Heterosandwich assay of nicotinic acetylcholine receptors

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HETEROSANDWICH assay of NICOTINIC ACETYLCHOLINE RECEPTORS

A Thesis
Submitted to the Faculty
of
Virginia Commonwealth University
by
Augustine Joseph Pagan IV

In partial Fulfillment of the
Requirements of the Degree
of
Master’s Degree

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Virginia Commonwealth University
Richmond, Virginia
To my parents for their unconditional support, care, love, and everything else they have given me.
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ABSTRACT

Using the technology afforded by Winschel et al., cyclen-1, a high affinity, strong complexation agent for 8-hydroxypyrene-1,3,6 trisulfonate and derivatives, a new assay has been developed for fluorescently labeling proteins of interest (POIs). Ligation of the endogenous ligand for nicotinic acetylcholine receptors (nAChRs), acetylcholine, using click chemistry afforded the triazole derivative of an alkynyl-acylcholine (compound 1) with 8-azidopyrene-1,3,6 trisulfonate (compound 2). Liposomes encapsulated with Rhodamine B were used to strengthen the initial fluorophore response of compound 2, using an anchored form of cyclen-1 complex. Using a palmitoyl tail as the lipophilic moiety for liposomal amplification, the subsequent response has a fluorophore ratio of up to 1:1 million, compound 2:Rhodamine B molecules. In vitro assay using compound 2 and cyclen-1 anchored liposomes with HEK-293 cells produced a positive binding response, allowing brightly colored fluorescent images of nAChRs upon the cellular membrane. A control for nAChR binding was performed using a co-culture of HEK-293 and endothelial cell lines. Control experiments show compound 2 and liposomes weak binding endothelial cells, however, this could be due to accumulation from another mechanism, more work is necessary to prove whether or not this is correct.
RATIONALE

Many fluorescent labeling technologies used today exhibit an affinity label in order to attach itself, whether covalently, or through non-bonding interactions, to the subject of interest. The attachment of fluorophores to various proteins and cell lines has led to improved visualization and understanding of protein dynamics, interactions and more, however, many of the techniques rely on one fluorophore per subject of interest. Even using fluorophores with exceptional quantum yields and emission spectra, a one molecule limit cannot detect proteins which are extremely low in abundance. A technique with the ability to amplify the fluorescent response of a low expression protein could improve the diagnosis and treatment of individuals, as precancerous cells are thought to exhibit low levels of certain membrane proteins.

Within this document, we describe a new technique for the fluorescent labeling of ligand gated ion channels and the subsequent amplification of the primary label using an affinity based liposome. By covalent attachment of two molecules, a ligand and a fluorophore, which bind to separate targets, we form a ‘heterosandwich’ compound which allows two binding events to occur simultaneously. The two binding events are 1) the ligand (acetylcholine) binding to its associated receptor and 2) the fluorophore (PTS) binding to a receptor-molecule (cyclen-1) which exhibits high binding affinity for the fluorophore. Amplification is based on the anchoring of the receptor, cyclen-1, into the bilayer of liposomes, with liposomes encapsulating a solution of fluorophore (with differing emission properties) within the aqueous pore.
Scheme. Deposition of the two molecules covalently linked, allowing two simultaneous binding events to occur. The binding of cyclo-1 with PTS (green molecule), and acetylcholine (black molecule) binding to an Acetylcholine Receptor protein.

Many new labeling technologies promote the use of fusion proteins to fluorescently label the protein of interest (POI) by fusing a suicide enzyme to the POI by vector technology. The suicide enzyme can then form a covalent bond with a ligand-fluorophore conjugate. These techniques can exhibit extremely high binding constants of the ligand to the suicide enzyme (HaloTag has a binding constant higher than that of strepavidin/avidin).

To test the methodology of this cascade system, we have chosen to use nicotinic acetylcholine receptors (nAChRs) as the POI, using the endogenous ligand acetylcholine (ACh) as one half of the heterosandwich complex.

Scheme. Structure of Acetylcholine.
The second half of the complex is a dye which binds with cyclen-1, a pyrene trisulfonate (PTS).

![Pyrene trisulfonate dyes. Y=OH, HPTS; Y=NH₂, APTS; Y=N₃, AzPTS.](image)

Azidopyrene trisulfonate (AzPTS) allows the use of click chemistry and can easily provide the heterosandwich complex.

![Conjugate of ACh and AzPTS, (AChPTS)](image)
This new molecule allows us to test the heterosandwich hypothesis \textit{in vitro} and provide us with feedback to establish this new methodology of labeling and fluorescent amplification of ligand gated ion channels.

\textit{Scheme.} Image depicting the cascade binding sequences between a Protein of Interest (blue, bright blue active site), ligand-fluorophore derivative (green), and a liposome anchored with cyclen-1 (red). The binding of AChPTS to AChR is labeled as 1. The binding of the liposome with a tethered cyclen-1 molecule is labeled as 2.
CHAPTER ONE

Fluorescence Microscopy and Small Molecule probes developed for biological assays

1.1 Introduction

Fluorescent molecules have become extremely powerful tools in the development of potent labeling agents; various cell lines, membrane proteins, and tumors can be tagged with fluorophores, visually detecting aspects of the biological milieu under investigation. Some fluorescent molecules exhibit the ability to be specifically quenched with another molecule or environmental change i.e. pH, small molecule reactants (H₂S), oxidoreductants and more. Fluorescent molecular tags were not instantly realized with the discovery of the phenomena of fluorescence. Small molecule tags were the first labels, antibodies were reacted with aromatic isocyanates in solution and allowed to behave in its proper biological environment. One of the first proteins found to auto fluoresce, Green Fluorescent Protein (GFP) were used on a large scale in labeling methods, however, GFP can inhibit natural activity due to the sterically demanding protein. The use of fluorophores has led to intriguing and exceptional results in biological breakthroughs, extending knowledge to new bounds.
Sometimes when fluorophores are used, an excess is afforded, saturating the solution/environment, affecting outcome and lowering the signal to noise ratio. The ability to quench fluorophores is possible, albeit, sometimes the quenching process can affect the outcome i.e. oxidoreductants, detergents, nonspecific-low potency quenchers. In this work we describe the ability to specifically and tightly bind a fluorophore attached to an endogenous ligand for various assays, labeling, surface chemistry, and the development of a new methodology for excisions/excisional biopsies.

1.2 Fluorescence Microscopy

Fluorescence microscopy has early beginnings, with various unrelated concepts coming together into what is now an exceptional and powerful instrument and technique. George Stokes, of Cambridge University, is considered to be one of the founders of fluorescent study, even coining the term “fluorescence” from the properties of one of the compounds he studied, CaF₂ (fluorite/fluorspar) which emitted red light upon irradiation with blue light⁴. His various studies (during and after 1852) also concluded a longer wavelength emission compared to the wavelength of excitation.⁴

During the development of higher precision microscope objectives, a relationship between the wavelength of light and the resolution was noticed, case in point, UV was the new goal, lowering the wavelength enhances resolution. While experimenting with
higher frequency excitations (blue & UV) the German scientist August Kohler noticed 'autoluminescence' (fluorescence) of certain tissues. Two breakthroughs in microscopy, cellular & molecular biology are two of the first staining assays introduced: 1) In 1941, Albert Hewett Coons successfully labeled a protein with a fluorescent dye, pneumococcal anti-serum with anthracene-isocyanate and fluorescein dyes, respectively. 2) By fluorescently labeling a secondary antibody, and a naked primary antibody, viral counterparts were identified in culture.³

The modern fluorescent microscope is based off the original design in the 60s, using a dichromatic mirror to reflect the excitation beam 90° into the sample solution, inducing fluorochrome emission. Dichromatic mirrors behave in a convenient way, reflecting higher energy light (shorter wavelength) and are seemingly transparent to longer wavelengths, providing, in spatial terms, an area for the detector related to the emission lamp. The image formed is gathered from multiple luminous points, or disks, of light, scattering light ubiquitously. The objective lens only captures a fragment of all light shining, and is directly proportional to the objective’s aperture at the imaging distance of the lens.
In order to capture a precision image, fluorochrome illumination is required to use a powerful, long lasting beam of light. All excitation wavelengths of the sample (fluorochrome) must also be accounted for and use of a proper lamp is necessary. Modern microscopes use a small range of high power illuminators, mercury, metal halide, and xenon lamps or lasers.

Fluorescence microscopy, as defined by name, uses fluorescence to image cells and molecules. Fluorescence is the incidental emission of longer wavelength light from shorter wavelength absorbed light. As light energy is absorbed by a fluorophore (light emitting molecule), electrons rise to a high energy singlet state and any excess energy is released as a lower energy photon than the corresponding excitatory photon.

Fluorescence lifetime is within the picosecond to nanosecond time scale of emission ($10^{-12}$ to $10^{-9}$ sec), the long lifetime release of photons is known as phosphorescence, on the order of hundredths of seconds ($10^{-2}$ sec) which also consists of an intersystem crossing of a singlet state electron to the triplet state. At the lowest energy state of a
molecule, electrons are referred to be in the ground state, or $S_0$. As electrons are energized by incidental light, they move from $S_0$ to $S_1$, $S_2$ and $S_n$ (excited singlet states). The emission of light compared to excitation wavelength from the fluorophore is referred to as the Stokes Shift of the molecule, with larger shifts being preferable for fluorescence microscopy as a low shift can induce large amounts of noise and hard to decipher emission spectra.

![Stokes Shift](image)

*Figure 1.2. The Stokes Shift phenomena, exaggerated for clarity and understanding*

Organic molecules exhibit fluorescent properties from a conjugated pi system, alternating double bonds or aromaticity is necessary for light emission. A general but not hard follow rule is the larger a pi system, the more red-shifted a molecule’s emission becomes, while smaller fluorescent molecules exhibit in the (closer to) blue region.
Fluorescence microscopy had humble beginnings, in the mid-nineteenth century, and has become one of the most sophisticated and powerful molecular biology techniques to date. Studying protein dynamics, transportation, labeling of disease and much more, the techniques presented are just some of the state-of-the-art advancements among this diverse area of cellular visualization.

1.3 Cellular Imaging Techniques using Fluorescent Molecules

The amount of progress in recent years has been astounding, with the advent of nucleotide base pair driven systems (Fluorescent in situ Hybridization), modified enzymes with suicidal tendencies, forming stable covalent bonds with a specialized ligand (HaloTag, SNAPtag, CLIPtag), and a reaction assisted fluorophore quench (β-Lactamase tag). Of these, some are vector based imaging techniques, requiring the use of transfection of the wild type polypeptide with a fusion gene.
1.4 Fluorescent tagging technologies

HaloTag:

HaloTag cell imaging technology is a modified form of haloalkane Dehalogenase (DhaA), which hydrolyzes alkyl halides into alcohols and halide anions respectively. A mutant form of DhaA creates a ‘locked’ covalent ester linkage between the nucleophilic Asp 106 residue and the haloalkane ‘tag’. This mutant form of DhaA replaces the WT amino acid of His272 to Phe272. As phenylalanine is a neutral amino acid, the hydrogen bonding/basic system created for enzymatic hydrolysis is disrupted, converting the enzyme into a high affinity covalent(suicide) partner from it’s natural function of enzymatic hydrolysis of alkyl halides.

Figure 1.3. DhaA enzymatic activity, WT (above), suicide enzyme (left)
Above, the wild type DhaA enzyme can perform its proper function of cleavage of the aspartate ester by the His272 gated base. In the image to the left, notice Phe272 substitution. In this instance, water can not be deprotonated as base shuffling is inhibited.

The haloalkane must be hydrophobic enough and long enough to fit into the binding pocket of DhaA for covalent bond formation to occur, which was found to be a six-carbon chain with the terminal ζ-C affording the electrophile.

![HaloTag reactive substrate](image)

**Figure 1.4. HaloTag reactive substrate**

**SNAP-tag:**

SNAP-tag technology is a mutant protein based on the DNA repair protein, O\(^6\)-alkylguanine-DNA alkyltransferase. The protein reacts specifically and quickly with benzylguanine (BG) derivatives, forming a thioether and releasing guanine in the process.\(^8,9\) To visualize a protein of interest, an expression vector is used to insert the gene of interest into a polypeptide gene containing a high affinity labeling site. This creates a fusion protein, the protein with the markable polypeptide on either the N- or C-terminus, dependent on protein of interest binding site and interactions, etc. This technique of polypeptide transfection is superior to fluorescent protein (FP) transfection, such as green fluorescent protein (GFP), as synthetic fluorophores which
bind to the polypeptide tag are paramount compared to natural peptide fluorophores. SNAP-tag was developed on the premise of the O6-alkylguanine-DNA enzyme, hAGT’s low specificity, including the binding of O6-benzylguanine, therefore, provides a a modular core for affinity labeling.9

SNAP-tag has led to the development of another high affinity fusion protein, based on reaction of benzyletosine derivatives as opposed to benzylguanines, known as CLIP-tag. This suicide enzyme can be used in conjunction with SNAP-tag to label to different POIs simultaneously. This tandem fluorescent labeling has proven to be a powerful technology for investigating protein-protein interactions.

β-Lactamase labeling:

β-Lactamase tag uses a similar approach as HaloTag and SNAPtag, transfection of a polypeptide, in this case, a β-Lactamase, and an appropriate tag (ligand) which covalently bonds to a fluorophore.10 The difference between the Halo and SNAP tags compared to β-Lactamase is the use of bacterial proteins and ligands, as β-Lactamases are not encoded within the human body, while Halo-tag and SNAPtag are modified versions of human proteins.

Mizukami et al. evolved the approach to use fluorogenic β-lactam molecules as well, fluorescently quenched molecules covalently attached to a β-lactam, a family of
cephalosporins. Cephalosporins are appropriate to use for the fluorogenic approach as a pi-system connects the β-lactam, forcing a resonant structure towards a fluorophore and leaving group.\textsuperscript{10}

Figure 1.5. \textit{β-lactamase quenching scheme}

Fusion proteins as described above, HaloTag, SNAPtag, and β-Lactamase tag, provide bio-orthogonality, high yields, and a variety of molecular tags, supplying a repertoire of biological capability not known before.

Live cell imaging is a valuable technique which offers the immediate appointment of use of the fluorescent molecule. Cell lines which exhibit similar properties to those which are either difficult to culture, or obtain, can be studied more efficiently. nAChR expressing HEK-293 cells were grown and assayed with the dye to distinguish the molecule’s binding properties \textit{in vitro}. 
**Fluorescent in situ Hybridization:**

Fluorescent in situ Hybridization (FISH), a labeling technique which detects the presence, or absence, of a specific DNA sequence in a particular chromosome. A probe is labeled with an affinity tag, such as biotin, and can be purified with antibodies, determining whether or not the sequence is present. The high specificity of FISH comes from the inherent binding of complementary sequences, allowing the detection of not one DNA but many RNA targets as well (mRNA, miRNA, lncRNA and more). FISH has been used in many different disciplines, genetic counseling, medicine, species identification, as well as the identification of chromosomal mutation and differentiation (genetic diagnostics). Before the advent of FISH, ISH was known, using radiolabeled nucleotides, yet too many drawbacks were noticed with disposal, safety, and cost. Probe sequences are synthesized through gene fragmentation of whole sequences through *in vitro* nick translation with DNase. The genetic sequence is ‘nicked’ with DNase, a nucleotide is removed by 5’ exonuclease activity and are replaced with dNTPs added into solution, some of which are biotinylated, DNA polymerase closes the nick with the new NTP and offers a newly tagged gene product.
CHAPTER TWO

Nicotinic Acetylcholine Receptors

2.1 Introduction

Nicotinic acetylcholine receptors (nAChRs) are widespread in organisms, present in both the peripheral and central nervous systems and neuromuscular junctions.\(^{15-29}\) The receptors in the brain are some of the most important proteins in life and proper health, dysfunction can lead to cognitive impairment, dementia, and radical neurological discontinuity.\(^{15-31}\) Diseases associate with the receptor include Alzheimer’s Disease, Parkinson’s, schizophrenia, muscular dystrophy, epilepsy, autism, nicotine addiction and many more.\(^{18,19,22,24,27,28,31}\)

Acetylcholine is the most studied endogenous substance, its inherent importance within the mammalian system as a neurotransmitter dictates rigorous investigation.\(^{15,16,19,21,23,25,31}\) Acetylcholine is released throughout the nervous systems, by both pre and post-ganglionic fibers of the parasympathetic and the somatic nervous systems. Neurons which release acetylcholine are known as cholinergic neurons. Two natural alkaloids, muscarine and nicotine, further order the receptors through the binding affinity of said molecules to the receptor. Receptors which bind muscarine predominantly, at a lower concentration, are known as muscarinic acetylcholine...
receptors (mAChRs), nicotinic acetylcholine receptors (nAChRs) bind nicotine preferentially.\textsuperscript{20}

![Figure 2.1. Structure of Acetylcholine]

A nAChR was the first isolated and purified neuroreceptor in its active form, from the torpedo stingray.\textsuperscript{26,32} nAChR is a pentameric transmembrane protein made up of a variety of 16 subunits, either homomeric (α only) or heteromeric pentamers of α, β, ε, and δ subunits.\textsuperscript{20,33,34,35} The ε subunit is however only found in growing (embryonic) neuromuscular junctions, when mature, is replaced with the δ subunit. Heteromeric pentamers can exist as binary, trinary, and quaternary complexes, up to four variable subunits forming one protein.\textsuperscript{16,20,34,36} Each subtype is genetically controlled and possesses its own affinity for acetylcholine and agonists/antagonists as well as functionality.

Molecules which behave similarly to acetylcholine, which stimulate the nervous system, are known as ‘cholinomimetic agents’.\textsuperscript{15,18,20} This class of compounds has the ability to bind agonistically to the receptor, performing the same function as acetylcholine, or the ability to inhibit such enzymes which destroy acetylcholine enzymatically through acetylcholinesterase (AChE).\textsuperscript{37}
Subtypes of nAChRs consist of the following: neuromuscular, ganglionic neuronal, central neuronal, and α7.15,17-19,20,23,25,29,38 Pentameric subunits bind together in a transmembrane fashion, with an amphipathic center which creates a hydrophilic pore that can be opened and closed, allowing cations to cross the membrane.16,20,34-36 Binding of acetylcholine occurs on the extracellular matrix side of the protein, two binding sites per protein, with sites located on the α subunits and the interface of αδ and αδ. All 16 subunit types consist of four moieties; 1) ~200 aa N-terminal extracellular domain; 2) three transmembrane domains; 3) a non-conserved cytoplasmic loop; and 4) C-terminal extracellular sequence of variable length.16,34,35,39,40

As nAChRs are pentameric and guided to open or close by the ligand acetylcholine, affording passage of selective cations (Ca^2+) through the membrane, nAChRs are a part of the pentameric ligand gated ion channel (pLGICs), the Cys Loop Superfamily.24,34,36,41,42 This family of ion channels play a large role in cellular communication and are highly conserved throughout species. Along with nAChRs, the Cys Loop superfamily includes 5-hydroxytryptamine receptors, α-aminobutyric acid (GABA_A&G) receptors, and Glycine (Gly) receptors. Cys Loop is derived from the conserved fifteen amino acid sequence with the first and fifteenth amino acid forming a disulfide bridge. This structure is shared by all members of the Cys Loop superfamily.33-36,39,40
Fast synaptic transmission relays information to nearby neurons quickly, releasing up to 3 million acetylcholine molecules in one transmission. nAChRs are believed to play a large role in many neurological diseases, including Alzheimer’s disease (AD), Parkinson’s, amyotrophic lateral sclerosis (ALS/Lou Gehrig’s disease), epilepsy, and schizophrenia among others.18,19,22,24,27,28,31

Nicotine, the stimulant in tobacco, is known to desensitize nAChRs and promote addiction.

2.2 Binding site of nAChRs

The receptor proteins can exist in a multitude of conformational states that are ligand dependent. From closed, being the most stable form, not allowing the passage of calcium ions, open, where a ligand binds to the receptor and causes a mechanical transformation allowing the opening of the pore, to the desensitized state, open or closed, this occurs more often with more than one acetylcholine bound on a protein.16,35,36,40

nAChRs contain two active binding sites which work in pseudo-tandem, one molecule of ACh provides ample opportunity for the pore to open. When two (both) sites interact with the neurotransmitter, a dramatic increase of ion pore opening occurs.
The neuromuscular junction, where some nAChRs are located, is the convergence of the musculature to the nervous system, stimulating and transferring information to and from the brain and skeletal muscle.\textsuperscript{17,21,23}

The binding of acetylcholine and agonists in the active site, the subunit interface, is known to be attributed to six loops, A-F, which contain many highly conserved aromatic residues as tyrosine and tryptophan. Loops A-C are contained within the ‘principal’ side of the active site, on one subunit. The remaining loops, D-F are on the complementary side, completing the interfacial binding site.\textsuperscript{43}

Trp143 (of loop B) affords stabilization of quaternary ammoniums and protonated tertiary amines in the binding pocket by a cation-\pi interaction. Aromatic moieties of
nAChRs agonists and antagonists generally increase the affinity of the ligand due to π-stacking, affording possible therapeutic agents.

All Cys loop endogenous ligands possess a cationic ammonium moiety, whether a quaternary amine (acetylcholine), or protonated primary amine (5-hydroxytryptamine). The stabilization of the cationic molecules are highly conserved throughout the Cys Loop superfamily, offering cation-π interactions with aromatic residues, phenylalanine, tryptophan, and tyrosine.

Figure 2.3. Close up of nAChR binding site. Imperative binding moieties are colored.
Loop C is known to have a significant conformational change upon binding, of either agonist or antagonist. As agonists bind to the active site, loop C “fully contracts”, while peptide inhibitors afford loop C a “fully extended conformation” and non peptide inhibitors release a transitional conformation. This C loop movement can be as much as 11Å determined by either agonistic or antagonistic binding.43

In the unbound AChR form, Loop C is above/over the the ligand binding site, and is close in space to the extracellular loop (ECL), a short sequence between transmembranes 2 & 3. Agonist binding leads to a wave of changes throughout the protein, moving Loop C closer down to the ligand and the extracellular loop is perturbed, allowing the ion channel to form.35,43

Water is also used as a stabilizer of the ligand in the binding pocket, using AChBP as a structural homologue and more easily characterized, assisting in H-bonding of various agonists including nicotine, varenicline, and lobeline43

2.3 Acetylcholine Esterase

Acetylcholine esterase (AChE) is a serine hydrolase which inhibits acetylcholine neurotransmitter action by hydrolyzing the molecule into choline and acetate. Expressed in cholinergic neurons, its role is to stop transmission of chemical signals. Acetylcholine is synthesized from acetyl CoA and choline. After synaptic vesicle
delivery to the synaptic cleft, the molecule either binds to its receptor then is enzymatically destroyed or is destroyed before receptor binding by the enzyme acetylcholine esterase.\textsuperscript{37}

\[ \text{Scheme 2.1. Reaction of Acetylcholine with ACh Esterase} \]

The enzymatic function is known as the catalytic triad of serine, histidine and glutamate.\textsuperscript{35,37} Glutamate-histidine acts as an anionic acid-base for serine which nucleophilically attacks the carbonyl of acetylcholine. Backbone glycine N-H protons are thought to stabilize the acetyl group transition state through hydrogen bonding. Catalytic activity is restored through another catalytic triad mechanism, this time using water as the nucleophile, hydrolyzing serine acetate.

Acetylcholine is synthesized in the body by cholinergic neurons, acetyl-CoA transfers an acetyl group to choline by choline acetyltransferase. A large portion of choline used for synthesis of acetylcholine comes from the recycling of choline from the decomposition of synaptic acetylcholine by AChE. Choline itself can be synthesized from serine through Serine Decarboxylase, S-Adenosyl methionine (methyl donor) and Choline N-methyl transferase (three times), and Choline Acetyltransferase (ChAT) in conjunction with Acetyl-S-CoA.
Brain nAChRs consist mainly of the heterolog α4β2 with stoichiometries of 2:3 and 3:2 respectively, and the homolog α7; while muscle nAChRs consist of various subunits and ratios.\textsuperscript{29,38,44} Neural acetylcholine receptors are known to be associated with a variety of mental illness including depression, of which, α4β2 is targeted in drug development and study. Hyperactivity of the cholinergic system relative to the adrenergic system can lead to depression, as the drug physostigmine, an acetylcholine esterase inhibitor, produces depressive symptoms in test subjects. As with AChE inhibitors, depressive symptoms occur from a build up of ACh at the synapse which leads to a long lasting desensitized state of the ion channel, allowing the unregulated passage of Na\textsuperscript{+}, K\textsuperscript{+}, and Ca\textsuperscript{2+}.

\textbf{2.4 The Neuromuscular Junction}

The parasympathetic nervous system (PNS) relays information from the central nervous system (CNS) in the brain to the body using nerves in the muscle, limbs, and organs. The PNS is much more easily prone to physical and toxic harm comparatively to CNS as CNS is protected by bone, skull and spinal cord, and chemically by the blood-brain barrier (BBB).\textsuperscript{17,21,25} The PNS is split into two classified systems, the somatic and autonomic nervous systems, control of voluntary skeletal muscle control and “involuntary” control of organs and bodily functions, respectively. The CNS is the part of the nervous system which constitutes the brain and spinal cord, coordinating information gathered from the peripheral nervous system to impact and direct action within the body.\textsuperscript{27}
The neuromuscular junction is the flux of chemical information connecting the nervous and muscular systems. Synapses between muscle fibers and efferent nerve cells release calcium in a process known as the excitation-contraction coupling of skeletal muscle. This leads to a chemical depolarization and ultimately, muscle contraction.23,31,42

Action potentials, or nerve impulses, occur when voltage gated ion channels open from a change in membrane potential, “firing” in sequence along the neuron, allowing a large flux of sodium ions into the cell. The electrochemical gradient changes and in doing so produces a larger electric current through the cellular membrane, after which, the ion channels swiftly close. Potassium channels then open and flow out of the cell, returning the cell to its resting electrochemical gradient.

When an action potential reaches the motor nerve terminal, ACh is released from synaptic vesicles into the synaptic cleft; synaptic vesicles containing ACh are ~100 nm in diameter and fuse with the cellular membrane, thus releasing the molecule.19,21,25 The neurotransmitters diffuse to the postsynaptic neuron and bind with acetylcholine receptors, completing the chemical message. Acetylcholine travels about 100 nm from where the vesicle fuses with the cellular membrane and releases the neurotransmitter, to the ACh receptor on the targeted cell.
CHAPTER THREE

Cyclen-Pyrene technology

3.1 Introduction

A selective, high affinity supramolecular complex, cyclen-1, was developed in 2005 by Winschel et al. to bind the fluorophore pH-sensitive dye, 8-hydroxypyrene-1,3,6-trisulfonate (HPTS or pyranine). Using a combination of factors including H-bonding, π-stacking of aromatic cores, enthalpy, and sodium ion chelation between esters, cyclen-1 exhudes a high affinity and selectivity for HPTS along with the others in the anionic dye series, 8-aminopyrene-1,3,6-trisulfonate (APTS), and pyrene tetrasulfonate (PTA), which are non-functionalized members of the Cascade Blue series of water soluble, non-toxic dyes.

3.2 Cyclen Receptor

Cyclen-1 is a strategically synthesized derivative of the cyclen core molecule. The suggested mode of binding is a chelation-type system where the pyrene molecule fills the space between the four arms, with the aromatic moieties π-stacking with the pyrene core. The palmitoyl side chain delivers an extra feature, the ability to anchor cyclen into the lipophilic membrane, while the toluidinyl moiety of cyclen provides
the molecule with a variety of possible functionalities. Through the manipulation of
this species we are able to selectively target a receptor and amplify it’s fluorescent
response. With this methodology we can move on to more selective agonists and
competitive antagonists of nicotinic acetylcholine receptors in order to develop a novel
and potent fluorescent labeling mechanism for the attenuation of nicotinically derived
maladies. As this new methodology is hoping to transform excisional biopsies and
operations, the potential to visibly label problematic receptors is of great use.

\[ R' \quad R \quad R \quad Z \]

\[ R' \quad O \quad O \quad NH \]

\[ Z' \quad NH_2 \]

*Figure 3.1. Cyclen-1 receptor*

Cyclen-1 is a promising molecule used in highly specific, non-covalent binding of a
fluorescent dye series, the pyrene trisulfonates/Cascade Blue series (and pyrene
tetrasulfonate). The dyes exhibit high quantum yield, absorptivity, water solubility,
and little/no toxicity.¹ Since the affinity and binding is not based upon covalent bonds,
and the complexation necessitates conditions containing protic solvents, a simple rinse
with aprotic solvent breaks the complex, releasing the pyrene based dye into solution.
Cyclen-1 HPTS complexation is afforded due to a variety of moiety factors, including
the esters at the ‘base’ of the cyclen core, which are thought to chelate a sodium ion in
solution, while a sulfonate of HPTS allows further stabilization due to ionic interactions. π-stacking of the toluidinyl (cyclen-1) & naphthyl (cyclen-2) moieties provides electron density into the severely electron withdrawn pyrene core. Protic solvents are necessary for binding to occur, while aprotic solvents rupture the association, freeing the two molecules into solution.

Even though cyclen-1 exhibits low solubility, attachment of a ‘support’ moiety is usually preferential anyway, rendering its aqueous characteristics minimal, just as used in strepavidin-biotin complexations, usually one or both are covalently bonded to another molecule for a particular study, foregoing the premise of unfortunate properties. To showcase the point, 1% cyclen-2 anchored liposomes are extremely stable and bind HPTS derivatives just as the naked affinity reagent.

3.3 Cascade Blue derivatives

This series of blue fluorescing, water soluble, highly anionic dyes exhibit high extinction coefficients (~2.5 x 10^4 M\(^{-1}\) s\(^{-1}\)) and a ~20-100 nm Stokes Shift. Cascade blue derivatives have been used as biologically orthogonal fluorophores for over two decades.\(^1\) With great fluorogenic properties as described before i.e., quantum yield, absorptivity, and aqueous solubility, the dyes can be used in a multitude of techniques and assays. The cascade series also exhibits membrane impermeability due to the high anionic character from the three sulfonate moieties.
Since the pyrene derivatives are bioorthogonal, *in vivo* and *in vitro* assays can be performed readily, with the exception of a recently published article detailing the complexation of HPTS and the central nervous stimulant, caffeine. Which would require the patient to refrain from consuming caffeine pre-surgery if a cyclen-pyrene tech excision methodology is used.
CHAPTER FOUR

Liposomes

4.1 Introduction

Liposomes, or unilamellar vesicles, are spherical constructs of amphipathic molecules, usually phospholipids. Consisting of a lipid bilayer, the polar heads of the molecules are hydrophilic and form interactions with water, while the hydrophobic tails coalesce together. As it consists of a bilayer, they contain a cavity of water on the interior. Forming a model of the lipid bilayer was the initial suggestion, when they were discovered in 1965, *Bingham et al.*47 However, throughout the years, liposomes have gained attention as highly efficient, versatile, stealth drug delivery vehicles, a modern day inverse biomimetic trojan horse.48-52 Liposomes are not only used in medicine, and have been developed and studied in a diverse array. Bioengineering has seen improvements with the use of the protective phospholipid bilayer, delivering genetic material into new species and cells for the introduction and use in a host.14,48

As one of the highest occurring phospholipids in the animal body membrane, phosphatidylcholine (PC) is an obvious choice for vesicle production and bioassays.
Liposomes, as synthetic constructs from commercially available biomolecules, have easily controlled properties: particle size, composition, encapsulated media, membranous targeting molecules, etc.

Lamellar vesicles are first formed through the hydration of amphipathic molecules. After hydration, formation of bilayers produces multilamellar vesicles (MLVs), essentially, liposomes within liposomes of various sizes. For more uniform size and a higher entrapment ratio, the method of extrusion or sonication can be employed. Extrusion uses pressure to force MLVs through a porous membrane to become a certain size, by rupturing larger MLVs, they are forced into a physically confined space and rip apart, re-coalescing on the other side of the membrane into smaller vesicles, while sonication affords 68nm unilamellar vesicles, also known as small
unilamellar vesicles (SUVs) <100 nm, large unilamellar vesicles range from >100 nm to ~3µm.

4.2 Liposomes as vehicles

Liposomes exhibit various advantages as drug carriers. Degradation resistance is inherent as the drug is trapped within an impermeable membrane, the bilayer and aqueous core are safe from biological conditions and potential molecular threats. As leakage is extremely slow due to poor diffusion, (pro)drugs and various molecules have longer half-lives, as do viral and genetic materials.\textsuperscript{14,51} Due to the higher stability of nucleotides, liposomes have also gained attention in the delivery of gene therapy.
and vaccines. The amphipathic character of liposomes allows the delivery of polar and non-polar molecules alike. Nonpolar molecules can exist within the lipid bilayer while polar molecules are solvated by the interior aqueous core. Since liposomes are made of biomolecules, they are preferential to many other types of diagnostic and therapeutic vehicles, such as: colloidal gold, nanoparticles, quantum dots, dendrimers, metallic nanoshells, micelles, nanospheres, and nanocapsules.\textsuperscript{55,56} Many of these systems are inherently toxic and or toxic from accumulation/aggregation.

An early liposomal development was the encapsulation of fluorescent molecules. As described before, liposomes are easily manipulated, nearly omniphilic for possible encapsulations, and as such, membrane anchored antibodies and proteins with high affinities can and have been incorporated which bind molecules/proteins of interest. This has led to the semi-cascade strategy of binding ligands and fluorophores leading to amplification of endogenous ligand bound to protein.

**Imaging:**

Liposomal imaging has also benefited from the introduction of radionuclides \textsuperscript{99m}Tc, \textsuperscript{67}Ga, and \textsuperscript{111}In.\textsuperscript{50} A cholesterol heavy diagnostic liposome using \textsuperscript{111}In, Vescan®, is used for imaging melanoma, sarcoma, and lymphoma tumors with high specificity and sensitivity. The radionuclides require significant stabilization, using such water soluble chelators, deferoxamine (DF) and nitriloacetic acid (NTA). Lipid anchored chelation exists as well, a product of stearylamine (SA) or phosphoethanolamine (PE)
with diethylene triamine pentaacetic acid (DTPA). Signal intensity has been increased with polychelating amphiphilic polymers (PAPs).

**EPR effect:**

Unilamellar vesicles can exhibit a variety of sizes, with a lower limit of 20 nm, in terms drug delivery, they are referred to as nanomedicines. This class size of medicine exhibits a special effect on tumors due to their tumultuous growth, known as the enhanced permeability and retention effect (EPR).\(^{55,57}\) This phenomenon occurs when tumors become large enough to require and develop their own blood supply. Unlike non tumorous blood vessels and biological infrastructure, tumors provide a crude framework with large gaps between capillary endothelium and afford poor lymphatic drainage. Poor drainage and gaps improve the retention of large particles like liposomes, engaging in EPR in a *passive* fashion. The gap between endothelium and interstitial space is normally 5-10 nm, while tumors possess gaps upwards of 100-780 nm, allowing a multitude of larger particles and macromolecules to aggregate.\(^{57}\) Even though non targeting nanomedicines rely on EPR to aggregate and have its most potent effect, liposomes are still the target of the reticuloendothelial system (RES), which uptakes particles, including vesicles, before the liposome can enter the tumorous cell. A suggested size limit for drug delivery vehicles is ~400 nm for highest uptake. To semi-solve this problem, stealth liposomes have been developed, the introduction of PEG to the vesicles affords a large shell of water around the particle,
blinding it from RES.\textsuperscript{58} This however also has inherent complications, because endocytosis into the cell of interest becomes more difficult.

Today's chemotherapeutics are highly toxic and efficiently destroy cancerous cells, yet pose a serious danger to the effected, the drugs themselves afford the most danger from their inherent activity. Which is why tissue and cellular targeting is so important, accuracy is nothing without precision, a drug is only effective if it only does its assigned 'job'. Fortunately, the maligned tissue offers a solution, tumor vasculature affords defective endothelial cells, wide lumen, lymphatics, and lack smooth muscle, all of which allows particles of a certain size range to potentially aggregate around the tissues, with gaps up to 4 μm in size. The effect is time dependent, the more time allowed to circulate through the blood, the higher the near-tumor concentration of particles. Renal excretion is inhibited, also due to size, prolonging the life of nano-drugs and relaying a difference on concentration of up to ten times compared to small molecular weight drugs.

**Functionalization:**
Multifunctional liposomes up the ante with a variety of triggers and ligands, able to direct themselves to the target and perform any number of functions. Cleavable PEG nearly perfects the usage of PEGylated liposomes, having the ability to remove PEG through simple hydrolysis of the ester linkage by esterases or low pH\textsuperscript{58}.\textsuperscript{58}
Targeting of liposomes is an exceptional technique, using covalently bonded ligands and or antibodies anchored to the vesicle, and is a common strategy for tumors and cancers as many exhibit over expressed receptors on the membrane surface.

Some of the most studied and important targets known in cancer to date are associated with membrane proteins and exhibit uptake mechanisms, including folate/folate receptor, transferrin/transferrin receptor, integrins, and human epidermal growth factor. These species have shown great promise in reverse oncogenesis.

**Transferrin Receptor targeting:**

Transferrin receptor is known to be upregulated in many cancerous cell lines, with an ability to cross the blood-brain barrier delivering anti-cancer medicines. Covalent linkage to transferrin has improved uptake of chemotherapeutics and a selective increase in cytotoxicity. Drug-resistant lines have also been shown to uptake transferrin-bound drugs, halting their developed response to counteraction.

Transferrin functions as a serum iron transport protein, 78 kDa glycoprotein, binding up to two Fe$^{3+}$ at a time, with a binding affinity higher than that of avidin/biotin, with a constant upwards of $10^{22}$ M$^{-1}$ at pH 7.4. As transferrin binds iron cation, a conformational change results, leading to a higher affinity for its receptor, transferrin receptor, with the diferric transferrin having upwards of a 100-fold increase in affinity compared to apotransferrin (non ferric binding transferrin).

Transferrin receptor levels are expressed higher on cells which proliferate more frequently, especially cancerous cells. The ferric transport proton has also become a
highly studied target for anticancer research purposes. Transferrin is also the main peptide used in the transportation of iron into the brain, as well as the passage of transferrin and anti-transferrin antibodies into the brain.

4.3 Immunoliposomes

Immunoliposomes are formulated by attaching antibodies to the surface, allowing targeting to cancerous cells. The vesicles have been developed in three different ways: type 1a exhibits antibodies bound to the bilayer; type 1b employ PEGylated liposomes with antibodies both attached to the bilayer; type II use PEGylated liposomes which have the antibodies covalently attached to the end of PEG.

![Figure 4.3. The three types of immunoliposome variations. Type 1a in the image uses single-chain variable fragment (scFv) in place of the entire antibody.](image)

Single-chain variable fragments (scFv) are fusion proteins which exhibit the smallest possible amino acid sequence containing the antigen binding site. scFv have been favored recently not only to their smaller size, but their formation from genetic engineering, easily allowing various amino acids to couple to the bilayer, in particular,
cysteine is generally used to replace a maleimide. Whole antibody liposomes have the
unfortunate problem of immunogenicity, being able to be taken up by macrophages
and certain liver cells, while scFv liposomes do not have this concern.

For the enhancement of a fluorescent response from the acetylcholine conjugate to
visibly mark the nAChR through confocal microscopy, we chose the encapsulation of
water soluble dyes with unilamellar vesicles at concentrations which can provide up to
thousands of times the brightness of a single bound molecule depending on
concentration of the encapsulated liposome.

We chose to use 400 nm as a suitable size for liposomes, determining whether or not
the anchored cyclen can sufficiently bind to PTS with such a large surface area in such
close proximity. Cyclen 1 was anchored to the bilayer through the introduction of a
palmitoyl side chain. As cyclen itself is not soluble in aqueous media, we hypothesize
the ‘arms’ of cyclen 1 are flattened out over the phosphate heads, giving a π-cation
interaction. This seems reasonable as the anisole derivative arm structure is similar to
tyrosine, which exudes a π-cation interaction with ammonium group of acetylcholine
in the binding pocket of nAChRs. The free liposome’s random movement through
solvent, will, upon close proximity to a bound ACh-PTS, bind tightly and anchor the liposome ‘above’ the nAChR, yielding a high fluorescent emission with light.

Figure 4.4. Image depicting the cascade binding sequences between a Protein of Interest (blue, bright blue active site), ligand-fluorophore derivative (green), and a liposome anchored with cyclen-1 (red).
CHAPTER FIVE

Synthesis of ACh derivatives and liposome encapsulation

5.1 Introduction

Possessing the technology to bind a fluorescent, membrane impermeable, small molecule affords the ability to develop a multitude of assays and investigations to better improve biological knowledge and quality of life. The decision to invent a small molecule director, for the use in excisional biopsies and procedures was/is being pursued. Methodology is crucial, and as such, the following new molecules and studies should offer a new strategy for the fluorescent visualization of tissues of interest i.e. malignant tissue/tumors. As nAChRs are so important in a variety of diseases and neurological disorders, compounded with the fact that nAChRs are some of the most studied receptors to date, we decided to use the protein as the foundation for ligand gated ion channel labeling and subsequent amplification.

The labeling molecule should possess similar receptor affinity and share as much structural integrity as possible. Therefore, a derivative of the endogenous ligand, acetylcholine, was synthesized with the intention for biological labeling activity. Since various studies throughout the years point out the fact that the trimethylammonium ‘head’ of acetylcholine is near perfected and is farthest into the receptor cavity, this moiety was preserved. The moiety chosen to alter was the acetate group, as many
different ligands have been synthesized based off this and does not inhibit functionality like the trimethylammonium head.

![Figure 5.1. Acetylcholine, endogenous ligand of nAChRs, base molecule](image)

5.2 Synthesis of Acetylcholine pentynoate. And Beyond.

Since the acetate tail of acetylcholine can be functionalized with minimal binding interference, to a pyrene trisulfonate, we decided to create a derivative of acetylcholine which possesses a terminal alkyne, to perform click chemistry. Using click chemistry requires an azido partner to couple with, as such, azidopyrene trisulfonate was first synthesized by Dr. Hartman in 2013 and was chosen as the second half of the heterosandwich molecule.
Scheme 5.1. Synthetic scheme of choline pentynoate and AChPTS. Choline pentynoate (first sequence).

4-pentynoic acid was added to a flame dried flask and potassium bicarbonate was added. The solids were dissolved in 200 μL of dry DMSO and stirred for 30 minutes. 5 equivalents of bromocholine bromide was dissolved in 1.2 mL DMSO and added to the flask under vigorous stirring at 50°C for 30 hours. It is noted that temperatures below 50°C afforded no reaction and temperatures above 50°C suffered heavy decomposition byproducts. Optimized reaction conditions were created through systematic treatment of the above compounds through various degrees of heating and
time, ultimately leading to the high number of equivalents of bromocholine bromide used. The use of AgO or AgCO₃ did not improve the yield but actually inhibited the reaction from moving forward. Silica gel column chromatography of gradient chloroform/methanol mixture lead to 134 mg of 2-(trimethylammonium)ethyl pent-4-y-noate (referred to as choline pentynoate).

The use of click chemistry made the process quite efficient, as the click partner (azide) of pyrene trisulfonate was first synthesized in 2013 by Matthew C.T. Hartman, 8-azidopyrene 1,3,6-pyrenetrisulfonate using Sandmeyer conditions with APTS, a cyclen-1 binding dye.²

![Scheme 5.2. AzidoPyrene synthesis as described by Dr. Matt Hartman.]

Azidopyrene trisulfonate was dissolved in water in a foil wrapped round bottom flask, copper sulfate and sodium ascorbate were added and stirred for ten minutes. Choline
pentyonoate was dissolved in water and added to the solution. The mixture was stirred for 18 hours at 45°C to afford the desired click product:

Along with the new ACh-fluorophore, cyclen-1 itself can only perform so much function. However, with the introduction of cyclen-1 into a trojan horse fluorophoric vehicle, the ability to enhance the fluorescence response up to thousands of times may be possible.

Manipulating the toluidyl moiety of cyclen-1, one can perform many chemistries, converting the aromatic amine into an amide, sulfonamide, urea, secondary amine, azide, carbamate etc. For our purposes, we chose amide formation with palmitoyl chloride, in order to envelope the cyclen molecule in the lipid bilayer of liposomes. Palmitoyl chloride is extremely reactive and possesses a relatively low boiling point.
(88°C), leading to easy purification and reaction conditions. It was hypothesized the long acyl chain would distribute itself among EYPC lipid tails, with the polar cyclen arms at the forefront of phase change.

![Diagram of reaction scheme]

Scheme 5.3. Reaction of cyclen-1 with palmitoyl chloride. Relative sizes are exaggerated for clarity of reactive group in reference.

**Liposomes:**

For the enhancement of a fluorescent response from the acetylcholine conjugate to visibly mark the nAChR through confocal microscopy, we chose the encapsulation of water soluble dyes with unilamellar vesicles at concentrations which will provide up to one million times the brightness of a single bound molecule. Unilamellar vesicles are particles made with molecules containing a hydrophilic ‘head’ and hydrophobic ‘tail’. In aqueous environment, the tails coalesce together and form a bilayer, similar to the biomembrane, with the hydrophilic heads on the interior and exterior, forming more favorable interactions with the solvent, water, including an aqueous core.
In this study, the unilamellar vesicle of choice are liposomes. Liposomes consist primarily of phospholipids, amphipathic molecules with an anionic glycerophosphate head. Liposomes have the ability to encapsulate a variety of molecules, hydrophobic, in the aqueous core, and hydrophobic molecules, within the lipid bilayer. Drugs have been encapsulated in liposomes to a great degree of success, as the liposomal doxorubicin improves efficacy and stability. As most unilamellar liposomal preparations are between 20 nm and 1 μm, the ‘solution’ is in reality a colloid.

We chose to use 400 nm as a suitable size for liposomes, determining whether or not the anchored cyclen can sufficiently bind to PTS with such a large surface area in such close proximity. Cyclen 1 was anchored to the bilayer through the introduction of a palmitoyl side chain. As cyclen itself is not soluble in aqueous media, we hypothesize the ‘arms’ of cyclen 1 are flattened out over the phosphate heads, giving a π-cation interaction. This seems reasonable as the anisole derivative arm structure is similar to tyrosine, which exudes a π-cation interaction with ammonium group of acetylcholine in the binding pocket of nAChRs. The free liposome’s random movement through solvent, will, upon close proximity to a bound ACh-PTS, bind tightly and anchor the liposome ‘above’ the nAChR, yielding a high fluorescent emission with light.

Phosphatidylcholine liposomes were prepared from egg yolk phosphatidylcholine (EYPC), 400 nm membrane through membrane extrusion conditions. Rhodamine B, a
water soluble, high emission dye in the high visible spectrum (red), was chosen as the indicative fluorescent marker to be used in the aqueous core of EYPC liposomes.

400 nm Cyclen-2 anchored liposomes were produced through 1.5% cyclen-2 in EYPC dissolved in 190 proof ethanol. Solvents were extricated via overnight oil pump evaporation. The mixture was hydrated with 50 nM Rhodamine B PBS buffer (2mL), pH 7.4 and 5 freeze/thaw cycles were performed, ensuring maximum containment. Extrusion through 400 nm membrane 21x lead to uniform vesicles which were purified through a Sephadex G-10 size exclusion column leading to a light purple milky solution (1.7mL) of cyclen-2 anchored 50nM Rhodamine B liposomes.

100 nm cyclen-2 anchored liposomes with 50 nM encapsulated Rhodamine B were made identically to 400 nm liposomes (the smaller 100 nm membrane was used for extrusion).
Figure 5.4. Image describing cyclen anchored liposome, with binding event of AChPTS. Pink sphere represents the liposome, orange elongated ‘U’ shows the cyclen receptor and the green shape shows AChPTS dye.
CHAPTER SIX

*in vitro* Assay of AChPTS with HEK-293 and Endothelial cell lines

6.1 Introduction

HEK-293 cells within the following studies over express nAChRs on the cell surface, more easily allowing establishment of our hypothesis. With the assay examining the binding of ACh-PTS to the membrane, along with the hypothesized cyclen anchored liposomes, the following steps include the *in vitro* introduction of ligand conjugated dye in the presence of different cell types, testing the hypothesis to an extent whether or not the acetylcholine conjugate can target cells of interest as designed. The co-culture of endothelial cells and previous assay will be performed, gaining insight into whether or not the acetylcholine conjugate somehow recognizes the new cells as a target.
6.2 HEK-293 binding assay with AChPTS and liposome

The first assay begins with a positive test for binding through visualization with fluorescence microscopy. If the ACh conjugate acts as an agonist/antagonist, the cells should be visibly ‘green’ with fluorescent molecules, indicative of binding.

The first trial shows promising results for the methodology. Green fluorescence can be noticed around the edges of cells, allowing for the continuation of the study (image B).

The overlap image produces a much better visualization of binding of the ACh conjugate (image C). Notice the blue arrows highlighting an increase in signal, located in one specific area of the cells. nAChRs, and most membrane proteins are thought to distribute themselves almost evenly throughout the membrane, however, these seem to aggregate in a certain manner. This phenomena could also be due to the nature of HEK cells themselves, as we know nAChRs at the neuromuscular junction are throughout the cell, ‘lining up’ to afford a cascade mechanism of cellular polarization.
Figure 6.1. First assay of over expressing nAChR HEK-293 cells. Bright field image of HEK-293 cells, image A. The introduction of 50 nM AChPTS dye after a five minute incubation, fluorescent mode, image B. Overlay of bright field and fluoro modes, image C; arrows indicate aggregation of nAChRs. Image D shows the incubation with 400 nM liposomes (encapsulated with Rhodamine B). Image E, overlay of A & D.
Concentration-dependent aggregation of liposomes (images D & E) upon the slide make it somewhat more difficult to gauge, however, noticing the same areas where green fluorescence is localized, shows binding of liposomes to the AChPTS bound to nAChRs on the membrane.

6.3 HEK-293/Endothelial co-culture binding assay with AChPTS and liposomes

The co-culture with endothelial cells is used as a control experiment, determining whether or not AChPTS preferentially binds with HEK-293 nAChR expressing cells, while endothelial cells do not bind the fluorescent marker.

Image F shows endothelial cells (elongated and ‘flat’) with HEK-293 cells (round/circular). Notice all HEK-293 cells exhibiting fluorescence. Only a couple endothelial cells seem to bind/aggregate the dye (cyan arrows). The rest of the cells fluorescing are most likely the HEK-293 line (image H).
Figure 6.2. HEK-293 and endothelial cell co-culture, bright field, image F. 50 nM AChPTS after a five minute incubation, image G. Overlay of F & G, image H. Blue arrows suggest labeled endothelial cells. Image I describes co-fluorescence of AChPTS and liposomes, Image J shows total liposomal fluorescence after AChPTS quench.
This image more clearly indicates HEK-cells exhibiting a fluorescent signal. Interesting is the co-fluorescence of both dyes in image I, AChPTS (green) and Rhodamine B (red). The endothelial cells in the foreground do not seem to show any fluorescence after a wash. As with previous images of HEK cells, these again afford a localized pattern of nAChR aggregation (cyan arrows).

A stoichiometry determination between liposomes and AChPTS was made using the culture in images I & J. In image I, cells were incubated for five minutes each with 100 nM AChPTS and then 50 nM Rhodamine B liposomes before imaging. A titration with liposomes was performed, until all green fluorescence (from AChPTS) was quenched. Image J shows 120 nM liposomes were sufficient to fully quench green fluorescence, determining a ~1:1 (120nM:100nM) binding ratio between liposomes and AChPTS.

An upper limit of fluorophore concentration was determined to be ~200 nM AChPTS and 200 nM liposomes. Images L and M show a high level of labeling/fluorescent signal on the cell cluster.
Figure 6.3. Cluster of HEK-293 and endothelial cells. Image K. 200 nM AChPTS incubation, image L. After addition of 200 nM liposomes, image M is formed.
6.4 Methodology

Using this assay as a stepping stone, we can assume from the following results that 1) binding of AChPTS is successful, 2) cyclen anchored liposomes behave as predicted and sufficiently bind the pyrene molecule, 3) fluorophore encapsulated liposomes help improve visualization 4) kinetics of both binding events are extremely quick, leading to a powerful message.

Excision technology:

Excision technology could be modified to use the methodology exploiting the interactions based on the cyclen/pyrene system. A visible detection method and sight directed procedure could greatly increase the precision compared to the current form, using a variety of diagnostics before and after surgery on the affected area (PET, FISH, immunohistochemistry, flow cytometry, CT scan, MRI, etc.). Since the methodology uses nontoxic substrates and fast identification, assistance with the physical detachment of tissues should be greatly improved.

The fluorescent tagging of excision able tissue can arise from the derivitization of a receptor ligand or antibody for the cell type. Using an HPTS core fluorophore attached to the cell identifiable ligand should behave as similarly as the cell studies performed in this work. The subsequent addition of encapsulated vesicles should heighten the
visible response and improve cellular differentiation, leading to a more sophisticated technique and proper removal of malignant tissues.

Using a sight directed aid, as described, will help reduce the invasiveness of the procedure, limiting exposure and lead to faster healing. The use of fluorescent media in medical procedures has minimal current study, and has been investigated somewhat using radiotracers, allowing a new type of methodology to expand the bounds of patient care.

Identification and localization of malignant tissue is imperative in the operative removal of the mass of cells (excision).

Figure 6.4. Depiction of envisioned excision using cyclen-pyrene technology with fluorescent amplification.

Neoplasms are generally targets for excisional biopsies and excisional operations, as the abnormal growths need to be tested for cancer as well as other skin diseases. Basal
and squamous cell carcinomas are known to behave in a manner which limit their full
detection, requiring methods to observe the delineation between cell types.

Microscopic excision technology is one the most successful methods of eliminating
malignant tissue from the body with physical means. Determining the physical edge of
harmful tissues thus requires a more sophisticated approach, delivering a visible
identifier/tag to the area.
CHAPTER SEVEN

Future Work

7.1 Introduction

Using our first assay used as a stepping stone for a new methodology has been successful. The modified endogenous ligand has shown to bind nAChRs along with the binding of the cyclen-1 anchored liposomes, reaching visual acuity.

7.2 Stronger binding models for the nAChR system

Even though acetylcholine is the endogenous substance for nAChRs, more potent species are available; nicotine, nicotine derivatives, and △-Bgtx are examples of possible molecules for applicable assays.

The cholinergic hypothesis of the onset of depression describes the hyperactivity of nicotinic receptors of the cholinergic system, compared to a hyperactive adrenergic system within the brain. As cholinergic receptors are overactive, levels of choline and hence acetylcholine are unregulated in depression brains. Since nicotinic receptors
are affected versus muscarinic, highly selective nAChR agonists and antagonists need to be used for study. One example is a derivative of nicotine, S1B-1508Y:

![Synthetic nicotine derivative, S1B-1508Y](image)

The only difference between this molecule and nicotine itself is the ethynyl moiety, yet possesses 1000 fold potency in receptor activation.³¹

![Possible synthetic scheme of SIB/AzPTS conjugate](image)
As shown above in the scheme, an additional acetylene moiety is installed, this is so that click chemistry can be performed, extending the range of this synthetic derivative with high affinity. The additional acetylene group can be added via the one step Cadiot–Chodkiewicz coupling reaction, or Glaser coupling with trimethylsilylacetylene.

The highest binding natural substrate, α-Bungarotoxin, can be conjugated to pyrene dye in the same manner. A suggested model is the following, pentynoic acid conjugated to ε-N of Lysine, which can be added to the N- or C-terminal of the peptide.

![Figure 7.2. AzPTS-Bgtx derivative](image)

7.3 Pyrene based system for various coupling reactions using SuFEx chemistry

Sulfur(VI) Fluoride Exchange (SuFEx) chemistry has early roots, however, its grandiose in becoming an extremely powerful coupling methodology is due to Dr. Barry Sharpless in the past year.60 His extensive work shows the ability for a new “click chemistry”, wide in scope, high yields, low impact, and easily purified products.
Palladium cross couplings with sulfonyl fluoride, as a replacement for triflate, easily accessible sulfonamides, sulfates, polymers, and more. SuFEx employs the use of a cheap, highly produced gas, Sulfuryl Fluoride, SO\(_2\)F\(_2\), and a molecule dubbed “the most perfect Michael acceptor ever found”, H\(_2\)C=CHSO\(_2\)F, ethenesulfonyl fluoride (ESF).\(^6\) Since the sulfur fluoride bond of sulfonyl fluorides is quite strong, the coupling is specific, using certain conditions depending on the chemistry wanted. Unlike sulfuryl and sulfonyl chlorides, the fluorides are extremely stable, decomposition and side reactions are eliminated, as well as the ability to better control the reaction.

Scheme 7.2. Ligation methods of HPTS using SuFEx chemistry.

The fluorosulfates (R-OSO\(_2\)F) can act as a replacement for the triflate group (OTf) and can also be used in palladium cross coupling reactions i.e. Suzuki, Heck, Sonogashira, enhancing the amount of chemistry the pyrene trisulfonates previously exhibited. Another extension, in scope and connection is the use of ESF, with the Michael addition being quick with generally 100% conversion of nucleophile, allowing the sulfonyl fluoride to react with another partner, possibly enzymes.
General polymerizations:

The employment of pyrene trisulfonates and pyrene tetrasulfonate in polymerizations can be possible with the new chemistries available. A four way sulfonyl linker can be afforded easily using the demonstrated transformation.

![Scheme 7.3. Scheme of PTA being polymerized via SuFEx.](image)

Using polymerization partners like diamines or diols, large net-like polymers, MOFs, and possibly dendrimers could be synthesized easily from affordable and accessible starting materials.
EXPERIMENTAL SECTION

All chemicals and solvents were purchased from either Sigma-Aldrich, Acros Organics or Fisher Scientific and used without further purification. Column chromatography was performed with silica gel 60 (230-400 mesh). All $^1$H NMR spectra were recorded on a Varian 300 MHz NMR spectrometer. All NMR chemical shifts (δ) were reported in parts per million (ppm) and were determined relative to the standard values for deuterated solvents.

2-(trimethylammonium)ethyl pent-4-ynoate: 4-Pentynoic acid (100 mg, 1mmol) and potassium bicarbonate (140 mg, 1 mmol) were dissolved in 200 μL of DMSO and stirred for 30 minutes. 2-(bromoethyl) trimethylammonium bromide (1.26 g, 5 mmol) was dissolved in 1.2 mL DMSO and the two solutions were combined and stirred at 50ºC for 30 hours. The solvent was concentrated under reduced pressure. The crude solid was purified via column chromatography, (silica gel, gradient CHCl$_3$/MeOH 8:1-4:1) to give 134 mg (0.507 mmol, 51%) of product. $^1$H NMR (300 MHz, DMSO) δ 3.44 (m, 1H); 3.15 (s, 9H); 2.51 (m, 2H); 2.31 (ddt, $J$= 9.5, 6.9, 1.5 Hz, 2H); 2.18 (td, $J$= 7.5, 7.1, 1.6 Hz, 2H); 1.22 (s, 2H). ESI-MS calc’d for C$_{10}$H$_{18}$NO$_2^+$ (M+) 184.13; found 183.403.

8-(4-(3-(2-(trimethylammonium)ethoxy)-3-oxopropyl)-1H-1,2,3-triazol-1-yl)pyrene-1,3,6-trisulfonic acid triammonium salt: 2-(trimethylammonium)ethyl
pent-4-ynoate (35 mg, 0.13 mmol), 8-azidopyrene-1,3,6-trisulfonic acid triammonium salt (12 mg, 0.05 mmol), copper sulfate (0.2 mg, 0.001 mmol), and sodium ascorbate (0.15 mg, 0.002 mmol) were dissolved in 2 mL millipore water and stirred for 18 hours at 45°C in the absence of light. The crude reaction was then purified by size-exclusion chromatography to afford 16 mg pure product (0.05 mmol). ¹H NMR (300 MHz, D₂O) δ 9.36-9.25 (m, 1H); 9.29 (s, 2H); 9.22-9.07 (m, 1H); 8.70 (s, 1H); 8.19 (s, 1H); 7.93 (d, J= 9.7 Hz, 1H); 3.16 (t, J= 0.9 Hz, 9H); 2.90-2.69 (m, 2H); 2.57 (t, J= 7.3 Hz, 2H); 2.49-2.38 (m, 2H); 2.36-2.29 (m, 2H). ESI-MS calc’d for (M²⁺) 332.03; found 332.23.
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APPENDIX

$^1$H NMR
AChPentynoate in DMSO-d_6

$^1$H NMR
AChPTS in D$_2$O

$^1$H NMR