previous work by Joanne Moszynski²² failed to observe any examples of this type of behavior in related oligoesters even though this question was investigated directly for many compounds. Current work by a colleague, Ye Zong²³, has shown that a similar behavior in panels c) and d) of Figure 3-4 are present in a series of unrelated oligoesters. This type of voltage-dependent activity could result in channels of longer duration forming at higher potentials, channels of larger diameter being stabilized at higher potentials, and/or insertion of more compounds at higher potentials leading to more channels in parallel. Whatever the origin in this system, the practical result is that the procedures described above can be used to attempt to kick start a system that appears to be inactive.
Figure 3-4. Voltage-dependent activity of HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$. a) and b): a single cycle of an episodic protocol showing conductance as a function of time and the applied potential changes. c) and d): averaged conductance as a function of applied potential; c) is the data from panel a) while d) is the data from panel b).
3.3 Testing the effectiveness of Pr-S-Hex-NH$_3^+$Cl as a fuel in the bilayer

Knowing the characteristics of HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$ in planar bilayer experiments, the effectiveness of Pr-S-Hex-NH$_3^+$Cl as a fuel to regenerate HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$ via a thioester exchange reaction could be evaluated. From the previous section, the activity of HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$ lasted around half an hour before disappearing, most likely breaking down to thiol. Using this, if the fuel was premixed into a 1M CsCl solution before introduction of HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$, could activity last longer than half an hour before disappearing? Figure 3-5 below illustrates the effect of the fuel on the activity of HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$. Initially, 90nmol of HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$ was injected and mixed into the system alongside 13.3µmol of Pr-S-Hex-NH$_3^+$Cl. Once activity was obtained, recording of the data began so HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$ addition was not indicated in Figure 3-5. The current channels look as though it cannot exceed 200pA but in actuality, the signals do not exceed the limit but when condensed the signals seem as though they are.
Figure 3-5. A diagram of current, voltage, capacitance, and average conductance over 30 minute intervals plotted against experiment time for HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$ premixed with Pr-S-Hex-NH$_3^{+}$Cl. Conditions: diPhyPC bilayer, 250µm aperture, Ag/AgCl electrodes, KCl junction solution with agar salt bridges, 1.6mM Cl$^{-}$NH$_3$-Hex-(CO)S-Pr in 1M CsCl at pH 8.2, with varying potential ±80-120mV.
The activity of HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$ was sustained for four hours with the presence of 1.6mM of Pr-S-Hex-NH$_3^+$Cl; this is an eight-fold duration increase, shown in Figure 3-5. This prolonged activity from the fuel confirms that thioester exchange reactions are capable of forming additional HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$ from HO$_2$C-Hex-ADip-Oct-SH. This draws a parallel with previous work in thioester exchange reactions, these exchange reactions were shown to occur readily and within half an hour. Since the erratic type activity and the conductance do not change throughout the course of this experiment, the source of this activity was determined to be coming from HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$ and proved that Pr-S-Hex-NH$_3^+$Cl was indeed effective as an external fuel.

### 3.4 Dissipative assembly of an ion transport system

With the activity of HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$ and HO$_2$C-Hex-Adip-Oct-SH established, the effectiveness of the fuel in the bilayer evaluated, and the protocol to maintain activity and bilayer stability configured, all the aspects of the dissipative assembly were ready to be put together. In order to produce a successful experiment, the right amount of HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$ had to be introduced into a stable bilayer while trying to establish activity quickly. Bilayer quality had to be constantly monitored and the applied potential had to be constantly adjusted to maintain activity if it was there and to stimulate activity if it had died off. Activity die-off needed to be confirmed once no more activity was noticed for 10 minutes at 200mV. After activity die-off, a limited amount of fuel would be added to re-establish activity without introducing any air into the system. Throughout the whole experiment the quality of the bilayer also had to be maintained.

Below in Figure 3-6 is an illustration of the first successful recording of a dissipative assembly system in which all these factors described were shown to be under control. This diagram shows a single cycle of activity regeneration and then activity die off. Once activity was obtained, recording of the data began so HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$ addition was not indicated in Figure 3-6.
Figure 3-6. A diagram of current, voltage, capacitance, and average conductance over 5 minute intervals plotted against experiment time. Showing one cycle of the dissipative assembly system. 22µmol of fuel was added. Conditions: diPhyPC bilayer, 250µm aperture, Ag/AgCl electrodes, KCl junction solution with agar salt bridges, in 1M CsCl at pH 8.2.

A total of 74 nmol of HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$ was injected into the system at before data recording and after a couple minutes at an applied potential of 200mV, activity was generated. As predicted, activity of HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$ began to die off after thirty
minutes and after twenty minutes of little to no activity, 5mg of Pr-S-Hex-NH$_3^+$Cl (solution in 100µL 1M CsCl; 2.2x10$^{-5}$mol; concentration in electrolyte if fully mixed would be 6.3mM) was injected. After approximately 5-10 minutes of fuel introduction, erratic type activity was observed. The activity level was around 400-1200pS for 20 minutes. In Figure 3-6, no higher conductance activity was detected; a conductance less than 2nS rules out the disulfide as the active species. When comparing activity grids of this system to the activity grids of HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$, the ranges of conductance and open duration are very similar; this is shown in Figure 3-7. After activity had died off again for HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$, unsuccessful attempts were made to re-establish the activity.

![Activity Grids](image)

Figure 3-7. Activity grids summarizing 100 minutes of the dissipative assembly system with one cycle (left). Compared to the activity grid of HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$ (middle) and HO$_2$C-ADip-Oct-SS-Oct-ADip-Hex-CO$_2$H (right).

With a recording of one cycle of the dissipative assembly system, more cycles of the dissipative assembly system were attempted. Figure 3-8 below shows a successful experiment of the dissipative assembly system going for three cycles. After a stable bilayer had been established, 8.4x10$^{-8}$mol of HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$ was injected into the system and after a couple minutes at an applied potential of 200mV, activity was generated. After activity had died down again, 5mg of Pr-S-Hex-NH$_3^+$Cl (solution in 100µL 1M CsCl; 2.2x10$^{-5}$mol; concentration in electrolyte if fully mixed would be 6.3mM) was injected to restore activity. Subsequently, 6mg and 8mg of Pr-S-Hex-NH$_3^+$Cl (solution in 100µL 1M CsCl, 2.7x10$^{-5}$mol and 3.6x10$^{-5}$mol, respectively) were injected for the die-off occasions after that. It should be noted that cycle 3 was established when the applied potential was at 200mV rather than 175mV for
the other two cycles. This could explain why the activity for the first two cycles lasted for only a short period of time. To confirm the activity of this experiment was not attributed to the disulfide, the activity grid of the third cycle was compared to that of HO₂C-Hex-Adip-Oct-S-Hex-NH₂₂; this is shown in Figure 3-9.

Figure 3-8. A diagram of current, voltage, capacitance, and average conductance over 5 minute intervals plotted against experiment time. Showing three cycles of the dissipative assembly system. Three injections of fuel were added in the order: 22µmol, 27µmol, and 36µmol. diPhyPC bilayer, 250µm aperture, Ag/AgCl electrodes, KCl junction solution with agar salt bridges, in 1M CsCl at pH 8.2.
3.5 Conclusions and future work

A dissipative assembly system in the form of an ion transport channel has been successfully designed and tested. The presence of argon gas was the key to this whole system being able to work. It was uncovered that the reason for the high activity behind the thiol was actually the disulfide instead of the thiol. This allowed the dissipative assembly system from previous work\textsuperscript{17} to be completed. The fuel was determined to be quite effective at thioester exchange reactions and reproduce/maintain activity. The presence of activity and inactivity during the cycles of the dissipative assembly system occurred as expected.

Future dissipative assembly systems should attempt to function in normal atmospheric conditions as those are the conditions in which natural systems function. If a rapid reduction reaction of the disulfide could be figured out, another on/off system in terms of ion transport could be developed. In this system in air, the reductant would hold the system in a functional state depending on the availability of the fuel, Pr-S-Hex-NH\textsubscript{3}+Cl. At some point the reductant itself would be exhausted and the system would revert to disulfide. Such a system would have two controlling conditions for stable functioning. Although still very simple, this is similar to a living cell in which several independent cycles are held away from global equilibrium through consumption of chemical energy sources and are interdependent on the availability of those sources.
4 Experimental

All the following samples, HO$_2$C-Hex-Adip-Oct-S-Hex-NH$_2$, HO$_2$C-ADip-Oct-S-Oct-ADip-Hex-CO$_2$H, and Pr-S-Hex-NH$_3^+$Cl, were available from prior work.$^{17}$ A Bruker AC300 (300 MHz, CDCl$_3$, room temperature, $^1$H) was used for the recording of NMR spectra. A Thermo Trace GC-DSQ was used to record ESI-MS spectra in negative mode. HPLC experiments were recorded on a HP Series 1100 with a Grace Davison “Alltima” RP C18 semi-prep (10mm x 150mm) column. All solvents were filtered with a Millipore sub-micrometer filter; solvents used are as follows: HPLC-grade acetonitrile, HPLC-grade methanol, HPLC-grade isopropanol, and deionized water. The elution gradient is as follows: at 0min, 5% methanol and 95% acetonitrile, to a gradual change to 15min, 50% methanol and 50% acetonitrile, to 16min, 5% methanol and 95% acetonitrile. The elution was monitored and recorded by UV at a wavelength of 254nm and fluorometrically with an excitation wavelength of 310nm and an emission wavelength of 330nm.

Bilayer Clamp Assay

A BC-525A bilayer clamp from Warner Instrument Corp. was used for all planar bilayer experiments. Data recording and analysis used ClampEx 8 and Clampfit 10 from Axon Instruments, respectively. The polystyrene cups used had 250µm diameter apertures and the lipid used was diphytanoyl phosphatidylcholine (diPhyPC). 200µL of 25mg/mL of lipid in chloroform was dried down with argon gas and re-dissolved in 200µL of decane. 1M CsCl in 10mM HEPES and 10mM TRIS was used as the electrolyte with a pH of 8.2. 0.5-1µL of the lipid solution was used to prime the aperture of the cup. The cup was then placed in a holding cell filled with 1M CsCl electrolyte. The circuit of the bilayer clamp was connected with agar salt bridges (containing KCl) and Ag/AgCl electrodes. In the electrolyte, 1-2µL of additional lipid was brushed over the aperture to form a bilayer, which was stabilized mechanically (lifting/dropping the cup and brushing the aperture) and monitored for half an hour. If the compound was not premixed, then at this stage the compound was either injected (around 5-10µL of solution with a 10µL syringe) 1cm from the aperture or brushed (1µL) onto the aperture. The bilayer stability was constantly monitored through capacitance tests and the protocols used to collect the data on ClampEx 8 are described in chapter 2.4. No stirring was done for any of the dissipative assembly experiments as stirring agitates the bilayer and there is a good chance the bilayer will
break. Due to a lack of significant mixing, the concentration of the fuel at the membrane is not the same with each injection. All the data signals were filtered with an 8-pole Bessel filter (1kHz) and a Gap-free protocol was used to collect the data. Fully stable bilayers never showed any signs of “activity”.

**Synthesis**

2-2: HO₂C-Hex-Adip-Oct-SH: In a Schlenk line, HO₂C-Hex-Adip-Oct-S-Hex-NH₂ (50mg, 0.0784mmol) was added to 3mL of a saturated bicarbonate solution. This solution was gently stirred and heated to 50°C for 24 hours; a white cloudy solution formed after the reactant had dissolved. Afterwards, the solution was cooled to room temperature then cooled further in an ice bath. At 0°C with vigorous stirring, 5mL of chloroform was added and the solution was acidified to pH 2 with 5M hydrochloric acid. The organic fraction was separated and dried with sodium sulfate. The solution was dried down under argon gas once to determine the yield of the reaction (14.8mg, 36%). Generally, this solution was kept in the chloroform solution and stored in the freezer at -20°C. All solvents used were bubbled with argon gas to prevent any oxidation.
References


