

Controlled Dopamine (D₁) Receptor Stimulation using Photo-Responsive Substrates

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“Progress is made by trial and failure; the failures are generally a hundred times more numerous than the successes; yet they are usually left unchronicled. The reason is that the investigator feels that even though he has failed in achieving an expected result, some other more fortunate experimenter may succeed, and it is unwise to discourage his attempts”

— Sir William Ramsay

From 'Radium and its Products', Harper's Magazine (Dec 1904), 110, No. 655,
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Abstract

Dopamine (2-(3,4-dihydroxyphenyl)ethylamine) is a key neurotransmitter involved in the formation of memory, and the process of learning. This is through the modulation of synaptic plasticity by the activation of cell surface receptors. The exact mechanism by which information from an external environment is processed and stored within our brain is still beyond our scientific knowledge. To determine how these processes work, we must first understand how a network of neurons communicates within a culture. In a model that enables development of our understanding of neuronal communication, continuous monitoring and stimulation of single cells using non-invasive methods is essential. For the development of this model, it is reasonable to treat monitoring and stimulation of cells as separate challenges. This thesis focuses on developing means that enable targeted stimulation of cells.

This project reports the design, synthesis and pharmacological data for novel photo-responsive conjugated dopamine analogues, tethered to surfaces, to control activity at the dopamine D₁ receptor. To achieve this, modifications to dopamine were made to identify functional groups, contributing to key binding sites found at the D₁ receptor. Synthesis of a photo-responsive spiropyran molecule and conjugation to dopamine using amide formation was used to control the availability of the pharmacophore to the D₁ receptor in solution, as well as on to glass substrates and nanoparticles. A reporter gene assay (CRE-SPAP) was used to determine the potency of the synthesised compounds.

It was shown that modification of the catechol and amino moieties of dopamine affect the potency of the compound at the D₁ receptor. Modification of the catechol resulted in no activity, whilst carbamate protection and *N*-alkyl modifications (aliphatic/aliphatic ether chains) at concentrations above 10⁻⁶ M resulted in generation of SPAP. Activation of the receptor was demonstrated using dopamine functionalised nanoparticles, successfully confirming that ligands can be immobilised on solid substrates and maintain their ability to activate surface receptors.

Controlled activation using the spiropyran dopamine analogue was also achieved at the D₁ receptor using both the soluble and surface bound compound. It was shown that activity was completely suppressed in the closed spiropyran state, whilst the open merocyanine isomer produced activity in the soluble form. When adhered on to a nanoparticle, the closed isomer showed a 17-fold reduction in effective concentration (50%) compared to the open isomer.

This work shows that control of the availability and potency of a neurotransmitter attached to a solid support system to activate a receptor on a cell surface is in principle possible. This paves the way for the design and synthesis of materials which act as photo-pharmaceuticals and enable high spatiotemporal control of the activation of dopamine D₁ receptors.

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Publications

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Chapter 1 - Introduction

Simple Living Artificial Brain (SLAB)

The 'Simple Living Artificial Brain' project or 'SLAB' for short, is a collaborative effort at the University of Nottingham. It aims to understand the underlying mechanisms of how information is processed in the brain by using minimal but essential components required for a cognitive process (i.e. a network of neurons and an environment to interact with (input)).

To achieve this, a simple experimental system where a cultured network of neurons would be able to exchange information dynamically with a virtual environment in real-time, analogous to how neurons interact within a neuronal network is required. Figure 1-1 gives an overview of all the components required for the SLAB system to operate. This shows a closed loop system, in which a cultured set of neurons under homeostatic control, integrates dynamically with a virtual environment. The concept envisages real-time action potential imaging, with high spatial awareness, and time resolved control of neuronal activation and neuromodulation. This is through the use of opto-responsive neurons and microfluidics. Ultimately, realisation of such a system could lead to a biological model to aid the development of treatments and cures for neurodegenerative diseases, such as Parkinson's, Alzheimer's and dementia.

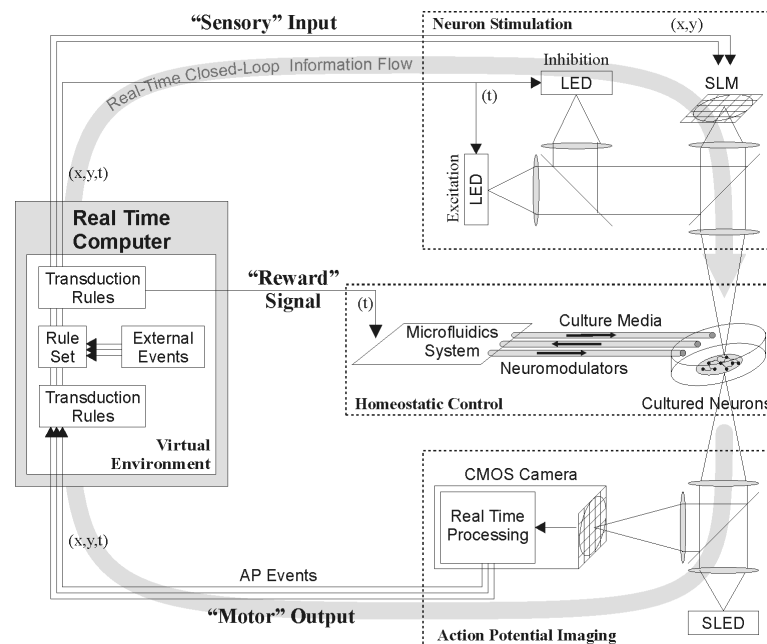


Figure 1-1 - Schematic of the simple living artificial brain concept showing a real-time closed loop, consisting of controlling sensory input and reward signalling to a cultured set of neurons, with simultaneous monitoring of cellular activation through action potentials (light emitting diode (LED), spatial light modulator (SLM), complementary metal-oxide semiconductor (CMOS) sensor and surface LED (SLED))

To understand how information is encoded and processed by a neuronal network, there is a need to stimulate and monitor each action potential generated from individual neurons in a large culture. Current technology such as multielectrode arrays and patch clamping techniques, which allows the synchronicity of measuring and stimulation of neurons in the simplest organism is a challenge (i.e. limits in resolution and culture size).

As the SLAB project is a large collaborative effort, this thesis solely focuses on one part of the project: designing a novel method for the stimulation of individual neurons. In doing so, we must explore the current methods of stimulation, which are specific to mechanisms currently thought to control memory and learning (synaptic plasticity), without the genetic modification of cells, and also a system which can control the activation of individual cells within a culture on demand.

This introduction presents information regarding neurodegenerative diseases, memory and learning, as well as critical information to understand how neurons are stimulated. Current state-of-the-art methods for neuronal stimulation with both their advantages and limitations, and methods we can employ to develop new neuron-stimulating systems is also discussed.

Neurodegenerative disease

Neurodegenerative diseases are incurable and debilitating conditions that result in the progressive degeneration and/or death of neurons. Etymologically, the prefix “neuro-” and termination “degenerative” refer specifically to nerve cells which are damaged over a long period of time, yet the term as a whole does not cover all types of diseases associated with the nervous system.¹ Przedborski, *et al*, describe the term as a large group of neurological disorders that ‘arise for unknown reasons and progress in a relentless manner’, preceded by ‘heterogeneous clinical and pathological expressions affecting specific subsets of neurons in specific functional anatomic systems’.¹ Diseases which are intensively researched and affect a multitude of people who are diagnosed every year include: Parkinson’s disease (PD)², Alzheimer’s disease (AD)³, and multiple sclerosis (MS).⁴

Full elucidation for the etiology of all neurodegenerative diseases currently plague research in determining cures and effective therapeutics. It has been shown that issues such as the formation of abnormal plaques/protein aggeragation⁵ caused by oxidative stress⁶ and loss of synaptic functionality⁷ lead to the disruption of common cellular pathways⁸ and subsequent neuronal dysfunction.

Parkinson's disease

Parkinson's disease (PD) was coined by French professor Jean-Martin Charcot between 1868-1881, building on the work entitled 'An Essay on the Shaking Palsy'⁹ conducted by James Parkinson in 1817 who first described the term 'paralysis agitans' for the symptoms associated with the disease. The disease causes symptoms such as bradykinesia (slowness of movement), tremors, rigidity and postural instability with patients developing dementia after several years.¹⁰

Specifically, PD is a neurodegenerative disorder attributed to the progressive loss of dopaminergic neurons (neurons which synthesise, store and contain receptors associated with the neurotransmitter dopamine).¹⁰ This occurs through the formation of abnormal protein aggregates (aggresomes) inside nerve cells known as Lewy bodies,^{11,12} and has been linked to mutations in proteins such as α -synuclein.¹³ α -Synuclein is a small protein (140 amino acids) containing an *N*-terminal alpha helical region, a hydrophobic central zone, and an acidic *C*-terminal region. Mutations in the gene encoding α -synuclein cause changes in the protein structure which alter the post-translational phosphorylation and solubility of α -synuclein, leading to the production of Lewy bodies.¹⁴ Mutations have been identified at positions Ala³⁰ and Ala⁵³, which are substituted to Pro³⁰ and Thr⁵³ respectively.

Although the physiological function of α -synuclein remains elusive, its proposed functionality is associated with vesicle dynamics at the presynaptic membrane including the controlled production, release and recycling of neurotransmitters such as dopamine.¹⁵ It has also been proposed to interact with enzymes in cultured cells to inhibit the production of dopamine, with overexpression of the protein reducing enzymatic activity of tyrosine hydroxylase¹⁶, a key enzyme in the production of dopamine.

These mutations contribute to the reduced production of dopamine, which causes an imbalance in stimulatory/inhibitory neuronal processes. This results in impaired functionality of sensory and physical attributes, including but not limited to, information processing networks¹⁷, movement (bradykinesia)¹⁸, and memory (dementia).¹⁹ The ability to manage some of the symptoms produced by PD has been demonstrated, with dopamine replacement²⁰ (typically levodopa is employed, a natural precursor to dopamine which crosses the blood brain barrier and is converted to dopamine) which also reduces the need for tyrosine hydroxylase¹⁶ and deep brain electrical stimulation (DBS) therapies²¹, but no cure or treatment for the regeneration of dopaminergic neurons have been shown to date.²²

For this to occur, we must develop an understanding of how researchers currently perceive the way the brain processes memory, and modulates learning and how we can expand this knowledge through the SLAB project.

Chemical biology

When developing systems which study the brain at a molecular level, it is essential to identify individual neuronal pathways and the way each neuron communicates and interacts with an external environment. To advance systems such as SLAB, we must collate information already known about the brain and understand specifics such as neurotransmission and neuromodulation.

Neurons encode and transmit messages intracellularly²³ at the synapse: electrically through connexions (gap junctions) and chemically through neurotransmitters such as dopamine and others shown in Figure 1-2.²⁴ They are supported by 'non-neuronal' cells which aid in the proper function of neurons. These include neuroglia which surround neurons and hold them in place, supplying nutrients and oxygen to neurons, insulating one neuron from another, destroying pathogens and removing dead neurons. A number of neuroglia exist, including but not limited to astrocytes, oligodendrocytes and Schwann cells.²⁵ While these cells are important for the function of a neuronal culture, the present work revolves around communication between cells and will therefore keep a main focus on neurons rather than neuroglia.

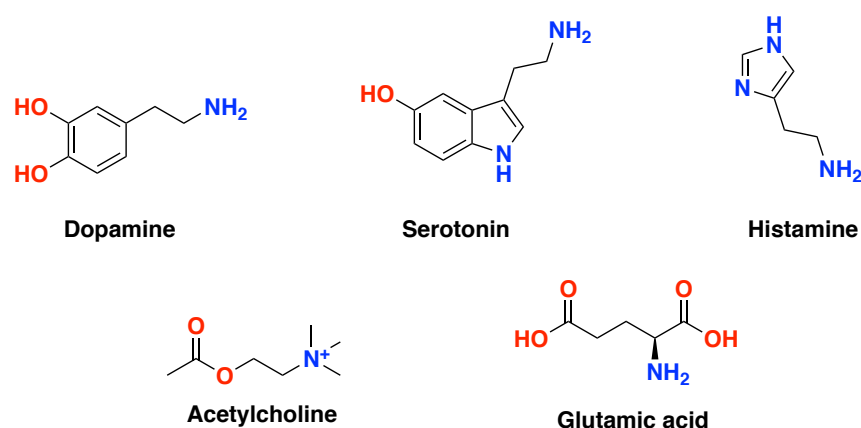


Figure 1-2 - The structure of common neurotransmitters found in the brain

The synapse

The synapse is an important area that plays a large role in transmitting electrical and chemical signals between neuronal cells. It consists of three areas: the presynaptic membrane (axon terminal), the synaptic cleft and the postsynaptic membrane (dendrite of another neuron)²⁶ as shown in Figure 1-3.

These three components work in tandem for signal transduction to occur. The diagram shows the propagation of a nerve impulse or action potential (1) causing an influx of calcium ions (2). A 'synaptic delay' defined as the time interval between peak of inward current through the presynaptic membrane and commencement of inward current through the postsynaptic membrane is then observed, before the release of neurotransmitters from synaptic vesicles into the synaptic cleft. This delay is typically observed between 0.3 and 0.5 ms.²⁷ For this to occur, the synaptic vesicles fuse with the presynaptic membrane, undergoing exocytosis of stored neurotransmitters (3). Activation of the receptors found on the postsynaptic membrane (4), cause an influx of sodium ions (5), which in turn leads to the postsynaptic potential (6) and finally the nerve impulse (7).

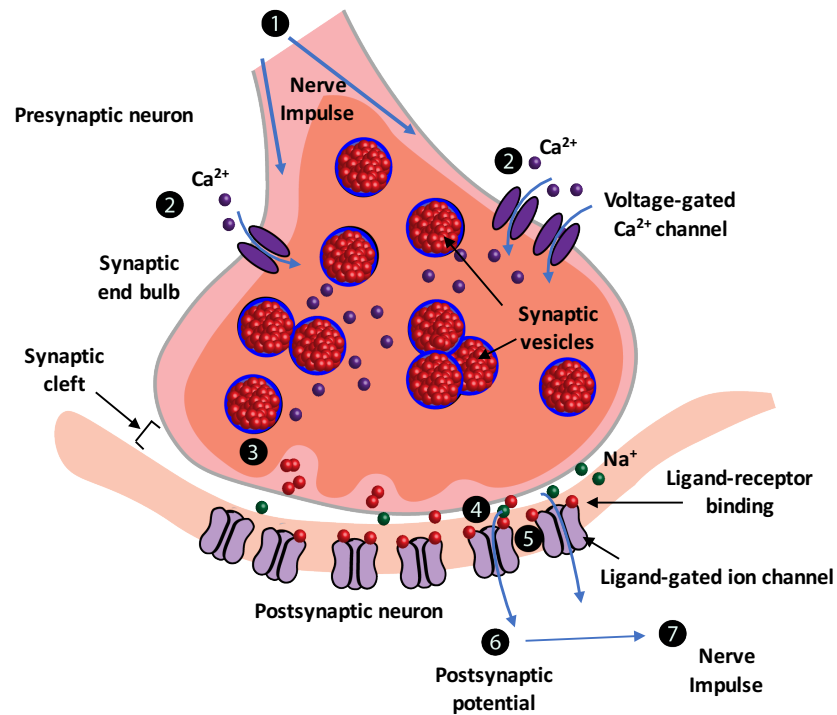


Figure 1-3 - Schematic of the synapse and process of communication between cells²⁶ (1) propagation of a nerve impulse or action potential, (2) influx of calcium ions (3) synaptic vesicles fuse with the presynaptic membrane, undergoing exocytosis of neurotransmitters (4) activation of the receptors found on the postsynaptic membrane (5) influx of sodium ions (6) postsynaptic action potential generation (7) resulting nerve impulse

Memory and learning (Information processing)

The synapse not only allows for communication between cells, it is also widely regarded as having an impact on memory and learning, importantly through a phenomenon known as plasticity.²⁸ Processing and storing information is the most fundamental feature of the mammalian central nervous system and synaptic plasticity is known to play a role in the mechanism behind memory and learning.^{29–31}

Synaptic plasticity

Plasticity, as a term in neuroscience can be traced back to as early as 1906, where Italian psychiatrist Ernesto Lugaro described the term as neurons changing in an adaptive fashion to enable psychic maturation, learning and even functional recovery after brain damage.³² Today, plasticity is known to describe the strength of the interactions between two neurons. This follows Hebbian theory, where repetitive neuronal stimulation tends to induce long lasting effects, as one neuron fires and stimulates another, the connection between the two strengthens.³³

Synaptic plasticity is often hypothesised as a physiological phenomenon that occurs when a specific pattern of neuronal activity strengthens or weakens a connection between two adjacent neurons, depending on the frequency of stimulation at the synapse. Physiological effects may include an increase or decrease in the number of receptors located on the postsynaptic membrane changing the sensitivity, synaptic efficacy and neuronal excitability that proceeds after initial stimulation.^{34,35} A variety of biological processes, including synaptic vesicle release and recycling¹⁵, neurotransmitter receptor trafficking³⁶, cell adhesion³⁷ and stimulus-induced changes in gene expression within neurons³⁸ have been used to study the molecular mechanisms that underlie changes in synaptic plasticity.³⁹ Plasticity has been typically studied in rodent models using the hippocampus, known to be critical in the formation

of memory. *In vivo* (implantation of electrodes⁴⁰), *in vitro* (dissociated cultures⁴¹) and *ex vivo* (hippocampal slices⁴²) have all been used for these studies.

Plasticity can be categorised into short-term (which acts in the timescale of tens of milliseconds) and long-term plasticity (which can last from minutes to hours).³² Short-term plasticity is achieved through temporal enhancements of a synaptic connection, which then in-turn decays rapidly back to its initial state. When the frequency of stimulation is high, this is known as long-term potentiation (LTP), the process in which a synaptic connection is strengthened. Low frequency stimulation, where the receptor is activated with shorter repetition intervals has been shown to actively weaken the synaptic connectivity, commonly known as long term depression (LTD). It has been shown that LTP and LTD are both dependent on postsynaptic elevations in intracellular calcium, which are controlled as a secondary effect through the activation of cell surface membrane receptors, in particular those associated with G-protein-coupled receptors (GPCRs).⁴³

Cell surface receptors

GPCRs are a family of large transmembrane proteins which protrude through the cell surface membrane seven times. These cell surface receptors detect molecules outside the cell and activate internal signal transduction pathways. Heterotrimeric G-proteins (α , β and γ) coupled to these receptors are activated upon ligand binding and act as a guanine nucleotide exchange factor, replacing guanosine diphosphate (GDP) with guanosine triphosphate (GTP). The G-protein's α subunit bound to GTP can then dissociate from the β and γ units, to further affect intracellular signalling processes (Figure 1-4). The signalling processes are determined from the α subunit ($G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha olf}$, $G_{\alpha q}$, $G_{\alpha o}$, $G_{\alpha t}$) shown in Table 1-1, with examples including but not limited to cyclic-adenosine monophosphate (cAMP) and phosphatidylinositol pathways.⁴⁴

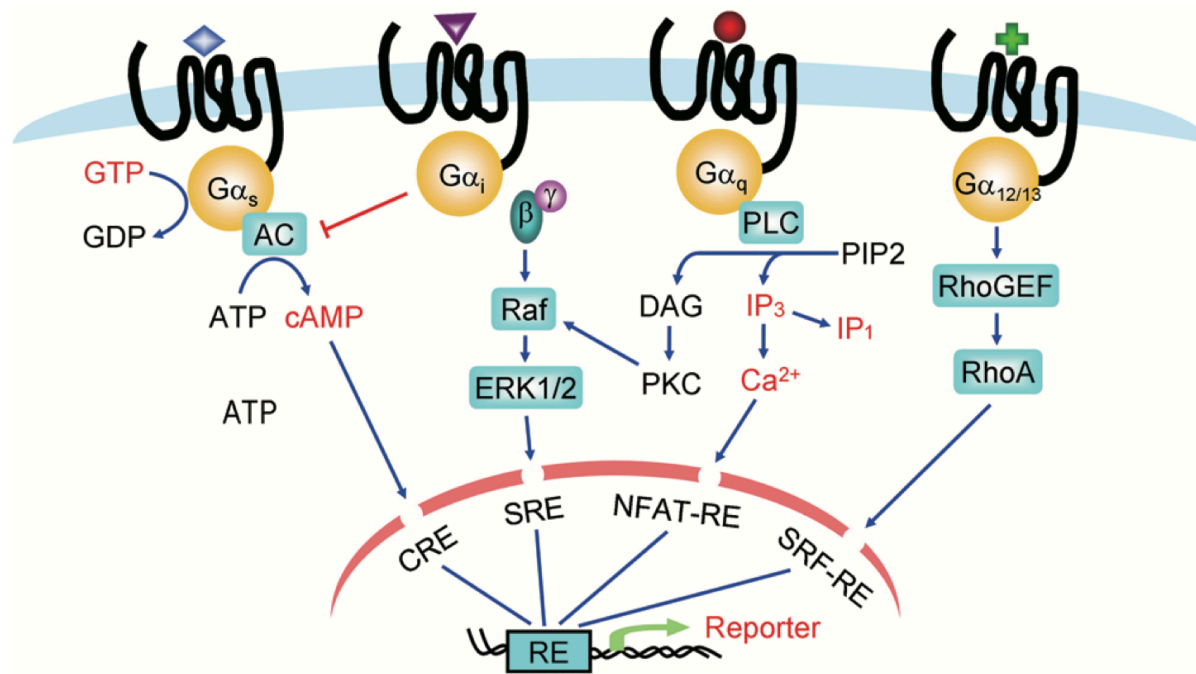


Figure 1-4 - Schematic representation of the major pathways activated by different G-proteins. In typical assays, the components marked in red are measured to determine activity (GTP - guanosine triphosphate; GDP - guanosine diphosphate; AC - adenylyl cyclase; ATP - adenosine triphosphate; cAMP - cyclic adenosine monophosphate; DAG - diglyceride; PKC - protein kinase C; IP_3 - inositol triphosphate; PLC - phospholipase C; CRE - cAMP response element; SRE - serum response element; NFAT-RE - nuclear factor of activated T-cells response element; SRF-RE - serum response factor response element, image reproduced from Zhang, et al)⁴⁵

Table 1-1 - G_{α} isoforms, associated effects and examples⁴⁶

Subtype	Associated Effector	2 nd Messenger	Receptor Examples
$G_{\alpha s}$	Adenylyl Cyclase	cAMP (increased)	β -Adrenergic (epinephrine) receptors; glucagon, serotonin, vasopressin
$G_{\alpha i}$	Adenylyl Cyclase K^+ channel	cAMP (decreased) Change in membrane potential	α_2 -Adrenergic receptor Muscarinic acetylcholine receptor
$G_{\alpha olf}$	Adenylyl Cyclase	cAMP (increased)	Odorant receptors in nose
$G_{\alpha q}$	Phospholipase C	IP ₃ , DAG (Increased)	α_1 -Adrenergic receptor
$G_{\alpha o}$	Phospholipase C	IP ₃ , DAG (increased)	Acetylcholine receptor in endothelial cells
$G_{\alpha t}$	cGMP phosphodiesterase	cGMP (decreased)	Rhodopsin (light receptor) in rod cells

Research conducted by Huang, *et al*, has demonstrated that plasticity in brain slices (visual cortex) can be regulated through a subset of GPCRs. These include stimulatory α subunits, $G_{\alpha s}$ (adenylyl cyclase - promotes LTP and suppresses LTD), and $G_{\alpha q}$ (phospholipase C - promotes LTD and suppresses LTP) with examples shown in Table 1-2 and Table 1-3.⁴³ Understanding the receptors and their associated effects can allow us to recognise which receptors we can target for the SLAB system, as well as what markers we can screen for to determine activation (i.e. cAMP).

Table 1-2 - $G_{\alpha s}$ protein coupled receptors⁴³

$G_{\alpha s}$ Receptor Type	Sub-category
Adrenergic	β_1 ; β_2 ; β_3
Dopaminergic	D_1 ; D_5
Histamine	H_2
Cholinergic – muscarinic acetylcholine	M_1
Serotonin	$5HT_4$; $5HT_6$; $5HT_7$

Table 1-3 - $G_{\alpha q}$ protein coupled receptors⁴³

$G_{\alpha q}$ Receptor Type	Sub-category
Adrenergic	α_1
Glutaminergic	$mGlu_1$; $mGlu_2$
Histamine	H_1
Cholinergic – muscarinic acetylcholine	M_1 ; M_3 ; M_5
Serotonin	$5HT_2$

Variations in the synaptic plasticity directly affects the number of receptors found on the cell surface based on the frequency of stimulation. This adds another dimension to cell stimulation, which typically follows the law of mass action.⁴⁷ This is the proposition that the rate of a chemical interaction is dependent and directly proportional to the activity and/or concentration of the reactants; in this case, the activity and concentration of the ligand to the receptor. This takes into consideration the affinity of the ligand (tendency of a ligand to bind to its receptor) and the efficacy of the ligand (measure of bound ligand to activate its receptor), otherwise known as the potency. Changes in synaptic plasticity can therefore affect what concentrations of ligand activate a cell surface receptor, which is of great interest in our search to understand memory and learning (strengthening of specific neurons).⁴⁸

Stimulating and monitoring neuronal networks

The use of primary cultures (cells that are cultured directly from a subject) is commonly employed for *in vitro* testing. Classically, cells for primary cultures for neurological studies are derived from dissociated rat neurons. Depending on the study, rat cortical⁴⁹, hippocampal⁵⁰ and spinal neurons⁵¹ are commonly employed. Mammalian cell lines such as Chinese hamster ovary (CHO)^{52–56} and human embryonic kidney (HEK)^{57–60} have also been utilised in studies which allow us to monitor the potency of synthesised drugs at individual GPCRs. These cells are modified with specific receptors using a transfection agent (such as Lipofectamine® LTX) and plasmid DNA dependent of receptor required.^{61–65} These can produce cells which exhibit receptors present on primary cultures such as dopamine D₁-D₅, and adrenergic β_{1-3} .

The ability to investigate neuronal activity has been readily demonstrated with the use of chemical methods such as GTP γ S binding assays⁶⁶, cAMP immunoassays (measuring cAMP directly produced in the cells using antibodies)^{67–69}, reporter gene assays^{70–72}, as well as electrophysiological techniques measuring changes in cell membrane potentials (action potentials) using multi electrode arrays (MEAs)⁵¹ and patch clamping.⁷³

Stimulation of neuronal cells has been achieved in a number of ways including chemical and electrical means as well as optogenetic approaches. These strategies are discussed below, with emphasis on the advantages and disadvantages relevant to the SLAB project. This will lead to discussions as to why a novel method is required for stimulation of the neurons.

Chemical methods

Neurotransmitters are typically employed to stimulate dissociated neuronal cultures. These molecules provide a type of 'natural' stimulation, as observed between synapses in the brain. Although neurons produce and store neurotransmitters in synaptic vesicles, external stimuli can be used to induce neuronal stimulation within a culture. Dosing neuronal cultures can be achieved using a number of methods, including but not limited to simple pipette loading (i.e. conducting cell assays)⁷⁴ or with the use of equipment which can control dosing with a timed release (i.e. auto dosing pump/microfluidic devices).⁴⁹ These approaches affect either the whole or a large part of the neuronal culture and are therefore limited in their spatial resolution. Stimulation of individual neurons has not yet been reported.

It has been demonstrated by Mourzina, *et al*, that limited spatial control can be achieved through the use of a non-invasive on chip microfluidic device (Figure 1-5). A solution is pumped through small pores, which are directed at specific neurons. Limitations to this method in controlling spatial dosing, include the diffusion of the compound upon release (activating neighbouring cells), alignment of cells to the pores as well as directing the neurotransmitter to millions of individual cells within a culture.

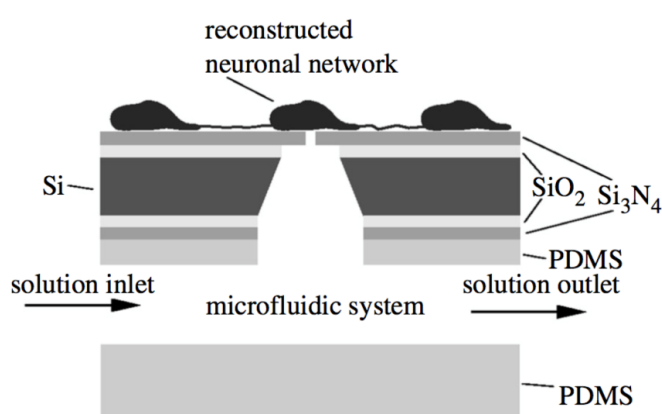


Figure 1-5 - Reconstructed neuronal network on a PDMS (polydimethylsiloxane) microfluidic device to provide localised neuronal activation (Si - silicon, SiO₂ - silicon dioxide, Si₃N₄ - silicon nitrate, reproduced from Mourzina, *et al*)⁴⁹

There are many advantages to chemical simulation such as temporal control, control of dosage amount and the ability to activate distinct signalling pathways dependent on the neurotransmitter. In understanding how a neuron interacts with its neighbouring cells, as well as its environment, we must be able to activate individual neurons. The limited ability to activate single neurons *in vitro* in a spatial manner is a problem. As ligand binding and activation is proportional to the number of receptor binding sites as well as the concentration, affinity and efficacy of the ligand⁷⁵, multiple neurons in a small vicinity can be activated with a diffusion of neurotransmitters.

Electrical methods

Most mammals encode and transmit information *via* action potentials. Changes in the membrane potential, allows for the influx and outflow of ions from the cell membrane, sending electrical pulses from one end of the neuron down through its axon to another within its network. These are generated naturally through chemical (i.e. neurotransmitters) and optogenetic (retina) activation but can be achieved artificially through electrical stimulation.

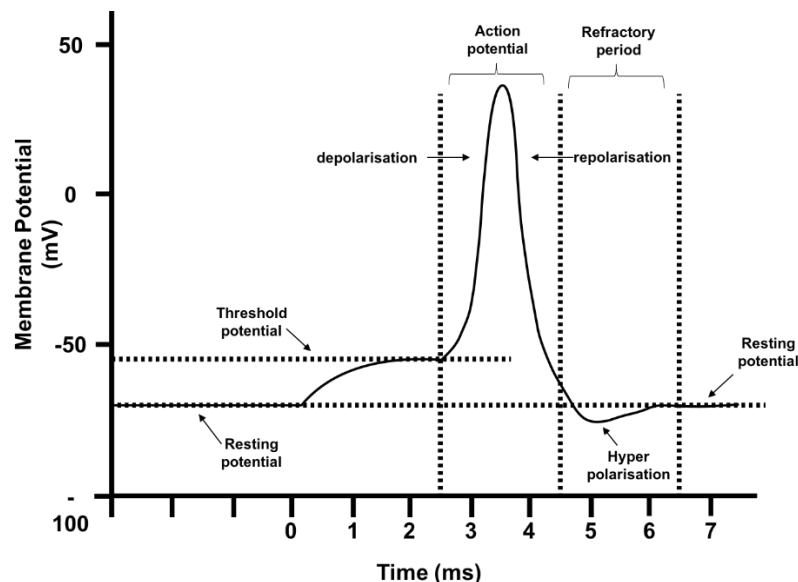


Figure 1-6 - Changes in membrane potential through the different stages of a neuron firing after cellular activation⁷⁶

All living cells maintain an electrical potential difference across their cell membranes; this value usually lies between -40 to -90 mV (known as the resting membrane potential).⁷⁷ Changes in this potential occur when cell surface receptors are activated. These include ionotropic (i.e. γ -aminobutyric acid, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and metabotropic (i.e. dopaminergic, adrenergic) receptors. Changes in intracellular and extracellular sodium and calcium levels, cause depolarisation of the cell membrane, generating an action potential (Figure 1-6) all within a few of milliseconds. This cascades through the neuronal network producing actions which control instances such as information processing and movement.^{23,46,78} Due to this electrical potential between the cell membrane, cells can be both stimulated and monitored using electrical methods. Two electrophysiological techniques exist which utilise the ability to both stimulate and monitor action potentials. These include MEAs and also single cell analysis (i.e. patch clamping) techniques.

MEAs use a non-invasive method that can be used for long term applications (i.e. months), by using electrodes arranged on a planar substrate, to detect electrical signals produced by neurons in the surrounding area.⁵¹ In order to extract information from single neurons, MEAs with a high density and spatiotemporal resolution are required, as well as the ability to monitor and isolate signals from each individual neuron. Hilgen, *et al*, have shown that spikes detected from each neuron can be identified within minutes using principle component analysis. Yet the ability to monitor independent action potentials in real time is still not possible for millions of cells in a large culture.⁷⁹ Stimulation of these neurons is also limited to those neurons which adhere onto the electrodes. In relation to the SLAB project, we require the stimulation and monitoring of millions of neurons, a limit which current MEAs cannot deal with.

Single cell analysis can overcome issues which relate to monitoring and stimulating individual cells by directly attaching the probe to a cell membrane, allowing the input/output of electrical signals to be conducted in real-time.⁷³ As disruption of the cell membrane occurs within this process, an exertion of stress can modify cellular processes, to give biased results in relation to proper cell function in its natural environment.⁸⁰ Due to the invasive nature of this technique, the cell membrane integrity is compromised and hence such measurements are typically only conducted for several hours at any time.⁸¹ Results obtained from such experiments may therefore not reflect occurrences in real neuronal networks. Furthermore, single cell analysis cannot be applied to a large number of cells in a culture simultaneously.

Optogenetics

Optogenetics is a semi-invasive technique which neuroscientists have used to control neuronal stimulation. This is conducted by the genetic modification and integration of light sensitive proteins into neuronal cultures which can be activated at specific wavelengths of light to trigger electro- and biochemical processes.⁸² Activation of receptors can be controlled with high spatio-temporal control making it an ideal model to use for the study of neurons. The ability of this approach to stimulate specific neurons in a time resolved manner has led to intense study and application of optogenetics over the last 5-10 years.⁸³ For example, Takahasi, *et al*, reported the transfection of rat cortical cells at 0.3 to 1.9%, with the light sensitive protein channelrhodopsin-2 (ChR2).⁸⁴ Successful activation of individual neurons (expressing the light responsive receptor) were shown using a 488 nm light source, subject to 20 ms of exposure. This lead to further activation of non-light sensitive neurons within the culture. Further to this, ChR2 channels transfected into dissociated neuronal cultures have been shown to be activated with short pulses of light (<1 ms) through the use of two photon lasers (at a depth of 150 um) by Andrasfalvy, *et al*.⁸⁵

A major disadvantage of an optogenetic approach to stimulate cells is that the cells must first be genetically modified. This may cause changes in cellular function, i.e. neurons modified with a light responsive receptor may not respond in a way that is identical to how they respond physiologically by themselves. This therefore would not allow us to understand the natural mechanisms present in a neuronal culture to understand how memory and learning is processed.

Theoretical advancements

Stimulation of cells can be achieved in a vast number of ways, yet only two methods show the ability for high spatio-temporal activation of individual cells, which are single cell analysis and optogenetics. Whilst patch clamping allows both single cell stimulation at a high resolution⁸¹, the invasive manner of protruding on/into the cell to stimulate and detect changes in electrical potential, limits the analysis to several hours rather than days, which is required to understand how the process of memory and learning occurs. Optogenetics allows long-term stimulation to be achieved, yet genetic modification of dissociated neuronal cultures change the natural mechanisms of the cells, which is not ideal to investigate the fundamentals of a natural process. Chemical stimulation is the most ideal due to its ability to activate a number of signalling pathways, but it lacks the ability for spatial control, even with microfluidic devices such as those shown by Mourzina, *et al.*⁴⁹

Development of a system which utilises the positives from chemical stimulation and optogenetics in a novel way can be aided through the use of responsive materials. Conceptually, cells would be cultured (unaltered genetically) on solid substrates, embedded with photo-pharmaceuticals activated on demand using different wavelengths of light. Responsive surfaces therefore may be the key to developing a system in which we can control cellular activation with non-invasive stimuli, and without the modification of a cell.

Responsive materials

Responsive materials are substrates that can change structure or function when subjected to a new environment.⁸⁶ This change is not limited to chemical properties of the surface but can also affect topographical features. A number of stimuli can influence changes in surface composition/functionality; these include external stimuli (i.e. light, temperature), as well as factors which can be secreted and produced from adherent cells (i.e. enzymes, pH). These can be seen in Figure 1-7. To generate responsive surfaces, stimuli responsive compounds can be tailored and implemented onto surfaces through self-assembled monolayers (SAMS) and/or polymer films. Most commonly these materials include glass (silica) and gold as planar/patterned substrates or nanoparticles.^{87–89}

Focusing on a non-invasive method which does not disturb the cell environment through electrical or biological changes (pH, enzyme and temperature) we turn our attention to light or optical stimulation. In choosing this stimulus, we can control different wavelengths of light at different intensities in a time resolved and highly directional manner.

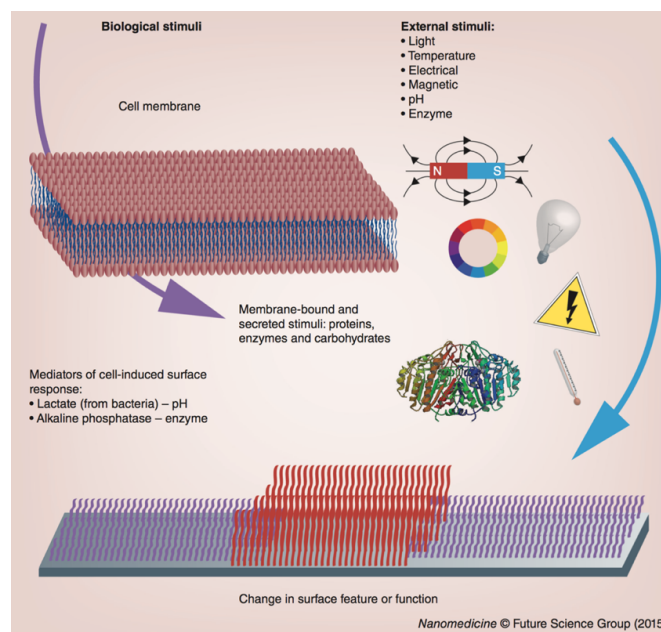


Figure 1-7 - Responsive material showing a change in surface feature or function when subject to a biological or external stimuli (reproduced from Dhowre, et al)⁵⁵

Optically responsive compounds

Optically responsive compounds undergo a variety of changes such as group polarity, charge, colour, shape and size after exposure to light.⁹⁰ Examples include compounds such as azobenzene, spiropyran, diarylethene and viologen (Figure 1-8).

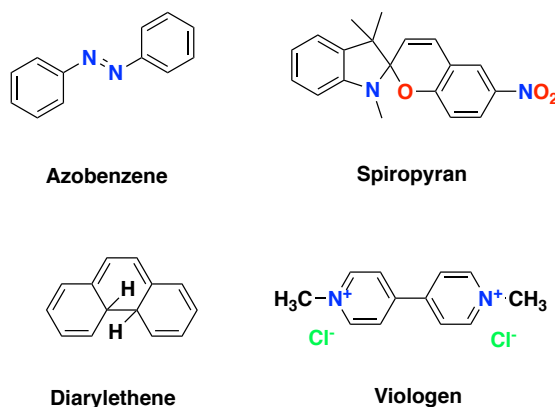


Figure 1-8 - Photo responsive molecules when subject to different wavelengths of optical stimuli can undergo changes in structure (i.e. charge, shape and size)

In the present context, azobenzene and spiropyran are the most interesting stimuli-responsive compounds because they have previously been used in biomedical applications^{88,91} and provide the possibility to be used as a molecular switch on a surface. This switch is possible from the reversible isomerisation they exhibit at different optical wavelengths.⁹² The importance of this reversible feature would conceptually allow the presentation and retraction of a biomolecule when conjugated. This is essential in developing a system which can activate a receptor on demand repeatedly without the modification of a cell surface receptor.

Using known or new chemistry to implement tethered photo-activated ligands on solid substrates rather than cell modification, we propose that we can control the presentation and retraction of a ligand with high spatio-temporal control critical to the project. Below we describe relevant uses of azobenzene and spiropyran both in solution and whilst tethered to either a surface or biological entity.

Azobenzene

Azo-compounds were typically employed as synthetic dyes and were not utilised in a biological setting until the late 1970s.⁶² Two conformational states exist, the *cis* and *trans* form (as shown in Figure 1-9) and can be switched using specific wavelengths of light.

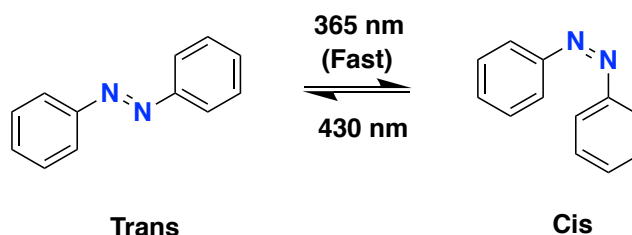
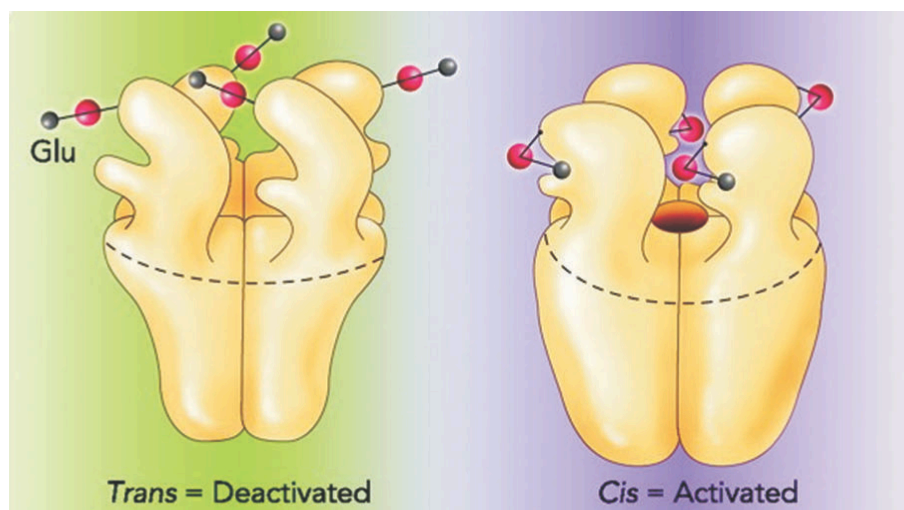


Figure 1-9 - Reversible *cis-trans* isomerisation of azobenzene dependant on exposure of two different wavelengths of light

Several studies have shown the use of azobenzene as a molecular switch in *in vitro* environments. Lester, *et al*, utilised the isomerisation as a tool to block acetylcholine receptor channels in *Electrophorus* electroplaques.⁹³ It was shown that the decay rate of open ion channels increased exponentially with the use of *N-p*-phenylazophenylcarbamylocholine (a light activated cholinergic blocking drug) in its *cis* form, whilst retaining a steady rate in its *trans* form. This was shown to occur within milliseconds of flashes of UV light at 360 nm, converting the *trans* form into the *cis* form.

Furthermore, Volgraf, *et al*, in 2005 conducted a study that had shown controlled activation of an ionotropic glutamate receptor, using an integrated azobenzene derivative coupled to a glutamate agonist.⁶¹ Upon illumination at 380 nm, the conformational switch (Figure 1-10) activates the glutamate receptor. This was confirmed by whole-cell patch clamping with the generation of an action potential. Importantly, this research shows that tethering light responsive moieties to agonists is possible without losing agonist activity.



*Figure 1-10 - Modification of a glutamate receptor using azobenzene coupled to a glutamate agonist. Activation of receptor occurs after exposure to UV light (380 nm), converting from the *trans* → *cis* isomer allowing successful binding of the immobilised ligand (reproduced from Volgraf, et al)⁶¹*

The reversible isomerisation of azobenzene has also been achieved on solid support systems. This has been for both cell adhesion, and also receptor recognition. Controlling cell adhesion was achieved using the presentation and retraction of RGD (a sequence of amino acids which promote cell adhesion) as described by Liu, *et al.*⁹⁴ It was shown that that cell adhesion of 3T3 fibroblasts was greater when the azobenzene was in its *trans* isomer than its *cis* form. This was due to the availability of the RGD to cell surface receptors, and was shown to be a reversible feature. This was demonstrated by cells adhered to the *trans* configured surface, and after a 10-hour exposure to 340-380 nm light of the surface, cells lifted. On exposure to 450-490 nm after 1 hour, cells once again adhered.

Zhang, *et al*, demonstrated the controlled binding of lectins to azobenzene-sugar (mannose and galactose) analogues immobilised on to solid substrates dependent on the presented photo-isomer. It was shown that in the *cis* isomer, the surface was dormant and no protein/cell adhesion was achieved, whilst in the *trans* form, an active state was achieved, promoting selective protein/cell adhesion.⁹⁵

Spiropyran

Spiropyran is a unique molecular switch that undergoes structural isomerisation in response to a variety of orthogonal stimuli. Light, temperature, pH, redox potential, and mechanical stress can all contribute to the isomerisation from the 'closed' spiropyran (SP) to the 'open' merocyanine (MC) isomer (Figure 1-11).⁸⁸

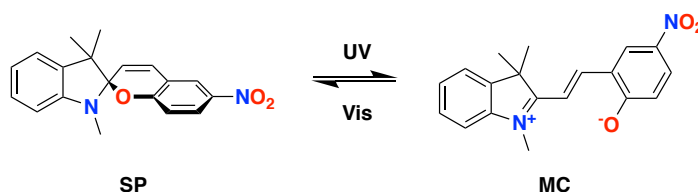


Figure 1-11 - Reversible isomerisation of spiropyran (SP) and merocyanine (MC) isomer using UV and visible light (365 nm and >430 nm respectively)⁸⁸

Isomerisation occurs upon exposure to UV light (C-O heterolytic conversion) or increased temperatures (through a 6 π electrocyclic ring opening step leading to the formation of a quinoidal resonance form) from a non-planar (SP), to a planar zwitterionic structure (MC)⁸⁸ as shown in Figure 1-12. The rapid isomerisation of SP→MC has been demonstrated by Zhang, *et al*, and as with azobenzene, switching of the spiropyran isomers was recorded in the femtosecond range using high intensity lasers.⁹⁶

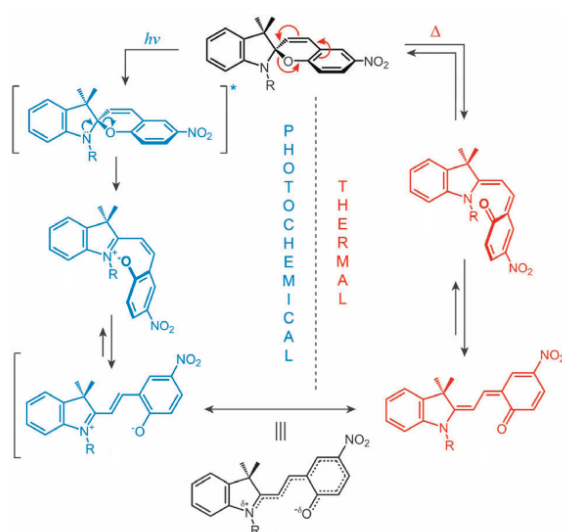


Figure 1-12 - Mechanism of photochemical and thermal conversion of spiropyran (reproduced from Klajn, *et al*)⁸⁸

Identification of the SP/MC isomers is typically conducted using UV/Vis spectroscopy. Spiropyran is comprised of two components, an 'indoline' and 'chromene' segment. Each have unique absorption bands at approximately 270-296 nm which corresponds to π - π^* electronic transition in the indoline portion of the spiropyran, and 320-350 nm for the 'chromene' segment. Upon isomerisation to the MC state, another absorption band is shown between the 500 to 600 nm range, resulted from conjugation between the indoline and chromene segment.⁹⁷ This can be seen in Figure 1-13.⁹⁸

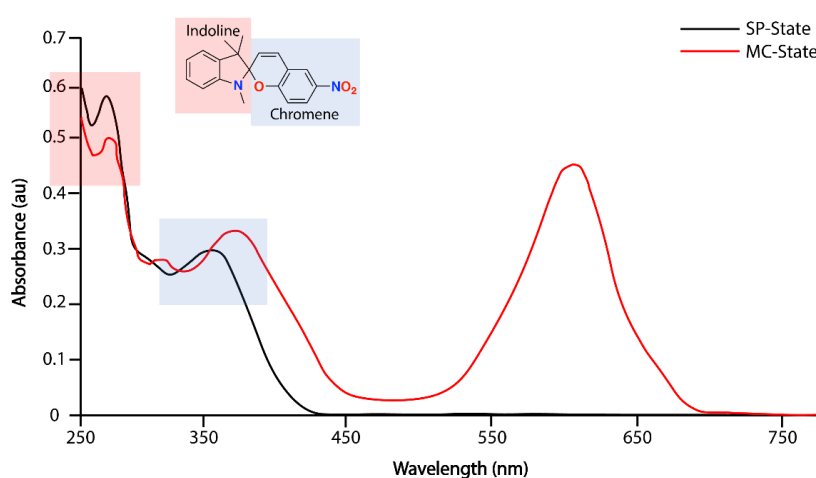


Figure 1-13 - UV absorption spectra of nitrospiropyrans in its SP and MC isomer in methanol during a single cycle of optical switching. Bands shown approximately at 280 nm and 350 nm represent the indoline and chromene segments (data obtained from Petchprayoon, et al)⁹⁸

Today, spiropyran has been used in many dynamic materials.⁸⁸ Attachments to polymers have yielded materials which can be used to control polymer/nanoparticle fluorescence⁹⁹⁻¹⁰¹, solubility¹⁰²⁻¹⁰⁴, aggregation/binding¹⁰⁵⁻¹⁰⁷, metal chelation¹⁰⁸⁻¹¹⁰ and changes in mechanical properties.^{111,112} Integration with biopolymers have shown the control of polypeptide conformation¹¹³⁻¹¹⁵ and enzyme activity.¹¹⁶⁻¹¹⁹ Finally, spiropyran has been implemented on to solid substrates to control surface wettability¹²⁰⁻¹²³, cell adhesion^{106,124-126} and also electrochemical properties.¹²⁷⁻¹³⁰

Examples of the uses of spiropyran have been described below. We have focused on instances where photo-isomerisation of the spiropyran has been used to modify specific physical properties (i.e. shape, size and polarity) to control porosity and surface adhesion of cells as well as to control biological activity.

Rosario, *et al*, explored the effect of photon-modulation of spiropyran surfaces in an attempt to control the flow of fluids in microfluidic devices.¹³¹ It was proposed that changes in surface wettability could allow the controlled transport of very small volumes. It was shown that water contact angles were reduced by 11-14° when a spiropyran functionalised glass surface was irradiated with UV light (365 nm) switching from the SP→MC isomer, whilst returning to a higher contact angle value when exposed to white light.¹³¹ In determining whether changes in surface properties aided in controlling fluid flow, capillary tubes modified with a spiropyran coating in the polar MC isomer were shown to have risen approximately 2.8 mm further than the closed SP isomer using water as a solvent.

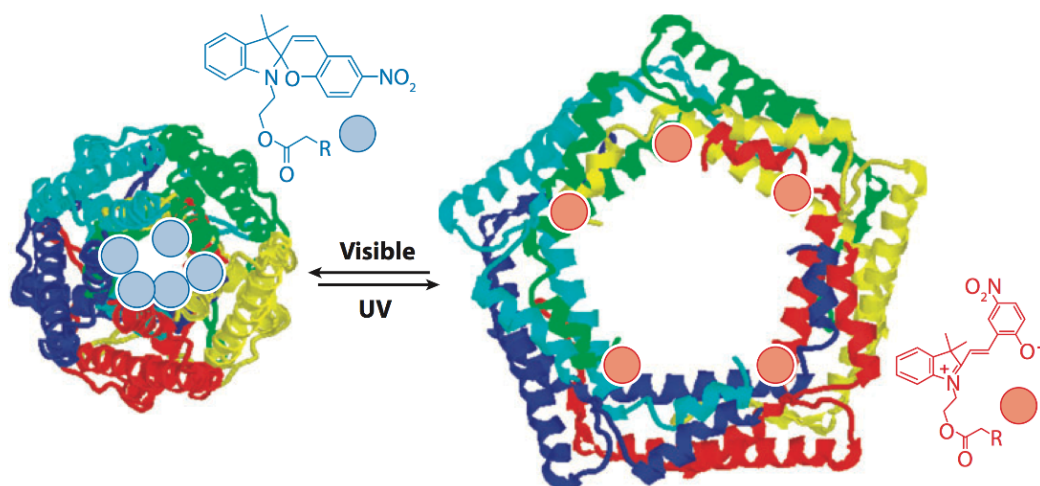


Figure 1-14 - Structural changes are observed on the mechanosensitive ion channel protein revealing a 3 nm pore upon isomerisation of the spiropyran using UV light (reproduced from Koçer, *et al*)¹¹⁵

Controlling structural properties of a biological pore using spiropyran was demonstrated by Koçer, *et al.*¹¹⁵ This was through the generation of a mechanosensitive ion channel, where the integration of spiropyran reversibly allowed the channel to act as a bio-hybrid nano-valve (Figure 1-14). The valve consisted of a channel protein of large conductance (MscL) from *Escherichia coli*. Modified with spiropyran, the channel can reversibly open and close a 3 nm pore when exposed to different wavelengths of light.

Controlling activity of α -chymotrypsin by using spiropyran-based protease inhibitors (Figure 1-15) was demonstrated by Zhang, *et al.*¹¹⁹ It was shown that dependent on the photo-isomer present, α -chymotrypsin activity could be controlled as determined by a colorimetric assay both in solution and when the spiropyran protease inhibitor was attached to a microstructure optical fibre. Using bovine α -chymotrypsin and an *in vitro* inhibition assay, inhibition constants (K_i) were determined graphically using a Dixon plot.¹³² It was shown that α -chymotrypsin inhibition was greater, when the spiropyran was in its non-polar SP isomer compared to its open polar MC isomer (5-fold).

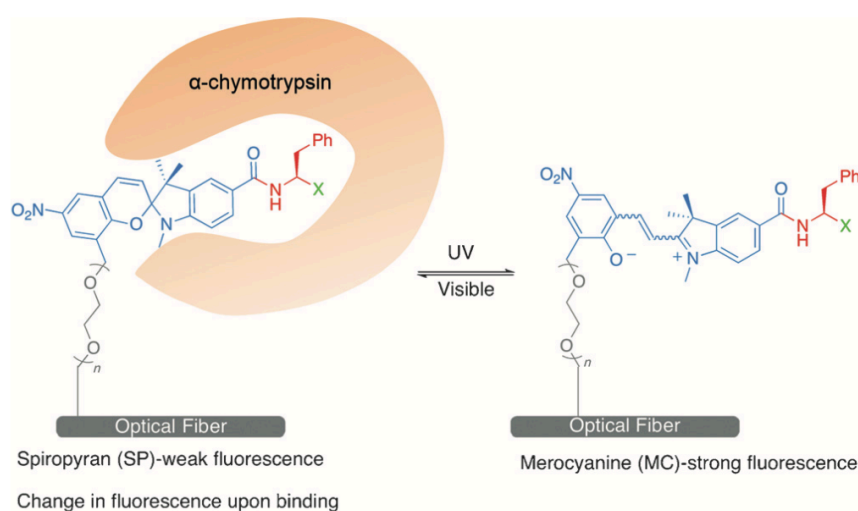


Figure 1-15 - Reversible binding of α -chymotrypsin to spiropyran coupled to an amino acid in both its SP and MC isomer (reproduced from Zhang, *et al.*)¹¹⁹

Both light responsive molecules discussed above (spiropyran and azobenzene), have specific advantages and disadvantages for the use in this thesis. The light-responsive surfaces developed here should only be active and stimulate neurons (i.e. adopt an extended configuration) when exposed to light; in their thermodynamically stable form, the molecules should remain contracted (i.e. no stimulation). This avoids the need for constant irradiation of the culture to suppress rather than activate neuronal stimulation.

As azobenzene in its thermodynamically stable isomer, is found in its extended *trans* form, we would need to keep the compound exposed to UV light for the use in our work, which is undesirable when working with cells. Spiropyran has two isomers, closed when exposed to white light and ambient conditions, whilst open after exposure to UV or increased temperature with reversible switching in the femtosecond range. The significance of this time scale gives promise for activation of neurons, as typically there is a 'synaptic delay' of 0.3 - 0.5 ms to generate action potentials between two neurons. When comparing the properties of both spiropyran and azobenzene, the ability of spiropyran to remain 'inactive' (i.e. contracted) under visible light as well as its very fast switching rate makes it the more suitable choice for the present work.

Cellular interactions and the Bio-interface

So far, we have described the ways cells are stimulated and communicate with other neighbouring cells in a culture as well as different types of responsive materials. A feature which has not been covered yet is the mechanisms with which a cell interacts physically with other cells and specifically with inorganic/organic substrates. Figure 1-16 gives a representation of a cell membrane showing a number of proteins and carbohydrates formed outside the cell membrane, commonly known as the extracellular matrix (ECM). As the non-cellular component present within all tissues and organs, the ECM provides essential scaffolding for cellular constituents, and initiates crucial biochemical and biomechanical cues that are required for tissue morphogenesis, differentiation and homeostasis.^{133–137} Similar to cell-cell interactions, surfaces have been developed to control specific biological cues. This interaction between a cell and a surface is known as the bio-interface and has been shown to play a large part in the determination of biological response of the cell on initial contact.¹³⁸

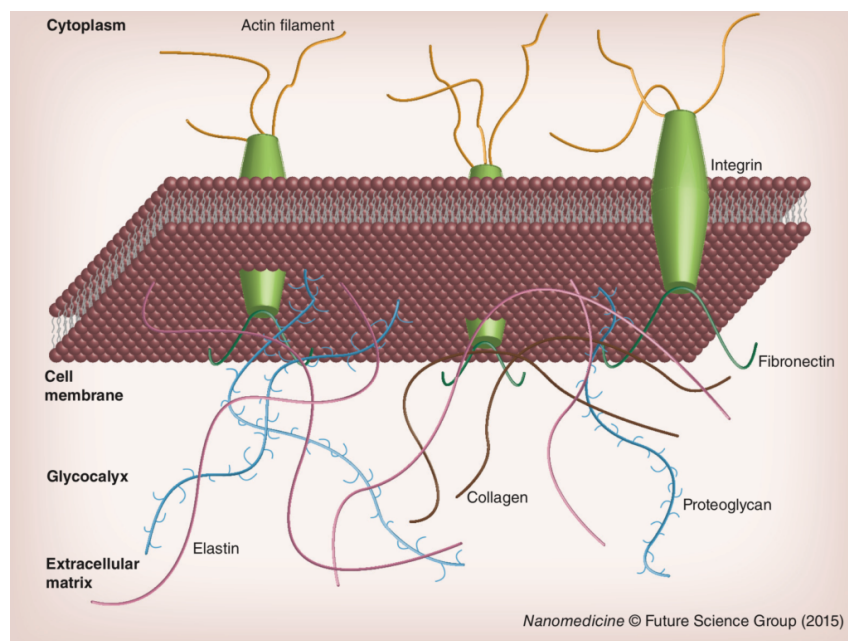


Figure 1-16 - Schematic of a cell membrane showing the connection between actin filaments of the cytoskeleton and the extracellular matrix through integrins (reproduced from Dhowre, et al)⁵⁵

Biological responses of cells in contact with inorganic/organic substrates have been attributed to both chemical and physical properties of the substrate itself.¹³⁸ Studies have shown a number of materials which can interact and mimic natural cellular environments, which include surfaces which evoke cellular responses such as adhesion⁸¹⁻⁸³, proliferation¹⁴²⁻¹⁴⁵, differentiation¹⁴⁶⁻¹⁴⁹ and migration.^{147,150-152} In the context of this work, we must find suitable chemical and physical properties (interfacial and bulk) of surfaces which not only allow the integration of a photo-responsive component to stimulate surface receptors but also to promote cell adhesion. Below we describe elements which have been explored for the interaction of cells with both planar substrates and also nanomaterials.

Planar substrates

The chemistry of planar substrates was shown to play a role in dictating multiple cell actions upon initial contact. Depending on the surface chemistry, a cell can undergo changes in morphology¹⁵³⁻¹⁵⁶, adhesion^{55,157-159}, migration^{153,160,161}, proliferation^{154,158,162,163}, and differentiation.^{154,164-166} Certain compounds and molecular structures such as RGD and IKVAV play pivotal roles in cell adhesion, whilst non-specific chemical properties such as charge and wettability can also contribute. It has been shown that positive charges on surfaces typically promote adhesion due to cells typically exhibiting a negative surface charge. For example, nitrogen based surfaces promote adhesion over carboxylic acid terminated surfaces.¹⁶⁷

The physical properties of a surface can play a large role in the ability for cells to interact with them, as well as control cell fate. These include topographical features such as roughness, and embedded patterns of varied dimensions.¹⁶⁸ It has been shown that cells respond to micron and nano sized features differently.¹⁶⁹ Typically, features which are several orders of magnitude smaller than the size of a cell (nano-sized features), play a larger role in affecting cell processes, such as cell adhesion^{170,171} and differentiation¹⁷²⁻¹⁷⁴.

Nanomaterials

Nanomaterials can be identified in three categories: materials that are nanostructured in the bulk¹⁷⁵; materials that have nanostructures on the surface (as mentioned previously for surface topography) and materials that contain nanostructured particles. Nanoparticles are defined as particles with dimensions up to 1000 nm and can be composed of a number of materials, including but not limited to latex^{176,177}, silica^{178–180}, titania^{181–183}, hydrogels^{184–186}, and various metals (i.e. gold, silver and iron).^{187–189} Nanoparticles have been used for a range of applications, such as drug delivery purposes and imaging. These applications heavily rely on the properties of the nanoparticles: considering composition, surface chemistry, size and also shape as shown in Figure 1-17.¹⁹⁰

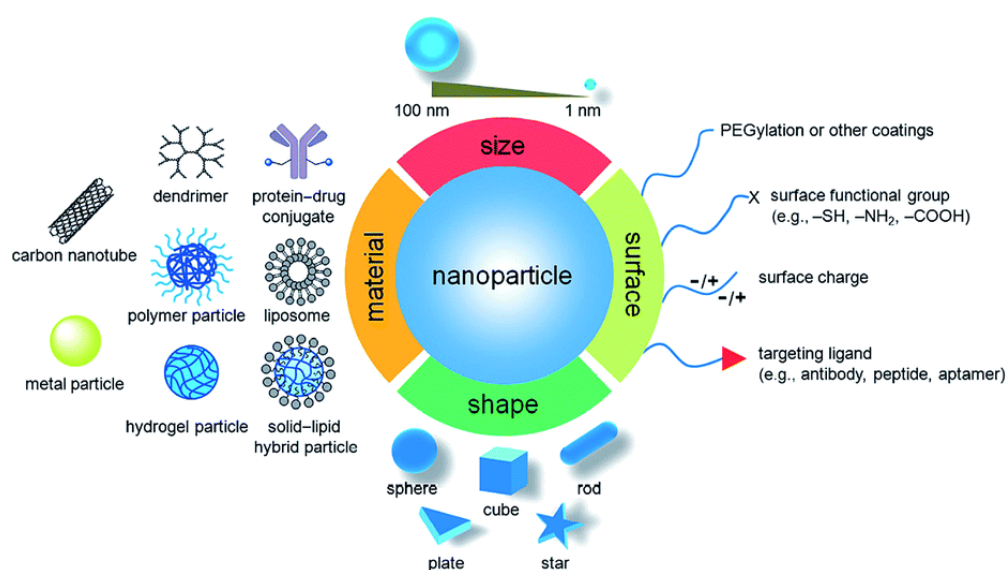


Figure 1-17 - Variable biophysical properties of nanoparticles including type, composition, surface chemistry, charge and shape (reproduced from Wang, et al)¹⁹⁰

Similar to how a cell interacts with a planar surface, the properties of the nanoparticle define its interaction, from surface receptor activation, to cellular uptake. As our project demands a way of stimulating surface receptors, nanoparticles must be small enough to diffuse freely through the extracellular matrix (< 200 nm)^{191,192}, as well as have a large surface area so that covalently bound ligands can be recognisable by cell surface receptors. This limits the

range in which we can operate within this thesis, with a compromise on nanoparticle size or ligand density must be made to navigate through to activate surface receptors.

After navigation through the ECM, the cellular responses which can be exhibited when a surface receptor is presented with a ligand coated nanoparticle is shown in Figure 1-18.¹⁹³ These interactions include the activation of surface receptors¹⁹⁴, endo/exocytosis of nanoparticles^{195–198}, as well as non-specific interactions with the surface membrane (phagocytosis^{199,200} and micropinocytosis²⁰¹). In particular, the mechanism associated when activating surface receptors, shows that receptor mediated endocytosis can readily occur with spherical nanoparticles in the range of 30 - 50 nm²⁰² which is further enhanced by surface chemistries which exhibit positive or negative charges (zeta potentials between +15 and 30 mV for positive particles, and between -40 and -15 mV for negative particles).²⁰³ As our functional groups are limited to spiropyran and dopamine, the size, shape and composition of the nanoparticle becomes essential to our work to avoid nanoparticle uptake.

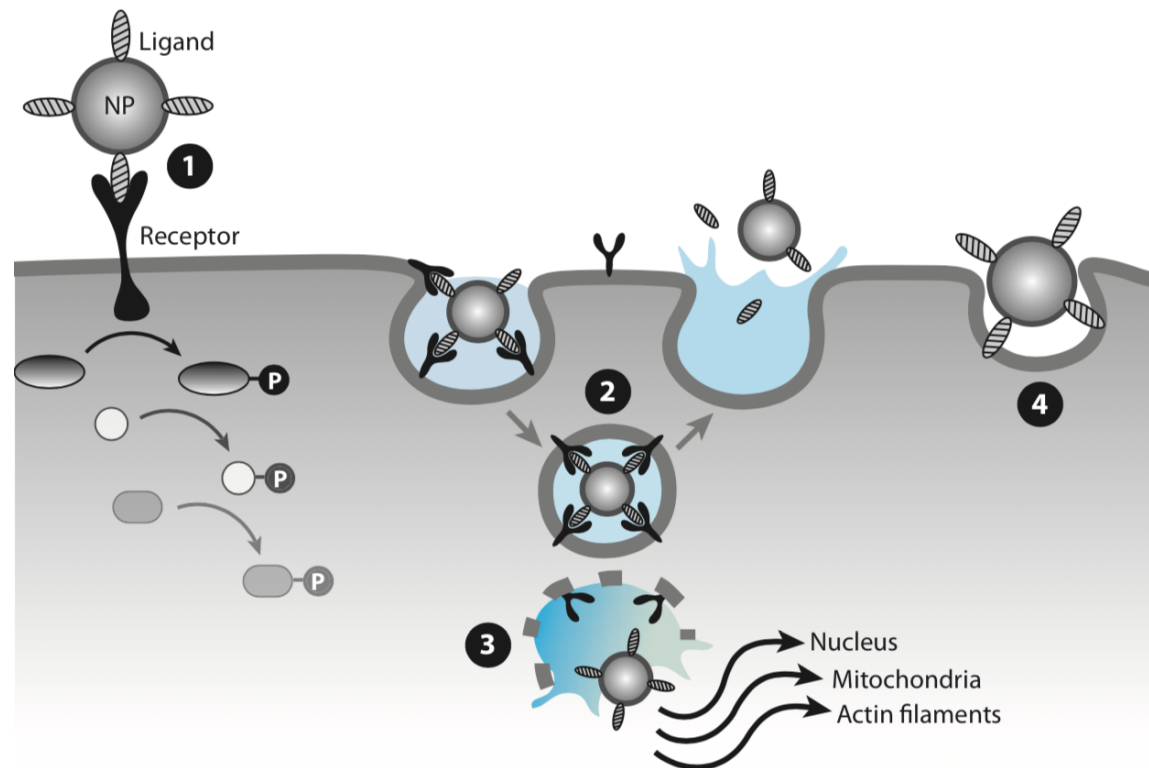


Figure 1-18 - Schematic showing ligand-coated nanoparticles interacting with the cell membrane. (1) Surface receptor activation, inducing a signalling cascade without entering the cell (2) receptor mediated endocytosis and exocytosis without leaving enclosed vesicle (3) nanoparticle targets subcellular structures (4) non-specific interaction (phagocytosis and micropinocytosis) with the cell surface membrane (reproduced from Albanese, et al)¹⁹³

Hypothesis

Stimulating and monitoring cellular activity is essential, both for improved understanding of how neuronal networks work, and as a means to treat damaged neuronal tissue or neurodegenerative diseases such as PD. To more fully understand the function of cells in neuronal networks, the ability to stimulate and record the activity of each individual neuron in a neuronal culture is necessary. This is challenging as current tools to stimulate individual neurons (patch clamping, microelectrode arrays) cannot be applied to millions of individual cells within a culture. We propose that stimuli-responsive materials can be used to stimulate neurons on demand, using external stimuli such as light in a highly spatial and temporal manner.

To generate a light-responsive neuron-stimulating platform, we first aim to synthesise a library of dopamine analogues that can retain the ability to activate dopamine receptors when linked to another side-group. We will specifically focus on the human D₁ receptor which has been shown to be associated with memory and learning.^{204–206} These dopamine analogues would then be coupled to a photo-responsive compound, in an attempt to control activity of the D₁ receptor. Finally, the photo-responsive neurotransmitter analogue would be immobilised on a surface to develop a functional bio-interfacial system which can stimulate individual neurons with high spatio-temporal control.

Concept

Development of a system like this requires three components (as shown in Figure 1-19): (i) a ligand known to stimulate and contribute to memory and learning (e.g. dopamine, highlighted in green), (ii) a photo-responsive unit (spiropyran, in blue), and (iii) the technology to incorporate these two materials on a surface (in red).

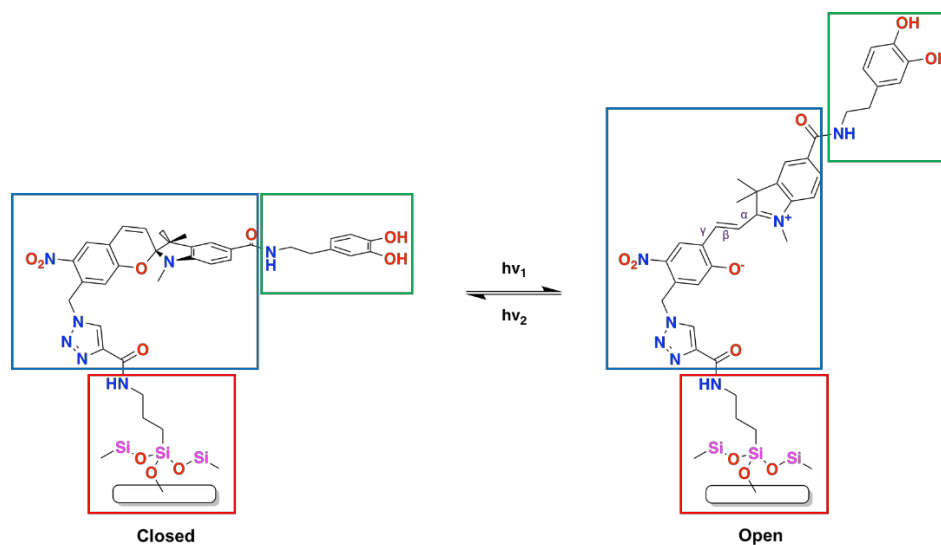


Figure 1-19 - Concept diagram showing surface modification and isomerisation of spiropyran to 'hide and reveal' dopamine on demand (red shows surface modification of silica based substrates with silanes, coupling of spiropyran is shown in blue through copper mediated click chemistry, and the green shows dopamine conjugation)

This concept shows a change in conformation for the spiropyran molecule, from the SP→MC isomer. Upon isomerisation, free rotation around the α , β and γ bonds as shown in the Figure 1-19, will in theory allow the bound dopamine to be more available to the D_1 receptor. Our concept shows modification at the amino moiety, but can alternatively be attached from the aromatic core or catechol (which is discussed in Chapter 3). Figure 1-20 shows our concept at the surface membrane. Dopamine is limited to a specific position when the photo responsive isomer is in its closed state, but flexible when in the MC isomer form.

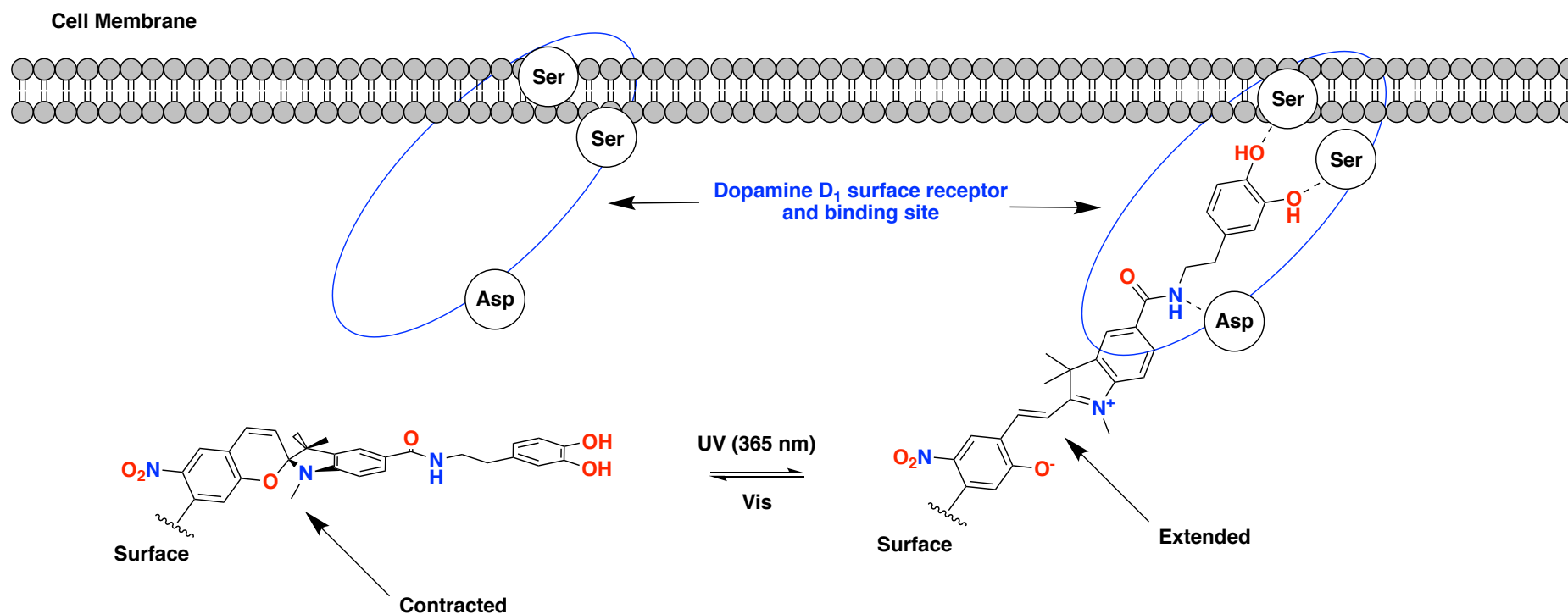


Figure 1-20 - The schematic conceptualises the ability for the spiropyran-dopamine photo-pharmaceutical to bind to the conserved amino acid residues (Ser¹⁹⁸, Ser²⁰² and Asp¹⁰³) found the D₁ receptor when in its open merocyanine isomer. This is controlled using different wavelengths of light to control isomerisation

Chapter 2 - Methods

Chapter 2 describes the tools and equipment used to synthesise and characterise the compounds and surfaces generated within this thesis. These include, microwave assisted organic chemistry, spectroscopic techniques (i.e. nuclear magnetic resonance, UV/Vis and infrared spectroscopy), spectrometric techniques (time of flight - secondary ion mass spectrometry) and combined techniques such as liquid chromatography mass spectrometry (LCMS).

Microwave assisted organic chemistry (MAOS)

Microwave assisted organic chemistry (MAOS) is a method which harnesses the ability of microwave irradiation (0.3 - 300 GHz) to assist in chemical reactions. MAOS often gets mistaken for having the ability to break chemical bonds assisting in synthetic chemistry. Due to its energy output of around 0.0016 eV at the frequency of around 2.45 GHz, this is not possible.²⁰⁷ It is actually used to apply heat and drive chemical reactions in an efficient and direct manner, with the ability to heat a reaction efficiently solely dependent on the phenomenon known as 'dielectric heating'. This is the ability for a specific material (solvent or reagent) to absorb microwave energy and convert it into heat as is determined by the so-called loss tangent (δ). Polar protic solvents tend to have high to medium loss tangents. The loss tangent (δ) can be derived from the Maxwell equation:

$$\tan \delta = \varepsilon''/\varepsilon' \quad \text{Equation 1}$$

Where ε'' - the dielectric loss (indicative of efficiency for converting electromagnetic radiation to heat), ε' - dielectric constant (ability of molecules to be polarised by the electric field). High $\tan(\delta)$ values correspond to efficient absorption, and lead to rapid heating of the medium.

The observed enhancements in the reaction kinetics for organic synthesis occur due to ionic conductance. Due to translational motion of electric charges, when an electrical field is applied, collision rates of ions increase and convert kinetic energy into heat as in typical reactions.

The focus in heating increases efficiency in MAOS, and the Arrhenius equation can give an insight on effects to the rate of reaction, when temperature is increased.

$$k = A * e^{-E_A/RT} \quad \text{Equation 2}$$

Where, k – rate of reaction, A – pre-exponential factor, e - exponential, E_A – activation energy, R – gas constant, T – temperature in Kelvin.

The overall process is more energy efficient than classical oil-bath/mantle heating. This can be shown in Figure 2-1 comparing conventional heating with microwave chemistry²⁰⁸ showing microwave heating is highly directional to inside the microwave inert materials (i.e. borosilicate).

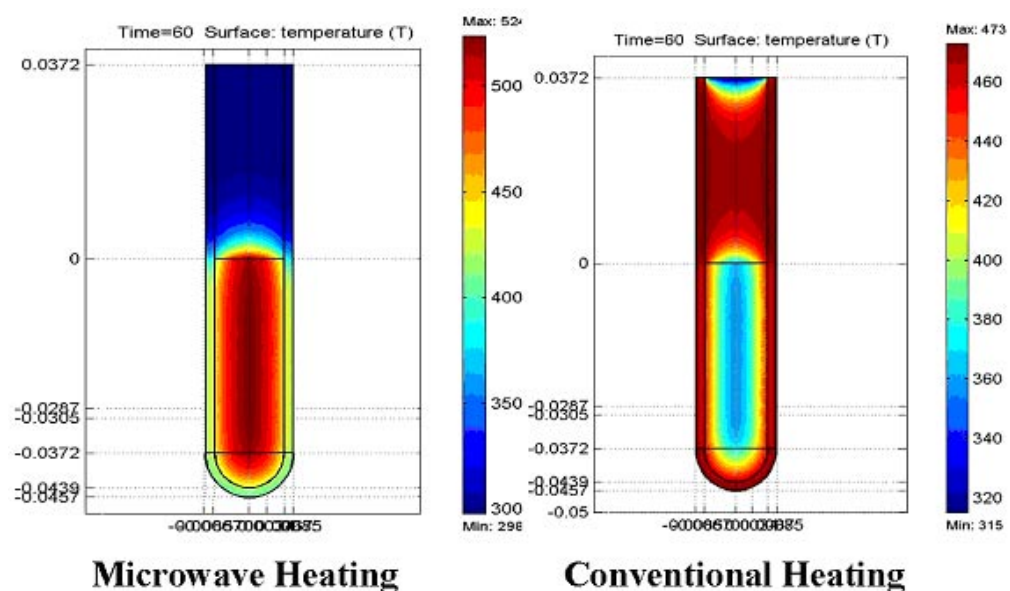


Figure 2-1 - Heat dissipation between vessel and solvent using microwave heating vs conventional heating²⁰⁸

Nuclear magnetic resonance spectroscopy (NMR)

Nuclear magnetic resonance (NMR) spectroscopy is a method to explore physical and chemical properties of a compound.²⁰⁹ It is a physical phenomenon of resonance transition between magnetic energy levels. This occurs when atomic nuclei are subject to an external magnetic field, absorb and re-emit electromagnetic radiation. This is then converted to a frequency spectrum after Fourier transformation. As a non-destructive technique, it is commonly used to analyse the structure and purity of both small biomolecules and large organic molecules (i.e. proteins). Figure 2-2 is a schematic of a typical NMR set up.

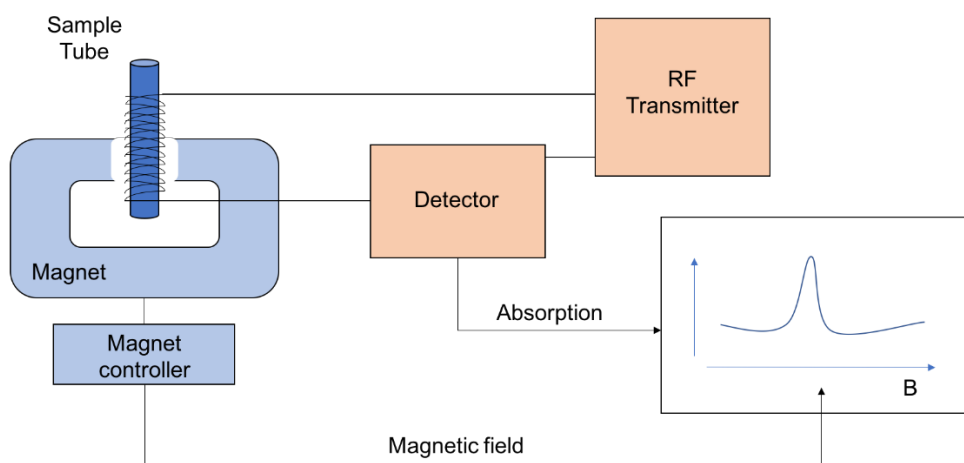


Figure 2-2 - Schematic of NMR apparatus

The basis of NMR relies on a property of a nuclei described as spin (I). This is a quantum effect which may be interpreted as a nucleus spinning about on its axis. Importantly, NMR-active nuclei such as ^1H , ^{13}C , ^{15}N , and ^{19}F exhibit an I value of $\frac{1}{2}$. Quantum mechanics states, that a nucleus with spin I will give us $2I+1$ possible orientations in a magnetic field (B_0). With a spin number of $\frac{1}{2}$ we get 2 possible orientations, with (α) and against (β) the magnetic field.

The energy between the two orientations in a magnetic field is shown by equation 3:

$$\Delta E = \gamma h B_o / 2\pi \quad \text{Equation 3}$$

where: B_o – external magnetic field, h – Planck's constant, and γ – gyromagnetic ratio.

Energy in the form of electromagnetic radiation (EM) is used to convert the α orientated nuclei, to the β state. Upon removal of the EM radiation, relaxation of the β state back down to the α state occurs, which causes a fluctuation in the magnetic field. This phenomenon is known as resonance, and can be detected and converted into the peaks we observe in an NMR spectrum. Typically, the solvent used for dilution (deuterated solvents) are used as internal standards, replacing the use of standards such as tetramethylsilane (TMS).²¹⁰

We can use NMR not only to detect single atomic NMR –active nuclei, but also to determine correlations between different NMR-active nuclei. This method is known as two-dimensional NMR. Identifications of spin-spin coupling between protons in ^1H NMR can be achieved through homonuclear correlation spectroscopy (COSY), whilst heteronuclear single-quantum correlation spectroscopy (HSQC) can correlate two different types of heteronuclear atoms separated with one bond, such as between ^1H and ^{13}C . To determine correlation between heteronuclear atoms 2-4 bonds away, heteronuclear multiple-bond correlation spectroscopy (HMBC) can be utilised.

Ultraviolet-visible spectroscopy (UV/Vis)

UV/Vis spectroscopy is an analytical method which uses electromagnetic radiation in the range of 190 - 800 nm to interact with matter to cause electronic transitions. Molecules containing π -electrons or non-bonding electrons (n-electrons) can absorb the energy in the form of ultraviolet or visible light to excite these electrons to higher anti-bonding molecular orbitals (Figure 2-3).²¹¹

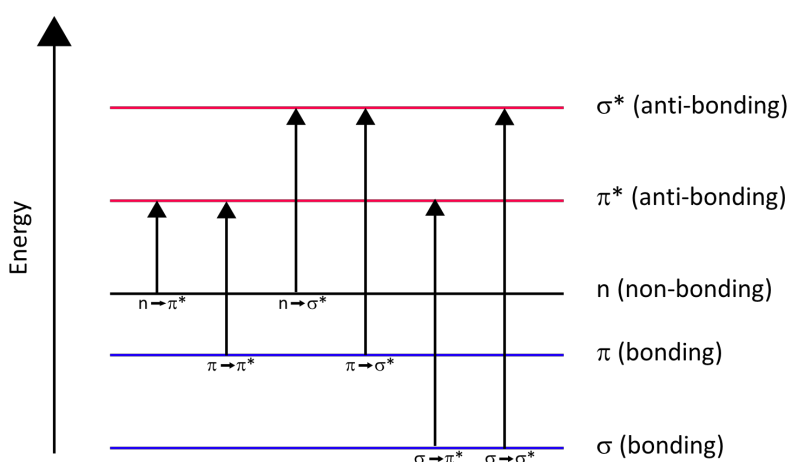


Figure 2-3 - Electronic transitions that can occur in organic molecules

The promotion of electrons usually occurs from the highest occupied molecular orbital (HOMO) to the lowest unoccupied molecular orbital (LUMO), and the resulting species is called an excited state. Absorption of this electromagnetic radiation at a specific wavelength is detected using an optical spectrometer, resulting in a spectrum of absorbance vs wavelength. The Beer Lambert law is commonly used to identify the linear relationship between absorption and concentration of a solution of a chromophore. From this, we can calculate the molar absorptivity (ϵ), which is used to compare different chromophores.

$$A = \epsilon cl \quad \text{Equation 4}$$

Where A – absorption, ϵ – molar absorptivity, c – concentration and l – path length

Infrared spectroscopy (IR)

Infrared spectroscopy (IR) is the study of vibrational transitions within a molecule, upon the absorption of infrared radiation. It is typically used in structure elucidation to determine specific functional groups, through molecular vibration.²¹² Vibration is the change in shape of a molecule which includes the stretching or bending of bonds as well as internal rotation around single bonds.

The fundamental vibrations are shown in Figure 2-4. Stretching vibrations involve the change in the inter-atomic distance between two bonds, whilst bending vibrations are characterised by the change in angle between two bonds. Characteristically, stretching vibrations appear at a shorter wavelength requiring a higher energy input whilst bending vibrations are typically found at longer wavelengths.

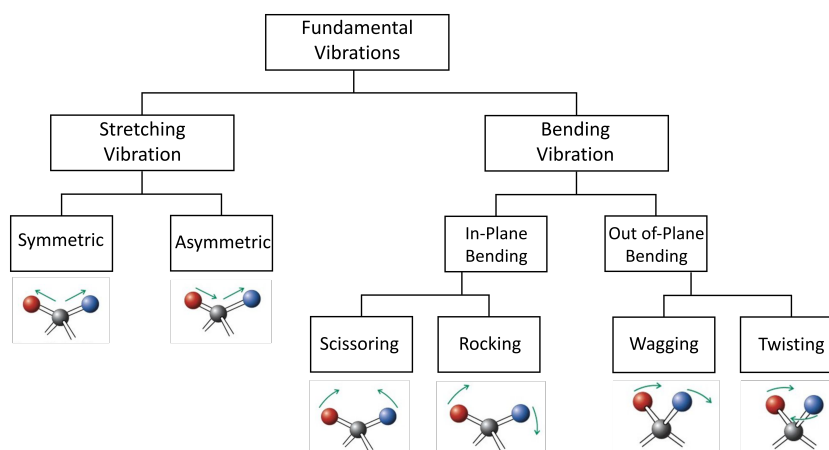


Figure 2-4 - Fundamental vibrations associated with Infrared absorption

For a molecule to absorb radiation, the vibrations or rotations must cause a net change in the dipole moment of the molecule. If the frequency of the radiation matches the vibrational frequency of the molecule then radiation will be absorbed, causing a change in the amplitude of molecular vibration which is recorded and converted through Fourier transformation to give an IR spectra.

Liquid chromatography mass spectrometry (LCMS)

Liquid chromatography mass spectrometry (LCMS) is a technique which combines the physical separation of molecular compounds (high performance liquid chromatography) with the mass analysis capabilities of a mass spectrometer. It utilises chromatographic methods to separate components based on their affinity for either the stationary or mobile phase. The detection of compounds is typically identified either through UV absorption, fluorescence or electrical conductivity.²¹³ Individual components are then ionised upon separation which can be achieved in a number of methods (e.g. electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI)), which then leads to the mass analyser (e.g. quadrupole, ion trap, time of flight and orbitrap mass spectrometers).²¹⁴

Each peak found on the chromatogram gives distinct mass fragments associated with said compound, this then allows us to determine the composition of samples, as well as the purity if a singular peak is obtained (Figure 2-5). This is limited to the absorption properties of compounds, as well as the limit of detection (LOD) which is dependent on the equipment used.

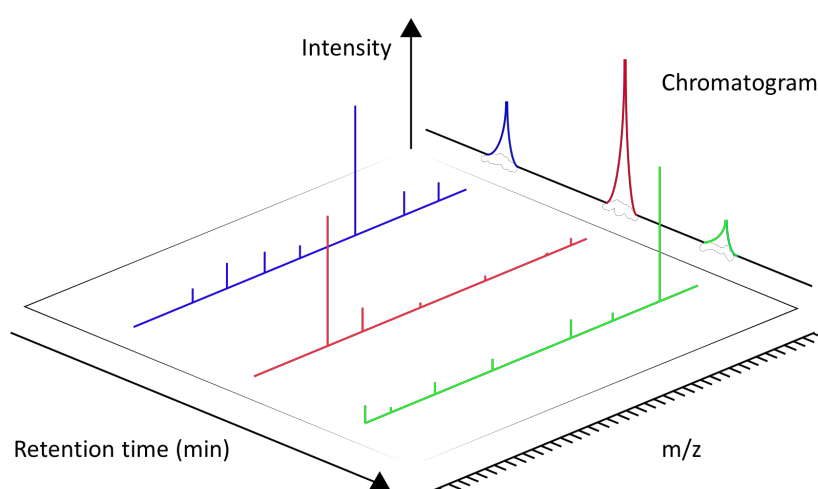


Figure 2-5 - Individual mass spectra are generated from peaks separated by liquid chromatography (each spectrum is linked to its respective peak by its colour)

Time of flight – secondary ion mass spectrometry (ToF-SIMS)

Time-of-flight secondary ion mass spectrometry (ToF-SIMS) is a very surface sensitive analytical technique, which can provide details on elemental and molecular information about surfaces. This includes thin layers, interfaces and bulk properties of a sample to give a full three-dimensional analysis.²¹⁵ It can be used as a semi-quantitative technique to determine certain molecular ions formed in the gas phase, after bombardment of the surface using a primary ion beam (i.e. bismuth, manganese, caesium and argon). Figure 2-6 shows the typical set up of a ToF-SIMS instrument.

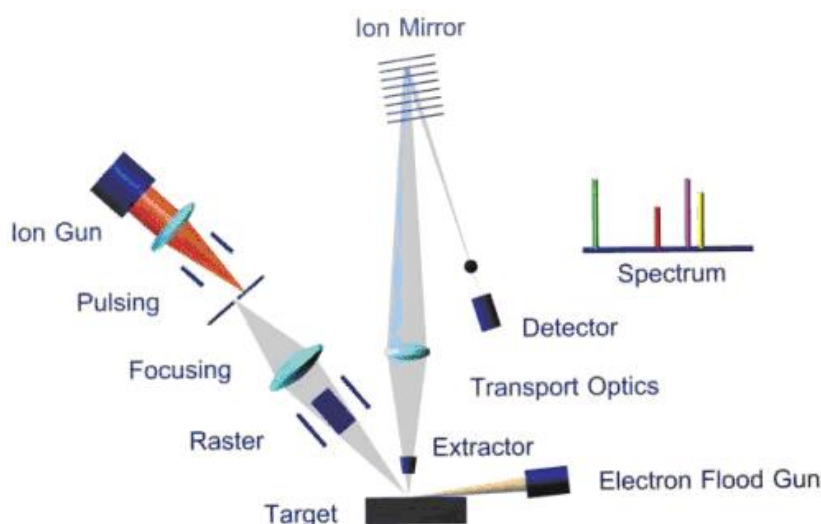


Figure 2-6 - ToF-SIMS Instrument schematic showing ion bombardment of the surface and extraction of reaction species formed to the mass spectrometer

Conducted under high vacuum, the generation of positive, negative and neutral ion species are formed *via* a 'collision cascade' that is triggered after the surface is hit by the ions of a primary beam and results in the generation and ejections of small fragments from the sample surface. Most of the emitted particles are neutral in charge, but a small proportion is also positively or negatively charged. The subsequent time-of-flight mass analysis of the emitted ions provides detailed information on the elemental and molecular composition of the surface.

Water contact angle (WCA)

The water contact angle (θ) is a measurement used to determine a surface's hydrophilic or hydrophobic properties. It is a precise measurement of the surface energy of the material (with a depth resolution of 0.33 to 1 nm) from the cohesive forces associated with water and the surface chemistry/topography.²¹⁶

The contact angle is defined geometrically as the angle formed by a liquid at the three-phase boundary where a liquid, gas and solid intersect (Figure 2-7). This is described by the Young equation:

$$\gamma_{SG} = \gamma_{SL} + \gamma_{LG} \cdot \cos\theta \quad \text{Equation 5}$$

Where, γ_{SG} – interfacial energy between solid-gas phase, γ_{SL} – interfacial energy between solid-liquid phase, γ_{LG} – interfacial energy between liquid-gas phase and θ – contact angle.

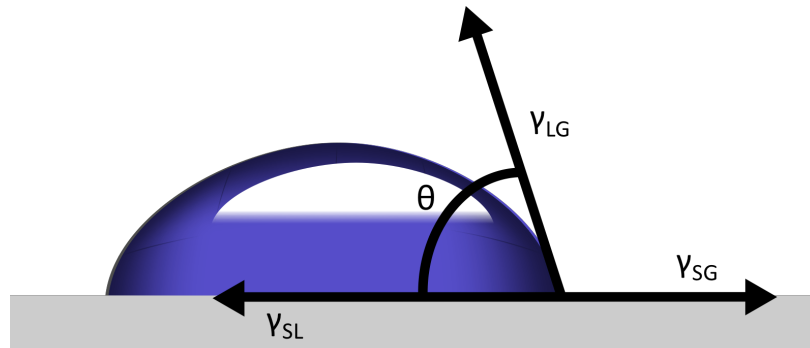


Figure 2-7 - Water droplet on solid substrate describing surface tension between the solid-liquid (SL), solid-gas (SG) and liquid-gas (LG) interface measure by angle θ

In general, it is determined that angles below 90° indicate surface wettability is favourable and is known as hydrophilic, whilst over the 90° angle, a surface is deemed to be hydrophobic.²¹⁰ Complete wettability occurs at 0° and surfaces have been known to be called super hydrophilic, whilst angles over 145° are known as super hydrophobic.

Chapter 3 - Synthesis and pharmacological studies of dopamine analogues at the D₁ receptor

Introduction

Neurotransmitters such as dopamine, norepinephrine, and serotonin (commonly classed as biogenic amines, Figure 3-1), play a critical role in the central and peripheral nervous systems.²¹⁷ Acting at the cell surface, these endogenous ligands, through ligand-receptor binding, lead to the activation of GPCRs. Understanding the structure and function of endogenous ligands, receptor crystal structures and ligand-target interaction is essential in the process for the discovery of new synthetic analogues.

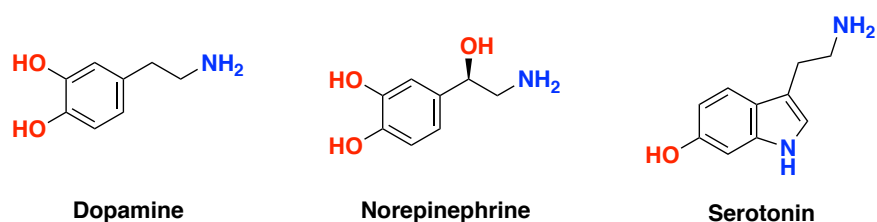


Figure 3-1 - Examples of biogenic amines that can activate surface receptors found on a cell membrane

This Chapter describes the modification of dopamine, in order to determine which functional groups are key in retaining agonist activity at the D₁ receptor. To do this, we first review the literature on dopamine interaction with the D₁ binding pocket, and in specific to which amino acid residues.

Dopamine - structure and function

Dopamine, a catecholamine, is a major neurotransmitter found in both the central nervous system (CNS) and peripheral nervous system (PNS). It is associated with a number of functions such as motor co-ordination²¹⁸, memory^{219–221}, reward and motivation behavior.²²¹ Naturally produced, dopamine is bio-synthesised from L-tyrosine (Figure 3-2) and is stored and transported in vesicular monoamine transporters 2 (VMAT2) from cellular cytosol to the synaptic cleft found between neurons. These vesicles operate under acidic conditions, preventing oxidation of the molecule. This is key to prevent the generation of plaques found in certain neurodegenerative disease such as PD and AD.²²²

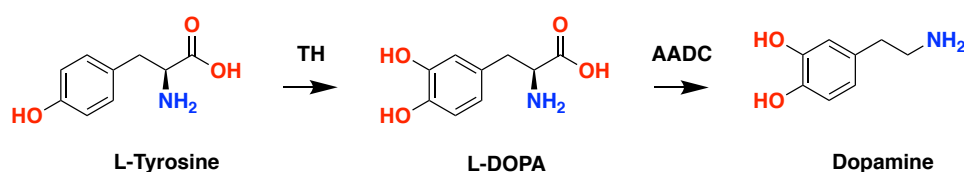


Figure 3-2 - Biosynthesis of dopamine from L-tyrosine (TH- tyrosine hydroxylase, AADC - aromatic-L-amino acid decarboxylase)²²³

Part of the GPCR 7 transmembrane domain family, dopamine exerts its effects at five metabotropic receptors (D₁-D₅). These are subdivided into two families, D₁-like (D₁ and D₅) which couple to stimulatory G-proteins (G_s) and D₂-like (D₂, D₃, and D₄) to inhibitory G-proteins (G_i).¹⁸

Figure 3-3 shows the cellular pathway and activation cascade when dopamine binds to D₁ type receptors. These are known to regulate synaptic plasticity, and hence were focused on within this Chapter.²²⁴

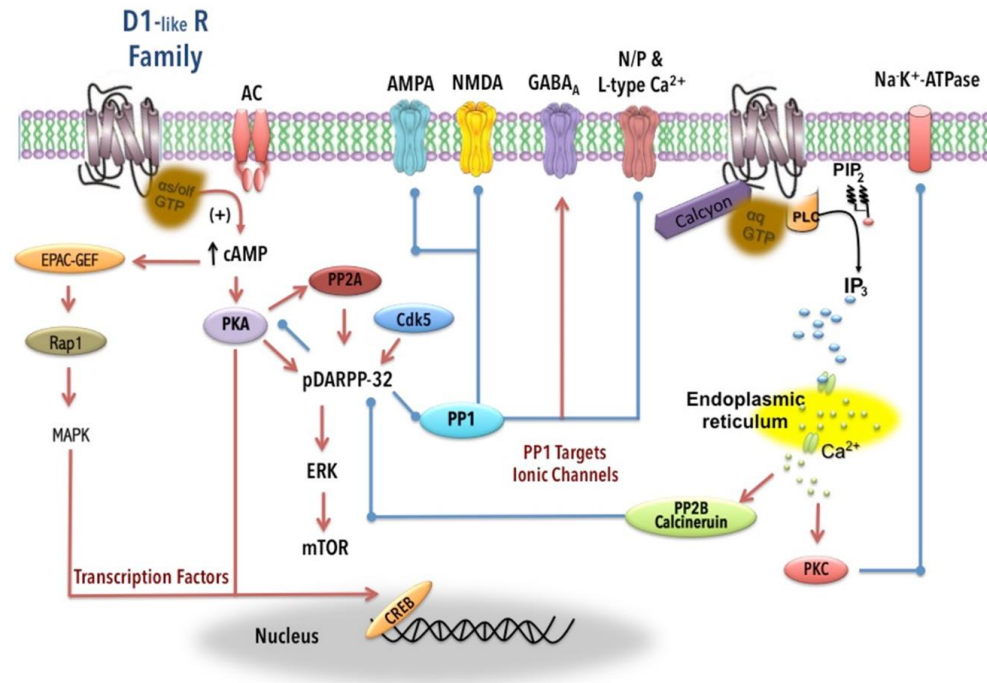


Figure 3-3 – Stimulatory (red) and inhibitory (blue) pathways of the dopamine D_1/D_5 receptor (cAMP - 3'-5'-cyclic adenosine monophosphate; PKA - protein kinase A; DARPP-32 - dopamine and cAMP regulated phosphoprotein; AC - adenylyl cyclase; PP1, PP2A or PP2B - protein-phosphatase; PKC - protein kinase C; PLC - phospholipase C; IP_3 - inositol triphosphate; mTOR - mammalian target of rapamycin; PIP_2 - phosphatidylinositol 2; MAPK - mitogen-activated protein kinase; EPAC-GEF - guanine-nucleotide-exchange factor of Rap1; AMPA - α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors; NMDA - N-methyl-D-aspartate; $GABA_A$ - γ -aminobutyric acid A; CREB - cAMP response element binding protein, reproduced from Rangel-Barajas, et al)¹⁸

Receptor-ligand binding

To date, there are no published crystal structures of the dopamine D₁ receptor. Identification of key interactions and functional residues, between agonists and the D₁ receptor have been conducted using site directed mutagenesis (a method which allows the specific and intentional changes to the DNA sequence of a gene or a gene product) and has been reinforced using homology modelling.^{58,225–231} Typically, homology models are based upon structural and sequence similarities with a defined crystal structure. Here, we use the human β_2 adrenergic receptor as our defined crystal structure, to obtain insight into the key binding interactions of the dopamine D₁ structure. Alignment of the genetic sequences for the human_DRD₁ (P21728), and human β_2 adrenergic receptor (P07550) as found on UniprotKB can be seen in Figure 3-4. This shows 134 identical positions, with 144 similarities, which can be defined using the Tailor classification system (a system which takes into consideration residue polarity, size, and functional group).²³²

P21728	DRD1_HUMAN	1	-----MRLLNTSAMDGTGLVVERDFSVRLTACFLSLLILSTLLGNTLVCAAVIR	50
P07550	ADRB2_HUMAN	1	MGQPGNGSAFLAPNGSHAPDHDTQERDEVVVGMGIVMSLIVLAIIVFGNVLVITAIK	60
			: : * * . . : . *** : . . : : : : : : : : : : : : : : : :	
P21728	DRD1_HUMAN	51	ERHLRSKVTNFFVISLAVSDLLVAVLVMPEKVAEITAGFNPPGSE-CNIWVAFDIMCSTA	109
P07550	ADRB2_HUMAN	61	ERLQ-TVTNYFITSACADIVMGLAVVPEGAHILMKMTFGNEWCEFTSIDVLGVTA	119
			*. : : : . : *	
P21728	DRD1_HUMAN	110	SILNLCVISVDRYWAISPPERYERKMTPKAIFLLISVAVTILSVLISEIPVOLSNNHAKPT	169
P07550	ADRB2_HUMAN	120	SIETLCVIAVDRYFATTSPEKYQSLITKNKARVILMVVIVSGITSELPIDQHWYRATHQ	179
			** . : *	
P21728	DRD1_HUMAN	170	SPSDGNATSLAETIDNCSSLSRTYAISSSVISFYIPVAIMIVTYTRIVRIQKQIRRTA	229
P07550	ADRB2_HUMAN	180	EAINC-----YANETCCDFFTNQAYAIASSIVSFYVPLVIMVFSRVFOEAKROLQKID	234
			. : . : *	
P21728	DRD1_HUMAN	230	ALERAARVAKNCOTITGNGKPEVCSQPESSKMSFKRETQVLKTLVIMGVVCCWLPEF	289
P07550	ADRB2_HUMAN	235	KSEGR-FHVONLSQVEQDGRTHGLRRSKT---CLKKHKALKTLGITMGTETLCWLPEF	290
			* . : . : . . : : . : : * * : * * . : : : : : : : : : : : *	
P21728	DRD1_HUMAN	290	LLNCLLPFCGSGETQPFCTDSNTFDVFNFGWANSNLNFIYAFNADFRKAESTLLCCYR	349
P07550	ADRB2_HUMAN	291	LVNIHVHIQ-----DNLIRKEVYILLNWIYVNSGFNPLIYCRSPDFRIAFQELLCLRR	344
			* : *	
P21728	DRD1_HUMAN	350	LCPATNNAIETVSIINNCAAMFSSHHEPRGSISKECNLVYLIPHAVGSSSEDLKKEEAAGI	409
P07550	ADRB2_HUMAN	345	SSLKAYG-NGYSNCGTIG-EQSCYHVEQEKENKLLCEDLPGETDFVGHQGTVPDNDISQ	402
			. : . : . : * * * . : * * . . . : : . : : . : : : . : : : *	
P21728	DRD1_HUMAN	410	ARPLEKLSPALSVILDYDITDVSLEKIQIPITQNGQHPT	446
P07550	ADRB2_HUMAN	403	GRNCST---NDSLL-----	413
			. * . . : *	

Figure 3-4 - CLUSTALO sequence alignment of human dopamine D₁ (P21728) and human β_2 adrenergic receptor (P07550) (* - indicates positions which have a single, fully conserved residue; : - indicates conservation between groups of strongly similar properties; . - indicates conservation between groups of weakly similar properties)

Figure 3-5 shows a theoretical model of the dopamine D₁ receptor generated using Swiss-model, based upon the β_2 adrenergic receptor (PDB: 5D5A) crystal structure.²³³ The model shows a visual representation of the seven-transmembrane structure, including both intra- and extra-cellular loops. This is one of many structures attributed to the dopamine D₁ receptor, and although the model is based on the β_2 adrenergic receptor (PDB: 5D5A) crystal structure, others have used different templates to develop homology models which are discussed below.

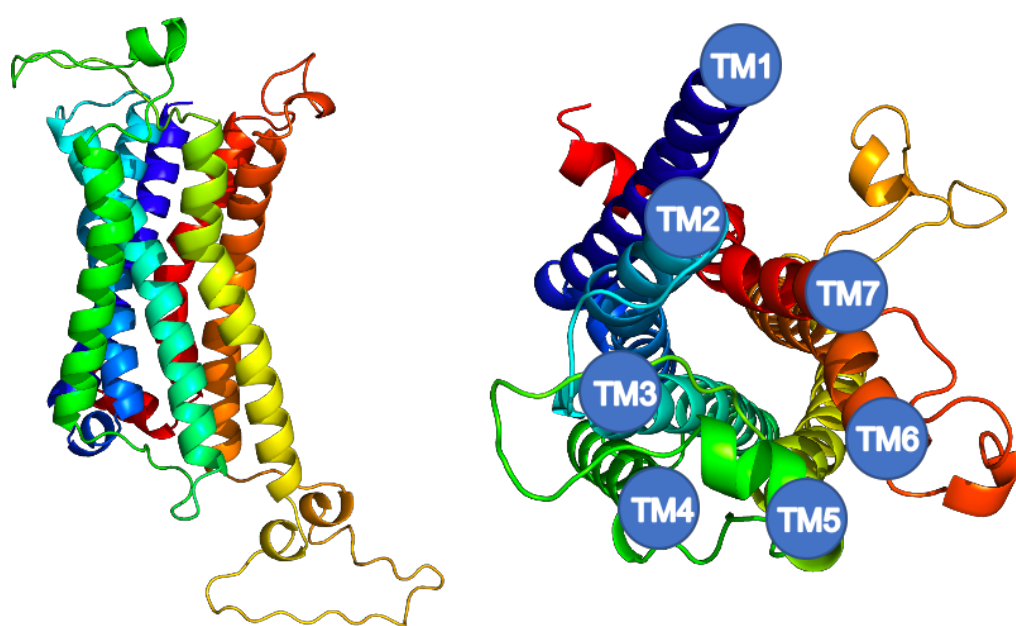


Figure 3-5 - Side and aerial view (showing transmembrane regions (TM)) of the D₁ dopamine receptor. Model created using Swiss-model with target sequence obtained from UniprotKB for the human_DRD₁ (P21728) and based upon the β_2 adrenergic receptor (PDB: 5D5A) crystal structure²³³

The first visual comparisons of GPCRs using computer modelling was conducted by Hibert, *et al*, who produced graphical representations of multiple transmembrane receptors. Primary sequence comparisons, secondary structure predictions and three-dimensional homology building, were built using bacteriorhodopsin as a template.²²⁵ This was to identify residues likely to be responsible for receptor affinity, selectivity, stereospecific and efficacy.

Modelling of the D₂ dopamine and β_2 adrenergic receptors using bacteriorhodopsin as a template indicated the importance of Ser⁵⁰⁴ and Ser⁵⁰⁷, for hydrogen bonding with the catechol found on the endogenous ligands. Asp³⁰⁶ was also identified as a key residue for the interaction with the amino moiety found on dopamine and adrenaline (formation of a salt-bridge, a combination of non-covalent bonds, ionic and hydrogen bonding).^{226,227} The models also indicated π - π interactions, between the aromatic rings present on the ligands and Phe⁵⁰⁸ and Phe⁶¹⁷ found on the 5th and 6th TM region respectively (as shown in Figure 3-6).

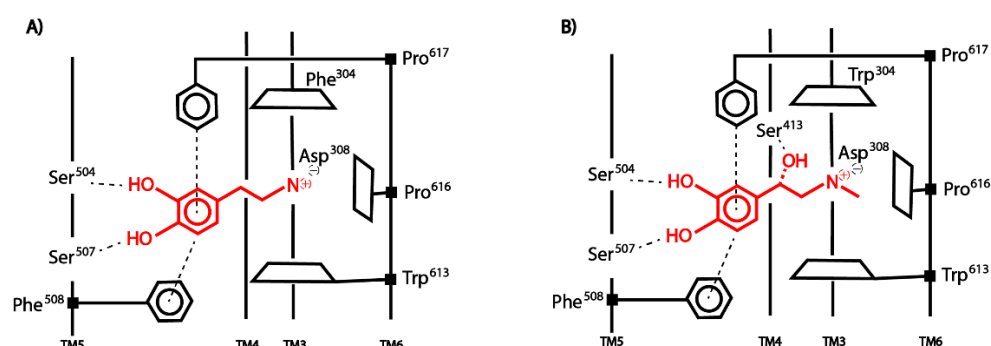


Figure 3-6 - Schematic representation of the interaction between D₂ dopamine (A) and β_2 adrenergic (B) receptors and their corresponding neurotransmitters – using bacteriorhodopsin as a template for homology models (reproduced from Hibert, *et al*)²²⁵

Today, the use of bacteriorhodopsin as a template has slowly diminished with the generation of homology models based on defined crystal structures. Filizola, *et al*, investigated the extent to which inactive crystal structures of GPCRs are suitable templates to build accurate homology models of the TM region of human class A GPCRs. This was for use in simple structure-based virtual screening aimed at computer-aided drug discovery. It was shown that rhodopsin based templates could only account for 1 to 2% of the modelling of GPCRs, whilst defined structures, which contain >30% sequence identities such as the mutant version of β_1 adrenergic receptor from *Meleagris gallopavo* (Mg_Adrb1_m23) could model 18% of all class A GPCRs transmembrane regions.²²⁸

Pollock, *et al*, were the first group to identify the importance of the serine residues found in the TM5 region (Ser¹⁹⁸, Ser¹⁹⁹ and Ser²⁰²) for catechol binding at the dopamine D₁ receptor.²²⁹ Working on the premise that two of the three serine residues when modified in the β_2 adrenergic receptor affected agonist binding and receptor activation²³⁰, modifications of the corresponding serine residues were made on the D₁ receptor. Alanine mutants were created using site directed mutagenesis, S198A, S199A and S202A. It was shown that when dopamine was used as an agonist, all three mutants played a role in decreased activity, whilst for other agonists such as benzazepine derivatives, only the S198A and S199A mutants led to reduced activity. Tomic, *et al*, utilising site directed mutagenesis, further confirmed that agonist activity is impaired when substitutions are made to Ser¹⁹⁸ and Ser²⁰² in TM5. It was also shown that agonist binding was affected when modifications were made on the TM3 region (Cys¹⁰⁶ and Ser¹⁰⁷), which may indicate they play a role in the formation of the ligand binding site.²³¹

Mente, *et al*,⁵⁸ further identified key interaction and binding residues by making mutations at 12 different orthosteric positions understood to be influential for ligand-receptor binding of dopamine and several agonists to the D₁ receptor. By using past literature and homology models generated in house (based upon the β_2 adrenergic receptor 3POG crystal structure²³⁴), the function of prototypical orthosteric D₁ agonists and partial agonists were tested. In order to gain a more detailed idea of the key interactions of multiple agonists and residues present in the D₁ binding site, a number of residues were modified. In addition to the conserved Asp¹⁰³, Ser¹⁹⁸, Ser¹⁹⁹ and Ser²⁰² amino acids, residues from the TM6 (Phe²⁸⁸, Phe²⁸⁹, Asn²⁹²), TM7 (Trp³¹⁸, Trp³²¹) and extracellular loops (ECL, Ser¹⁸⁸, Leu¹⁹⁰) were subject to mutagenesis. Focusing on the catecholamine-based ligands studied, interactions with the conserved amino acid residues remained crucial to binding. Whilst additional key interactions were identified at: Phe²⁸⁸, Phe²⁸⁹ (TM6) and Leu¹⁹⁰ (ECL) contributing to π - π interactions, as well Asn²⁹² (TM6) *via* catechol hydrogen bonding.

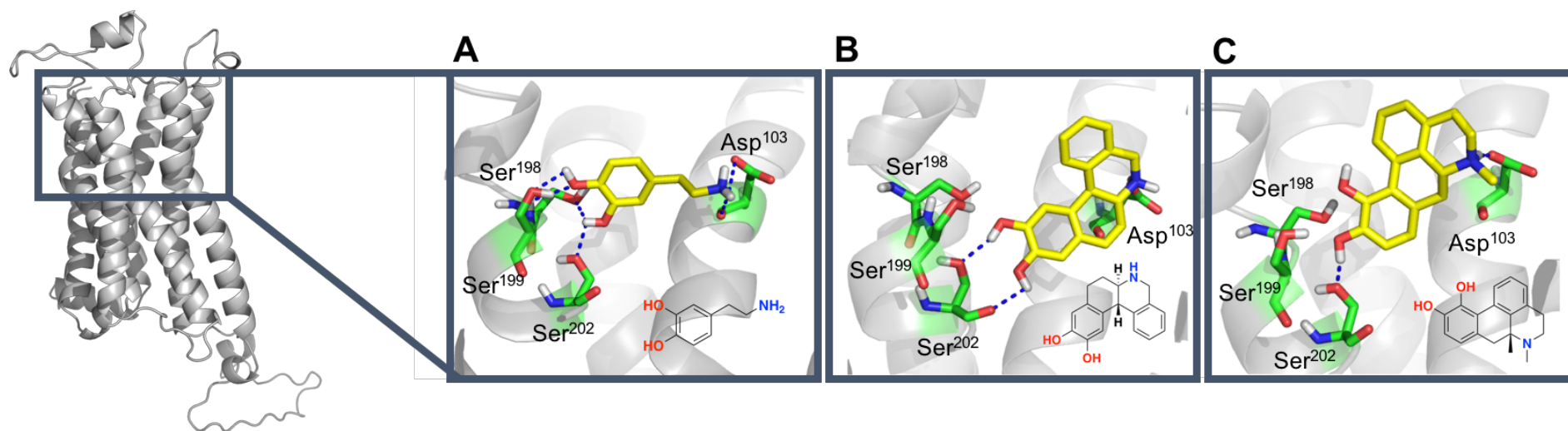


Figure 3-7 - D₁ homology model (built upon the 3P0G β_2 adrenergic receptor crystal structure)²³⁴ with dopamine (A), apomorphine (B) and dihydrexine (C) binding visualised with the conserved Ser¹⁹⁸, Ser¹⁹⁹, Ser²⁰² and Asp¹⁰³ residues found on the TM5 and TM3 regions respectively. As shown, the increased presence of aromatics causes a change in the flexible binding pocket, but does not allow complete docking. Interactions are observed with the catechol and amine for all agonists (reproduced from Mente, et al)⁵⁸

The interactions of dopamine, apomorphine^{235–237} and dihydrexine^{238–241} with the conserved amino acid residues (Asp¹⁰³, Ser¹⁹⁸, Ser¹⁹⁹ and Ser²⁰²) in the binding pocket using the homology model are shown in Figure 3-7 as predicted by Mente, *et al.* The binding of the two agonists differ from the endogenous ligand, due to their larger size and functional groups present, showing that the receptor binding site is flexible to different agonists, to allow successful activation.

A summary of the interactions can be seen in Figure 3-8. This shows key interaction sites of dopamine, and residues present on the D₁ receptor. It shows, key polar interactions on the TM5 region (Ser¹⁹⁸, Ser²⁰²), hydrophobic interactions (π - π) with TM6 (Phe²⁸⁸), and the formation of a salt-bridge in the TM3 region (Asp¹⁰³). We must now explore current agonists, identify the key functional groups of the agonist dopamine, as well as determine an ideal modification point to retain potency at the D₁ receptor.

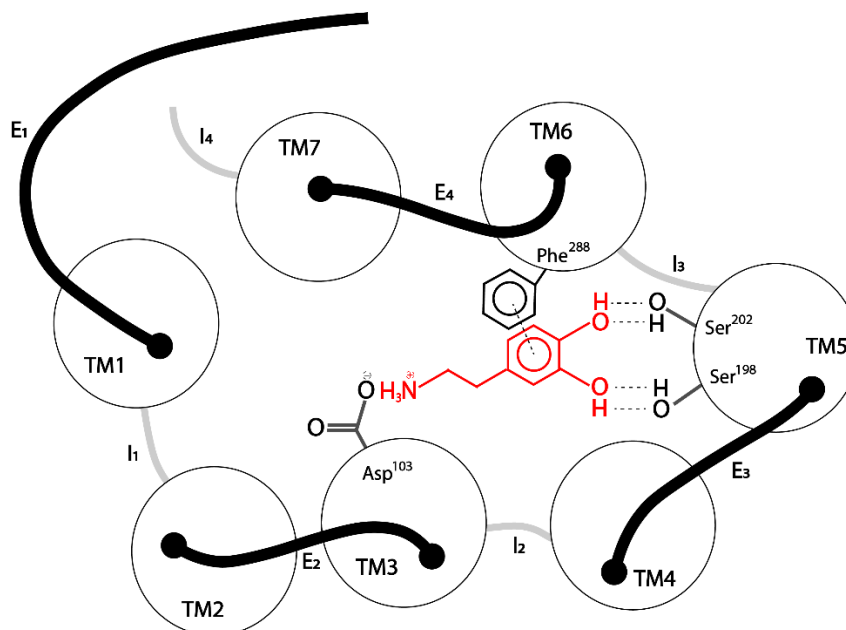


Figure 3-8 - Summary of the key residues and interactions in the binding of dopamine to the human D₁ receptor (E₁: extracellular N-terminal domain; E₂₋₄: extracellular loops; I₁₋₃: intracellular loops; I₄: intracellular C-terminal domain; TM1-7: transmembrane regions, adapted from Strosberg, *et al*, for the β_2 adrenergic receptor)²⁴²

Dopamine D₁ ligands

Structurally, several agonists exhibit functional groups similar to dopamine. Dihydropyridine²⁴³ and apomorphine^{235–237} retain the catechol group, with an amino group situated two carbons away from the aromatic core structure, like dopamine as shown in Figure 3-9 (pink dashed line). Some ligands have a different scaffold such as the ergoline based agonists which can still readily activate the dopamine D₁ receptor, yet do not contain groups such as the catechol.²⁴⁴ This suggests that to modify dopamine, we must retain the catechol and either modify from the amino or aromatic group to retain potency at the D₁ receptor.

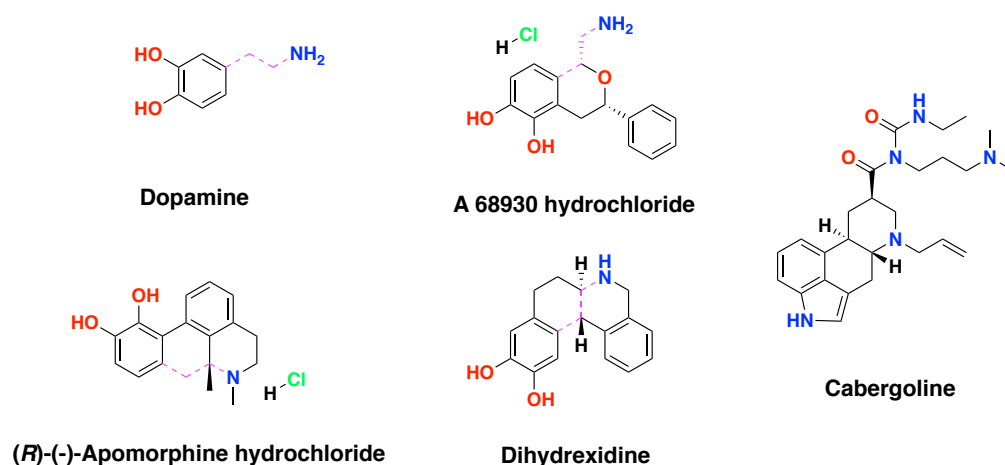


Figure 3-9 - Selective D₁ agonists which show similarities in distance from the catechol and amino moieties (as shown in pink) and also the ergoline based agonist showing the absence of a catechol

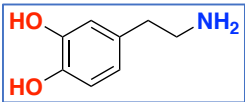
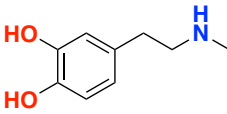
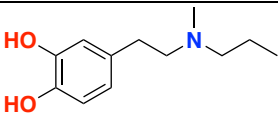
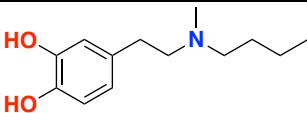
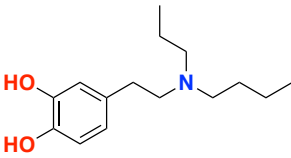
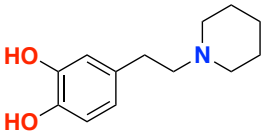
Agonists for the D₁ receptor have been extensively researched with Cannon, *et al*, focusing on the modification of the endogenous dopamine ligand²⁴⁵, and Andersen, *et al*, on synthetic agonists and their selectivity for and potency at the D₁ receptor.²⁴⁶

Dopamine modification

Amine

Cannon, *et al*, in 1983 comprehensively reviewed modifications of dopamine at the amino moiety.²⁴⁵ Emphasis was made on the modification of dopamine at the *N*-terminal, synthesising *N*-*N*-dialkylated dopamine conjugates, conducted by Ginos, *et al*, as shown in Table 3-1.²⁴⁷

Table 3-1 - Net cAMP generated for the stimulation of mouse caudate nuclei as identified by Ginos, *et al*, for the *N*-*N*-dialkylated dopamine analogues (*n*=6)²⁴⁷

Compound	Net cyclic AMP generated <i>pmol</i> (mean $\pm$ SEM)
	21.0 $\pm$ 2.8
	24.3 $\pm$ 2.4
	5.1 $\pm$ 1.4
	14.4 $\pm$ 1.7
	18.8 $\pm$ 2.6
	0

The analogues shown were used to measure the cAMP generated in mouse caudate nuclei after a single dose of 10 μ M per compound (n=6).²⁴⁷ The net cAMP was determined by subtracting the mean amount of cAMP produced by the non-stimulated controls from each mean amount obtained from measurements of stimulated production of cAMP. It was shown that cAMP production was retained for the *N*-alkyl analogues. This included mono-alkylation using a methyl group as well as *N,N*-dialkylated molecules with larger aliphatic chains (propyl and butyl). Critically, as a single high concentration of 10 μ M for each compound was used in this study, limited information could be extracted from these measurements.

Dopexamine (Figure 3-10) is another prime example of a synthetic *N*-alkyl analogue of dopamine. It has been administered to stimulate β_2 adrenergic receptors to treat heart failure after heart surgery,²⁴⁸ although there has been no reported literature for the use on dopaminergic receptors.

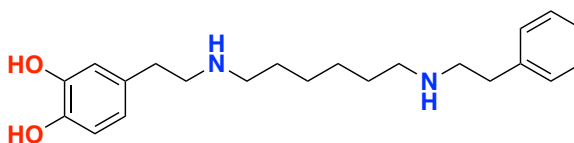


Figure 3-10 - Dopexamine - a stimulatory drug used at the β_2 adrenergic receptor to treat heart failure after surgery

Aromatic modification

The conjugation of neurotransmitters from the aromatic core, can give an option for surface immobilization without tampering with the bioactive functional groups i.e. the catechol and amino moieties found on dopamine. Dopamine, when subject to specific conditions such as exposure to tyrosinase²⁴⁹, electrolysis²⁵⁰ and an increased pH²⁵¹, undergoes conversion to its quinone form, causing intramolecular cyclisation (Figure 3-11).

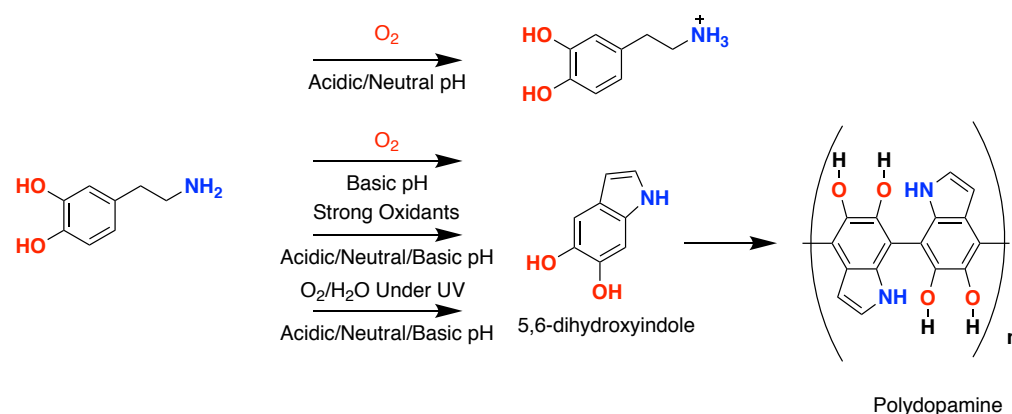


Figure 3-11 - Conditions which dictate the oxidation of dopamine (adapted from Du, et al)²⁵²

This cyclisation occurs through a Michael addition mechanism which is shown in Figure 3-12, and has been used to react dopamine with amine/sulfur terminated compounds.^{249,252}

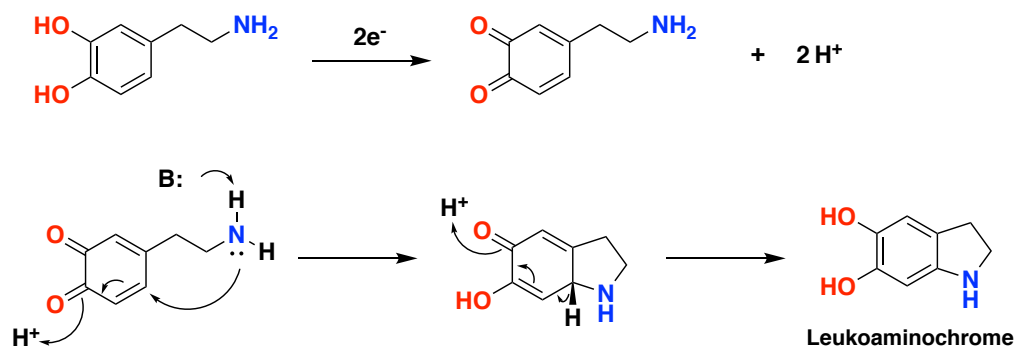


Figure 3-12 - Internal cyclisation of dopamine through the 1,4-Michael addition mechanism to form a leucoaminochrome

Mitchell, *et al*, have generated several dopamine analogues using sulfur terminated molecules, utilising the quinoidal form through electrolysis. The voltage dependant synthesis, formed a thio-ether bridge between the sulfur and aromatic core of dopamine although at low yields (<30%). This method was used for the coating of antigens on biosensors for the raising of specific antibodies.²⁵⁰

Another method which demonstrated the modification of dopamine from its aromatic core, was conducted by Funder, *et al*.²⁵³ This was for the use as a tool for aptamer selection, using systematic evolution of ligands by exponential enrichment (SELEX). Using a multistep synthetic approach, they developed a PEG-azide dopamine analogue capable of attaching to a nanoparticle (Figure 3-13).

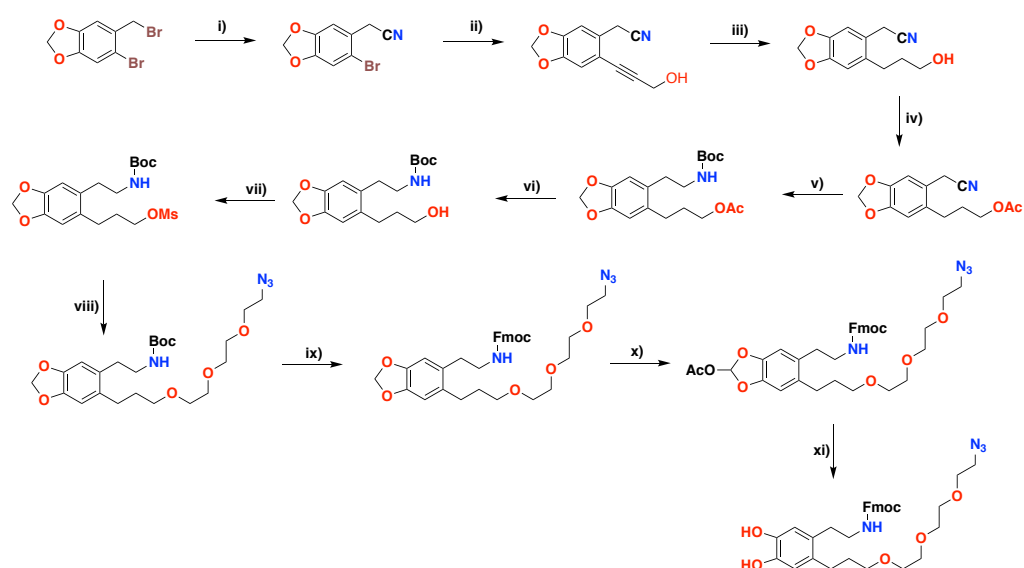


Figure 3-13 – Synthetic route for PEG-azide dopamine analogue as synthesised by Funder, *et al*²⁵³ (i) NaCN, DMF, rt, 92%, (ii) 10 mol% $\text{Cl}_2\text{Pd}(\text{PPh}_3)_2$, Cul, Et_3N , CHCCH_2OH , THF, 80 °C, 27% (iii) 20 mol% Pd/C, H_2 , MeOH/EtOAc, rt (iv) Ac_2O , pyridine, rt (v) NaBH_4 , Boc_2O , $\text{NiCl}_2(\text{H}_2\text{O})_6$, MeOH, rt (vi) Na (s), MeOH, rt, 48% (4 steps) (vii) MsCl , Et_3N , Et_2O , 0 °C (viii) $\text{HO}(\text{C}_2\text{H}_4)\text{O}(\text{C}_2\text{H}_4)\text{O}(\text{C}_2\text{H}_4)\text{N}_3$, NaH, 0 °C to rt, DMF, 55% (ix) 1) 4 M HCl in dioxane, 2) Fmoc-Osu, DIPEA, DCM, rt, 60% (2 steps) (x) $\text{Pb}(\text{OAc})_4$, PhH, 80 °C (xi) HCl, MeOH, 30% (2 steps)

These examples exist as a means for other applications rather than for the activation of dopamine receptors. As this was the case, and there had been some evidence of dopamine receptor activation when the primary amine was modified, in this work we focused on analogues modified from this functional group.

Measuring GPCR activity

GPCR signalling consists of a series of spatial and temporal events, and an assay must take into consideration immediate or cascaded actions. Measuring immediate events after receptor activation reduce incidences of false positives, whilst measuring further down the signal cascade will enhance signal-to-noise ratio due to signal amplification.⁴⁵ Table 3-2 shows a comprehensive list adapted from Zhang, *et al*, of the current technology used to screen for GPCRs activity.⁴⁵ The assays identified include radio-ligand/tagged binding^{67–69}, G-protein dependent (GTPγS⁶⁶, CRE/MRE^{70–72}, cAMP^{243,246,254}, calcium^{255–257} and IP₃^{258–260}), G-protein independent (receptor trafficking^{261–263}, β-Arrestin recruitment^{264–266}, label-free assay^{267–269}) and receptor dimerization assays.^{270–273} It also includes the GPCR subtypes suitable for each assay, as well as the pros and cons for each method.

Assays which have been reported in literature to determine agonistic efficacy at the dopamine D₁ or G_{αs} subtype in general have typically been shown to be associated with G-protein dependent assays, specifically measuring the upregulation of cAMP. This is evident from a number of literature sources including the work conducted by Clark, *et al*, who assessed the affinity and efficacy of three tetracyclic conformationally restricted analogues of SKF38393²⁵⁴; Anderson, *et al*, for benzazepine and isoquinoline derivatives²⁴⁶, and Lovenberg, *et al*, to determine the efficacy of dihydrexidine.²⁴³ Alternatively, efficacy of agonists at different G_{αs} subtype receptors have been assessed using gene reporter assays as evident from the work described by Hill, *et al*, for the measurement of the β₂ adrenoceptor.⁷²

Table 3-2 – Summary of GPCR screening technologies (reproduced from Zhang, et al)⁴⁵

Assay classification	Commonly used assay	Commercial (HTS) technology (Company)	Pros	Cons	Suitable GPCRs
Receptor binding assay	Radio-ligand binding assay;	Filtration assay (PerkinElmer), SPA (GE Healthcare or PerkinElmer),	High-throughput; less interference; obtain agonist and antagonist in one assay	Availability of radio-ligand; generation of radioactive waste; need secondary functional assay	any GPCR
	Other tagged-ligand binding	DELFIATM TRF (PerkinElmer), LanthaScreenTM system (Invitrogen), Tag-liteTM system (Cisbio), Filtration assay (PerkinElmer), SPA (GE Healthcare or PerkinElmer), DELFIATM GTP Binding Assay (PerkinElmer)	Non-radioactive; high-throughput; obtain agonist and antagonist in one assay	Availability of tagged-ligand; need secondary functional assay	any GPCR
G-protein dependent assays	GTPγS binding	Filtration assay (PerkinElmer), SPA (GE Healthcare or PerkinElmer), DELFIATM GTP Binding Assay (PerkinElmer)	Functional cell-free assay; discrimination between full or partial agonists, neutral antagonists, inverse agonists, allosteric regulators	Relatively low signal to background window	G _{ai/o}
	CRE/MRE reporter assay		Homogenous assay; high-throughput; large signal to background window	Need to know the coupling mechanism, not good for orphan GPCR	any GPCR
	cAMP assay	SPA cAMP assay (GE Healthcare or PerkinElmer), HitHunterTM (DiscoverX), AlphaScreenTM (PerkinElmer), Fluorescence polarization-based cAMP kits (PerkinElmer, Molecular Devices and GE Healthcare), HTRF™-based cAMP (Cisbio), cAMP Glosensor™ (Promega)	High-throughput; very sensitive	Need to know the coupling mechanism, not good for orphan GPCR	G _{as} , G _{ai/o}

	Ca ²⁺	Fluo-3 or Fluo-4 (Invitrogen) and FLIPR™ automated real-time fluorescence plate readers (Molecular Device)	High-throughput; functional assay for live cells; obtain agonist, antagonist and allosteric modulator in one assay	Fluorescent interference from compounds; not good for inverse agonist and slow binding agonist	G _{αq} (with G _{α15/16} , could be universal)
	IP _{1/3}	SPA IP ₃ assay (PerkinElmer), AlphaScreen™ (PerkinElmer), HitHunter™ Fluorescence Polarization (DiscoverX), HTRF IP-One™ (Cisbio)	High-throughput; functional assay for live cells; good for slow binding ligands	Limited industrial validation	G _{αq} (with G _{α15/16} , could be universal) any GPCR
G-protein independent assays	Receptor trafficking	Cellomics ArrayScan™ (Thermo Scientific), INCell™ Analyzer 3000 (GE Healthcare), Opera™ (Evotec Technologies), Acumen™ Explorer (TTP Lab Tech)	Image based, high-content; functional assay for live cells; generic method for all GPCRs	Image based, relatively low throughput	any GPCR
	β-Arrestin recruitment assay	Transfluor™ (Molecular Device), BRET/FRET - Tango™ (Invitrogen), PathHunter™ (DiscoverX)	High-throughput; image or non-image based; functional assay for live cells; useful in biased signal detection; generic method for all GPCRs	Affinity for β-arrestin binding varies among GPCRs; less sensitive; need further pathway analysis	any GPCR
	Label-free assay	BIND™ RWG biosensors (SRU Biosystems), Epic™ RWG biosensors systems (Corning Inc), ECISTM (Applied Biophysics), xCELLigence™ System (Roche and ACEA Biosciences), CellKey™ (MDS)	Label-free functional assay in native live cells; summation of all cellular events	Possibly higher false positive and false negative rates; need special instrument and costly microplate; need further pathway analysis	any GPCR
Receptor dimerization assay		BRET/FRET - PathHunter™ (DiscoverX), Tag-lite™ (Cisbio)	GPCR heterodimers are considered new pharmacological targets	Very artificial system, cannot assess GPCR dimerization in native state	any GPCR

HTS – high-throughput screening; SPA - scintillation proximity assay; TRF - time-resolved fluorescence; GTP - guanine triphosphate; cAMP - 3',5'-cyclic adenosine monophosphate; CRE – cAMP response element; MRE – metal response element; BRET - bioluminescence resonance energy transfer; FRET - fluorescence resonance energy transfer

Chemistry – synthetic approach

A variety of synthetic approaches to developing dopamine analogues modified at the amino group were considered and researched. A comprehensive review on the formation of secondary amines conducted by Salvatore, *et al*, aided in the understanding of the synthetic methods available. The formation of a secondary amine can be accomplished in many ways, with a few examples displayed in Figure 3-14.²⁷⁴

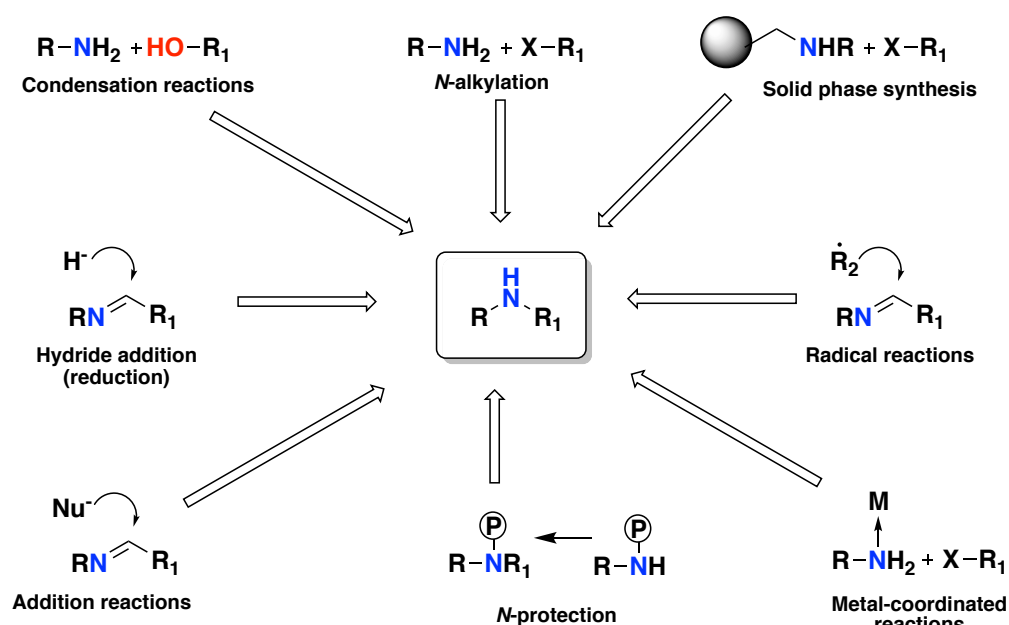


Figure 3-14 - Synthetic routes for the formation of secondary amines as reviewed by Salvatore, *et al*²⁷⁴

The methods used for the modification of dopamine in literature have common features such as the protection of the catechol (to prevent oxidation) and the formation of amides and imines which are then reduced. This has been shown by Connor, *et al*, for the use as a cannabinoid ligand (formation of *N*-arachidonoyl dopamine)²⁷⁵, as well as Karlson, *et al*, for the use as a sclerotizing agent for insect cuticles as researched back in 1962.²⁷⁶

Ginos, *et al*, used an alternate method which employed the use of 3,4-dimethoxyphenylacetic acid, which was converted into the corresponding acid chloride with the use of thionyl chloride. From this, a primary amino group readily reacted with the acyl chloride, forming an amide. Reduction of this species then allowed the formation of a secondary amine. Finally, the use of hydrogen iodide and acetic acid were used to remove the methoxy protecting groups on the catechol as shown in Figure 3-15.

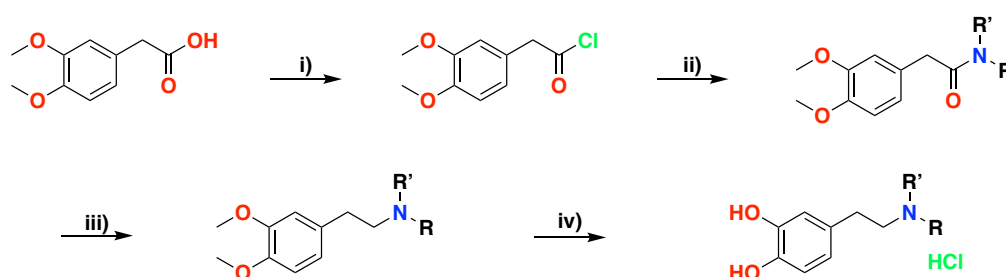


Figure 3-15 - Reaction scheme for the synthesis of N-alkylated dopamine analogues as conducted by Ginos, *et al*²⁴⁷, i) SOCl_2 , ii) $\text{RR}'\text{NH}$, iii) Diborane, THF, iv) a, 57% HI, Ac_2O , b, HCl

Reductive amination

It has been shown that the reactions between β -arylethylamines and carbonyl compounds can lead to the formation of 1, 2, 3, 4-tetrahydroisoquinolines (as shown in Figure 3-16) known as the Pictet-Spengler condensation.²⁷⁷

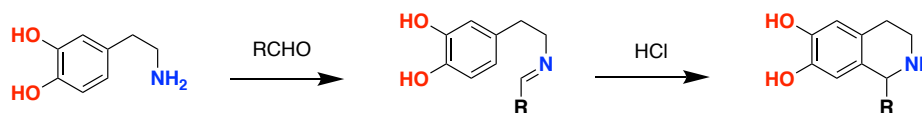


Figure 3-16 - Pictet Spengler condensation²⁷⁷ (salsolinol, $\text{R}=\text{CH}_3$)

Dopamine was shown to form a tetrahydroisoquinolines with an aldehyde by Lichman, *et al*, for the synthesis of salsolinol ($\text{R}=\text{CH}_3$)²⁷⁸, which has been shown to be potential neurotoxin for the dopaminergic system in the human and rat brain.^{279,280}

***N*-Alkylation**

Although no reported reactions utilising direct *N*-alkylation have been identified using dopamine as a starting medium, primary amines have readily been used to form secondary amines. Typically, the use of alkyl/aryl halides or organo-sulfonates are commonly employed to generate secondary amines in the process known as the 'Hofmann alkylation' or *N*-alkylation (Figure 3-17). Due to the nucleophilicity of the amine, they can be effectively used in S_N2 reactions commonly in the presence of a base.

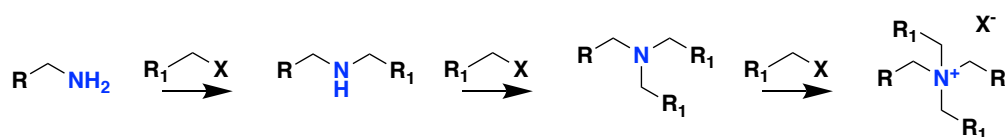


Figure 3-17 - Hofmann alkylation from primary to quaternary amine

Focusing on secondary amine formation, the synthetic value of this technique has been limited, giving rise to mixtures of secondary, tertiary amines and quaternary ammonium salts. Although methods have been used to promote the production of the secondary amine, such as the use of excess amine in relation to alkyl halide or sulfonates, reaction yields have been known to vary depending on the amine. The removal of excess amine can be time consuming, can often be difficult and is wasteful of what can be expensive starting materials.

Condensation reactions

Condensation reactions are another alternative to synthesizing secondary amines, although these typically involve using expensive transition metal catalysts. Hydrogen transfer can also be catalysed by a base, but require elevated temperatures or extensively long reaction times.

Aims and Objectives

The aim of this Chapter focuses to determine modifications at key functional groups on dopamine, to retain activity at the D₁ receptor with surface attachment in mind. In doing so, dopamine is modified from the catechol and amine moieties with protecting groups and from the amino group with aliphatic/aliphatic ether chains using *N*-alkylation. Figure 3-18, shows the modification sites of dopamine conducted within this Chapter.

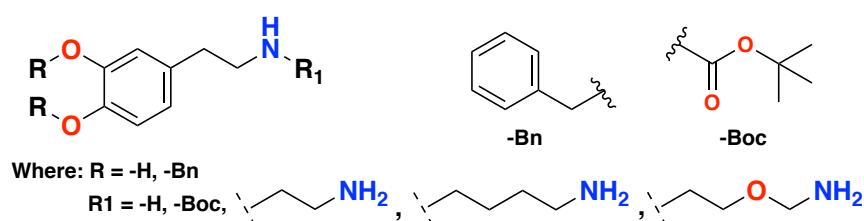


Figure 3-18 - Overview of dopamine analogues synthesised within this Chapter

Due to the limited literature on the modification of dopamine, to determine key functional groups essential for potency at the D₁ receptor, a protection strategy was used to test whether the catechol or amine played a larger role in activation of the D₁ receptor. To do this, *O*-benzyl (Bn), and *tert*-Butyl carbamates (Boc) were utilised on the catechol and amino group respectively to investigate if modification at the catechol, would suppress activity/potency at the D₁ receptor.

Using the catechol protected analogues, modification of the amino moiety was then conducted, using a variety of tethers of varying length and chemistry using microwave assisted *N*-alkylation. Linkers were prepared using *N*-Boc protection, and activated to organo-sulfonates. Deprotection of the *O*-Bn and *N*-Boc protected analogues were then conducted to achieve our complete library. Finally, a secreted placental phosphatase assay (SPAP) was used to determine potency at the D₁ receptor.

Results and Discussion

Dopamine protection

Oxidation of dopamine is readily observed under basic conditions (as identified by Du, *et al*), which is why a protection strategy was employed at the catechol to avoid internal cyclisation and polymerization.²⁵² This negated the formation of the quinoidal structure (Figure 3-19), preventing the 1,4-Michael addition to occur (internal cyclisation).

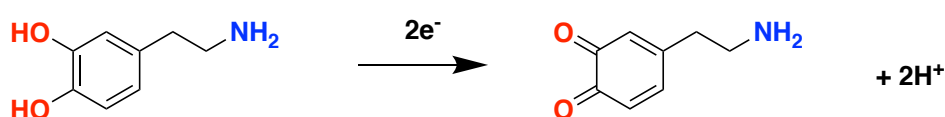


Figure 3-19 - Formation of dopamine-o-quinone

O-Benzyl (Bn) protection was identified as the ideal group to protect the catechol due to its resistance to basic conditions, for which the proposed *N*-alkylation reaction would be conducted. Figure 3-20 shows the reaction scheme to which dopamine was protected.

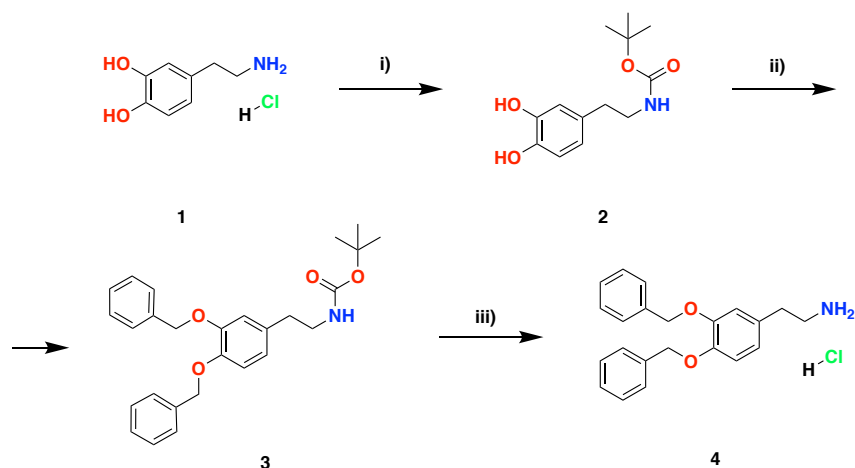


Figure 3-20 - Reaction scheme for the formation of tert-Butyl (3,4-dihydroxyphenethyl) carbamate (2), tert-Butyl (3,4-bis(Benzyloxy)phenethyl) carbamate (3), and 2-(3,4-bis(Benzyloxy)phenyl) ethan-1-amine hydrochloride (4), (i) Boc_2O , Et_3N , N_2 , RT, 12Hr (60%); (ii) $BnBr$, K_2CO_3 , N_2 , RT, 12Hr (84%); (iii) HCl (2 M), Et_2O (94%)

To achieve Bn protection, the amino group on (2-(3,4-dihydroxyphenyl)ethylamine) was initially protected using Boc. The protection was conducted using di-*tert*-Butyl dicarbonate (methanol, inert atmosphere, overnight, RT) to create *tert*-Butyl (3,4-dihydroxyphenethyl) carbamate (**2**). A maximum yield of 60% was obtained for this reaction, which was far less than reported in literature, which was quantitative (99%) as reported by Geiseler, *et al*, in 2012.²⁸¹ The use of degraded di-*tert*-Butyl dicarbonate may contribute to this low yield, in addition to washing with 1 M HCl to remove any unreacted amine. Although the concentration of the HCl (1 M) is low, and has been shown to remove unreacted amines in the aqueous phase using liquid-liquid extraction, as the *tert*-Butyl is labile to acidic conditions we may deprotect some of the generated product and convert it back to its original reactant (dopamine) and also *tert*-Butyl alcohol. Compound **3**, *tert*-Butyl (3,4-*bis*(Benzyloxy)phenethyl) carbamate was then synthesised successfully using benzyl bromide. In comparison to Geiseler, *et al*, we saw a rise in yield from their reported literature value from 63% to 84%.²⁸¹ Finally, deprotection of the *N*-Boc protection was conducted under acidic conditions (diethyl ether: HCl (2 M)), to generate compound **4**, 2-(3,4-*bis*(Benzyloxy)phenyl) ethan-1-amine hydrochloride with an excellent yield of 94%. This was greater than the yields obtained by Fernandez, *et al*, which reported yields of 81%, and similar to Geiseler, *et al*, reporting quantitative yields.^{281,282} In both of these examples a combination of TFA/CH₂Cl₂ was used resulting in the TFA salt in comparison to our hydrochloride salt.

Synthesis of the dopamine analogues were confirmed using ¹H and ¹³C NMR and LCMS. Figure 3-22 shows the ¹H NMR spectra for each of the compounds synthesised as well as the dopamine hydrochloride purchased from Sigma Aldrich (full assignments can be found in the experimental section within this Chapter). From literature, we can confirm the NMR spectra correlate to the synthesised dopamine analogues, **2**, **3** and also **4**.^{281–283} A small shift in reported values is observed, which can be explained due to variable experimental conditions, i.e. solvent, temperature and concentration.^{209,284}

Synthesis of compound **2** was confirmed by the peak generated at 1.42 ppm, which was indicative of the 9 protons from the *tert*-Butyl protection. The absence of acidic protons confirmed that this was not due to unreacted di-*tert*-Butyl dicarbonate. Excess *tert*-Butyl alcohol which is a by-product from the reaction (typically found at 1.03 to 1.40 ppm dependent on the deuterated solvent used) is also absent.²⁸⁵ The $[M-H]^-$ at m/z 252 is observed for compound **2** within the LCMS spectra. In addition to this, mass fragments in the positive spectra were also identified, in relation to the phenomenon known as the McLafferty rearrangement.²⁸⁶ Figure 3-21 shows the rearrangement and subsequent ionisation of the formed species. In our analysis, we can see a peak in reference to the first step at an m/z of 198, $[M-tert\text{-Butyl}+2H]^+$, further confirming the successful Boc protection.

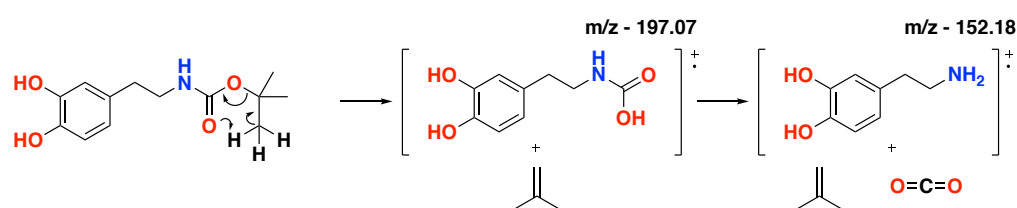


Figure 3-21 - McLafferty rearrangement and ionisation of the Boc protection group using LCMS analysis

The generation of peaks (found on compounds **3** and **4**) at 5.09 ppm and 7.39 ppm are representative of CH_2 groups, and aromatic protons respectively, confirming the presence of the benzyl ether. Whilst compound **3** exhibits the Boc protection group, after deprotection under acidic conditions, we not only see the peak disappear from 1.42 ppm, we see the addition of an acidic proton in the spectra at 8.06 ppm indicating a hydrochloride salt forming as seen before in the dopamine hydrochloride (**1**) spectra. Once again we can confirm the product mass peaks using LCMS, 456.15 $[M+Na]^+$ for compound **3**, and 334.10 $[M+H]^+$ for compound **4**.

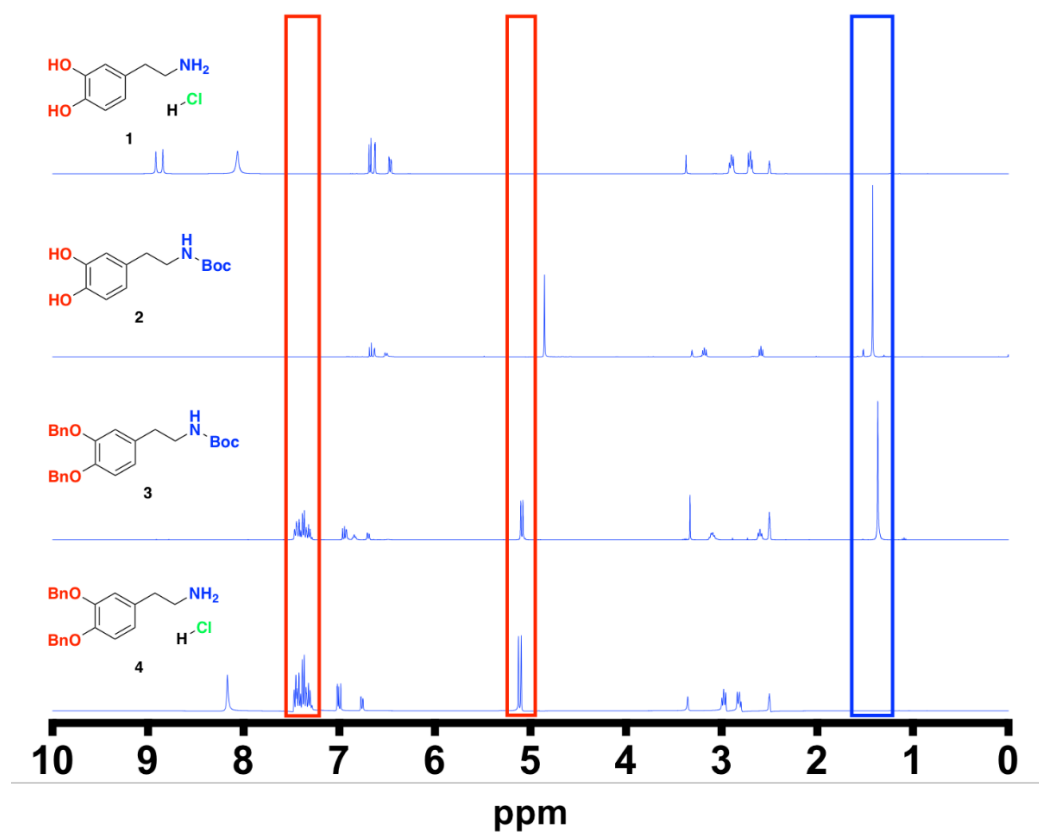


Figure 3-22 - ^1H NMR of dopamine analogues (**1-4**). Shifts at 1.42 ppm (blue) are representative of the CH_3 ($\times 3$) found on the tert-Butyl protection, whilst the shifts at 5.09 and 7.39 ppm (red) represent successful O-benzyl protection (CH_2 and aromatic protons respectively)

Linker selection

To successfully synthesise the extended dopamine analogues, specific functional groups were required on the chosen linkers with varied length and chemistry. *N*-Alkylation was the chosen method in this Chapter, due to literature indicating a Pictet-Spengler type reaction occurring when dopamine is used in imine formation, between an aldehyde and its primary amino group.²⁷⁷ As *N*-alkylation reactions utilize alkyl halides or sulfonates to alkylate a 1°, 2° or 3° amine, one of the two functionalities were required on our chosen linkers.

Linkers which exhibited a primary amino and alcohol group were chosen. Conversion of the alcohol to an organo-sulfonate would allow *N*-alkylation to dopamine, whilst the amine would be used as functional group to tether to a surface. This was chosen over other groups due to the ease of surface immobilisation, using di-isopropylcarbodiimide chemistry with carboxylic acid terminated surfaces and other compounds, as discussed in Chapter 4.

A variation in length and electronegativity was chosen. Two chain lengths were selected, a two (ethanolamine) and five carbon (5-amino-1-pentanol) aliphatic chain. An aliphatic ether (2-amino(ethoxy)ethanol)) was also chosen to see if the hydrophobicity of the linker played a role in the activation of and potency at the D₁ receptor. These linkers can be seen in Figure 3-23.

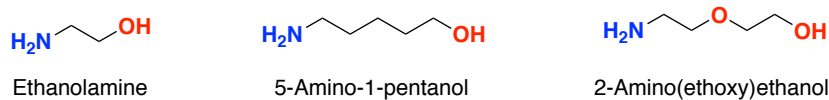


Figure 3-23 - Linkers selected for the N-alkylation reactions, exhibiting amino groups for surface attachment, and alcohol groups to generate organo-sulfonates

Protection and activation of linkers

In order to utilise the linkers, protection (*N*-Boc) and activation (*O*-tosylation) was required. Successful Boc protection was confirmed by ^1H and ^{13}C NMR, where an additional CH_3 peak with an integral of 9 at 1.37 ppm (using $\text{DMSO-}d_6$) representative of the *tert*-Butyl group (compounds **5a-c**, Figure 3-24) was observed. This was complemented by the formation of a peak at 28 ppm in the ^{13}C NMR spectra. In terms of yield, we achieved 97%, 88% and 91% for compounds **5a-c** respectively. The procedure used followed the same method for Boc protection of dopamine (Figure 3-24).²⁸³

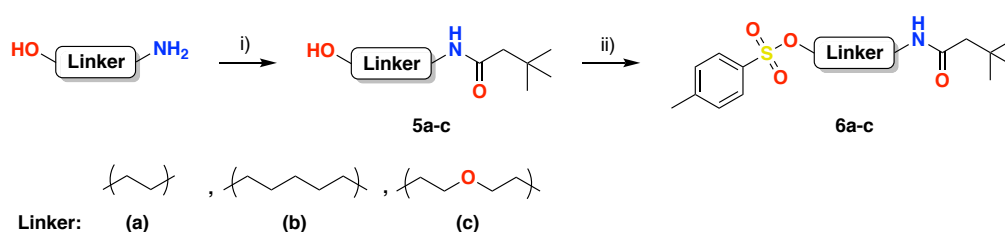


Figure 3-24 - Reaction scheme for *N*-Boc protection (**5a-c**) and synthesis of corresponding sulfonate esters (**6a-c**) (i) Boc_2O , Et_3N , N_2 , RT, 12Hr (a: 97%, b: 88%, c: 91%); (ii) CH_2Cl_2 , Et_3N , N_2 , 0 °C to RT, 24Hr (a: 68%, b: 44%, c: 71%)

Activation of the linkers were required due to the low reactivity of the alcohol. As hydroxide ions are not very good leaving groups, alcohols have limited uses in substitution reactions. However, activation can occur using Lewis or Brønsted acids which allows the hydroxide ion to leave as water. Conversion to a leaving group such as organo-sulfonates (mesylate, tosylate, or triflates) can allow nucleophilic substitution to occur readily due to the low pKa value of approximately -1 to -3 in comparison to the values of the alcohols at ~ 17 .²⁸⁷

Here, *p*-toluenesulfonyl chloride (TsCl) was used to convert the alcohol to the corresponding sulfonate ester.²⁸⁸ Successful synthesis of the organo-sulfonates was achieved through nucleophilic substitution, and were purified using column chromatography. The addition of the sulfonate group was confirmed through ¹H NMR and LCMS to generate compounds **6a-c**.

Figure 3-25 shows the changes in ¹H NMR spectra for the ethanolamine linker on the addition of the Boc protecting group (**5a** - *tert*-Butyl (2-hydroxyethyl)carbamate) as well as tosylation of the hydroxyl moiety (**6a** - 2-((*tert*-Butoxycarbonyl)amino)ethyl 4-methylbenzenesulfonate). As previously mentioned, the addition of the peak at 1.37 ppm with an integral of 9 is indicative of the Boc protection group, whilst peaks at 7.77/7.48 and 2.42 ppm represent aromatic peaks and methyl protons respectively for the addition of the sulfonate.²⁸⁸ Upon substitution of the alcohol to the tosylate an up-field shift is observed in both the CH₂ peaks, showing modification has occurred in a neighbouring environment. This change is representative of the electron density near the two protons, changing from an alcohol, to a group which contains several heteroatoms including two additional oxygen and a sulfur atom. The up-field shift (to the right) is caused by shielding of the protons from the external magnetic field.²⁸⁹

Fragments found at 333.05 (theoretical - 333.14) and 631.20 (theoretical - 631.22) mass charge units on the LCMS were found to be representative of the [M+NH₄]⁺ and [2M+H]⁺ ions for compound **6a**.

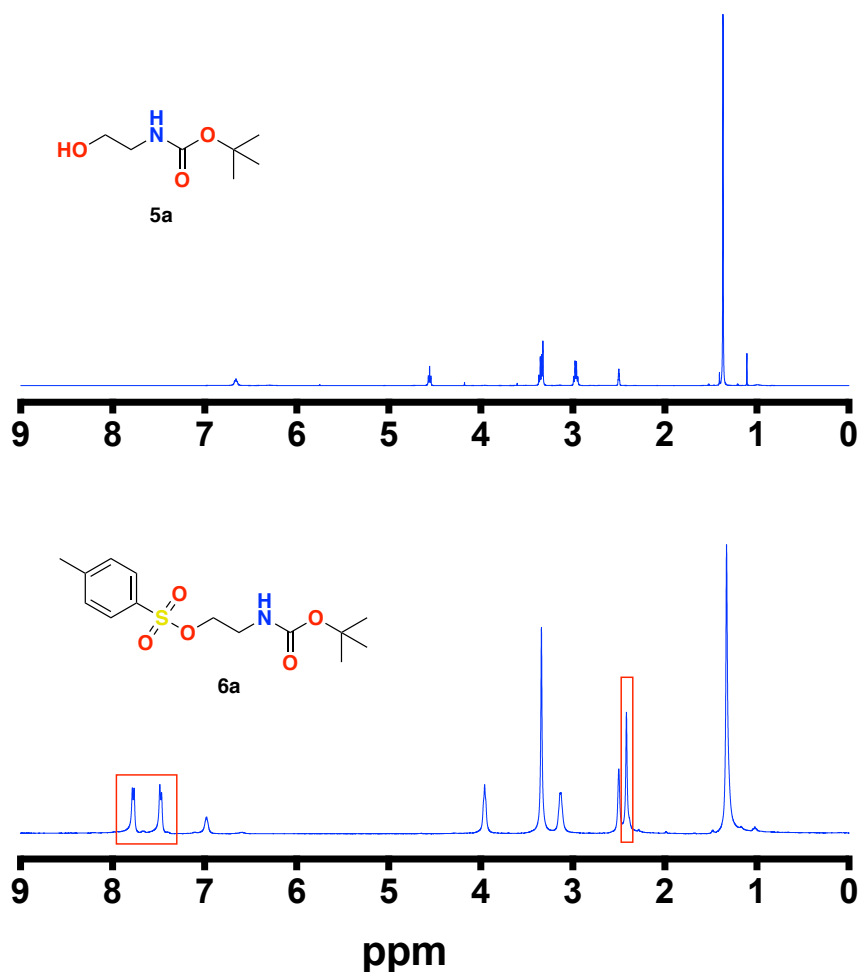


Figure 3-25 - ^1H NMR of tert-Butyl (2-hydroxyethyl)carbamate (**5a**), and 2-((tert-butoxycarbonyl)amino)ethyl 4-methylbenzenesulfonate (**6a**) showing the addition of peaks at 7.77/7.48 and 2.42 ppm before and after organo-sulfonate conversion

Successful protection and activation was also achieved for the other two linkers, confirmed by NMR and LCMS (data for the compounds can be found in the experimental section within this Chapter) which matched reported literature values. The yields obtained were much lower, at 68% (80%)²⁹⁰, 44% (83%)²⁹¹ and 71% (85%)²⁹² respectively for compounds **6a-c**. The lower yields may have been achieved due to experimental set up and also the ability to keep the reaction between 0-5 °C. Hydrolysed *p*-toluenesulfonyl chloride may have also been a cause for these low yields.

***N*-Alkylation - microwave synthesis**

Microwave synthesis has been shown to be an effective method in a number of chemical reactions including *N*-alkylation.²⁰⁷ As no reported literature on dopamine *N*-alkylation exists, a method was developed. Using a microwave active solvent, a base known to be used in *N*-alkylation and also varied temperatures to determine changes in reaction mixture composition over a set time period, we proceeded as below.

A screening experiment was conducted utilizing *N,N*-di-isopropylethylamine (DIPEA) as a base, with acetonitrile (MeCN - a solvent commonly used in microwave chemistry)²⁰⁸ and the starting materials 2-((*tert*-Butoxycarbonyl)amino)ethyl 4-methylbenzenesulfonate (**6a**) and 2-(3,4-*bis*(Benzyloxy)phenyl) ethan-1-amine hydrochloride (**4**) at three temperatures (40, 80 and 120 °C) for 5 minutes. This was done to see whether we could utilize microwave chemistry to synthesize a fully protected, novel dopamine-linker conjugated analogue, *tert*-Butyl (2-((3,4-*bis*(Benzyloxy)phenethyl)amino)ethyl)carbamate - (**7a**, Figure 3-26).

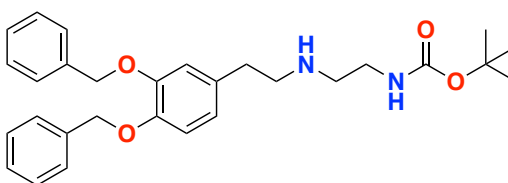


Figure 3-26 - *tert*-Butyl (2-((3,4-*bis*(Benzyloxy)phenethyl)amino)ethyl)carbamate (**7a**)

Figure 3-27 shows the data obtained using LCMS of the reaction mixture when compound **4** was reacted with **6a** (80 °C, DIPEA), showing evidence that the desired compound **7a** was synthesised. Peak assignments for the spectra are shown in Table 3-3. Synthesis of **7a** was evident in the LCMS spectra, found at 3.8 minutes with a mass to charge ratio of 477.25 indicative of the $[M+H]^+$ ion fragment.

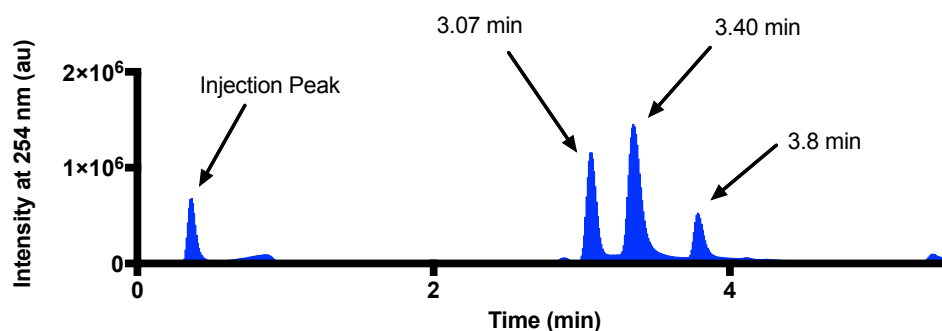


Figure 3-27 - Reaction mixture as identified by LCMS showing elution times and profiles of 2-(3,4-bis(Benzyloxy)phenyl) ethan-1-amine hydrochloride (**4**, 3.40 min), 2-((tert-Butoxycarbonyl)amino)ethyl 4-methylbenzenesulfonate (**6a**, 3.07 min), and mono-alkylated product (**7a**, 3.80 min) with reaction conditions: DIPEA, MeCN, 80 °C, 5 minutes (95% water:5% acetonitrile, to 100% acetonitrile over 6 minutes)

Table 3-3 – LCMS peak assignments of the product obtained after the N-alkylation of 2-(3,4-bis(Benzyloxy)phenyl) ethan-1-amine hydrochloride (**4**) with 2-((tert-Butoxycarbonyl)amino)ethyl 4-methylbenzenesulfonate (**6a**) to generate mono-alkylated product (**7a**) (DIPEA, MeCN, 80 °C, 5 minutes) (95% water:5% acetonitrile, to 100% acetonitrile over 6 minutes)

Elution Time	Mass	Structure
3.40	334.10 [M+H] ⁺ , 667.35 [2M+H] ⁺	(4)
3.07	333.05 [M+NH ₄] ⁺ , 631.20 [2M+H] ⁺	(6a)
3.80	477.25 [M+H] ⁺ , 421.15 [M-tert-butyl] ⁺	(7a)

Figure 3-28 shows the peak integrals for compound **7a**, in relation to the reactions conducted at 40, 80 and 120 °C. As shown, no peak was observed at the lower temperature, whilst at 120 °C a product was observed. As this experiment was only conducted once, we could take limited information as to why higher temperatures produced a lower absorbance value. We predict this is due to either degradation, or *bis*-alkylation of compound **4**.

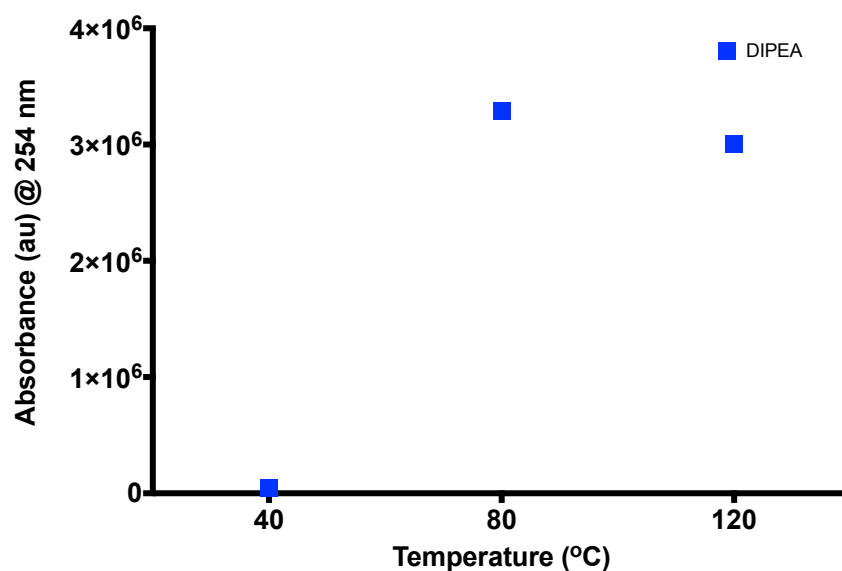


Figure 3-28 - Comparison of LCMS peak intensity at 3.8 minutes (representative of the mono-alkylated product, **7a**) for *N*-alkylation of 2-(3,4-bis(Benzyloxy)phenyl) ethan-1-amine hydrochloride (**4**) with 2-((*tert*-Butoxycarbonyl)amino)ethyl 4-methylbenzenesulfonate (**6a**) at 40, 80 and 120 °C for 5 minutes (*n*=1)

After demonstrating that we could successfully *N*-alkylate dopamine using microwave chemistry, a strategy was devised to optimise the reaction. This initially included the use of different bases at 40, 80 and 120 °C at 5 minutes until a trend could be established. Upon identification of a suitable base, and temperature, time increments from 5 minutes to 1 hour was used. The ratio of reagents was kept the same throughout, with the amine and organo-sulfonate linker at 1:2, whilst the base was at 2 equivalents.

'Hünigs base' commonly known as DIPEA, potassium carbonate (K_2CO_3) and caesium carbonate (Cs_2CO_3) were the chosen for optimisation. Each base was chosen for their typical use in *N*-alkylation reactions as well as for specific properties such as the 'caesium effect', which is known to control chemoselectivity but more importantly for its high compatibility with a wide range of functional groups as described by Salvatore, *et al*, aiding in direct *N*-alkylation's of amines.²⁹³

These non-nucleophilic bases are typically employed for kinetic deprotonation, abstracting a proton to aid in nucleophilic addition/substitution reactions. They are commonly known to be moderately basic, with the pKa of the conjugate acid typically around 10-13. These were chosen in favour of bases which had higher pKa values for the conjugate acids (i.e. sodium hydride -17 and lithium di-isopropylamide - 35-40) due to fears of deprotonation of the *N*-Boc protected linker, causing unwanted side reactions.

In optimizing the reaction conditions, the ratio of starting materials (compounds **4** (34 mmol, 11.54 mg) and **6a** (68 mmol, 20 mg)) was kept the same throughout the various conditions attempted. The amount of base was provided at 2 equivalents to compound **4**. MeCN (250 μ l) was used as the solvent in each instance, and the variable conditions were analysed using LCMS. The peak area of the given product was normalized by total count and compared to determine optimal conditions.

Compound **6a** has previously shown to be problematic under basic and elevated temperatures, undergoing cyclisation to form 2-oxazolidinone as shown by Curran, *et al* (Figure 3-29). It was shown that upon using conditions relative to *N*-alkylation conditions, it has been the only known example where a *tert*-Butyl protecting group can be removed under basic conditions.²⁹⁴ For this reason, it was chosen for our optimisation reactions, to try and determine a synthetic protocol, minimising side reactions, and maximising product yields.

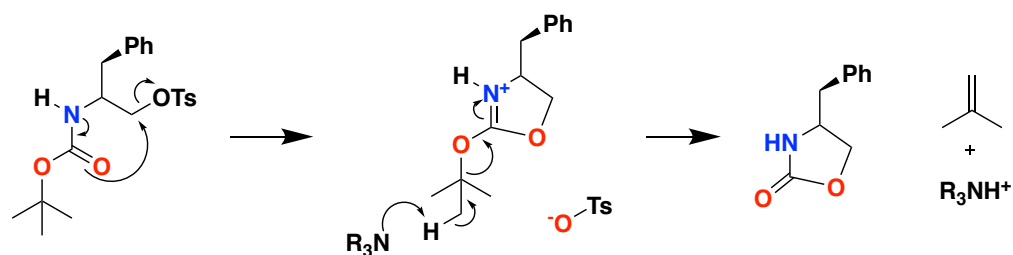


Figure 3-29 - Mechanism for the formation of 2-oxazolidinone from 2-((tert-Butoxycarbonyl)amino)ethyl 4-methylbenzenesulfonate (**6a**) under basic conditions as proposed by Curran, et al.²⁹⁴

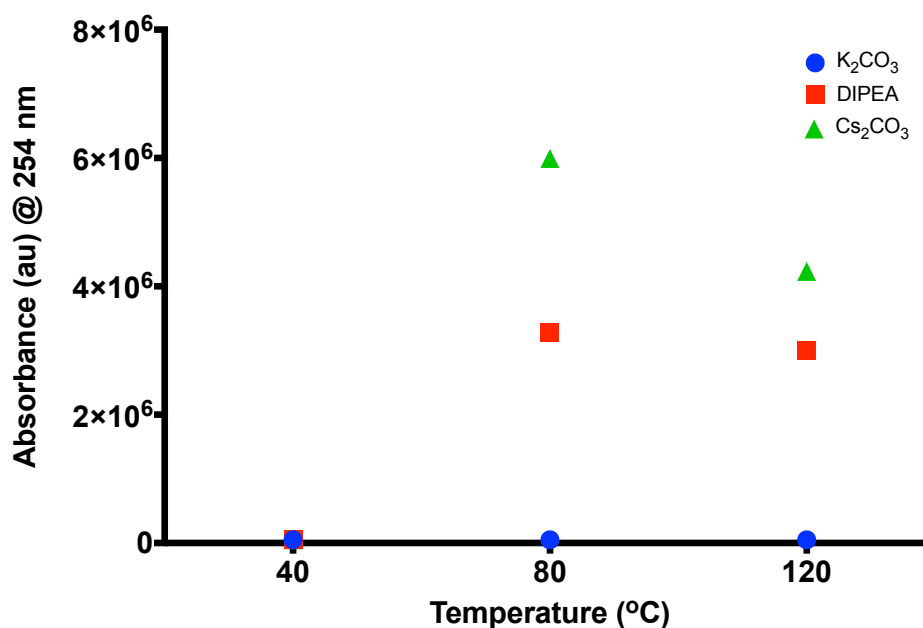


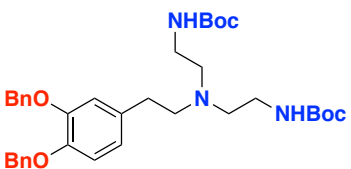
Figure 3-30 - Comparison of LCMS peak intensity at 3.8 minutes (representative of the mono-alkylated product, **7a**) for N-alkylation of 2-(3,4-bis(Benzyloxy)phenyl)ethan-1-amine hydrochloride (**4**) with 2-((tert-Butoxycarbonyl)amino)ethyl 4-methylbenzenesulfonate (**6a**) for 5 min, at 40, 80, and 120 °C, using K₂CO₃, DIPEA and Cs₂CO₃ as a base in MeCN (n=1)

Figure 3-30 shows the peak areas generated using LCMS for the mono-alkylated product (**7a**) using three different bases, after 5 minutes microwave exposure at 40, 80 and 120 °C (with acetonitrile as the solvent). The generation of a peak at 3.8 minutes (representing the mono-alkylated product, **7a**) was compared using peak intensity. A larger peak area indicated the presence of a higher amount of product within the reaction mixture. It was shown that

caesium carbonate at 80 °C produced the largest amount of **7a** (5.99×10^6 au) in comparison to the other bases and temperatures. DIPEA indicated the presence of a mono-alkylated product peak at both 80 and 120 °C (3.28×10^6 and 3.00×10^6 au respectively). No peak area was obtained for the potassium carbonate, indicating that no product was generated within the reaction mixture. Solubility issues were observed using potassium carbonate as a base, which may have led to the lack of product formation.

In addition to the product peak, peaks representative of the *bis*-alkylated, and also impurities generated from the reaction were seen for the caesium carbonate and DIPEA as a base at both 80 and 120 °C. Table 3-4 categorises these peaks, with the m/z and elution times.

Table 3-4 - Additional peaks observed in the LCMS spectra for the optimisation of mono-alkylated product (7a) using DIPEA or caesium carbonate as a base >80 °C

Elution Time (mins)	Mass	Structure
2.50	431.25	Unidentified impurity peak
4.27	620.35 [M+H] ⁺	<i>bis</i> -alkylated 

A comparison of the peak area ratios of the starting materials, mono-alkylated, *bis*-alkylated and impurity generated in the reactions using DIPEA and caesium carbonate, normalised by total count was shown in Table 3-5. Figure 3-31 gives an indication of trends observed at different reaction temperatures for DIPEA and caesium carbonate as a base.

Table 3-5 - Reaction composition (%) for the N-alkylation of 2-(3,4-bis(Benzyloxy)phenyl) ethan-1-amine hydrochloride (**4**) with 2-((tert-Butoxycarbonyl)amino)ethyl 4-methylbenzenesulfonate (**6a**) using DIPEA and Cs₂CO₃ as a base (5 min, 40, 80 and 120 °C) to generate the mono-alkylated product (**7a**) as determined by LCMS

Condition (Base, °C)	4 (%)	Free base 4 (%)	6a (%)	7a (%)	bis (%)	Impurity (%)
DIPEA, 40	0.0	61.8	38.2	0.0	0.0	0.0
DIPEA, 80	0.0	53.4	30.0	16.7	0.0	0.0
DIPEA, 120	35.8	31.6	3.1	13.4	0.0	16.1
Cs ₂ CO ₃ , 40	6.9	43.3	38.0	0.0	0.0	11.8
Cs ₂ CO ₃ , 80	9.0	35.3	30.6	25.1	0.0	0.0
Cs ₂ CO ₃ , 120	22.7	32.2	7.3	17.3	6.5	14.0

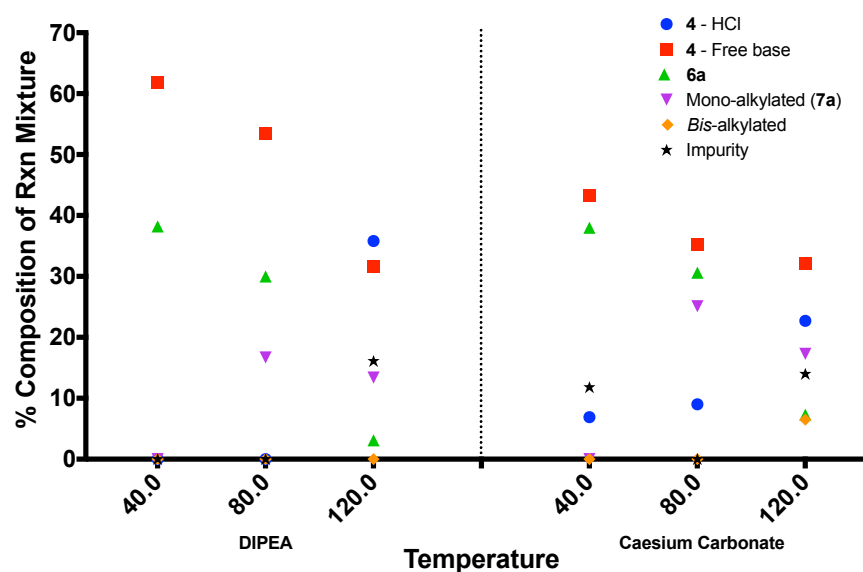


Figure 3-31 - Reaction percentage composition for the N-alkylation of 2-(3,4-bis(Benzyloxy)phenyl) ethan-1-amine hydrochloride (**4**) with 2-((tert-Butoxycarbonyl)amino)ethyl 4-methylbenzenesulfonate (**6a**) using DIPEA and Cs₂CO₃ as a base (5 min, 40, 80 and 120 °C, n=1)

As shown in Figure 3-31, we see a general trend that a larger amount of compound **4** is consumed when we increase temperature for both bases, whether this is to its free base or to generate the mono/*bis*-alkylated product. At the highest temperature, it is shown that conversion to the free base is limited, which affects the reactivity with the tosylated linker.

The same observation can be made for compound **6a**. A larger consumption of the material can be seen as temperature increases. Although in this case, the generation of an impurity peak starts to form, which can be due to the lack of **4** in its free base form to react with. The mono/*bis*-alkylated products also appear at 80 and 120 °C, with increased temperature, comes a higher kinetic rate of reaction with the tosylated linker, generating a smaller amount of impurity.

We can conclude that the generation of the mono-alkylated product is more favourable in the reaction conducted at 80 °C using caesium carbonate as a base. The data shows a higher consumption of starting material with the highest percentage composition of the mono-alkylated product, with no visual observation of the *bis* and impurity peaks.

LCMS spectra were then obtained at time points of 5, 10, 30, 45 and 60 minutes using Cs₂CO₃ at 80 °C for further optimisation. Table 3-6 gives us the percentage composition for each time point, and the data can be visualised in Figure 3-32.

Table 3-6 - Reaction composition (%) for the N-alkylation of 2-(3,4-bis(Benzyloxy)phenyl) ethan-1-amine hydrochloride (**4**) with 2-((tert-Butoxycarbonyl)amino)ethyl 4-methylbenzenesulfonate (**6a**) using Cs₂CO₃ as a base for 5, 10, 30, 45 and 60 minutes at 80 °C, to generate the mono-alkylated product (**7a**) as determined by LCMS

Condition (min)	4 (%)	Free base 4 (%)	6a (%)	7a (%)	bis (%)	Impurity (%)
Cs ₂ CO ₃ (5)	9.0	35.3	30.6	25.1	0.0	0.0
Cs ₂ CO ₃ (10)	40.2	18.4	18.3	10.1	0.0	13.0
Cs ₂ CO ₃ (30)	9.2	23.9	11.6	27.2	10.7	17.4
Cs ₂ CO ₃ (45)	23.2	21.3	5.2	29.4	8.5	12.5
Cs ₂ CO ₃ (60)	4.4	24.0	4.4	27.8	8.9	30.5

There was no obvious linear trend in the synthesis of the target compound **7a** observed in Figure 3-32 with an increase in the reaction time, but there was evidence of the tosylated linker reducing as time progressed. This cannot only account for the increase in yield for the mono alkylated products but also for the impurity as well.

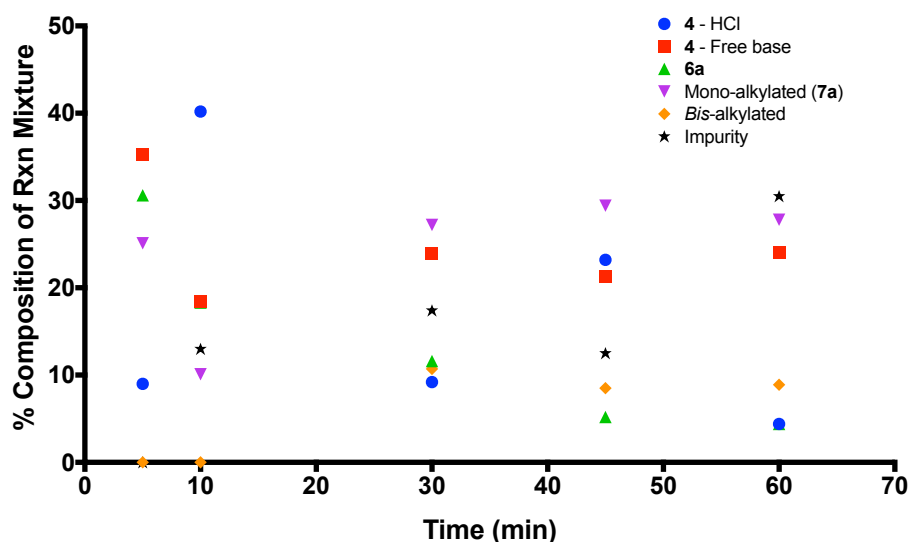


Figure 3-32 - Reaction percentage composition for the N-alkylation of 2-(3,4-bis(Benzyloxy)phenyl) ethan-1-amine hydrochloride (**4**) with 2-((tert-Butoxycarbonyl)amino)ethyl 4-methylbenzenesulfonate (**6a**) using Cs₂CO₃ as a base at 80 °C, for 5, 10, 30, 45 and 60 minutes (n=1)

The optimal condition that results in the maximal amount of product formed while minimising the amount of impurities was shown to be at 45 minutes. This was therefore used as the ideal conditions for further synthesis (as shown in Figure 3-33).

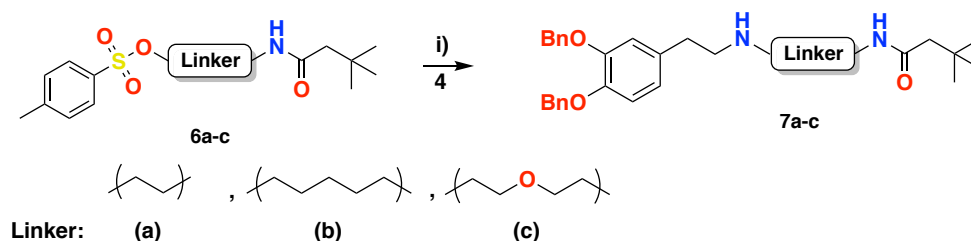


Figure 3-33 - N-alkylation of 2-(3,4-bis(Benzyloxy)phenyl) ethan-1-amine hydrochloride (**4**) with **6a-c**, i) μW , 80°C , 45 min (**7a**: 24%, **7b**: 49%, **7c**: 46% after purification)

Column chromatography was used to isolate the product from the reaction mixture. Co-elution of the product peak and the 2-oxazolidinone impurity formed in the *N*-alkylation reaction was observed with a selection of solvent systems, at different percentages per solvent utilised (e.g. dichloromethane or chloroform; methanol, ethyl acetate: hexane, ethyl acetate: petroleum ether, ethyl acetate: pentane, toluene). Isolation of the product was achieved in two steps. Utilising column chromatography, the 2-oxazolidinone and product was co-eluted using neutralised silica, with ethyl acetate (99%): Et₃N (1%) as the solvent system. Recrystallisation of the product was achieved using toluene and petroleum ether, with the target compound remaining in solution. Evaporated to dryness, a light-yellow compound (**7a**) was obtained at a yield of 24%.

Upon successful synthesis and purification of compound **7a**, microwave synthesis was used to successfully synthesise the other extended dopamine analogues, achieving yields with isolation of *tert*-Butyl (5-((3,4-bis(Benzyloxy)phenethyl)amino)pentyl)carbamate (**7b**) at a 49% yield, and *tert*-Butyl (2-(2-((3,4-bis (Benzyloxy)phenethyl)amino) ethoxy)ethyl) carbamate (**7c**) at 46%.

Figure 3-34 shows the ^1H NMR of compounds **7a-c**. Peaks were assigned as expected, with integrals for each synthesised compound relating to a mono-alkylated product. The lack of an acidic proton also confirmed binding at the *N*-terminus, which was further confirmed by the lack of peaks associated with the tosylate.

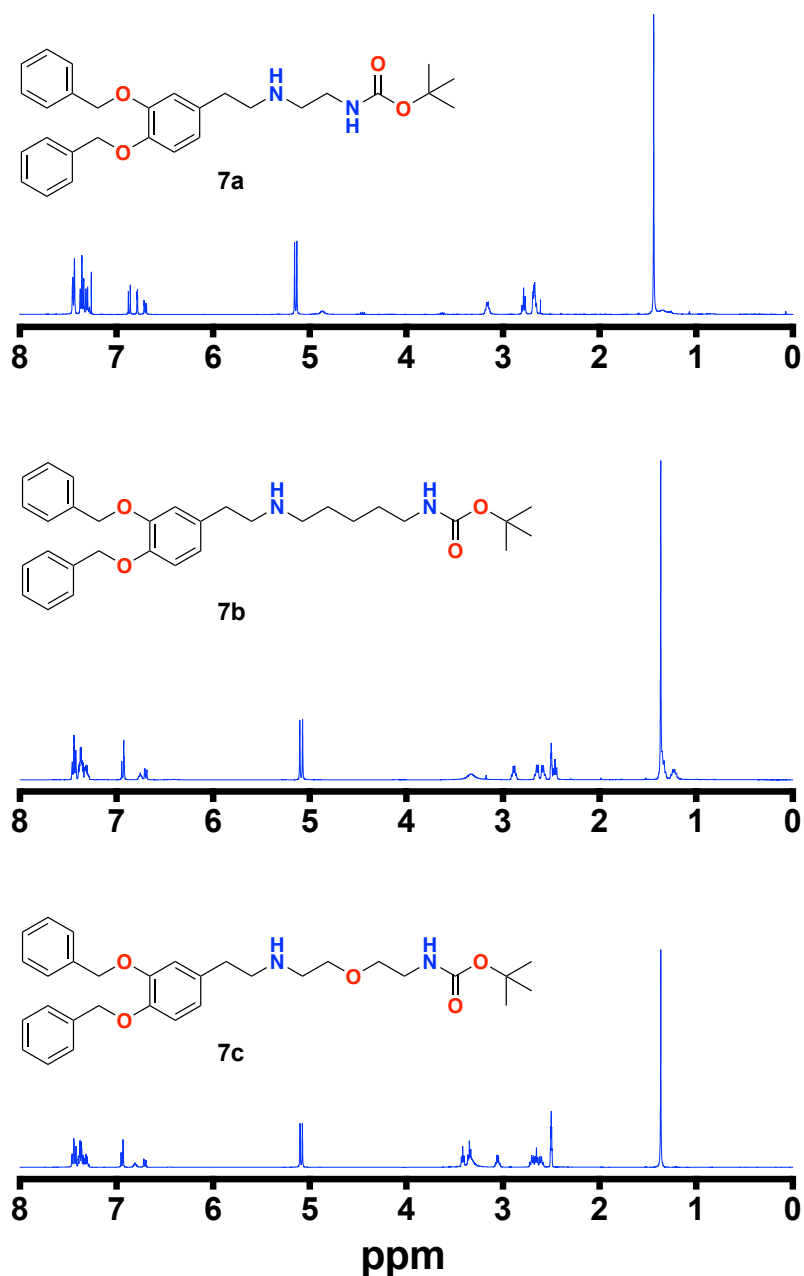


Figure 3-34 - ^1H NMR for compounds **7a-c** showing successful *N*-alkylation through the use of microwave chemistry

N-Boc deprotection

Efficient de-protection of the Boc group was achieved using a combination of dissolving the compound in methanol, with the addition of excess 2 M HCl in di-ethyl ether for 1 hour. This was conducted twice to ensure the compound did not precipitate from solution as a salt prior to the de-protection due to the secondary amine which was formed in *N*-alkylation. The generation of the benzyl protected, di-hydrochloride salts was evident from the ^1H NMR, and were produced at quantitative yields. Boc deprotection was confirmed with the disappearance of the CH_3 peak at 1.37 ppm (with an integral of 9 protons) representative of the *tert*-Butyl group. Figure 3-35 shows an example of the Boc deprotection for compound **7c**.

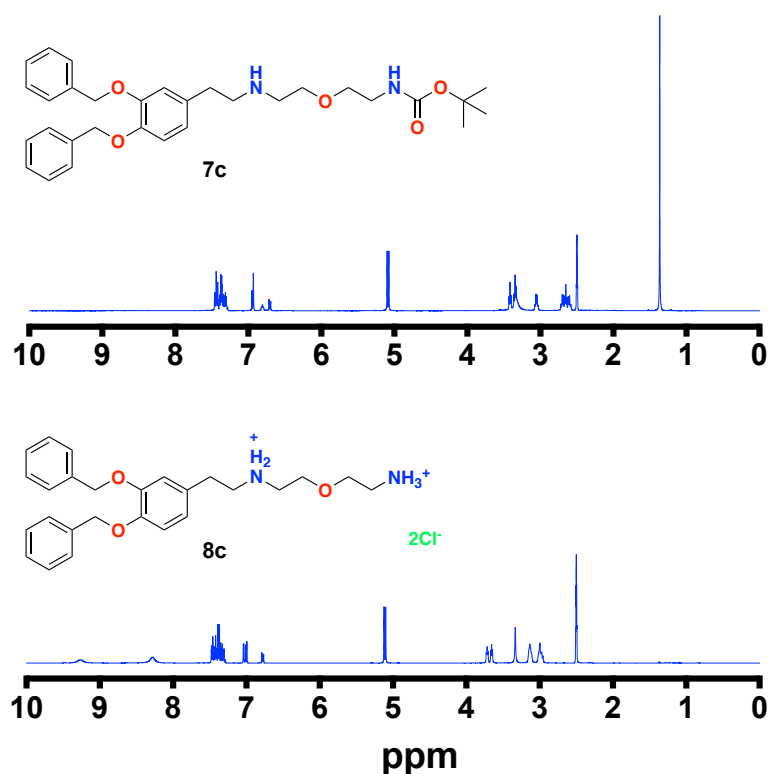


Figure 3-35 - ^1H NMR showing Boc deprotection of tert-Butyl (2-(2-((3,4-bis (Benzyloxy)phenethyl)amino) ethoxy)ethyl) carbamate (**7c**), to generate 2-(2-Aminoethoxy)-N-(3,4-bis(Benzyloxy)phenethyl)ethan-1-amine dihydrochloride (**8c**). Absence of the CH_3 peak (1.37 ppm), and additional proton peaks confirm synthesis of the dihydrochloride salt (8.28, and 9.26 ppm)

O-Benzyl deprotection

Benzyl ethers are highly stable and resistant to many reaction conditions, which makes it ideal as a protection group for the catechol under the *N*-alkylation conditions. Conditions for removal, requires hydrogenation or acidic medium at high temperatures.²⁹⁵ The desire to keep the Boc protection, which is labile under acidic conditions, hydrogenation was used for deprotection.

A series of reactions were conducted using 10% w/w palladium on carbon and hydrogen. From the outset, we observed (using LCMS) deprotection of the benzyl group for all three compounds using methanol and water as a solvent at a 9:1 ratio. Deprotection of compound **7a**, as shown by the LCMS spectra (Figure 3-36) shows the development of two peaks, at 1.79 and 2.90 minutes. These were in relation to the product peak, and the generation of toluene within the reaction. A change in LCMS baseline profile can be explained by a low concentration of the components found in the solution analysed.

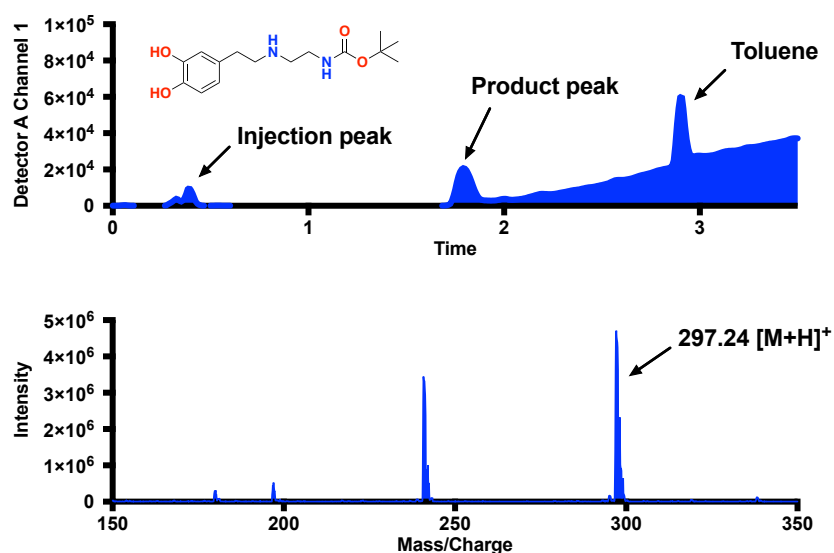
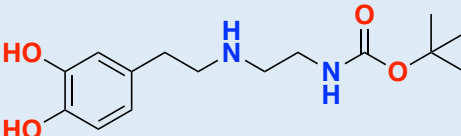
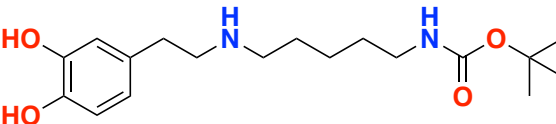
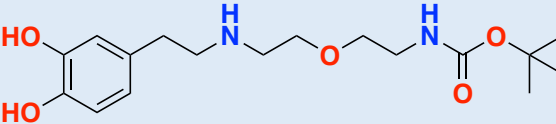


Figure 3-36 - LCMS spectra for O-benzyl deprotection of compound **7a** using Pd/C, H₂ and MeOH: Water (9:1) showing formation of a toluene peak (2.94 min) and mass fragment associated with the [M+H]⁺ of the deprotected compound at 1.79 min

Table 3-7 shows the *O*-Bn deprotection of compounds **7a-c**, and their respective mass to charge ratios and elution times as identified by LCMS.

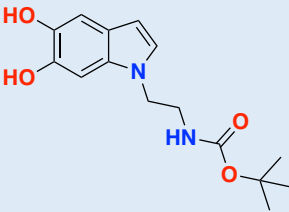
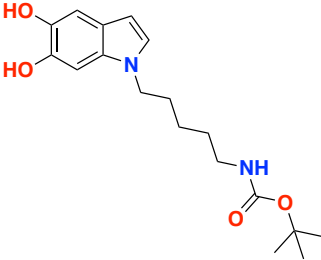
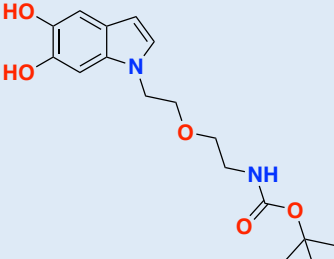
*Table 3-7 – Identified product peak elution times and mass fragments (LCMS) for *O*-Bn deprotection of compound 7a-c using Pd/C, H₂ and MeOH: Water (9:1)*

Elution time (min)	m/z	Compound
1.79	297.24 [M+H] ⁺	
2.04	339.26 [M+H] ⁺	
1.97	341.23 [M+H] ⁺	

With successful deprotection observed in the LCMS spectra, isolation of the material was conducted using filtration through Celite (to remove the palladium from the reaction mixture). Evaporation of the solvent for all three compounds resulted in a black sticky substrate, which indicated catechol oxidation. Analysis using LCMS, mass values obtained could be explained for the generation of cyclic products.

It was concluded that the generation of these materials was due to the exposure to atmospheric conditions, as well as the aqueous component of the solvent mixture. The increased elution times are due to an increased LCMS run time. This was utilised to check that there was no overlap of peaks giving rise to both the desired and oxidised compounds. This method changed the elution profile from 95% water:5% acetonitrile to 100% acetonitrile over 15 minutes instead of the standard 6 minutes.

Table 3-8 - Elution times, mass fragments and suspected structures (indicating cyclisation of the desired compounds) as determined by LCMS for *O*-Bn deprotection of compound **7a-c** using Pd/C, H₂ and MeOH: Water (9:1)

Elution time (min)	m/z	Compound
4.26	293.20 [M+H] ⁺	
5.203	335.18 [M+H] ⁺	
4.53	337.18 [M+H] ⁺	

In an attempt to prevent oxidation, different solvent systems were used, as well as controlling exposure to atmospheric conditions upon isolation. These conditions involved using methanol, ethyl acetate and acetic acid as alternate solvents with the removal of water as a solvent in the process.

Efficiency of *O*-Bn deprotection, was monitored by LCMS using the product peaks generated at 1.79, 2.04 and 1.97 minutes (for compounds **7a-c**). It was shown that using methanol and ethyl acetate at 100%, greatly reduced the deprotection efficiency with each method taking over 3 days to produce little product. As opposed to ethyl acetate, methanol as a solvent continued to generate a higher intensity peak for the deprotection of compounds **7a-c**, over a period of a week culminating in full deprotection.

The application of a constant nitrogen stream was applied to the reaction mixture upon removal of the palladium on Celite, and prior to solvent evaporation. After the solvent was removed, the formation of black sticky compounds was once again observed, indicating as above (cyclisation of the compound).

Endeavouring to retain the Boc protection, whilst preventing oxidation of the molecules, we attempted to utilise an acid as a solvent. Acetic acid was used as it is not strong enough to deprotect the Boc protection, but would be able to reduce the ability for the compound to oxidise in atmospheric conditions. Acetic acid on its own and a combination of acetic acid: methanol was used to see if we could negate the oxidative effects upon solvent evaporation. Using LCMS to determine the reaction species forming upon conducting the reaction, we observed the generation of new molecular ion peaks over the period of a week which did not relate to the masses of the three desired deprotected compounds. Due to this, the synthetic strategy was evaluated, and due to the complications in generating a Boc protected molecule, universal deprotection was sought.

Universal deprotection

Trifluoroacetic acid (TFA) has been shown to both deprotect Boc²⁹⁶ and benzyl groups at extended time periods and at elevated temperatures.²⁹⁷ Due to the low yields achieved and complexity of generating the dopamine-linked molecules, we used *tert*-Butyl (3,4-*bis*(Benzyloxy)phenethyl) carbamate (**3**) as a control, to deprotect down to dopamine. This was to determine the efficiency and ability to deprotect the compounds using this method. To understand the amount of deprotection occurring, a calibration plot was generated using a concentration (mM) vs total area counts (au/uV) for dopamine. This was used to calculate the percentage conversion between the starting material and final deprotected product.

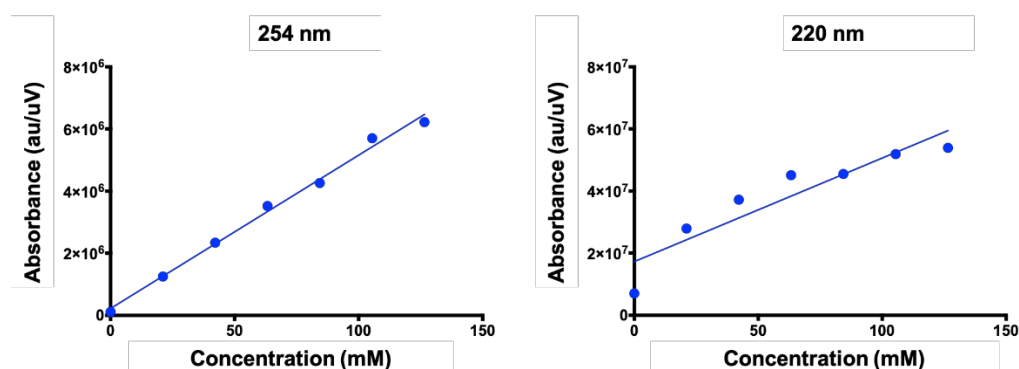


Figure 3-37 - Concentration vs absorbance for dopamine hydrochloride using LCMS at two detector wavelengths (1 - 254 nm, $r^2=0.99$), (2 - 220 nm, $r^2=0.85$) ($n=1$)

A concentration range from 0 to 130 mM of dopamine hydrochloride was monitored at two different detector traces at: 254 nm and 220 nm. The trace at 254 nm showed a linear trend ($r^2= 0.99$) in comparison to the 220 nm detector ($r^2= 0.85$) as shown in Figure 3-37. Using the equation generated ($Y=49347X +220571$) for the 254 nm detector, we could identify the amount of compound **3** converted to **1** using peak area.

An initial screening for the universal deprotection of **3** was conducted at three different temperatures: 40, 60 and 100 °C for 5 minutes using the microwave reactor. Instant deprotection was observed of the Boc protection group, with limited deprotection of the *O*-Bn group.

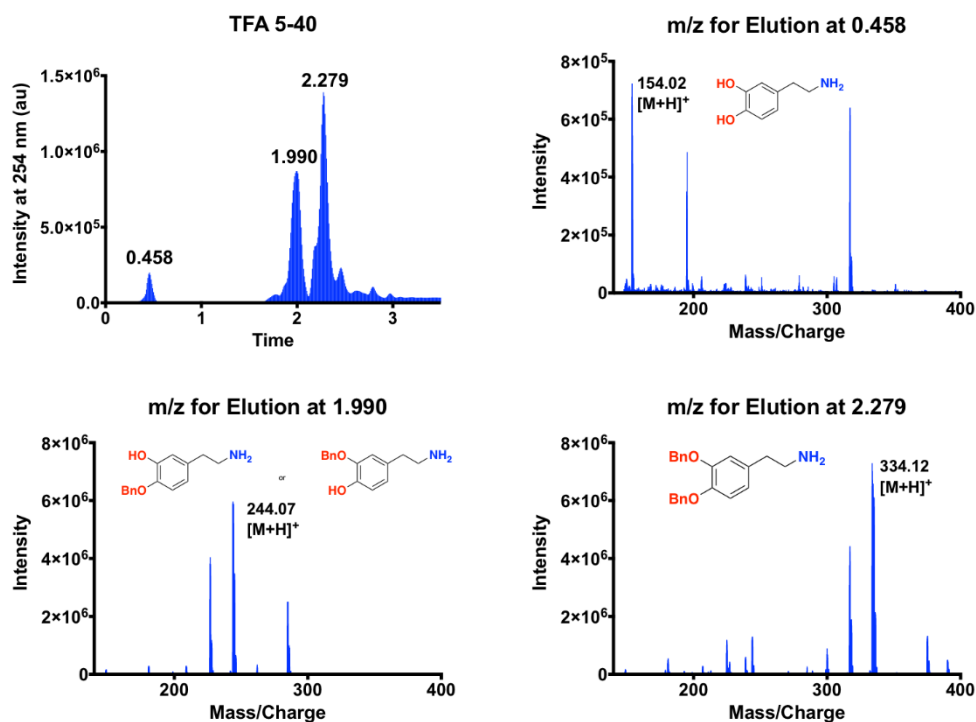


Figure 3-38 - Universal deprotection of compound **3** using 100% TFA at 40 °C for 5 minutes. LCMS Spectrum shown in top left, *m/z* spectrum obtained from the peaks at 0.458, 1.990 and 2.279 minutes are shown with corresponding structures

It was shown that after 5 minutes at 40 °C, peaks associated with a fully deprotected compound ($[M+H]^+$, 154 *m/z*), partial benzyl deprotected compound ($[M+H]^+$, 244 *m/z*) and Boc deprotected ($[M+H]^+$, 334 *m/z*) could be seen in the LCMS (Figure 3-38). An increase of temperature yielded a higher fully deprotected compound, but was limited to a maximum conversion of 24%. To aid removal of the protection group, scavengers were employed. The scavenger acts to remove or de-activate impurities and unwanted side products formed in the reaction, in this case to act as a soft nucleophile to aid in the removal of *O*-Bn protecting group.²⁹⁸ Two scavengers were employed, triisopropylsilane (TIS) and thioanisole (TAS) (Figure 3-39).

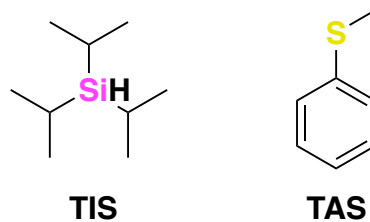


Figure 3-39 - Structure of triisopropylsilane (TIS) and thioanisole (TAS) common scavengers which act as soft nucleophiles for the removal of O-Bn protecting groups

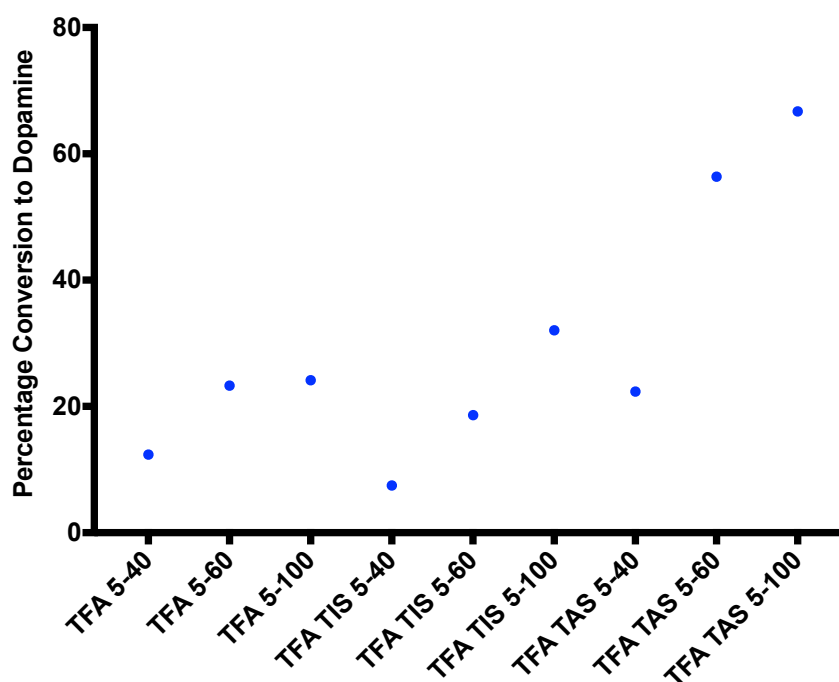


Figure 3-40 - Percentage conversion of compound **3** to dopamine (TFA salt) using trifluoroacetic acid (TFA) with thioanisole (TAS) and triisopropylsilane (TIS) as scavengers at 40, 60 and 100 °C (n=1)

Figure 3-40 shows that upon addition of TIS and TAS, an increase in conversion when subject to 100 °C, 33% and 66% respectively within 5 minutes occurs. Using this as a baseline, we then further attempted to optimize the reaction using TAS at the elevated temperature, for an increased period of time. Figure 3-41 shows percentage conversion when TAS was used at 100 °C at 5, 10, 30, 45 and 60 minutes. As time was increased, the percentage conversion also increased.

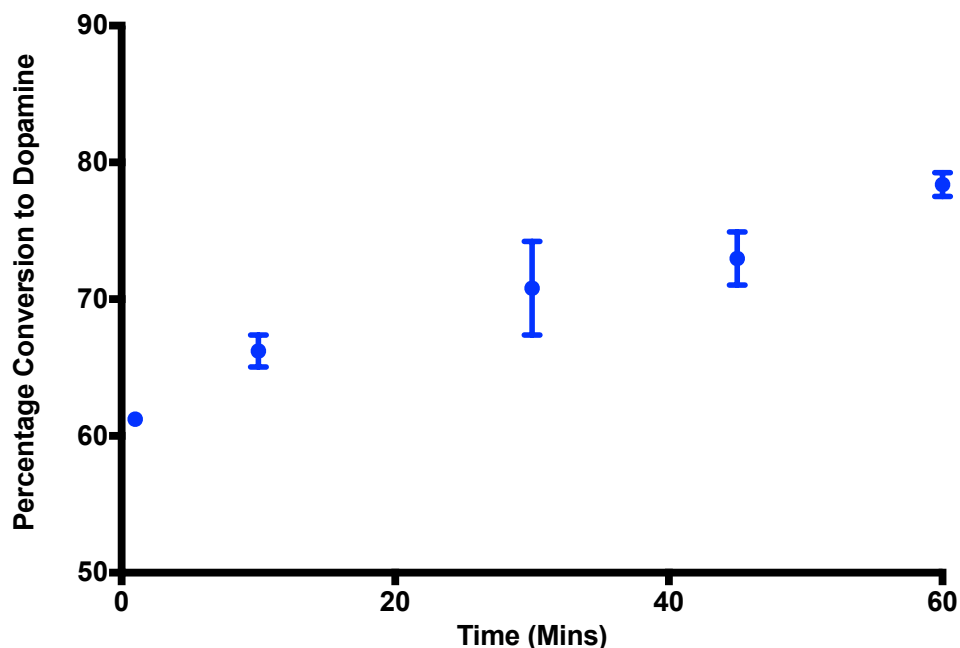


Figure 3-41 - Deprotection of compound **3** at 100 °C from 1-60 minutes using thioanisole as a scavenger ($n=3$, error bars represent SD)

Isolation of the target compound was found to be an issue with this method of deprotection. Due to partial deprotection occurring and the formation of the TFA salt, the product was hard to separate using thin layer chromatography. Elution on LCMS was shown to be too close to the injection peak, making it hard to separate with techniques such as preparative HPLC.

As benzyl deprotection of compounds **7a-c** was shown to be successful, until isolation using hydrogenation, we attempted to deprotect the hydrochloride salts (**8a-c**). As dopamine is typically stabilised in the salt form, we aimed to prevent oxidation from occurring. This followed the reaction scheme shown in Figure 3-42.

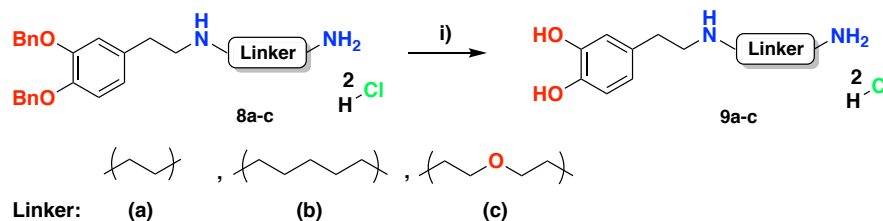


Figure 3-42 - Reaction scheme of benzyl deprotection for compounds **9a-c**, i) Pd/C, H_2 , MeOH, 100 psi (**9b** -64%, **9c** - 48%)

A clean deprotection was achieved using compounds **8a-c**, in a typical hydrogenation reaction using methanol as a solvent, in a high-pressure vessel (100 psi) overnight resulting in the formation of toluene as a by-product. Care was taken to shield the product from environmental conditions, due to the property of the catechol to oxidise, and their hygroscopicity in relation to the functional groups and hydrochloride salt. It was shown that the stability of the ethanolamine-dopamine analogue (**9a**) was still prone to oxidation due to the exposure of methanol and elevated temperatures under solvent reduction. Isolation of compound **9a** was unsuccessful, observing discolouration and film formation in the round bottom flask after drying. Figure 3-43 shows degradation of the compound when analysed in deuterated methanol, even after flushing the NMR tube with nitrogen and backfilling to be shielded from environmental factors.



Figure 3-43 - Degradation of 4-(2-((2-aminoethyl)amino)ethyl)benzene-1,2-diol (**9a**)

Deprotection of compounds **8b** and **8c** were successfully achieved using the method above, to yield 4-(2-((5-Aminopentyl)amino)ethyl)benzene-1,2-diol (**9b**) at 64% and 4-(2-((2-(2-Aminoethoxy)ethyl)amino)ethyl)benzene-1,2-diol at 48% (**9c**) (stored in an inert atmosphere at -20 °C). As benzyl deprotection using hydrogenation typically produce quantitative yields, it was concluded that on deprotection of the catechol, chelation between the palladium and dopamine analogue may occur, causing a loss of product through filtration on Celite. Whilst there is no published evidence of the chelation of palladium and dopamine, there is evidence of catechols readily chelating with a number of metals including copper²⁹⁹ and iron.³⁰⁰ Analysis of compounds **9b** and **9c** was conducted using NMR and LCMS. Benzyl deprotection was identified from the lack of aromatic protons and CH₂ group associated with the protection group found in the ¹H NMR, whilst a reduction in the mass for all compounds of m/z 182 was observed, resulting in the formation of toluene.³⁰¹

Biology – Structure activity relationship for dopamine derivatives

GPCR ligand discovery relies on the ability to measure how, the quantity required and structural properties of a ligand to activate receptors found on cell surfaces. To achieve this, a simple and high throughput assay was required, specifically focusing on $G_{\alpha s}$ based receptors, for the dopamine D_1 receptor. Typically, assays which have been used to measure dopamine agonist activity at the D_1 receptor focus on G-protein dependent assays. These assays can include measuring a number of markers such as the hydrolysis of GTP to GDP, the upregulation of cAMP and activation of cAMP dependant pathways such as protein kinases (PKA) and cAMP response elements⁴⁵ as previously discussed.

Here, Chinese hamster ovary cells stably expressing the dopamine D_1 receptor (CHO- D_1) and a cyclic 3',5' adenosine monophosphate response element-secreted placental alkaline phosphatase (CRE-SPAP) reporter gene was used to evaluate the pharmacological properties of the compounds synthesised. This assay was chosen due to its high throughput, specificity for the D_1 receptor, simplicity and its enhanced signal-to-noise ratio due to measuring downstream, reducing the likeliness of false positives. Developed by Hill, *et al*, for the study of GPCRs, it has previously shown great use in determining agonistic activity at the β_2 adrenoceptor.⁷²

Generation of SPAP is achieved through a cascade effect, when a ligand binds to a cell surface GPCR, an increase of intracellular cAMP is observed leading to the activation of the cAMP response element (Figure 3-44). It is easy to quantify as it is secreted from cells, and can be measured by sampling the culture medium.

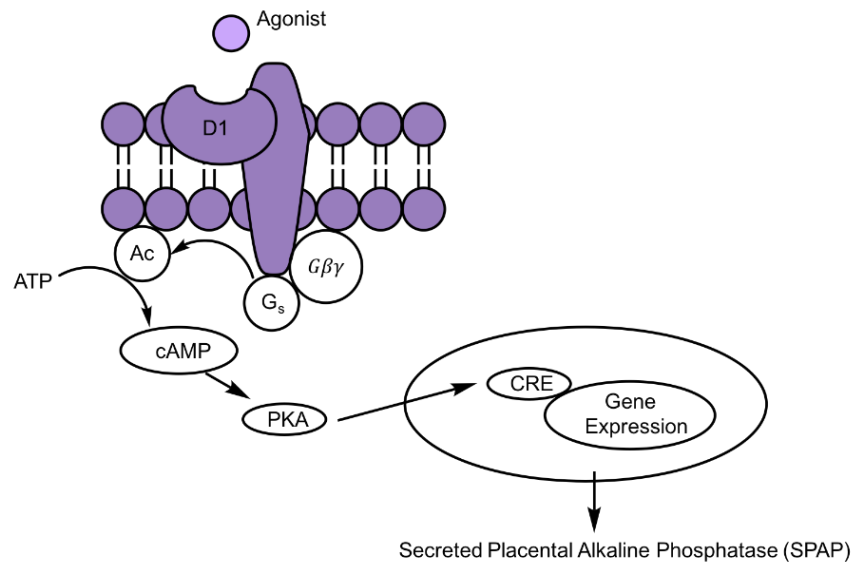


Figure 3-44 - SPAP generation pathway⁷² (D₁- receptor, Ac - acetylcholine, ATP - adenosine triphosphate, G_s - isoform, Gβγ - heterotrimeric proteins, cAMP - cyclic adenosine monophosphate, PKA - protein kinase A, CRE - cAMP response element)

It can also be separated from other endogenous alkaline phosphatases due to its stability up to 65 °C, where other enzymes are destroyed, SPAP remains. Detection of the generated SPAP can be achieved by the hydrolysis of *p*-nitrophenol phosphate, causing a colour change that can be quantified using a colorimetric plate reader at 405 nm.

Dopamine control

Dopamine undergoes oxidation in aqueous conditions, to which nature overcomes this by storing the neurotransmitter in acidic vesicles before it is released (VMAT2 – acidic vesicles).⁵⁹ Due to the nature of our experiment, we must incubate the CHO-D₁ cells with our target compound for 4 hours at an elevated temperature (37 °C) which may cause oxidation of the compound.

To counter act this, we tested the use a free radical scavenger to prevent oxidation of the dopamine whilst conducting the SPAP assay. Ascorbic acid, which is more commonly known as vitamin C, was used due to its ability to act as an anti-oxidant.³⁰² Figure 3-45 shows the method of radical scavenging of ascorbic acid. It has been shown by Sutor, *et al*, that using ascorbic acid was an effective method to reduce mass oxidation of dopamine.³⁰³

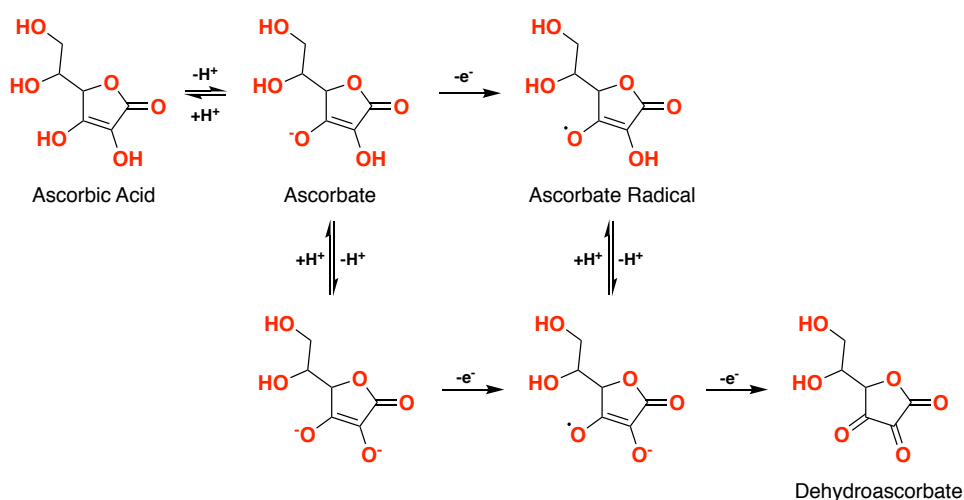


Figure 3-45 - Structure and reductive method of action of ascorbic acid³⁰²

In essence of this, we compared dopamine with and without ascorbic acid (40 μM) per well to determine the difference in effective concentration at 50% (EC_{50}). The results are shown in Figure 3-47, with the data obtained from 5 experiments ($N=5$), with a repeat of 3 wells per concentration ($n=3$). As each plate contained its own control samples, the data was basal corrected, and then averaged to give the data shown.

Forskolin (Figure 3-46) a diterpene of the labdane family, was used as the positive control at a concentration of 10^{-4} M and is known to directly activate the adenylyl cyclase pathway. Activation of adenylate cyclase using forskolin, results in increased levels of intracellular cAMP and elicits responses which have been proposed to be dependent of cAMP as a secondary messenger.³⁰⁴

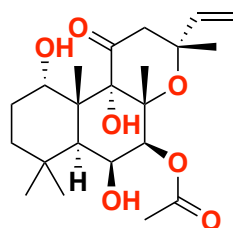


Figure 3-46 - Structure of forskolin

The EC_{50} for compound **1** was shown to be at 4.6×10^{-8} M and 7.5×10^{-7} M in the presence and absence of ascorbic acid, respectively. This indicated the stability of the dopamine was higher in the assay conditions when buffered with 40 μ M of ascorbic acid. The significance of this result shows that the efficacy of dopamine is higher when buffered using an acid in our assay, which is similar to how it is released naturally from acidic vesicles (VMAT2). The use of ascorbic acid was applied to all further assays in this thesis to prevent oxidation of our synthetic derivatives.

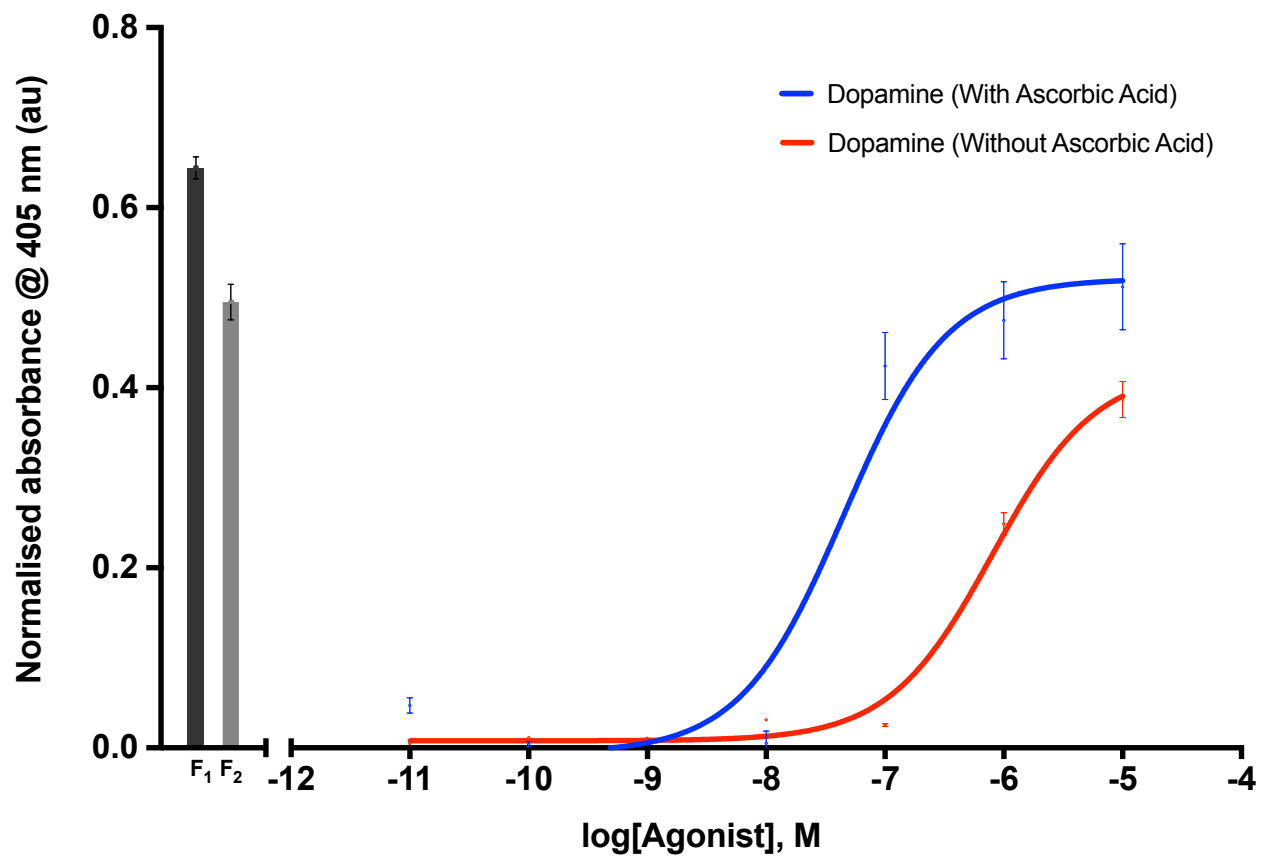


Figure 3-47 - Dose response of compound **1** with and without ascorbic acid for the dopamine D₁ secreted placental alkaline phosphatase assay. Positive controls of forskolin are also shown as F₁ and F₂ which represent assays with and without ascorbic acid present (n=3, N=5, error bars represent SEM)

Dopamine protected analogues

In theory, activity at the dopamine D₁ receptors should be suppressed when the catechol and amine of the endogenous dopamine ligand is modified. This is from observations made by Swaminath, *et al*, who examined the differences in mechanism of activation of the β_2 adrenoceptor by catechol agonist, isoproterenol, and non-catechol agonist, salbutamol.³⁰⁵ It was shown that despite the structural similarities of the salbutamol and catecholamine agonists, the aromatic ring of the salbutamol occupies a different space in the β_2 adrenoceptor, and thereby induces a functionally different active state, leading to reduced potency. This also suggests flexibility in the receptor, and the possibility of achieving more than one active state through different interactions between receptors and ligands.

Figure 3-48 and Figure 3-49 shows proposed binding and activity from the SPAP assay for compounds **1-4** respectively. The suppressed activity of compound **2** can be explained by both the structure and polarity of the compound. Typically, the primary amine binds to an aspartic acid on the TM3 region, which causes a salt bridge to form.³⁰⁵ When modified as a carbamate, a change in size to fit in the binding pocket occurs as well as the lack of charge. Hydrogen and ionic bonding of the system changes, and the formation of the salt bridge is not possible. Although this is the case, the carbamate can still interact with the aspartic acid through hydrogen bonding and with the catechol still intact we see activity, although at a reduced level (EC₅₀ of 3.71×10^{-6} M for the carbamate compared to 4.47×10^{-8} M for dopamine).

A complete reduction in potency at the D₁ receptor is shown for compound **3**. Modification at the catechol suppresses all activity and no SPAP is generated above the basal level. This supports theories in which the catechol plays a large role in activity. The lack of activity may also be a result of the size of the benzyl groups, which are too large to fit within the D₁ binding pocket.

A negative response is observed for when the catechol is protected and the primary amine remains free, **4**. This response may indicate the possibility of an antagonistic/inverse agonistic effects at the D₁ receptor, reducing the absorbance below the basal level. Cytotoxic effects, which are due to high concentrations causing apoptosis of the cells, and reducing the SPAP levels generated at the basal level could also be a cause for this result.

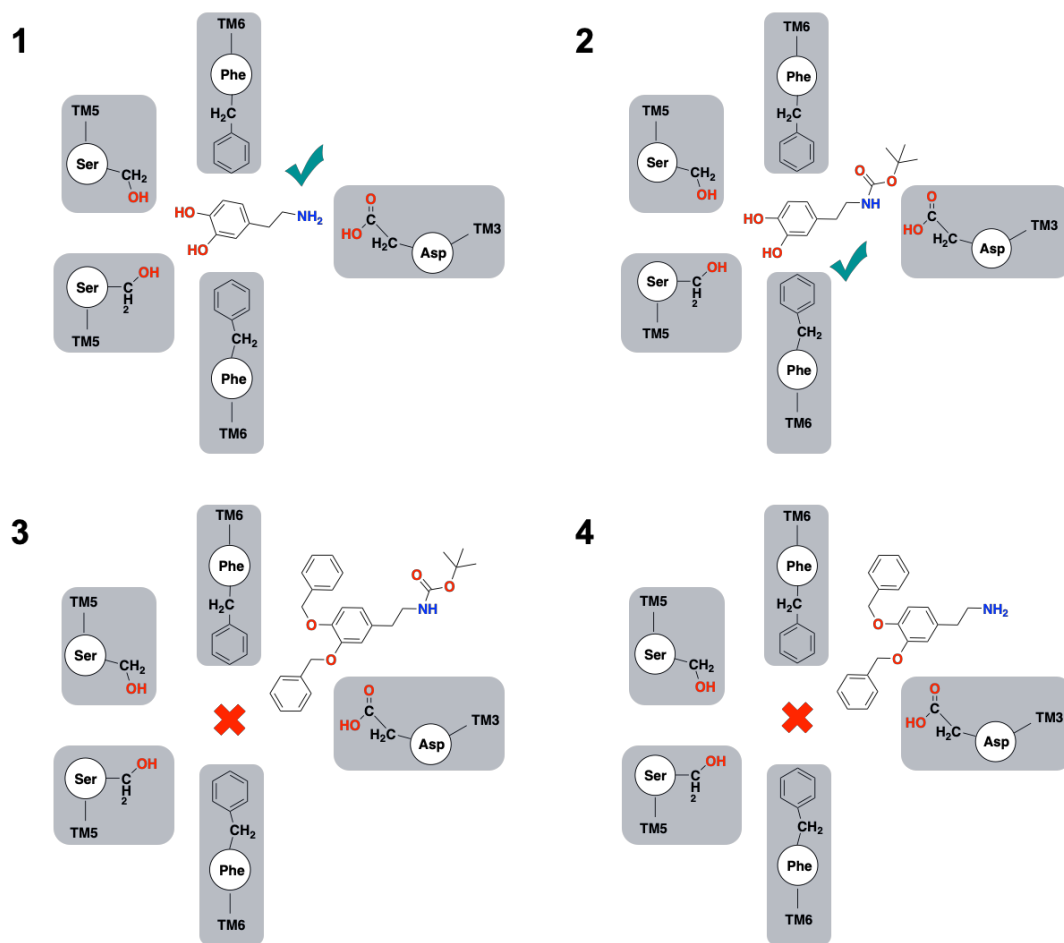


Figure 3-48 - Proposed binding interactions of compounds **1-4** and the D₁ receptor. Compounds **1** and **2** are small enough to fit into the adaptable binding pocket, whilst compounds **3** and **4** fail to activate the receptor due to the large catechol preventing interactions with the serine residues on TM5 (green tick represents potency at the D₁ receptor, whilst red cross marks no activity above basal level in the SPAP assay)

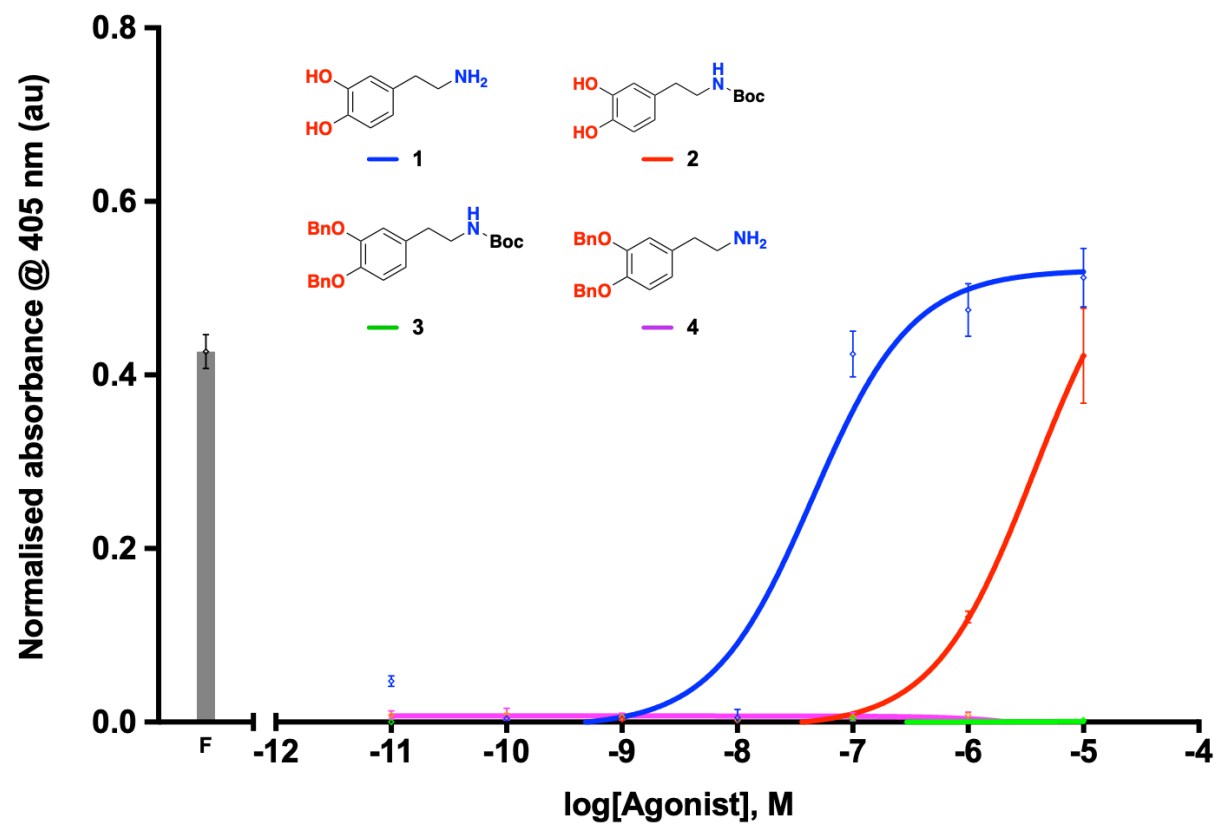


Figure 3-49 - Dose response of dopamine analogues **1-4** for the dopamine D_1 secreted placental alkaline phosphatase assay. Positive control of forskolin is shown as F ($n=3$, $N=5$, error bars represent SEM)

Extended dopamine analogues

The proposed binding and activity from the SPAP assay for compounds **7a-c** is shown in Figure 3-50 and Figure 3-51. It was expected that these compounds would produce no activity at the D₁ receptor, similar to the results observed for compound **3**. As expected, the compounds were not potent in activating the D₁ receptor, which is due to the ability for the compounds to fit and subsequently interact with the binding sites within the receptor.

At high concentrations, we observe for all the three compounds an absorbance lower than the basal level. This is similar to compound **4**, and it is to be believed that this effect is caused due to the improper function of the cell, or antagonistic/inverse agonistic effects. Although this is the case, further tests would need to be utilised to determine the cause for this effect. These tests may include assays which monitor the reduction of cAMP from pre-stimulated cells, as well as cell viability assays such as 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT) or (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS).

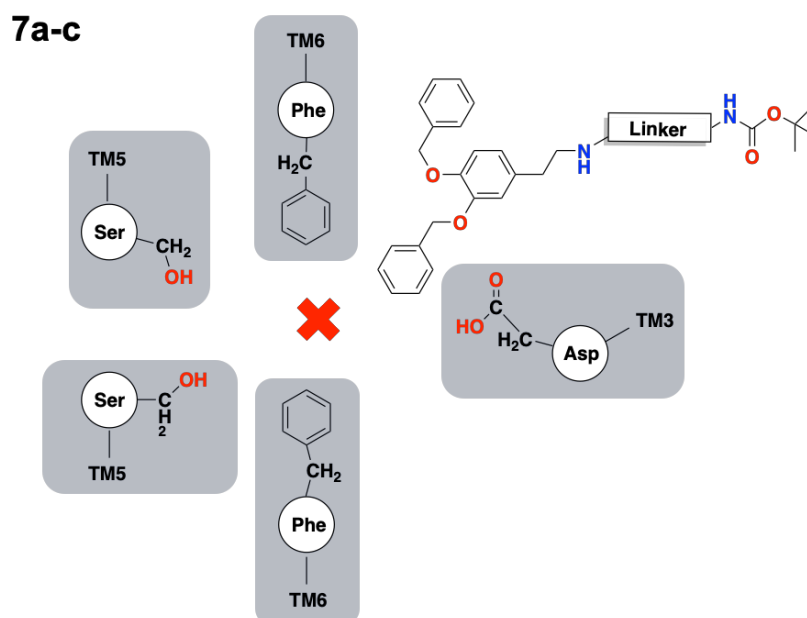


Figure 3-50 - Generic proposed binding interactions of compounds **7a-c** and the D₁ receptor (red cross marks no activity above basal level in the SPAP assay)

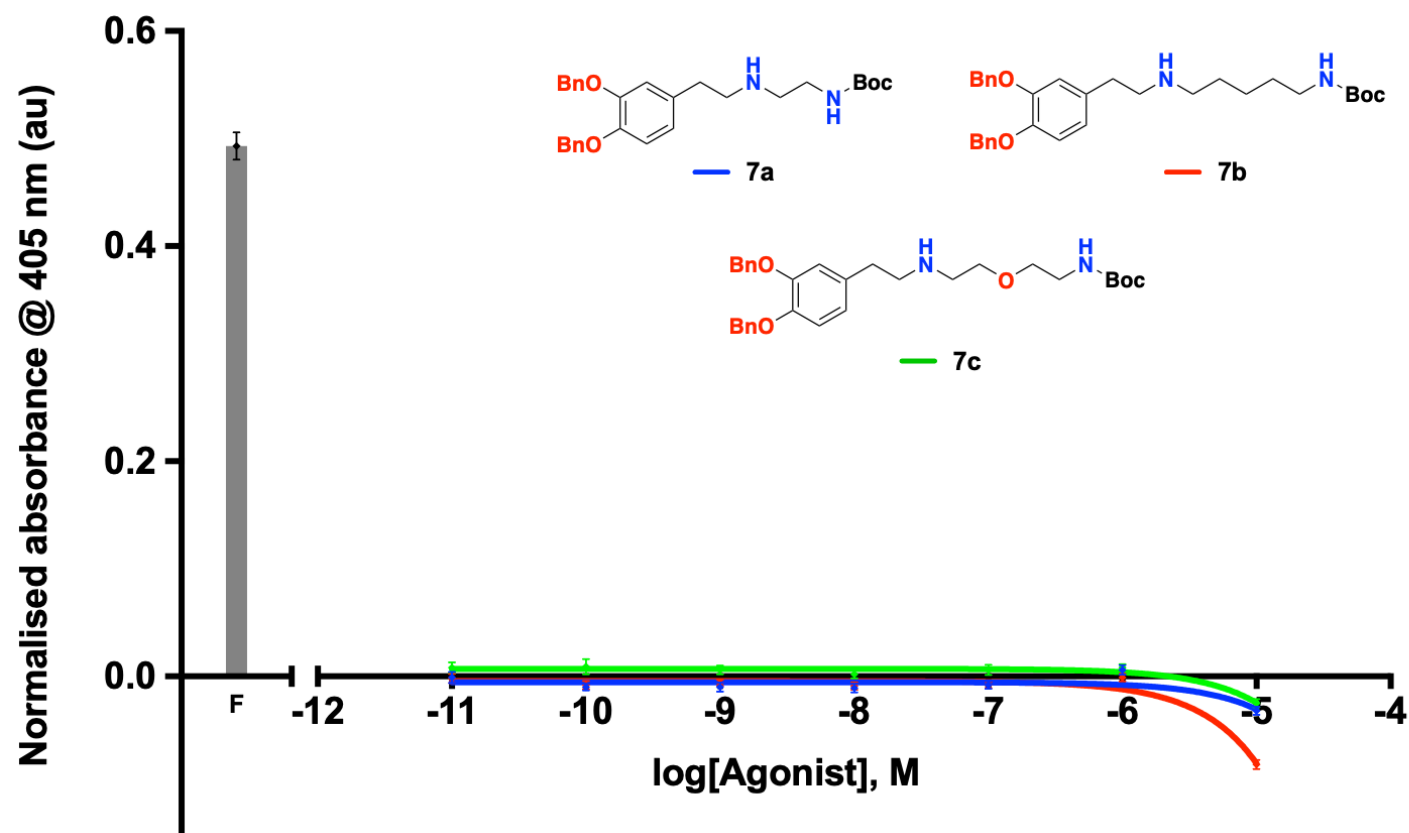


Figure 3-51 - Dose response of extended dopamine analogues **7a-c** for the dopamine D_1 secreted placental alkaline phosphatase assay. Positive control of forskolin is shown as F ($n=3$, $N=5$, error bars represent SEM)

Figure 3-53 shows the activity for compounds **8a-c**. Little activity is observed for each compound up to concentrations of 1×10^{-5} M. Higher concentrations were not investigated (in order to retain a range which contributed to a similar potency to dopamine as well as due to solubility of the molecule) and there are therefore insufficient data points to determine an EC_{50} value with confidence. This gives us an indication that the biogenic amine still plays a role in the activation of the receptor. This is either from the secondary amine that is formed within the reaction or the free terminal amino group still able to bind to the aspartic acid on the TM3 region. This can be visualised in Figure 3-52, where the extended chain fits into the binding pocket instead of the pharmacophore of dopamine. To determine this further, additional compounds would need to be synthesised and tested at the D_1 receptor. These compounds may relate to agonists which do not specifically exhibit the catechol, such as the ergoline derivatives.

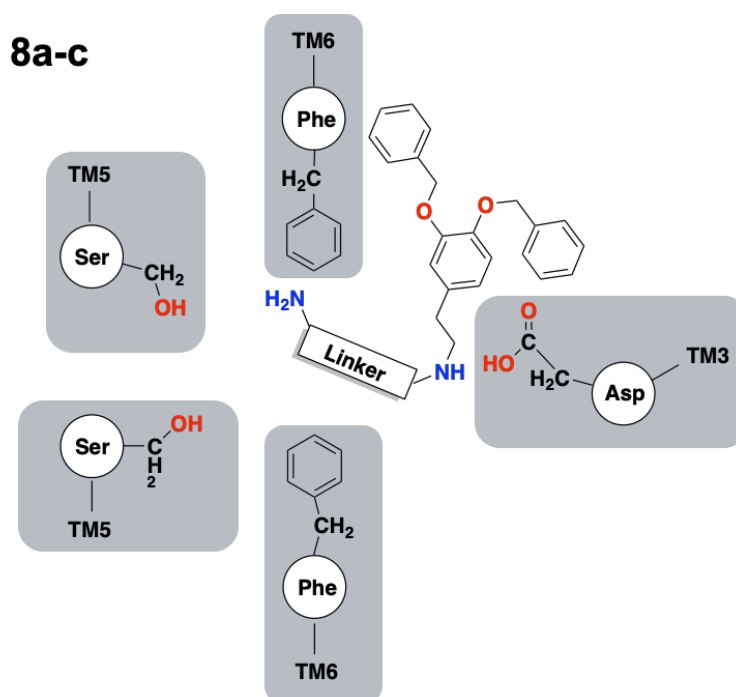


Figure 3-52 - Generic proposed binding interactions of compounds **8a-c** and the D_1 receptor. As shown, the amine is suspected to interact with the TM3 Asp¹⁰³ to generate low levels of SPAP in the reporter gene assay

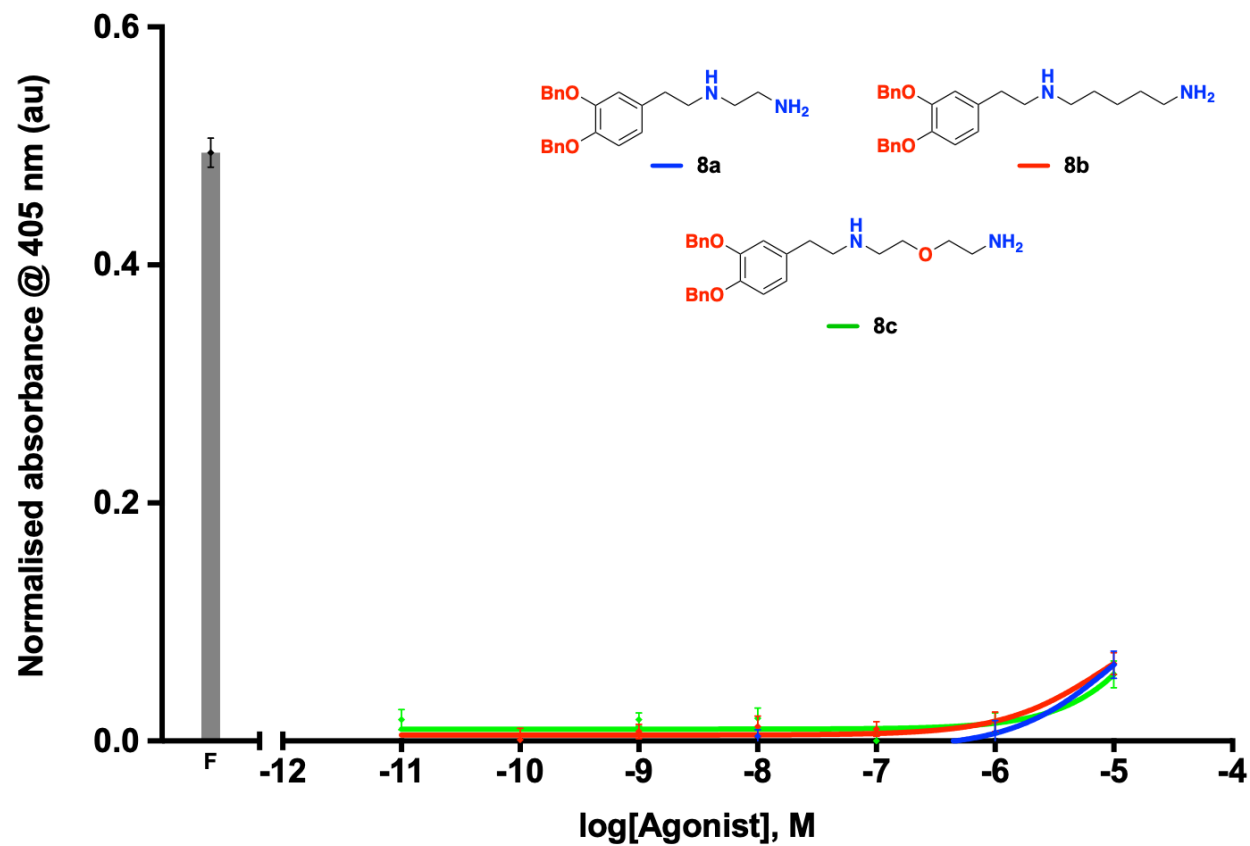


Figure 3-53 - Dose response of compounds **8a-c** for the dopamine D_1 secreted placental alkaline phosphatase assay. Positive control of forskolin is shown as F (n=3, N=5, error bars represent SEM)

Figure 3-54 shows the proposed binding of compounds **9b** and **9c**, whilst the activity from the gene reporter assay is shown in Figure 3-55. Successful activation of the D₁ receptor was shown, although activity was suppressed in comparison to the endogenous ligand. Calculations of the EC₅₀ values were once again limited, due to the data points obtained above basal level. A higher absorbance is observed for the aliphatic chain, over the aliphatic ether at a concentration of 1x10⁻⁵ M. This might be due to the flexibility in the linker chain, with changing the ability for the compound to rearrange and adapt to the binding pocket.

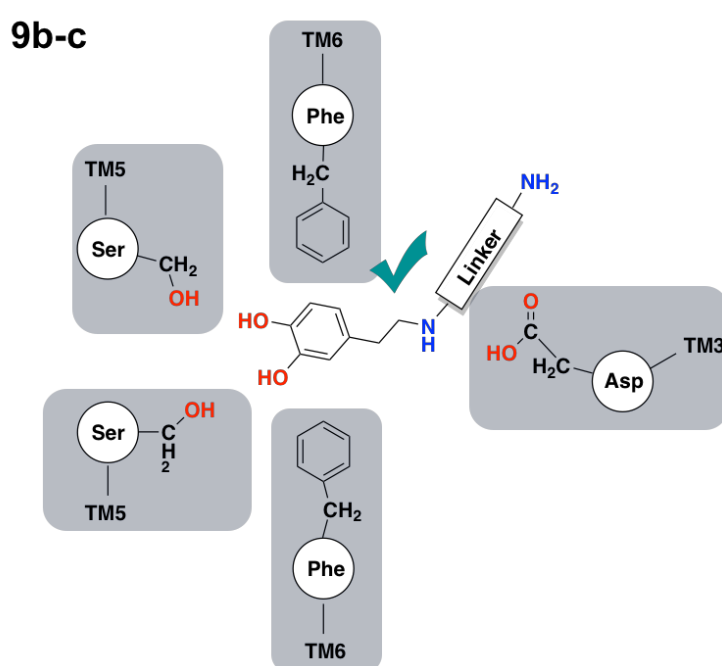


Figure 3-54 - Generic proposed binding interactions of compounds **9b-c** and the D₁ receptor. Catechol interacts with Ser¹⁹⁸ and Ser²⁰² (TM5) and secondary amine through hydrogen bonding with the Asp¹⁰ (TM3) to generate SPAP in the gene reporter assay

We can conclude modification of the amino moiety of dopamine retains activity at the D₁ receptor when modified as a secondary amine (**9b-c**) or as a carbamate (**2**), with the later at a higher potency. This is significant, and will allow us to use modifications at this site to control activity at the D₁ receptor when attached to a photo-responsive element, as well as being adhered on to a solid support system.

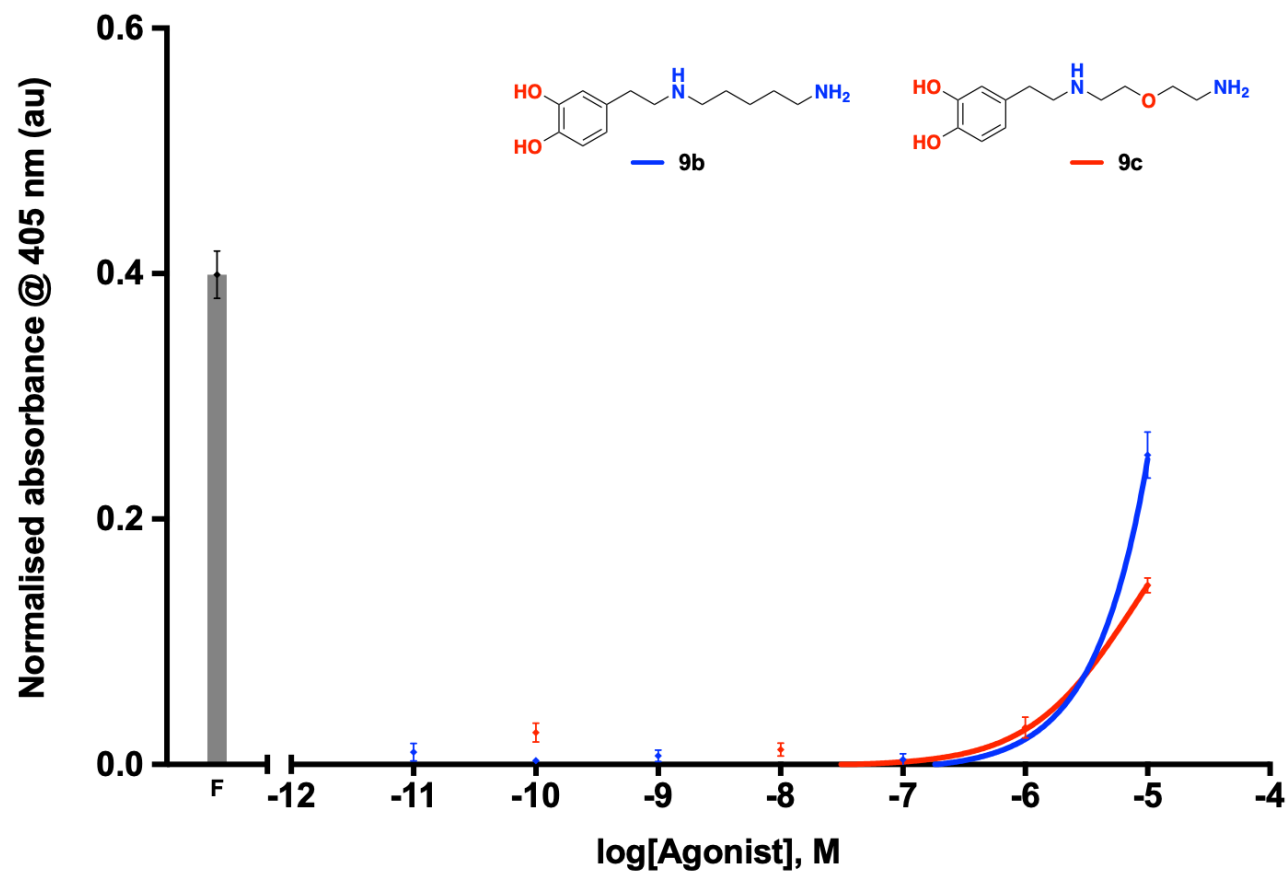


Figure 3-55 - Dose response of compounds **9b-c** for the dopamine D_1 secreted placental alkaline phosphatase assay. Positive control of forskolin is shown as F ($n=3$, $N=5$, error bars represent SEM)

Conclusion

A library of analogues was synthesised, characterised and tested for potency at the dopamine D₁ receptor using dopamine as a base structure. This was conducted in order to understand the key functional groups present on the endogenous ligand, and whether modification at specific sites caused suppression of potency at the D₁ receptor.

Successful modification of dopamine was achieved at both the amino and catechol moieties. Using Boc protection, the primary amino group was initially modified (**2**, 60%) to allow benzyl protection of the catechol (**3**, 84%). Deprotection of the Boc group (**4**, 96%), generated a compound which was used successfully for *N*-alkylation. *N*-Alkylation was conducted using three different linkers with varied length and chemistry, which were protected at one terminal (*N*-Boc) and activated for nucleophilic substitution at the other (*O*-Ts). This successfully generated *N*-Boc and *O*-Bn dopamine-linker analogues (**7a-c**, 24%, 49%, 46%). Generation of the Boc deprotected salts was achieved using a combination of hydrochloric acid and diethyl ether (**8a-c**, 98%, 96%, 97%).

The deprotection of the benzyl groups present on the catechol proved difficult when *N*-Boc protection was to be retained, hence universal deprotection of the *N*-alkylated dopamine analogues was conducted. Deprotection of the compounds was achieved under high pressure over a short time scale, although upon deprotection, the extended small chain dopamine analogue, oxidation was observed. This reduced the final compounds to the two long chain dopamine analogues, the 5-carbon aliphatic (**9b**, 64%) and aliphatic ether (**9c**, 48%) chains.

CRE-SPAP was used to identify the potency of the synthesised analogues at the D₁ receptor. It was shown that activity was affected when dopamine was modified at both and individually from the catechol and primary amino group.

No activity was observed for compounds **3** and **4**, whilst negative values below the basal level were observed for compounds **7a-c**. This observation allowed us to identify the importance of the catechol on dopamine, and the ability to retain activity at the D₁ receptor. We can identify the large benzyl protecting group may play a pivotal role restricting access into the binding pocket, as well as preventing the catechol interactions with the conserved serine residues found on the TM5 region (Ser¹⁹⁸, Ser²⁰²). Restrictions posed by the carbamate, and *N*-alkyl derivatives can also affect binding with the aspartate on the TM3 region (Asp¹⁰³) as identified by compounds **2** and **9b-c**, showing activity above 10⁻⁵ M. As the free catechol can interact with the serine residues, the synthesised analogues interact with the Asp¹⁰³ residue through hydrogen bonding, instead of the typical salt bridge formation like compound **1**.

Compounds **8a-c** show minor SPAP production when the catechol is protected at high concentrations. This was attributed to structural properties of the molecule, so that the linker and amino groups present, are still able interact with the Asp¹⁰³ residue resulting in some activity. Further studies are required to determine this, including the synthesis of alternate agonists which do not contain the catechol. These would be based on agonists such as the ergoline derivatives.

Activation of the D₁ receptor was evident when modification was conducted at the primary amino group using both a carbamate and through *N*-alkylation using small aliphatic/aliphatic ether chains. Although at reduced potency (as expected), modification from the amino groups was then used as an anchor point for both modification with a photo-responsive unit (Chapter 4), and also for surface immobilisation (Chapter 5) for the ability to control activity at the D₁ receptor.

Experimental

All Chemicals were purchased from Sigma Aldrich (England) unless specified and solvents were obtained from Fisher Scientific (England). All compounds were used as received without any further purification.

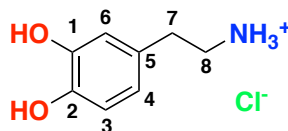
^1H NMR (400.26 MHz) and ^{13}C NMR (100.66 MHz) were conducted on a Bruker AVIII HD 400 MHz system with a 5 mm BBFO+ probe. Solvent peaks were used as reference values³⁰⁶, ^1H NMR: CDCl_3 at 7.26 ppm, $\text{DMSO-}d_6$ at 2.50 ppm, and CD_3OD at 3.31 ppm. ^{13}C NMR: CDCl_3 at 77.16 ppm, $\text{DMSO-}d_6$ at 39.52 ppm, and CD_3OD at 49.00 ppm.

Liquid chromatography mass spectrometry was performed on a Shimadzu LCMS-2020 system with MS2020 single quad mass spec in dual polarity mode. Using a C18 reverse phase column (50 x 2 mm), UV detection was conducted at two channels, 220 and 254 nm. A linear gradient 5% - 95% solvent B over 6 minutes was used for the short run, whilst an extended run lasted 15 minutes. Solvent A: 0.1% formic acid in water; solvent B: MeCN.

Chromatography - reaction courses and product mixtures were routinely monitored by TLC on silica gel (60, F_{254}) using ethyl acetate: hexane: triethylamine (**A**), methanol: dichloromethane: acetic acid (**B**) and toluene (**C**). Compounds were visualized with ninhydrin/ Δ , Iodine staining or just using UV light at 254 or 365 nm. Column chromatography was conducted using silica gel 60 angstrom pore size, 35-70 microns, with amino compounds eluted using neutralized silica pre-soaked in ethyl acetate with 1% triethylamine (Et_3N).

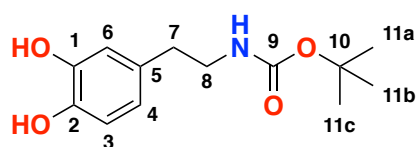
Synthesis and analysis of dopamine analogues

Dopamine - (2-(3,4-dihydroxyphenyl)ethylamine) hydrochloride (1)



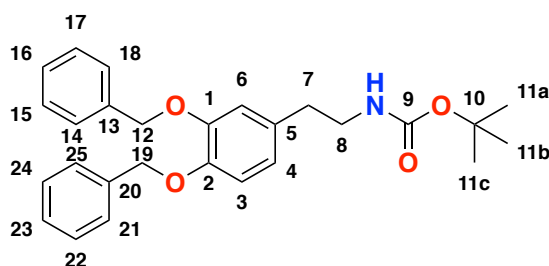
M.P. 127-128 °C; ^1H NMR (DMSO- d_6): δ 8.92 (s, OH (**10**), 1H), 8.84 (s, OH (**11**), 1H), , 8.06 (s, NH_3^+ , 3H), 6.68 (d, J = 8.0 Hz, Ar-H (**C3**), 1H), 6.63 (d, J = 2.0 Hz, Ar-H (**C6**), 1H), 6.47 (dd, J = 8.0, 2.1 Hz, Ar-H (**C4**), 1H), 2.90 (dt, J = 9.7, 6.4 Hz, $\text{CH}_2\text{-NH}$, 2H), 2.70 (dt, J = 9.6, 6.4 Hz, $\text{CH}_2\text{-Ar}$, 2H); ^{13}C NMR (101 MHz, DMSO- d_6): δ 145.3 (**C1**), 144.1 (**C2**), 127.9 (**C5**), 119.2 (**C4**), 116.1 (**C6**), 115.8 (**C3**), 40.3 (**C8**), 32.4 (**C7**); **LC-MS, Eluted at:** 0.4 min: m/z = $\text{C}_8\text{H}_{12}\text{NO}_2$ [$\text{M}+\text{H}$] $^+$ calcd 154.08; found 153.95, $\text{C}_{10}\text{H}_{15}\text{N}_2\text{O}_2$ [$\text{M}+\text{MeCN}+\text{H}$] $^+$ calcd 195.11; found 195.00, $\text{C}_{16}\text{H}_{23}\text{N}_2\text{O}_4$ [$2\text{M}+\text{H}$] $^+$ calcd 307.00; found 307.10, $\text{C}_8\text{H}_{10}\text{NO}_2$ [$\text{M}-\text{H}$] $^-$ calcd 152.07; found 152.05, $\text{C}_8\text{H}_9\text{KNO}_2$ [$\text{M}+\text{K}-2\text{H}$] $^-$ calcd 190.05; found 190.05, $\text{C}_{16}\text{H}_{20}\text{N}_2\text{O}_4$ [$2\text{M}-2\text{H}$] $^-$ calcd 304.90; found 304.90; R_f = 0.63 (**B**, 10:90:1)

Synthesis of *tert*-Butyl (3,4-dihydroxyphenethyl) carbamate (2)²⁸³ - A solution of dopamine hydrochloride (**1**) (3 g, 15.8 mmol) in methanol (40 ml) was prepared and stirred under N₂ for 5 minutes. Triethylamine (1.1 eq, 2.43 ml) was added to the solution followed by Boc-anhydride (1.1 eq, 3.79 g). The mixture was stirred under N₂ for 12 hours. The solvent was then removed under reduced pressure. The remaining residue was dissolved in CH₂Cl₂ (40 ml) and washed three times with 5 ml of each of 1.0 N HCl and brine. The organic layer was then dried over anhydrous magnesium sulfate. After filtration, the organic phase was reduced down to 5 ml, and triturated with cold hexane. A white precipitate was formed and collected by filtration. (2.419 g, 60%)



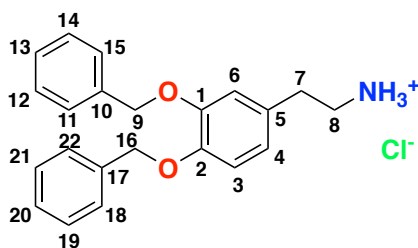
M.P. 136-138 °C; **¹H NMR** (Methanol-*d*₄) δ 6.67 (d, *J* = 8.0 Hz, Ar-H (**C3**), 1H), 6.63 (d, *J* = 2.1 Hz, Ar-H (**C6**), 1H), 6.51 (dd, *J* = 8.0, 2.1 Hz, Ar-H (**C4**), 1H), 3.25 – 3.13 (m, CH₂-NH, 2H), 2.59 (dd, *J* = 8.3, 6.6 Hz, CH₂-Ar, 2H), 1.52 (d, *J* = 2.4 Hz, NH, 1H), 1.42 (s, CH₃, 9H); **¹³C NMR** (Methanol-*d*₄) δ 158.4 (**C9**), 146.1 (**C1**), 144.6 (**C2**), 132.1 (**C5**), 121.0 (**C4**), 116.9 (**C6**), 116.3 (**C3**), 79.9 (**C10**), 43.4 (**C8**), 36.6 (**C7**), 28.8 (**C11a-c**); **LC-MS, Eluted at:** 2.37 min: *m/z* = C₁₃H₂₄N₂O₄ [**M+H+NH₄**]²⁺ calcd 136.08; found 136.95, C₉H₁₂NO₄ [**M-*tert*-Butyl+2H**]⁺ calcd 198.08; found 197.95, C₂₆H₃₉N₂O₈ [**2M+H**]⁺ calcd 507.26; found 507.25, C₁₃H₁₈NO₄ [**M-H**]⁻ calcd 252.12; found 252.00, C₁₄H₂₀NO₆ [**M+FA-H**]⁻ calcd 298.12; found 297.95, C₂₆H₃₇N₂O₈ [**2M-H**]⁻ calcd 505.25; found 505.15; R_f = 0.27 (**A**, 30:70:0)

Synthesis of *tert*-Butyl (3,4-*bis*(Benzyloxy)phenethyl) carbamate (3**)**²⁸¹ - *tert*-Butyl (3,4-dihydroxyphenethyl) carbamate (**2**) (2 g, 7.8 mmol) was dissolved in a round bottom flask coated with foil in DMF (50 ml). K₂CO₃ (6.65 eq, 7.25 g) was then added and the system was protected under N₂. Benzyl bromide (2.5 eq, 2.34 ml) was then added slowly to the solution. After stirring at room temperature for 24 hours, the resulting solid was filtered and washed with ether (100 ml). Filtrate collected, and washed with ice water (25 ml) and brine. Organic layer was dried over anhydrous magnesium sulfate and concentrated to 50 ml. After storage at -20 °C for 5 hours, a white precipitate formed and was collected through vacuum filtration (2.901 g, 84%)



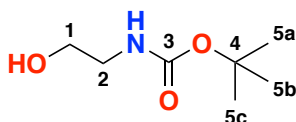
M.P. 107-109 °C; ¹H NMR (DMSO-*d*₆) δ 7.60 – 7.21 (m, Ar-H (OBn), 10H), 6.95 (d, *J* = 8.2 Hz, Ar-H (**C3**), 1H), 6.93 (d, *J* = 1.9 Hz, Ar-H (**C6**), 1H), 6.84 (t, *J* = 5.7 Hz, NH, 1H), 6.70 (dd, *J* = 8.2, 1.9 Hz, Ar-H (**C4**) 1H), 5.09 (d, *J* = 8.8 Hz, CH₂-(OBn), 4H), 3.20 – 3.00 (m, CH₂-NH, 2H), 2.60 (t, *J* = 7.5 Hz, CH₂-Ar, 2H), 1.37 (s, CH₃, 9H); ¹³C NMR (DMSO-*d*₆) δ 155.5 (**C9**), 148.2 (**C1**), 146.6 (**C2**), 137.5 (**C20**), 137.3 (**C13**), 132.6 (**C5**), 128.3 (**C22**, **C24**), 128.3 (**C15**), 128.2 (**C17**), 127.7 (**C16**), 127.6 (**C23**), 127.5 (**C14**, **21**), 127.4 (**C18**, **25**), 121.1 (**C4**), 115.1 (**C6**), 114.7 (**C3**), 77.4 (**C10**), 70.3 (**C12**), 70.1 (**C19**), 41.5 (**C8**), 34.9 (**C7**), 28.2 (**C11a-c**); **LC-MS**, **Eluted at:** 4.03 min: *m/z* = C₂₇H₃₁NNaO₄ [**M**+Na]⁺ calcd 456.21; found 456.15, C₂₉H₃₄N₂NaO₄ [**M**+MeCN+Na]⁺ calcd 497.24; found 497.20, C₅₄H₆₃N₂O₈ [**2M**+H]⁺ calcd 867.46; found 867.45, C₂₈H₃₂NO₆ [**M**+FA-H]⁻ calcd 478.00; found 478.10; R_f = 0.53 (**A**, 30:70:0)

Synthesis of 2-(3,4-bis(Benzyloxy)phenyl)ethan-1-amine (4)²⁸¹ - A solution of *tert*-Butyl (3,4-bis(Benzyloxy)phenethyl) carbamate (**3**) (0.5 g) in DCM (1.8 ml) was added to an excess of hydrochloric acid (2 M) in diethyl ether (10 ml). After stirring for 1 hour, a white solid was formed and collected using vacuum filtration. (0.41 g, 96%).



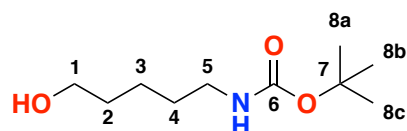
M.P. 160-162 °C; ¹H NMR (DMSO-*d*₆) δ 8.17 (s, NH₃⁺, 3H), 7.51 – 7.26 (m, Ar-H (OBn), 10H), 7.02 (d, *J* = 2.0 Hz, Ar-H (**C3**), 1H), 6.99 (d, *J* = 8.2 Hz, Ar-H (**C6**), 1H), 6.76 (dd, *J* = 8.2, 2.0 Hz, Ar-H (**C4**), 1H), 5.11 (d, *J* = 11.4 Hz, CH₂-(OBn), 4H), 2.98 (dd, *J* = 9.4, 6.4 Hz, CH₂-NH, 2H), 2.82 (dd, *J* = 9.4, 6.4 Hz, CH₂-Ar, 2H); ¹³C NMR (DMSO-*d*₆) δ 148.4 (**C1**), 147.1 (**C2**), 137.4 (**C10**), 137.3 (**C16**), 130.4 (**C5**), 128.3 (**C12**, **14**), 128.3 (**C19**, **21**), 127.8 (**C13**), 127.7 (**C20**), 127.6 (**C11**, **15**), 127.4 (**C18**, **22**), 121.2 (**C4**), 115.1 (**C6**), 114.7 (**C3**), 70.2 (**C9**, **16**), 39.2 (**C8**), 32.5 (**C7**); LC-MS, Eluted at: 2.07 min: *m/z* = C₂₂H₂₄NO₂ [**M**+H]⁺ calcd 334.17; found 334.10, C₂₄H₂₇N₂O₂ [**M**+MeCN+H]⁺ calcd 375.20; found 375.15, C₂₄H₂₆N₂NaO₂ [**M**+MeCN+Na]⁺ calcd 397.18; found 397.15, C₄₄H₄₇N₂O₄ [**2M**+H]⁺ calcd 667.34; found 667.35; R_f = 0.47 (**B**, 10:90:0)

Synthesis of *tert*-Butyl (2-hydroxyethyl)carbamate (5a)²⁸³ - A solution of ethanolamine (2 g, 32.7 mmol) in methanol (15 ml) was prepared and stirred under N₂ for 5 minutes. Et₃N (1.1 eq, 8.27 ml) was added to the solution followed by Boc-anhydride (1.1 eq, 3.64 g). The mixture was stirred under N₂ for 12 hours. The solvent was then removed under reduced pressure. The remaining residue was dissolved in CH₂Cl₂ (40 ml) and washed three times with 5 ml of each of 1.0 N HCl and brine. The organic layer was then dried over anhydrous magnesium sulfate. Solvent removed under reduced pressure to form a light-yellow oil (5.12 g, 97%).



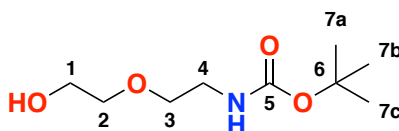
¹H NMR (DMSO-*d*₆) δ 6.66 (t, *J* = 5.8 Hz, NH, 1H), 4.56 (t, *J* = 5.6 Hz, OH, 1H), 3.35 (dd, *J* = 11.8, 5.8 Hz, CH₂-OH, 2H), 2.97 (q, *J* = 6.2 Hz, CH₂-NH, 2H), 1.37 (s, CH₃, 9H); ¹³C NMR (DMSO-*d*₆) δ 155.6 (C3), 77.4 (C4), 60.0 (C1), 42.6 (C2), 28.2 (C5a-c); R_f = 0.32 (A, 30:70:0)

Synthesis of *tert*-Butyl (5-hydroxypentyl) carbamate (5b)²⁸³ - A solution of 5-amino-1-pentanol (3 g, 29 mmol) in methanol (15 ml) was prepared and stirred under N₂ for 5 minutes. Et₃N (1.1 eq, 4.45 ml) was added to the solution followed by Boc-anhydride (1.1 eq, 6.98 g). The mixture was stirred under N₂ for 12 hours. The solvent was then removed under reduced pressure. The remaining residue was dissolved in CH₂Cl₂ (40 ml) and washed three times with 5 ml of each of 1 N HCl and brine. The organic layer was then dried over anhydrous magnesium sulphate. Solvent removed under reduced pressure to form a light-yellow viscous oil (5.21 g, 88%)



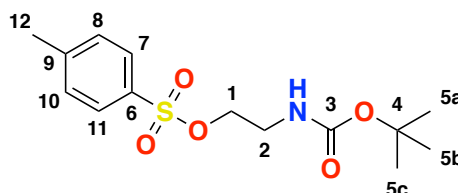
¹H NMR (DMSO-*d*₆) δ 6.74 (t, *J* = 5.7 Hz, NH, 1H), 4.32 (t, *J* = 5.2 Hz, OH, 1H), 3.36 (td, *J* = 6.5, 5.1 Hz, CH₂ (C1), 2H), 2.88 (td, *J* = 7.0, 5.8 Hz, CH₂-(C5), 2H), 1.37 (s, CH₂ (C2, 4), CH₃-(C8a-c), 13H), 1.30 – 1.18 (m, CH₂ (C3), 2H); ¹³C NMR (DMSO-*d*₆) δ 155.5 (C6), 77.2 (C7), 60.6 (C1), 32.2 (C5), 31.3 (C4), 29.4 (C2), 28.3 (C8a-c), 22.8 (C3); R_f = 0.31 (A, 50:50:0)

Synthesis of *tert*-Butyl (2-(2-hydroxyethoxy)ethyl)carbamate (5c)²⁸³ - A solution of 2-amino(ethoxy)ethanol (3 g, 28.5 mmol) in methanol (15 ml) was prepared and stirred under N₂ for 5 minutes. Et₃N (1.1 eq, 4.37 ml) was added to the solution followed by Boc-anhydride (1.1 eq, 6.85 g). The mixture was stirred under N₂ for 12 hours. The solvent was then removed under reduced pressure. The remaining residue was dissolved in CH₂Cl₂ (40 ml) and washed three times with 5 ml of each of 1.0 N HCl and brine. The organic layer was then dried over anhydrous magnesium sulfate. Solvent removed under reduced pressure to form a light-yellow oil (5.37 g, 91%)



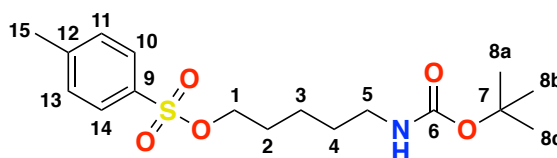
¹H NMR (DMSO-*d*₆) δ 6.76 (t, *J* = 5.8 Hz, NH, 1H), 4.56 (t, *J* = 5.5 Hz, OH, 1H), 3.51 – 3.44 (m, CH₂-(C1)-OH, 2H), 3.43 – 3.34 (m, CH₂-(C2, 3) 4H), 3.06 (q, *J* = 6.0 Hz, CH₂-(C4)-NH, 2H), 1.37 (s, CH₃, 9H); ¹³C NMR (DMSO-*d*₆) δ 155.6 (C5), 77.6 (C6), 72.0 (C2, 3), 69.2 (C1), 60.2 (C4), 28.2 (C7a-c); R_f = 0.73 (A, 70:30:0)

Synthesis of 2-((*tert*-Butoxycarbonyl)amino)ethyl 4-methylbenzenesulfonate (6a)³⁰⁷ - In a round bottom flask, *tert*-Butyl (2-hydroxyethyl) carbamate (**5a**) (2.079 g, 10.1 mmol) was dissolved in CH₂Cl₂ (10 ml) at 0 °C. Tosyl chloride (1.2 eq, 2.31 g) was then added followed by DMAP (1 mg). Et₃N (1.5 eq, 2.07 ml) was then added slowly to the mixture, which was stirred at 0 °C for 2 hours and a further 24 hours at room temperature. The resulting solution was then washed with 10% citric acid (2 x 25 ml) to remove excess Et₃N (low concentration to prevent removal of Boc protecting group) and NaHCO₃ (2 x 25 ml) to remove and hydroxylate the excess tosyl chloride, followed by brine (25 ml). Organic layers were combined and dried using magnesium sulfate, and then concentrated under reduced pressure obtaining a light-yellow oil. The oil was dissolved in minimal EtOAc and triturated with hexane until the formation of a cloudy suspension. The solution was then cooled at -20 °C for 1 hour, and then stirred until a white solid formed. Product was collected by vacuum filtration (2.198 g, 68%)



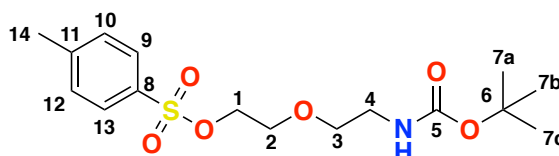
M.P. 64-66 °C; **¹H NMR** (DMSO-*d*₆) δ 7.77 (d, *J* = 7.9 Hz, CH-(**C7,11**), 2H), 7.48 (d, *J* = 8.0 Hz, CH-(**C8, 10**), 2H), 6.98 (t, *J* = 6.0 Hz, NH, 1H), 3.96 (t, *J* = 5.5 Hz, CH₂-(**C1**), 2H), 3.13 (q, *J* = 5.7 Hz, CH₂-(**C2**), 2H), 2.42 (s, CH₃-(**C12**), 3H), 1.33 (s, CH₃, 9H); **¹³C NMR** (DMSO-*d*₆) δ 155.5 (**C3**), 144.9 (**C6**), 132.2 (**C9**), 130.1 (**C8, 10**), 127.6 (**C7, 11**), 78.0 (**C4**), 69.1 (**C1**), 28.1 (**C5a-c**), 21.1 (**C2**); **LC-MS, Eluted at:** 3.07 min: *m/z* = C₁₄H₂₅N₂O₅S [**M+NH₄**]⁺ calcd 333.14; found 333.05, C₂₈H₄₃N₂O₁₀S₂ [**2M+H**]⁺ calcd 631.22; found 631.20; R_f = 0.33 (**A**, 30:70:0)

Synthesis of 5-((*tert*-Butoxycarbonyl)amino) pentyl 4-methylbenzenesulfonate (6b)³⁰⁷ - In a round bottom flask, the *tert*-Butyl (5-hydroxypentylcarbamate) (**5b**) (2 g, 9.8 mmol) was dissolved in CH₂Cl₂ (10 ml) at 0 °C. Tosyl chloride (1.2 eq, 2.25 g) was then added followed by DMAP (2 mg). Et₃N (1.5 eq, 2.01 ml) was then added slowly to the mixture, which was stirred at 0 °C for 2 hours and a further 24 hours at room temperature. The resulting solution was then washed with 10% citric acid (2 x 25 ml) to remove excess Et₃N (low concentration to prevent removal of Boc protecting group) and NaHCO₃ (2 x 25 ml) to remove and hydroxylate the excess tosyl chloride followed by brine (25 ml). Organic layers combined and dried using magnesium sulfate, and then concentrated under reduced pressure. Oil obtained re-dissolved in minimal ethyl acetate (EtOAc) and titrated with hexane until a cloudy suspension was obtained. Solution cooled at -20 °C for 1 hour, and then stirred until a white solid formed, which in turn was collected by vacuum filtration (0.77 g, 44%)



M.P. 48-50 °C; **¹H NMR** (DMSO-*d*₆) δ 7.78 (d, *J* = 7.9 Hz, Ar-H-(**C10, 14**) 2H), 7.48 (d, *J* = 7.9 Hz, Ar-H-(**C11, 13**), 2H), 6.74 (t, *J* = 5.9 Hz, NH, 1H), 3.98 (t, *J* = 6.3 Hz, CH₂-O, 2H), 2.83 (q, *J* = 6.5 Hz, CH₂-NH, 2H), 2.42 (s, CH₃-(**C15**), 3H), 1.54 (p, *J* = 6.6 Hz, CH₂-(**C13**), 2H), 1.36 (s, CH₃, 9H), 1.32 – 1.01 (m, CH₂-(**C2, 4**), 4H); **¹³C NMR** (DMSO-*d*₆) δ 155.5 (**C6**), 144.8 (**C9**), 132.5 (**C12**), 130.1 (**C11, 13**), 127.5 (**C10, 14**), 77.3 (**C7**), 70.9 (**C1**), 28.7 (**C5**), 28.2 (**C8a-c**), 27.8 (**C4**), 22.1 (**C3**), 21.1 (**C15**); **LC-MS, Eluted at:** 3.40 min: *m/z* = C₃₄H₅₅N₂O₁₀S₂ [**2M+H**]⁺ calcd 715.32; found 715.30, C₁₃H₁₉NO₅S [**M-*tert*-Butyl+H**]⁺ calcd 301.10; found 301.95; C₁₂H₁₉NO₃S [**M-Boc+H**]⁺ calcd 257.11; found 257.95; R_f = 0.35 (**A**, 30:70:0)

Synthesis of 2-(2-((*tert*-Butoxycarbonyl)amino)ethoxy)ethyl 4-methylbenzenesulfonate (6c)³⁰⁷ - In a round bottom flask, *tert*-Butyl (2-(2-hydroxyethoxy)ethyl)carbamate (**5c**) (3.163 g, 15.4 mmol) was dissolved in CH₂Cl₂ (15 ml) at 0 °C. Tosyl chloride (1.2 eq, 3.52 g) was then added followed by DMAP (crystal). Et₃N (1.5 eq, 3.15 ml) was then added slowly to the mixture, which was stirred at 0 °C for 2 hours and a further 24 hours at room temperature. The resulting solution was then washed with 10% citric acid (2 x 25 ml) to remove excess Et₃N (low concentration to prevent removal of Boc protecting group) and NaHCO₃ (2 x 25 ml) to remove and hydroxylate the excess tosyl chloride followed by brine (25 ml). Organic layers were combined and dried using magnesium sulfate, and then concentrated under reduced pressure to form a light-yellow oil (3.934 g, 71%).



¹H NMR (DMSO-*d*₆) δ 7.85 – 7.71 (m, Ar-H-(**C10**, **12**), 2H), 7.48 (d, *J* = 8.1 Hz, Ar-H-(**C9**, **13**), 2H), 6.73 (t, *J* = 5.8 Hz, NH, 1H), 4.15 – 4.05 (m, CH₂-(**C3**), 2H), 3.61 – 3.46 (m, CH₂-(**C1**), 2H), 3.34 – 3.27 (m, CH₂-(**C2**), 2H), 3.00 (q, *J* = 6.0 Hz, CH₂-NH, 2H), 2.42 (s, CH₃-(**C14**), 3H), 1.36 (s, CH₃, 9H); ¹³C NMR (DMSO-*d*₆) δ 155.5 (**C5**), 144.9 (**C8**), 132.4 (**C11**), 130.1 (**C10**, **12**), 127.6 (**C9**, **13**), 77.6 (**C6**), 69.9 (**C3**), 69.1 (**C2**), 67.5 (**C1**), 39.4 (**C4**), 28.2 (**C7a-c**), 21.1 (**C14**); LC-MS, Eluted at: 3.10 min: *m/z* = C₃₂H₅₀N₂O₁₂S₂Na [2M+Na]⁺ calcd 741.26; found 741.25, C₂₇H₄₃N₂O₁₀S₂ [2M-Boc+H]⁺ calcd 619.28; found 619.20, C₁₂H₁₇NO₆S [M-*tert*-Butyl+H]⁺ calcd 303.08; found 303.90, C₁₇H₁₉NO₅S [M+FA-H]⁻ calcd 404.13; found 403.95; R_f = 0.54 (A, 50:50:0)

N-Alkylation

A CEM discover (microwave synthesizer) was used for the microwave reactions. General parameters were set to 250 W of microwave power, whilst time and temperature were adjusted according to optimisation procedure. A 5 ml microwave glass vial with vacuum seal lid was used in all experiments.

Optimisation of N-alkylation reactions:

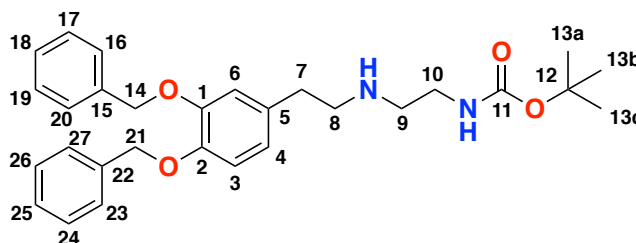
(2-((*tert*-Butoxycarbonyl)amino)ethyl 4-methylbenzenesulfonate (**6a**, 20 mg), and 2-(3,4-*bis*(Benzyloxy)phenyl)ethan-1-amine hydrochloride (**4**, 11.75 mg) were placed in a microwave vial (5 ml) with dry acetonitrile (250 μ l). The chosen base for the reaction was added at 2 equivalences to compound **4** which corresponded to potassium carbonate (8.22 mg), DIPEA (11.06 μ l) and caesium carbonate (20.70 mg). Reactions were conducted with the following procedure for 5 minutes at 40, 80 and 120 °C.

Analysis was conducted using LCMS, diluting 50 μ l of the reaction mixture further into 200 μ l of acetonitrile for analysis. The peak area of the given product was normalized by total count and compared to determine optimal conditions.

Reactions were conducted as above, at 80 °C for 5, 10, 30, 45 and 60 minutes using caesium carbonate as the base. Optimal conditions were found to be at 80 °C for 45 minutes, and were used for the synthesis of the fully protected dopamine linked analogues as shown below.

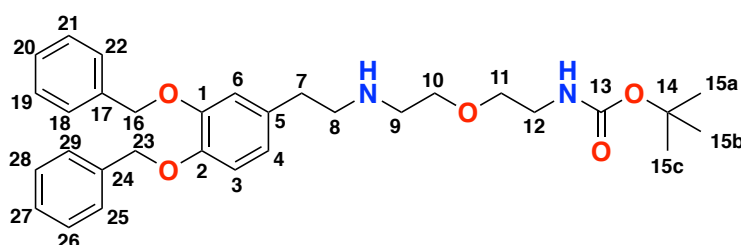
***tert*-Butyl (2-((3,4-*bis*(Benzyloxy)phenethyl)amino)ethyl)carbamate (7a) -**

(2-((*tert*-Butoxycarbonyl)amino)ethyl 4-methylbenzenesulfonate (6a) (240 mg), caesium carbonate (248 mg) and 2-(3,4-*bis*(Benzyloxy)phenyl)ethan-1-amine hydrochloride (4) (141 mg) were placed in a microwave vial (5 ml) with dry acetonitrile (3 ml). Reaction was conducted at 80 °C for 45 minutes, to which then the reaction mixture was concentrated using reduced pressure. Column chromatography (EtOAc (99%): Et₃N (1%)) was used to isolate the product and 2-oxazolidinone side product. The side product was isolated through recrystallization using toluene and petroleum ether, whilst the product remained in solution. After evaporation of the solvent, the product was obtained as a light yellow solid. (45 mg, 24%)



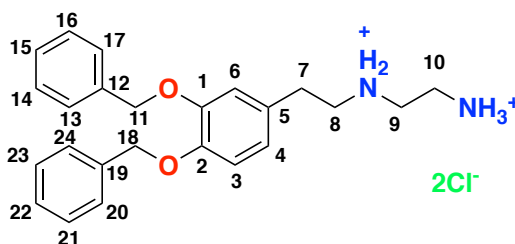
¹H NMR (Chloroform-*d*) δ 7.48 – 7.27 (m, Ar-H (OBn), 10H), 6.87 (d, *J* = 8.1 Hz, Ar-H-(C3), 1H), 6.78 (d, *J* = 2.0 Hz, Ar-H-(C6) 1H), 6.70 (dd, *J* = 8.1, 2.1 Hz, Ar-H-(C4), 1H), 5.14 (d, *J* = 9.2 Hz, CH₂-(C14, 21) 4H), 4.87 (s, NH-(CH₂)₂, 1H), 3.16 (q, *J* = 5.8 Hz, CH₂-(C10) 2H), 2.83 – 2.75 (m, CH₂-(C8), 2H), 2.68 (dt, *J* = 6.0, 2.6 Hz, CH₂-(C7, 9), 4H), 1.44 (s, CH₃, 9H); ¹³C NMR (Chloroform-*d*) δ 156.2 (C11), 149.0 (C1), 147.6 (C2), 137.6 (C15), 137.5 (C22), 133.5 (C5), 128.6 (C17, 19, 24, 26), 127.9 (C18, 25), 127.5 (C16, 20, 23, 27), 121.7 (C4), 116.0 (C6), 115.5 (C3), 79.3 (C12), 71.7 (C14), 71.5 (C21), 50.8 (C8), 49.1 (C9), 40.4 (C10), 35.9 (C7), 28.6 (C13a-c); LC-MS, Eluted at: 2.40 min: *m/z* = C₂₉H₃₇N₂O₄ [M+H]⁺ calcd 477.27; found 477.25, C₂₅H₂₉N₂O₄ [M-*tert*-Butyl+H]⁺ calcd 421.25; found 421.15, C₂₄H₂₉N₂O₂ [M-Boc+H]⁺ calcd 377.25; found 377.10; R_f = 0.25 (A, 99:0:1)

Synthesis of *tert*-Butyl (2-(2-((3,4-*bis* (Benzyloxy)phenethyl)amino)ethoxy)ethyl carbamate (7c) - 2-(2-((*tert*-Butoxycarbonyl) amino)ethoxy)ethyl 4-methylbenzenesulfonate (6c) (274 mg), caesium carbonate (248 mg) and 2-(3,4-*bis*(Benzyloxy)phenyl)ethan-1-amine hydrochloride (4) (141 mg) were placed in a microwave vial (5 ml) with dry acetonitrile (3 ml). Reaction was conducted at 80 °C for 45 minutes, to which then the reaction mixture was concentrated using reduced pressure. Column chromatography (EtOAc (99%): Et₃N (1%)) was used to isolate the product. Fractions of the product were then pooled and evaporated to dryness under reduced pressure. The product was obtained as a white solid. (93 mg, 46%)



¹H NMR (DMSO-*d*₆) δ 7.49 – 7.26 (m, Ar-H (OBn), 10H), 6.97 – 6.92 (m, Ar-H- (C3, 6), 2H), 6.81 (t, *J* = 5.7 Hz, NH, 1H), 6.71 (dd, *J* = 8.1, 2.0 Hz, Ar-H-(C4), 1H), 5.09 (d, *J* = 9.6 Hz, CH₂-(C16, 23), 4H), 3.42 (t, *J* = 5.6 Hz, CH₂-(C11) 2H), 3.35 (t, *J* = 6.0 Hz, CH₂-(C10), 2H), 3.05 (q, *J* = 5.9 Hz, CH₂-(C12), 2H), 2.77 – 2.53 (m, CH₂-(C7, 8, 9), 6H), 1.37 (s, CH₃, 9H); **¹³C NMR** (DMSO-*d*₆) δ 155.6 (C13), 148.2 (C1), 146.5 (C2), 137.5 (C17), 137.4 (C24), 133.4 (C5), 128.3 (C19, 21, 26, 28), 127.7 (C20), 127.6 (C27), 127.5 (C18, 22), 127.4 (C25, 29), 121.0 (C4), 115.1 (C6), 114.7 (C3), 77.6 (C14), 70.2 (C16), 70.1 (C23), 69.6 (C10), 69.0 (C11), 51.0 (C8), 48.5 (C9), 39.7 (C12), 35.3 (C7), 28.2 (C15a-c); **LC-MS, Eluted at:** 2.35 min: ***m/z*** = C₃₁H₄₁N₂O₅ [M+H]⁺ calcd 521.29; found 521.30, C₂₇H₃₃N₂O₅ [M-*tert*-Butyl+H]⁺ calcd 465.23; found 465.15, C₂₇H₃₃N₂O₅ [M-Boc+H]⁺ calcd 421.24; found 421.15, C₃₂H₄₃N₂O₇ [M+FA-H]⁻ calcd 565.28; found 565.05; R_f = 0.22 (A, 99:0:1)

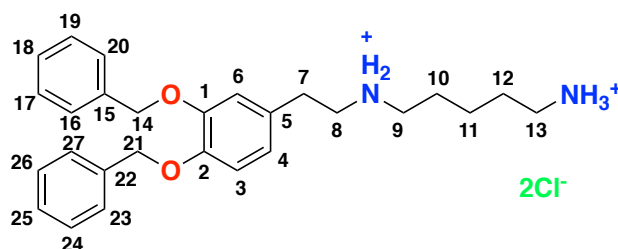
Synthesis of N^1 -(3,4-bis(Benzyloxy)phenethyl)ethane-1,2-diamine dihydrochloride (8a) - 7a (100 mg) was dissolved in methanol (1 ml) in a round bottom flask equipped with a magnetic stirrer. To the flask, diethyl ether. HCl (2 M, 5 ml) was added. After one hour, the solvent was evaporated and the process repeated. The precipitated product was then collected and dried to obtain the desired products as a white dihydrochloride salt. (93 mg, 98%)



^1H NMR (Methanol- d_4) δ 7.51 – 7.26 (m, Ar-H (OBn), 10H), 7.04 (d, J = 2.0 Hz, Ar-H (C3), 1H), 7.01 (d, J = 8.2 Hz, Ar-H (C6) 1H), 6.85 (dd, J = 8.2, 2.0 Hz, Ar-H (C4) 1H), 5.13 (d, J = 14.3 Hz, CH_2 -(C11, 18) 4H), 3.35 (dd, J = 5.4, 1.9 Hz, CH_2 -(C8, 9) 4H), 3.32 – 3.23 (m, CH_2 -(C7), 2H), 2.98 (dd, J = 8.7, 6.9 Hz, CH_2 -(C10), 2H); ^{13}C NMR (Methanol- d_4) δ 149.2 (C1), 148.2 (C2), 137.3 (C12, 19), 129.3 (C5), 128.1 (C14, 16, 21, 23), 127.5 (C15, 22), 127.3 (C13, 17, 20, 24), 121.6 (C4), 115.7 (C6), 115.5 (C3), 71.1 (C11, 18), 49.2 (C9), 44.3 (C8), 35.4 (C10), 31.5 (C7); LC-MS, Eluted at: 1.6 min: m/z = $\text{C}_{24}\text{H}_{29}\text{N}_2\text{O}_2$ [$\text{M}+\text{H}$] $^+$ calcd 377.33; found 377.10, $\text{C}_{48}\text{H}_{57}\text{N}_4\text{O}_4$ [$2\text{M}+\text{H}$] $^+$ calcd 753.44; found 753.40.

***N*¹-(3,4-bis(Benzyloxy)phenethyl)pentane-1,5-diamine dihydrochloride (8b)**

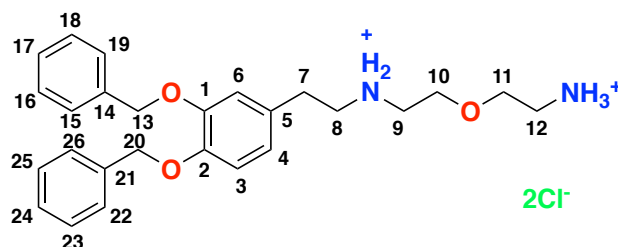
– **7b** (100 mg) was dissolved in methanol (1 ml) in a round bottom flask equipped with a magnetic stirrer. To the flask, diethyl ether. HCl (2 M, 5 ml) was added. After one hour, the solvent was evaporated and the process repeated. The precipitated product was then collected and dried to obtain the desired products as a white dihydrochloride salt. (91 mg, 96%)



¹H NMR (DMSO-*d*₆) δ 9.10 (s, NH₂⁺, 2H), 8.01 (s, NH₃⁺, 3H), 7.51 – 7.24 (m, Ar-H (OBn), 10H), 7.02 (d, *J* = 2.0 Hz, Ar-H (**C3**), 1H), 7.00 (d, *J* = 8.2 Hz, Ar-H (**C6**) 1H), 6.77 (dd, *J* = 8.2, 2.0 Hz, Ar-H (**C4**), 1H), 5.11 (d, *J* = 8.2 Hz, CH₂-(**C14**, **21**) 4H), 3.07 (s, CH₂-(**C8**), 2H), 2.89 (dd, *J* = 10.4, 6.4 Hz, CH₂-(**C7**, **13**) 4H), 2.77 (s, CH₂-(**C9**), 2H), 1.61 (dq, *J* = 29.5, 7.7 Hz, CH₂-(**C10**, **12**), 4H), 1.38 (p, *J* = 7.6 Hz, CH₂-(**C11**) 2H); ¹³C NMR (DMSO-*d*₆) δ 148.4 (**C1**), 147.1 (**C2**), 137.3 (**C15**, **22**), 130.2 (**C5**), 128.4 (**C17**, **19**, **24**, **26**), 127.7 (**C18**, **25**), 127.5 (**C16**, **20**, **23**, **27**), 121.2 (**C4**), 115.1 (**C6**), 114.8 (**C3**), 70.2 (**C14**, **21**), 47.7 (**C9**), 46.3 (**C8**), 38.3 (**C13**), 31.0 (**C7**), 26.3 (**C12**), 24.7 (**C10**), 22.8 (**C11**); LC-MS, Eluted at: 1.7 min: *m/z* = C₂₉H₃₉N₃O₂ [M+MeCN+2H]²⁺ calcd 230.65; found 230.60, C₂₇H₃₅N₂O₂ [M+H]⁺ calcd 419.26; found 419.20.

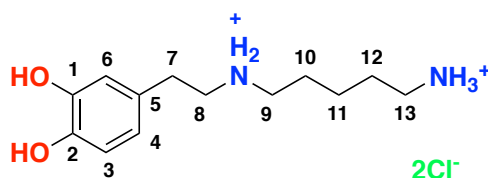
2-(2-Aminoethoxy)-N-(3,4-bis(Benzyloxy)phenethyl)ethan-1-amine

dihydrochloride (8c) – 7c (100 mg) was dissolved in methanol (1 ml) in a round bottom flask equipped with a magnetic stirrer. To the flask, diethyl ether. HCl (2 M, 5 ml) was added. After one hour, the solvent was evaporated and the process repeated. The precipitated product was then collected and dried to obtain the desired products as a white dihydrochloride salt. (92 mg, 97%)



¹H NMR (DMSO-*d*₆) δ 9.26 (s, NH₂⁺, 2H), 8.28 (s, NH₃⁺, 3H), 7.52 – 7.24 (m, Ar-H (OBn), 10H), 7.04 (d, *J* = 2.0 Hz, Ar-H (C3) 1H), 7.00 (d, *J* = 8.2 Hz, Ar-H (C6) 1H), 6.78 (dd, *J* = 8.2, 2.0 Hz, Ar-H (C4), 1H), 5.11 (d, *J* = 9.0 Hz, CH₂-(C13, 20) 4H), 3.75 – 3.69 (m, CH₂-(C11) 2H), 3.65 (dd, *J* = 5.6, 4.3 Hz, CH₂-(C10), 2H), 3.13 (q, *J* = 5.7 Hz, CH₂-(C8, 12), 4H), 2.99 (dq, *J* = 11.7, 6.9, 5.7 Hz, CH₂-(C7,9), 4H); **¹³C NMR** (DMSO-*d*₆) δ 148.9 (C1), 147.5 (C2), 137.8 (C14), 137.7 (C21), 130.8 (C5), 128.8 (C16, 18, 23, 25), 128.3 (C24), 128.2 (C17), 128.1 (C15, 19), 127.9 (C22, 26), 121.6 (C4), 115.6 (C6), 115.3 (C3), 70.7 (C13, 20), 66.8 (C10), 65.6 (C11), 48.2 (C8), 46.7 (C9), 39.1 (C12), 31.3 (C7); **LC-MS, Eluted at:** 1.17 min: ***m/z*** = C₂₈H₃₇N₃O₃ [M+MeCN+2H]²⁺ calcd 231.64; found 231.50, C₂₆H₃₃N₂O₃ [M+H]⁺ calcd 421.24; found 421.15, C₅₂H₆₅N₄O₆ [2M+H]⁺ calcd 841.48; found 841.48, C₂₆H₃₂ClN₂O₃⁻ [M+Cl]⁻ calcd 455.20; found 455.00.

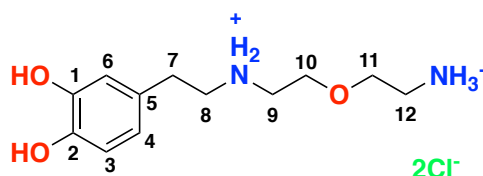
4-(2-((5-Aminopentyl)amino)ethyl)benzene-1,2-diol (9b) – 8b (40 mg) was placed in a high-pressure tube, equipped with a stirrer bar. A solution of methanol (5 ml) with palladium on carbon (10% w/w) (4 mg) was added. Sealed within the high-pressure chamber, the vessel was flushed with hydrogen twice and then set to a pressure of 100 psi. The mixture was stirred overnight. Contents were then transferred using a syringe into an argon back filled round bottom flask with suba-seal®. Palladium was removed through Celite using methanol to filter through the compound. Solvent was then removed under reduced pressure to yield a white crystalline solid. (11 mg, 64%)



^1H NMR (Methanol- d_4) δ 6.74 (d, J = 8.0 Hz, Ar-H (**C3**), 1H), 6.71 (d, J = 2.1 Hz, Ar-H (**C6**), 1H), 6.60 (dd, J = 8.0, 2.2 Hz, Ar-H (**C4**), 1H), 3.19 (dd, J = 9.2, 6.7 Hz, CH_2 -(**C7**), 2H), 3.07 – 2.98 (m, CH_2 -(**C9**), 2H), 2.95 (t, J = 7.7 Hz, CH_2 -(**C13**), 2H), 2.86 (dd, J = 9.2, 6.7 Hz, CH_2 -(**C8**), 2H), 1.80 – 1.65 (m, CH_2 -(**C10**, **12**), 3H), 1.48 (p, J = 7.7 Hz, CH_2 -(**C11**), 2H); ^{13}C NMR (Methanol- d_4) δ 145.4 (**C1**, **2**), 127.5 (**C5**), 119.6 (**C4**), 115.4 (**C6**), 115.3 (**C3**), 49.0 (**C9**), 48.1 (**C8**), 39.0 (**C13**), 31.4 (**C7**), 26.6 (**C12**), 25.3 (**C10**), 23.1 (**C11**); LC-MS, Eluted at: 0.33 min: m/z = $\text{C}_{17}\text{H}_{30}\text{N}_4\text{O}_2$ [$\text{M}+2\text{MeCN}+2\text{H}$] $^{2+}$ calcd 161.11; found 161.00, $\text{C}_{13}\text{H}_{23}\text{N}_2\text{O}_2$ [$\text{M}+\text{H}$] $^+$ calcd 237.16; found 236.95, $\text{C}_{13}\text{H}_{21}\text{N}_2\text{O}_2$ [$\text{M}-\text{H}$] $^-$ calcd 237.16; found 236.95, $\text{C}_{26}\text{H}_{43}\text{N}_4\text{O}_4$ [$2\text{M}-\text{H}$] $^-$ calcd 475.33; found 475.15.

4-(2-((2-(2-Aminoethoxy)ethyl)amino)ethyl)benzene-1,2-diol (9c) –

8c (40 mg) was placed in a high-pressure tube, equipped with a stirrer bar. A solution of methanol (5 ml) with palladium on carbon (10% w/w) (4 mg) was added. Sealed within the high-pressure chamber, the vessel was flushed with hydrogen twice and then set to a pressure of 100 psi. The mixture was stirred overnight. Contents were then transferred using a syringe into an argon back filled round bottom flask with suba-seal®. Palladium was removed through Celite using methanol to filter through the compound. Solvent was then removed under reduced pressure to yield a white crystalline solid. (11 mg, 48%)



¹H NMR (Methanol-*d*₄) δ 6.76 (s, Ar-H (**C3**), 1H), 6.74 (d, Ar-H (**C6**), 1H), 6.63 (dd, *J* = 8.0, 2.0 Hz, Ar-H (**C4**), 1H), 3.83 (t, *J* = 4.8 Hz, CH₂-(**C11**), 2H), 3.78 (t, *J* = 4.9 Hz, CH₂-(**C10**), 2H), 3.37 (s, CH₂-(**C12**), 2H), 3.30 (t, *J* = 4.9 Hz, CH₂-(**C8**), 2H), 3.22 (q, *J* = 4.7 Hz, CH₂-(**C9**), 2H), 2.97 (dd, *J* = 9.9, 6.2 Hz, CH₂-(**C7**), 2H); **¹³C NMR** (Methanol-*d*₄) δ 146.6 (**C1**), 145.5 (**C2**), 129.2 (**C5**), 121.0 (**C4**), 116.8 (**C6**), 116.7 (**C3**), 67.8 (**C11**), 66.8 (**C10**), 50.5 (**C8**), 49.4 (**C9**), 40.5 (**C12**), 32.6 (**C7**); **LC-MS, Eluted at:** 0.35 min: *m/z* = C₁₄H₂₅N₃O₃ [M+MeCN+2H]²⁺ calcd 141.59; found 141.25, C₁₂H₂₁N₂O₃ [M+H]⁺ calcd 241.15; found 241.00, C₁₂H₁₉N₂O₃ [M-H]⁻ calcd 238.16; found 238.90, C₁₂H₂₀ClN₂O₃ [M+Cl]⁻ calcd 275.11; found 274.90.

Biological procedures

Chinese hamster ovary cell culture (dopamine D₁)

Cells were generously donated by Prof. Jillian Baker (Faculty of Medicine & Health Sciences, University of Nottingham) at passage 12. Cells were cultured in complete media (DMEM-F12 with 10% fetal calf serum (FCS) and L-glutamine (5 mM)) at 37 °C in an atmosphere of 5% CO₂ until 70% confluent in an uncoated T75 tissue culture flask. After removing the media, cells were washed with PBS (5 ml) and incubated with trypsin (1 ml) for approximately 3 minutes to detach the cells from the flask. Cells were then transferred to a universal tube (30 ml) containing fresh complete media (10 ml). Centrifuged for 5 minutes at 1000 rpm, the supernatant was discarded.

Three procedures that were conducted after centrifugation are as followed. i) split, ii) prepare for the SPAP assay, and iii) to store under liquid nitrogen.

- i) Cells were re-suspended in fresh complete media (10 ml), and were then split 1:10 into uncoated T75 culture medium flasks with fresh complete media (20 ml) until 70% confluency and process repeated as above until passage 20.
- ii) Cells were re-suspended in fresh complete media (10 ml) and cells were counted using a haemocytometer. For one 96 well plate, cell suspension was diluted to 35000 cells/ 100 µl. 100 µl was then dispensed into each well of a clear 96 well plate using an Eppendorf multipipette fitted with a 10 ml tip, set to 0.5. Cells were then incubated at 37 °C in an atmosphere of 5% CO₂ for 24 hours, and then serum starved for a further 16 hours using fresh serum free media (DMEM-F12 with L-glutamine (5 mM), 100 µl).
- iii) Pellet was suspended in DMSO and FCS with 1 ml transferred to a Nalgene cryo vial. This vial was then placed in a Mr. Frosty at -80 °C overnight before being stored under liquid nitrogen.

Secreted placental alkaline phosphatase (SPAP) assay

Dilutions of the synthesised compounds were made at 10 mM in DMSO resulting in less than 0.01% of DMSO for the final concentrations in the assay and with a concentration range of 10^{-5} to 10^{-11} M. A stock solution of forskolin (10^{-3} M) was used as a positive control and basal levels were also monitored as a baseline for SPAP production without any agonists. A stock solution of ascorbic acid was made up in double distilled water (ddH₂O) at 7 mg/ml. For an individual plate, 10 µl was then added to fresh serum free media (10 ml). Aspiration of the media found in the clear 96 well plates was then conducted, replacing the media with 90 µl of the ascorbic acid doped serum free media.

10 µl of the diluted compounds were then incubated for 4-hours, at 37 °C, 5% CO₂. After the 4-hour period, plates were removed from the incubator, each well aspirated and replaced with 40 µl of pre-warmed, non-doped fresh serum free media and placed back in the incubator for a further hour. After completion of this stage, the 96 well plates were placed in an oven at 65 °C for 30 minutes. *p*-Nitrophenol phosphate (1.85 g) was then prepared in diethanolamine (DEA) buffer (50 ml), and was added to the plates once they had cooled to room temperature (100 µl per well). Plates were then placed in an incubator at 37 °C without CO₂ for 20 minutes until a yellow colour developed. Plates were run on a colorimetric plate reader at 405 nm to obtain details on the level of SPAP per well. Analysis of the SPAP assay was conducted using Graphpad prism using a non-linear regression fitting, stimulatory [agonist] vs response (three variables). Prior to this, each 96 well plate was normalised using the basal control on the plate.

Chapter 4 - Spiropyran for the use in Photo-pharmacology

Introduction

The field of photo-pharmacology has been of interest over the last 20 years³⁰⁸, but has come into focus in the last 5 years with advancements in the use of photo-responsive compounds.^{309,310} In theory, we can control pharmaceuticals remotely using photo-labile protecting groups on ligands, or by using responsive compounds to control the activity of a compound at specific receptors in a dynamic manner.

Photo-pharmacology can provide a much better spatio-temporal resolution for the introduction and delivery of drugs than conventional methods. This is through the ability to load, distribute or deliver ligands to specified targets and control activation, on demand using non-invasive optical stimuli.³⁰⁹ This can overcome issues such as non-specific ligand binding, target delivery (similar to a prodrug) and control compound toxicity in biological environments. In addition, it could potentially be used as a method to control the activation of neurons in a culture as required by the SLAB system.

Development of photo-responsive bio-ligands require two components, a photo-responsive element (activated using a specific wavelength) that can modulate availability or activity of a ligand and the conjugated ligand itself. Broichhagen, *et al*, in 2015 described the challenges associated with photo-pharmacology, identifying the importance of finding the correct photo-switch, making sure its toxicity is not an issue in the system required, and also the photochemical isomerisation is efficient in the required environment.³¹⁰

In addition to the introduction into photo-responsive compounds found in Chapter 1, which described structure and function and the premise to work in biological environments, we expand on the use of these compounds. We describe the uses specifically in terms of GPCRs, as well as focus on the role spiropyran can play for photo-pharmaceuticals.

Controlled activation of the A_{2A} adenosine receptor has been demonstrated by Bahamonde, *et al*, with the use of a diazo compounds linked to a non-selective adenosine agonist.³¹¹ The research showed that upon isomerisation both *cis* and *trans* forms of the adenosine azo-derivative could act as a full agonist for the adenosine A₃ receptor, whilst as a partial agonist (in the *trans* form) and antagonist (*cis*-form) at the adenosine A_{2A} receptor. Although this method does not control activation on demand, it does show that different photo-induced states of photo-responsive compounds linked with ligands can activate different receptors, and dependent on isomeric conformation control different cellular processes.

Although not at a GPCR, it has been shown that spiropyran can be used in an effective manner to change the activity of the antibiotic ciprofloxacin. Velema, *et al*, had shown that upon irradiation of spiropyran with UV light (365 nm) to form its MC isomer, a larger antibacterial activity was observed in comparison the SP isomer. This successfully shows that spiropyran can be used as a photo-responsive element in controlling the activity of a drug in a biological system, which makes it promising for the use in our system.³¹²

Chemistry – synthetic approach

Spiropyran derivatives

The general structure of spiropyran is shown in Figure 4-1. Modifications have been made at both the indoline and chromene segments, prior to the synthesis of spiropyran. These derivatives have been used in a number research projects, including but not limited to imaging (modifying optical properties)³¹³, drug delivery^{119,314,315} and surface attachment.^{87,112,316–318}

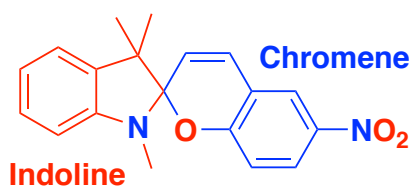


Figure 4-1 - Typical structure of spiropyran identified by its indoline and chromene segments

Modifications to the indoline has primarily focused on generating functional groups which can be used to attach to a number of compounds and surfaces. These structures are either modified from the amino group, or aromatic component. Modifications at the amino group include aliphatic chains terminated in alcohols^{319,320}, acrylates^{321–324} and carboxylic acids.^{325–328} Alternatively, modifications at the aromatic group have shown compounds which exhibit halides^{329–331}, alcohols^{332–334}, amines^{335–337} and carboxylic acids^{119,329,338}.

Synthetic derivatives of spiropyran where modifications have been made at the chromene segment typically focus on modifying the optical properties or switching parameters of the compound. There have also been instances where modifications have been made to attach onto other compounds. These include substituting the nitro group to alcohols^{339–341}, halides^{342–344} and also aromatics.^{344–346} Modifications at the aromatic have also been conducted, to tether onto surfaces and other compounds including but not limited to alcohols^{347–349}, and azides.^{119,350}

As from the previous Chapter, modification of dopamine from the primary amino group allows the compound to retain potency at the D₁ receptor. Due to this, we must establish methods which dopamine can be attached to spiropyran. These can include *N*-alkylation (as discussed and conducted in Chapter 3) as well as amide formations which will be discussed below.

Amide formation

The formation of an amide bond has been shown to be achieved through direct condensation of an amino group and acid at high temperatures (160 – 180 °C), but often is incompatible with the other functional groups present on starting materials, causing unwanted side reactions and degradation of products. Typically, upon mixing an acid and amine, salt formation is thermodynamically favourable, so the activation of the acid is generally required, to form acyl halides, synthetic anhydrides, and activated esters. In particular, focus of one-pot solutions have proven popular due to simplicity and high reaction yields.³⁵¹ This follows the *in situ* generation of the activated carboxylic acid in the presence of an amine. These one-pot methods employ the use of coupling agents such as carbodiimide or phosphonium/uronium salts.

Carbodiimide coupling reagents

Commonly used coupling reagents include dicyclohexyl carbodiimide (DCC), diisopropyl carbodiimide (DIC) and 1-ethyl-3-(3'-dimethylamine) carbodiimide HCl salt (EDC) as shown in Figure 4-2.

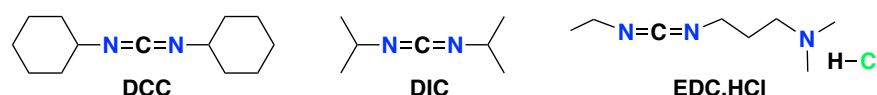


Figure 4-2 - Chemical structures of DCC, DIC, and EDC coupling reagents

These are used to generate *O*-acylisourea anhydride intermediates, which can directly react with amines, to yield the desired amides and urea by-product dependent on the coupling reagent.³⁵² DCC and EDC are typically employed in solution-phase chemistry due to the water soluble urea by-product, whilst DIC is commonly employed for solid-phase chemistry, with the resulting urea soluble in organic solvents such as dichloromethane.

Uronium/phosphonium salt based coupling reagents

The use of compounds such as *N*-[(Dimethylamino)-1*H*-1,2,3-triazolo-[4,5-*b*]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HATU) and 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) are regularly employed in the formation of amide bonds in 'one-pot' reactions.³⁵³ Like the carbodiimide, the driving force of the reaction is to form the urea by-product. An *in situ* activation of the acid is achieved through the formation of an ester using the coupling agent, which then readily reacts with the amine, to form the amide.

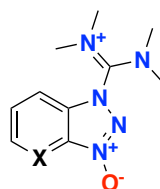


Figure 4-3 - Structure of HATU/HBTU coupling reagent ($X = N$ (HATU), $X = C$ (HBTU))

Aims and Objectives

The aim of this Chapter is to develop a photo-responsive neurotransmitter analogue that can be immobilised on to a solid substrate, which can reversibly activate the dopamine D₁ receptor. In doing so, we focus on the synthesis, photo-physical characterisation and pharmacology of the photo-responsive unit spiropyran and its conjugation with dopamine. Harnessing the structural and physiochemical characteristics of the spiropyran-dopamine analogue, we aim to control activation at the dopamine D₁ receptors in solution.

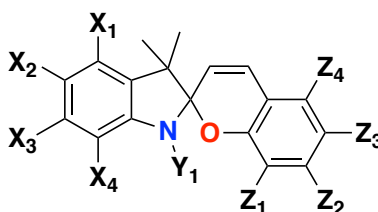


Figure 4-4 -Possible spiropyran modification sites for surface attachment and dopamine conjugation (X₁-X₄, Y₁ - indole, Z₁₋₄ chromene)

As shown in Figure 4-4, different modification sites are present on the spiropyran. Using the photo physical properties of spiropyran, dopamine would be attached to either the chromene or indole segment, whilst surface attachment to the opposite. To do this, spiropyran analogues were synthesised using conventional methods as well as the use of microwave chemistry, exhibiting carboxylic acid (X₂), amino groups (Y₁) on the indole, as well as a hydroxyl group (Z₃) and azide (Z₁) moieties on the chromene segment. Bio-conjugation of the dopamine was then conducted.

Analysis of the photo-responsive molecule was conducted using both chemical and physiological methods (NMR, LC-MS and UV/Vis spectroscopy). Pharmacological activity was assessed using the SPAP assay as in Chapter 1 to determine controlled activation and potency at the D₁ receptor.

Results and Discussion

Synthetic strategy

Spiropyran has been used in a variety of scenarios, from chemical to biological applications⁸⁸ but at the time of developing a dual functional derivative, reported literature on the material focused on a single anchor point. Due to this, we used two methods which modified the spiropyran precursors (chromene and indoline) to synthesise 3-(6-hydroxy-3',3'-dimethylspiro[chromene-2,2'-indolin]-1'-yl) propanoic acid (**11b**). In 2015, a dual functional spiropyran molecule was developed, by Zhang, *et al* (8-(azidomethyl)-1',3',3'-trimethyl-6-nitrospiro[chromene-2,2'-indoline]-5'-carboxylic acid, **15**). This was then synthesised and used to further develop this project.¹¹⁹

Synthesis and photo physical characterisation of 3-(6-hydroxy-3',3'-dimethylspiro[chromene-2,2'-indolin]-1'-yl) propanoic acid (**11b**)

In synthesising a dual functional spiropyran, we took inspiration from two spiropyran derivatives. The first from Stumpel *et al*, forming a phenolic group capable of forming an ester at the chromene end of the spiropyran.³⁵⁴ The second from the amino group found on the indoline segment as described by Baumann, *et al*.³⁵⁵ This is shown in Figure 4-5, where the carboxylic acid would tether to a surface, and the phenolic group to the desired neurotransmitter (as an *N*-alkyl derivative of dopamine or through an ether functionality using a linker).

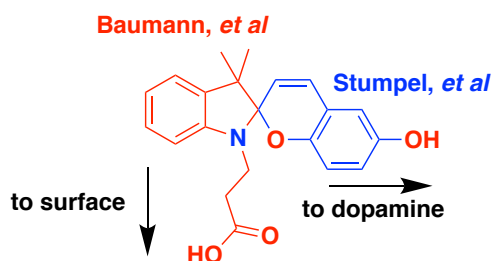


Figure 4-5 - Novel dual functional spiropyran designed to couple to dopamine through an ester/amine bond at the phenolic group and a carboxylic acid to terminal surface amino groups (**11b**)

Two spiropyran derivatives were synthesised as shown in Figure 4-6. Using the method devised by Baumann, *et al*, 2-hydroxy-5-nitrobenzaldehyde (**a**) was initially used as a control, to confirm the synthetic protocol conditions. Photo physical characterisation was also conducted for comparison to our desired phenolic derivative (using 2,5-dihydroxybenzaldehyde (**b**)).³⁵⁵

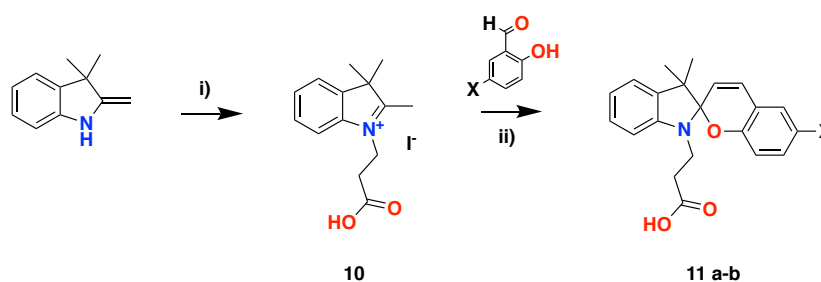


Figure 4-6 - Synthesis of spiropyran derivatives i) 3-iodo-propionic acid, reflux 24h, (98%) ii) methylethylketone, piperidine, reflux, 5h (dark) (X = NO₂ (**11a**, 71%), OH (**11b**, 57%))

Successful synthesis of both the -nitro and -hydroxyl terminated spiropyran was achieved, although the synthetic yields were lower for 3-(6-hydroxy-3',3'-dimethylspiro[chromene-2,2'-indolin]-1'-yl) propanoic acid (**11b**, 57%) than 3-(3',3'-dimethyl-6-nitrospiro[chromene-2,2'-indolin]-1'-yl) propanoic acid (**11a**, 71%). This difference in yield may have been due to the electronegativity of both benzaldehyde exhibiting the nitro group in comparison to the hydroxyl group in the para/ortho position away from the aldehyde. In comparison to literature, a yield of 71% for compound **11a** is relatively low, where it has been shown that a number of articles suggest yields in the range of 80-95%.^{356–358}

Structural analysis was conducted through ¹H NMR (Figure 4-7) and ¹³C NMR (found in the experimental), with mass analysis coming through LCMS. The structure of **11a** was consistent with data obtained by Baumann, *et al*.³⁵⁵ NMR was used to confirm the successful synthesis of **11b**. It was also shown that the compound was hygroscopic in nature, as after drying in a vacuum oven overnight at 40 °C, and freeze-drying, compound **11b** retains water as identified from the peak found at 3.33 ppm.

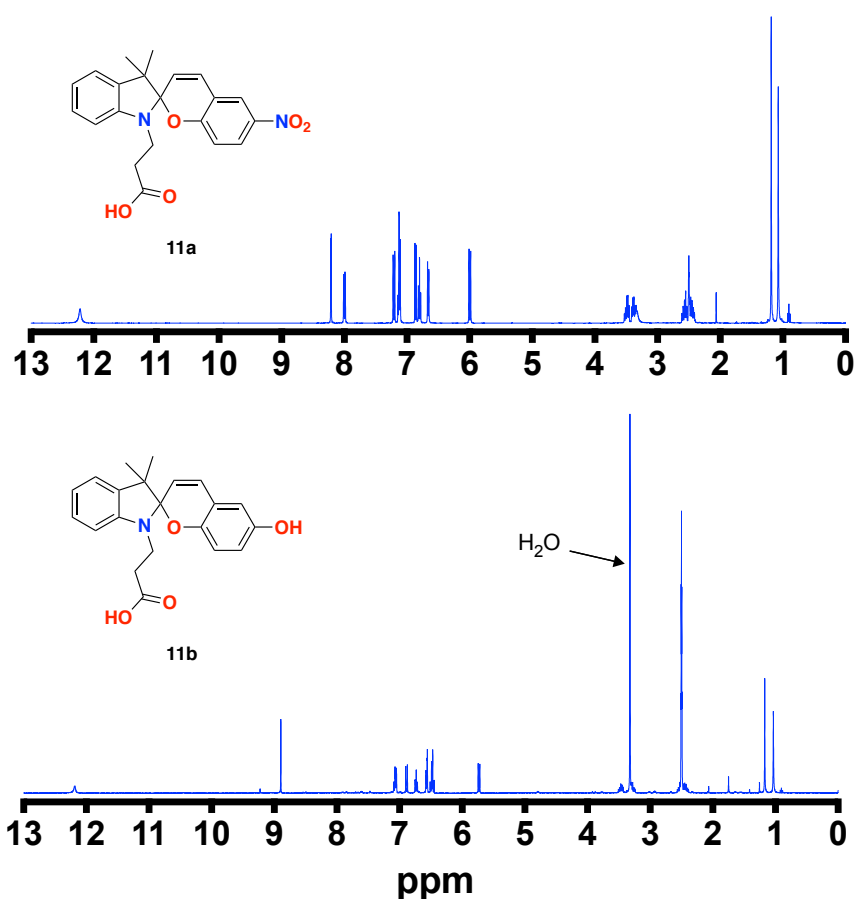


Figure 4-7 - ^1H NMR of spiropyran derivatives with -nitro and -hydroxyl functionalised chromene segment showing water retention in compound **11b** after freeze drying

Mass analysis of the compounds show their corresponding $[\text{M}+\text{H}]^+$ ion fragments, at m/z 381.22 (theoretical: 381.14) and m/z 351.95 (352.15) for compounds **11a** and **11b** respectively.

The switching of compounds **11a-b** from the SP→MC isomer, was conducted using an 8 W UV lamp (365 nm), and from the MC→SP isomer using white light. UV/Vis spectroscopy, was used to determine changes in structure and which isomer was present under specific wavelengths of light. This would allow us to determine whether our synthesised novel spiropyran would be able to act as a molecular switch in our desired system.

Photo-physical characterisation

It is commonly known, that upon isomerisation of spiropyran, a large red shift is observed when the molecule is switched from the closed SP isomer to the zwitterionic MC form.⁸⁸ This colour change is highly dependent on the structural aspects of the spiropyran as well as contributions from solvent interactions.³⁵⁹

Photochemical isomerisation involves heterolytic cleavage of the C-O bond between the indoline and chromene components, known as the spiro-junction.⁸⁸ Figure 4-8 shows the UV/Vis spectra obtained for our control **11a** (0.05 mM) in DMSO after exposure to white and UV light (365 nm) for 10 minutes. We can readily identify components within the UV spectra for both the indoline and chromene segment. There are two characteristic absorbance bands seen in our spectra for both the open and closed forms at 270 nm and 350 nm. This is consistent with literature, where the band at 270 nm corresponds to the π - π^* electronic transition in the indoline portion of the spiropyran, and the band at 350 nm corresponds to the chromene moiety. The open form has an additional characteristic band at 560 nm, which is due to conjugation between the indoline and chromene segments.^{97,360}

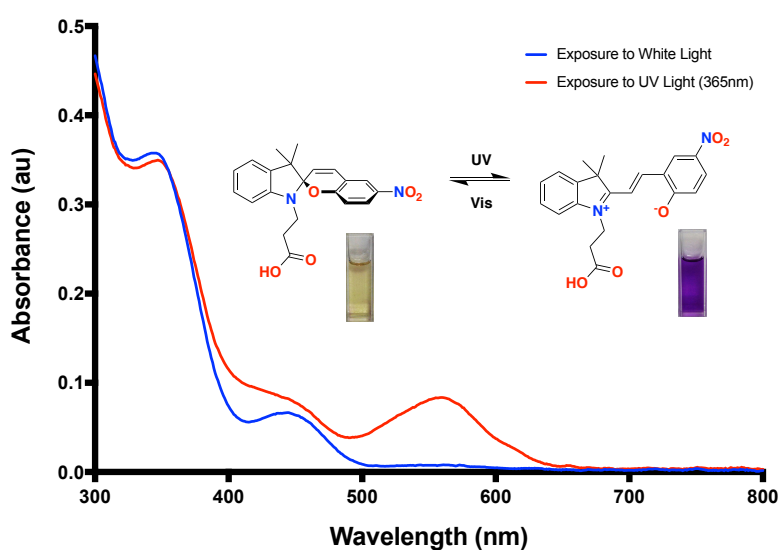


Figure 4-8 - UV/Vis spectra for 3-(3',3'-dimethyl-6-nitrospiro[chromene-2,2'-indolin]-1'-yl) propanoic acid (**11a**) showing the SP and MC state

Reversible switching of **11a** was demonstrated by irradiating the closed SP isomer with UV light (365 nm) for 15 minutes to obtain the MC isomer, and then white light for 15 minutes to reverse the process. Changes in absorbance were recorded at 560 nm, representing the presence of the conjugated MC isomer. This is shown in Figure 4-9, where reversible switching was observed back and forth for four cycles with no obvious degradation. This was the maximum switches attempted in house, but it has been shown in literature that this can extend up to 10 cycles and beyond.^{88,119}

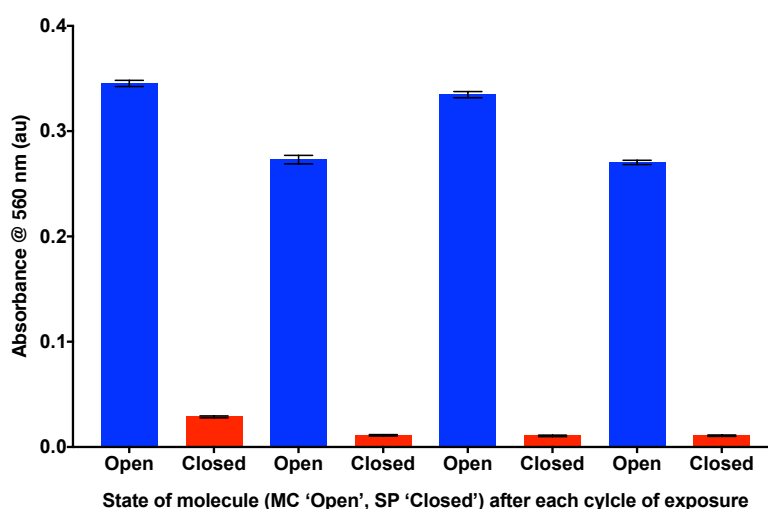


Figure 4-9 - Changes in absorbance at 560 nm as 3-((3',3'-dimethyl-6-nitrospiro[chromene-2,2'-indolin]-1'-yl) propanoic acid (**11a**) is irradiated with UV light (365 nm) and white light for 15 minute cycles ($n=3$)

The UV/Vis spectra of **11b** (0.05 mM) in DMSO showed no visual change at the band around 560 nm upon UV light conditioning, with the compound remaining colourless in solution, indicating the presence of the SP isomer. As switching was not observed in DMSO, due to the hygroscopic nature of **11b**, water was used as a solvent to identify switching capabilities. Upon addition of **11b** to spectroscopic grade water (0.05 mM), a purple solution was instantly observed. The change in colour was a result of conjugation between the indole and chromene segments, forming the open MC isomer. Unlike **11a**, irradiation with white light did not cause reversion back to the SP isomer as identified by the lack of a colour change, back to a colourless solution as observed with **11a**.

This could be explained through effects of resonance stabilisation, where a stable quinoidal structure is formed due to electron donation of the phenolic group (which has replaced the nitro moiety). To more fully understand the switching parameters of the compound and verify the lack of photo-responsiveness, the compound was subject to acidic/alkali conditions. As shown in Figure 4-10, this was to confirm both the presence of the MC isomer (which has previously been used to identify the presence of the isomer in literature) and to see whether we could switch the compound using light in its MCH^+ isomer (pH 2). This was chosen to match experimental conditions identified by Kanamori, *et al* who showed that in the MCH^+ isomer derivatives can undergo $\text{MC} \rightarrow \text{SP}$ isomerisation using white light.³⁶¹

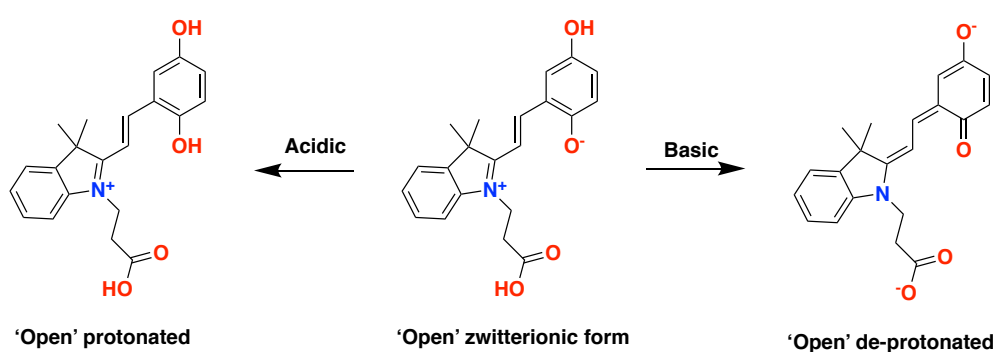


Figure 4-10 - Different merocyanine states of 3-(3',3'-dimethyl-6-nitrospiro[chromene-2,2'-indolin]-1'-yl) propanoic acid (**11b**) at pH = 2, 7 and 12

Figure 4-11 shows UV/Vis spectra obtained for **11b**, under different pH conditions. The zwitterionic MC isomer, is shown with a band at 580 nm. Upon protonation (MCH^+), a red-shift is observed giving an orange solution, whilst a blue-shift occurs when the compound is deprotonated. Upon irradiation of the compound with white light when in the MCH^+ state, switching back to a colourless solution (indicating the presence of the SP isomer) was still not observed. As reversible isomerisation was not shown to be possible using different wavelengths of light, an alternate solution was required for our system.

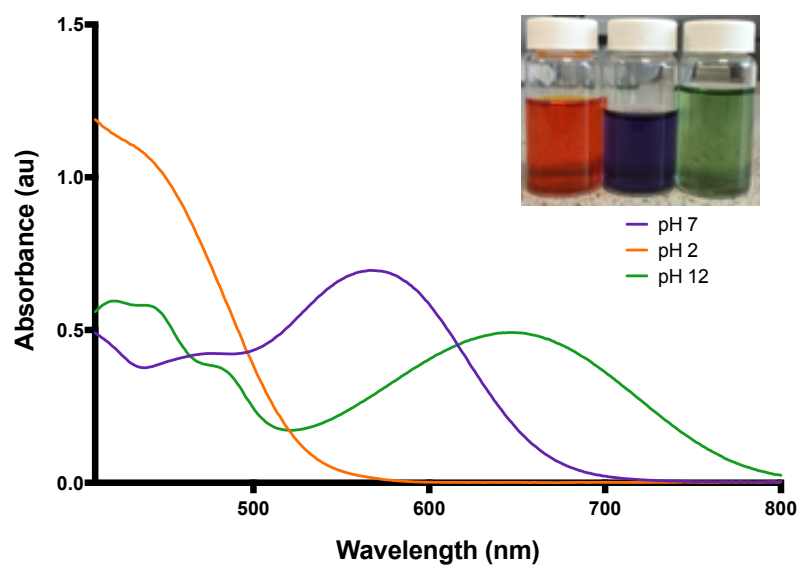


Figure 4-11 - UV/Vis spectra of compound **11b** at different pH levels in spectroscopic grade water

Synthesis and photo-physical characterisation of 8-(azidomethyl)-1',3',3'-trimethyl-6-nitrospiro[chromene-2,2'-indoline]-5'-carboxylic acid (**15**)

The ability to couple spiropyran to an optical fibre, and incorporate an amino acid residue which reversibly controlled binding to α -chymotrypsin was demonstrated by Zhang, *et al.*¹¹⁹ Whilst attachment to the amino acid residue was conducted from the indoline segment using amide coupling, surface attachment was conducted in one of three methods. These methods included: hydroxyl terminated molecules, directly reacting with piranha treated optical fibres (condensation reaction); activated carboxylic acids with surfaces presenting terminal amines (amide coupling); and copper mediated azide-alkyne cycloaddition (click chemistry). In our system, we selected the azide-alkyne click system for surface coupling, which was to avoid unwanted side reactions with the amino group on dopamine. The spiropyran derivative used for this project is shown in Figure 4-12.

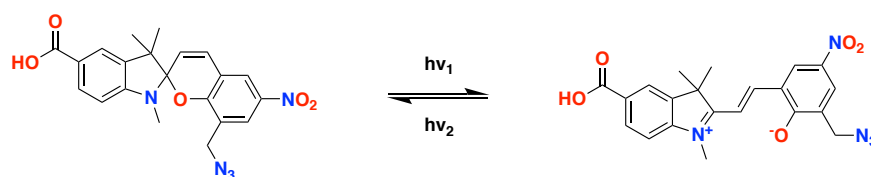


Figure 4-12 - Structural isomerisation of spiropyran with azide and carboxylic acid functionalities developed by Zhang, *et al.*¹¹⁹

To attain compound **15**, the synthetic procedure in Figure 4-13 was followed. Firstly, using nucleophilic substitution (S_N2), 3-chloromethyl-5-nitrobenzaldehyde was converted to form 3-azidomethyl-5-nitrobenzaldehyde (**12**) to develop an anchor for surface tethering. Synthesis of 3,3-dimethyl-3*H*-indole-5-carboxylic acid (**13**) and 1,3,3-trimethyl-2-methyleneindoline-5-carboxylic acid iodide (**14**) was then conducted to form the indole component, exhibiting an aromatic carboxylic acid to be used to couple with the amino group on dopamine. Synthesis of the photo-responsive unit, 8-(azidomethyl)-1',3',3'-trimethyl-6-nitrospiro[chromene-2,2'-indoline]-5'-carboxylic acid (**15**) was then conducted using compounds **12** and **14** utilising microwave chemistry.

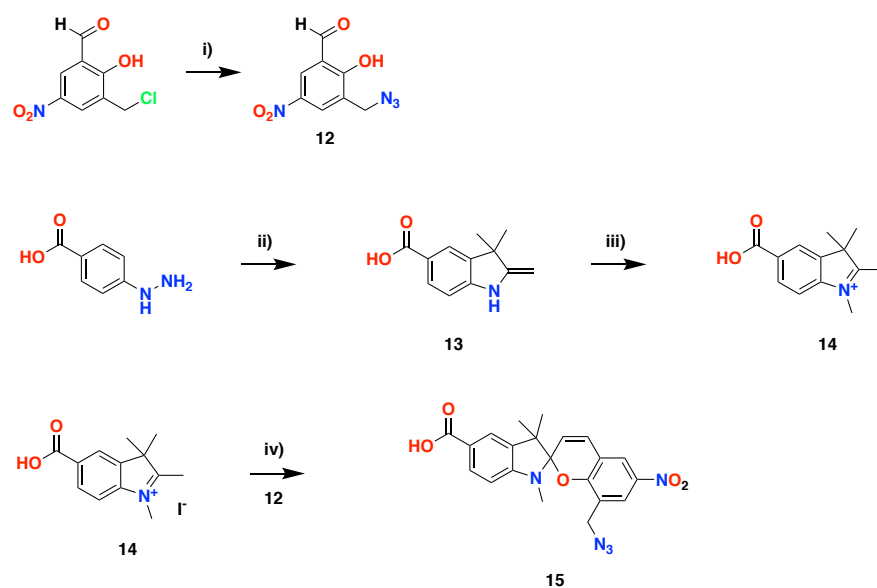


Figure 4-13 - Reaction pathway for the synthesis of the compound **15** (i) NaN₃, DMSO, (78%) ; (ii) 4-hydrazinobenzoic acid, 3-methyl- 2-butanone, EtOH, H₂SO₄, reflux, (53%); (iii) toluene:MeCN (2:1), MeI, reflux, (62%); (iv) 10min, uW, Et₃N, (72%)

Successful conversion of the alkyl halide into the azide functionality (**12**) was supported by ¹H NMR, ¹³C NMR and LCMS, and with comparison to literature values.¹¹⁹ Figure 4-14 shows the ¹H NMR obtained, and displays a shift from 4.9 to 4.6 ppm, in regard to the adjacent CH₂ peak, when nucleophilic substitution occurs. A shift in the NMR at 8.75 ppm for the aromatic proton in the ortho-position to the CH₂-N₃ is also observed, confirming successful substitution. LCMS further confirmed the mass and purity of compound **12**, giving m/z values of 223.90 and 220.85 for the [M+H]⁺ and [M-H]⁻ ions respectively.

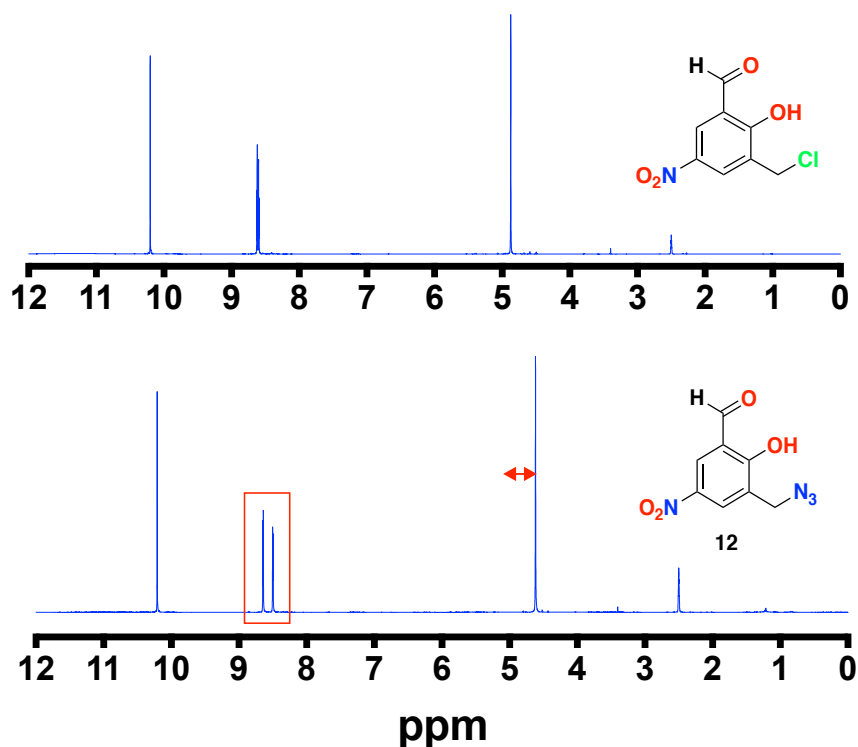


Figure 4-14 - ^1H NMR of 3-chloromethyl-5-nitrobenzaldehyde and 3-azidomethyl-5-nitrobenzaldehyde (**12**) showing an up-field shift in CH_2 protons from 4.9 to 4.6 ppm (red arrow), and change in aromatic protons at 8.75 ppm (highlighted in red) when alkyl chloride is replaced with an azido functionality

The reported literature yield of compound **12** was 76%; this was a crude product which was used without purification. In our case, research into the explosive nature of azides³⁶² showed that using the equation below we could determine how stable our synthesised azide was and whether we could purify our compound.

$$(N_{\text{Carbon}} + N_{\text{Oxygen}}) / N_{\text{Nitrogen}} \geq 3 \quad \text{Equation 6}$$

where N – is the number of specified heteroatoms. When the value is greater than or equal to 3, this signifies a stable compound.

Using this equation, we calculated the value of compound **12** to be 3, concluding that the compound would be stable enough to undergo purification. 2D thin layer chromatography was used to determine both the R_f value and whether the compound would degrade on silica upon purification.

An R_f value of 0.7 was obtained for compound **12**, retaining the impurity at the baseline using toluene as a solvent. A second run of the TLC plate in toluene at a rotation of 90° showed that a single peak was once again observed at an R_f value of 0.7, indicating the compound was stable on silica. As the impurity was retained on the baseline, a silica plug was used to obtain a pure compound with a yield higher than reported at 78%.

Synthesis of **13** was achieved using Fisher indole synthesis, converting aryl hydrazones into indoles, using a Brønsted acid as a catalyst (sulfuric acid, 98%).³⁶³ Successful synthesis of the product was determined using ^1H NMR, ^{13}C NMR and LCMS (m/z 204.00, $[\text{M}+\text{H}]^+$ ion), which all corresponded with reported literature values. Reported yields were much higher than achieved, from 91% down to 53%.¹¹⁹ Purity of starting materials, such as the sulfuric acid may have been a cause in the low yields obtained in our experimental set-up.

The reaction of **13** with methyl iodide, generated compound **14** as the iodide salt. Identification through LCMS showed the generation of a mass fragment at m/z 218.06 (theoretical: 218.11), which was in relation to the $[\text{M}]^+$ ion, whilst an additional methyl peak was observed at 4.01 ppm (^1H NMR) in comparison to compound **13**. Further to this, the spectra obtained, corresponded with literature values obtained by Zhang, *et al.*¹¹⁹ Whereas quantitative yields were reported in literature, here a yield of 62% was obtained. This was believed to be due to loss of material during the isolation of the product.

Synthesis of **15** from the method reported by Zhang, *et al.*, showed poor yields achieving a 13% yield after preparative HPLC.¹¹⁹ Using MAOS, a much-improved method was developed (with low reaction times, and high yields). Collating reaction conditions found in literature for the synthesis of different spiropyran derivatives (giving yields between 13% and 89%), we could determine parameters in which a higher yield should theoretically be able to be obtained. Common parameters for these reactions included polar

protic/aprotic solvents (i.e. ethanol, butanone), high temperatures (reflux conditions), and for periods longer than 3 hours. A number of bases were also employed, such as piperidine and triethylamine, whilst a number of reactions suggested containing the reaction vessel in the dark throughout the synthesis, others did not.^{88,92,119,364,365}

We aimed to use MAOS to shorten these times to minutes rather than hours, using a microwave active solvent. It was shown that using MeCN, with Et₃N at 40 °C for 10 minutes, produced a pure product which precipitated out of solution with a 72% yield. In an attempt to optimise this reaction, changes in reaction time and temperature were made. Time increments showed no increase in yield up to 1 hour, but an increase in temperature, yielded a dark red sticky solution from which no product precipitated.

Due to the complex structure of **15**, identification of the proton environment was aided by homonuclear correlation spectroscopy (COSY) and heteronuclear single-quantum correlation spectroscopy (HSQC) as shown in Figure 4-15 - and Figure 4-16. Successful synthesis and conformation of the structure was confirmed by comparing with literature values produced by Zhang, *et al.*¹¹⁹

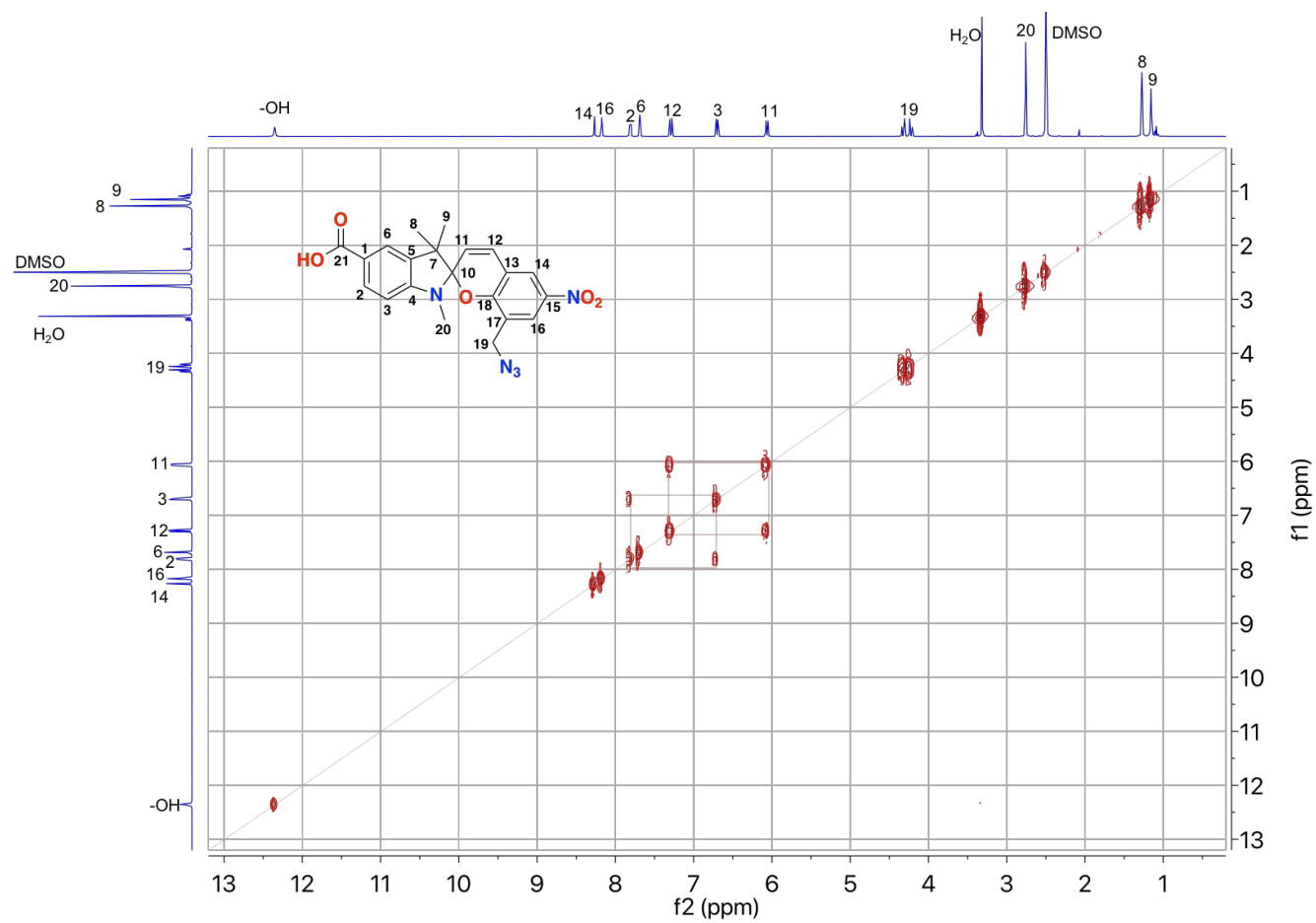


Figure 4-15 - COSY NMR of 8-(azidomethyl)-1',3',3'-trimethyl-6-nitrospiro[chromene-2,2'-indoline]-5'-carboxylic acid (**15**) (proton vs proton)

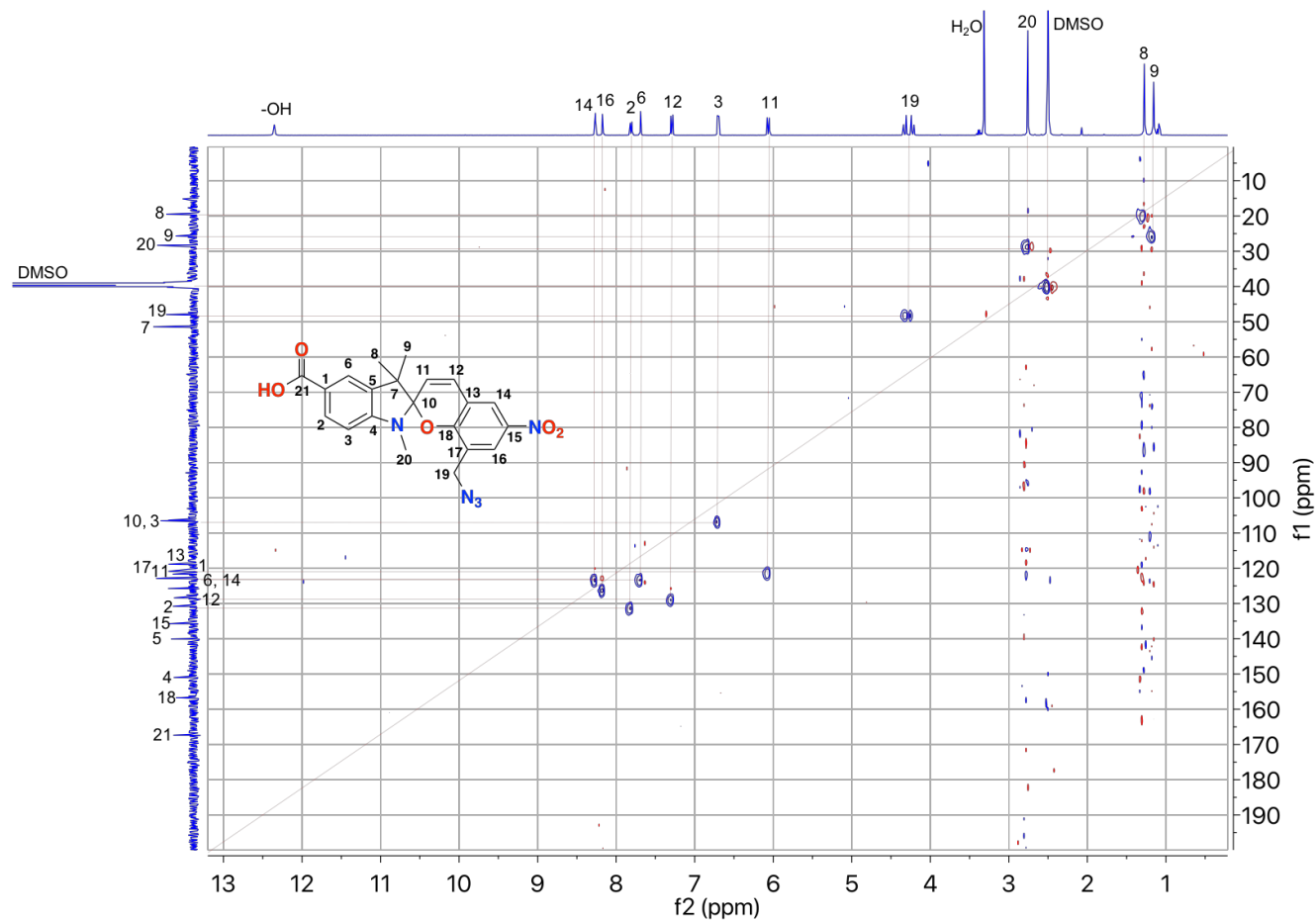


Figure 4-16 – HSQC NMR of 8-(azidomethyl)-1',3',3'-trimethyl-6-nitrospiro[chromene-2,2'-indoline]-5'-carboxylic acid (**15**) (proton vs carbon)

Photo-physical characterisation

The photo-physical characteristics of **15** was explored using indicator free Dulbecco's media - DMEM F12 with the addition of L-glutamate (5 mM) as a solvent. This was to understand the behaviour of the photo-responsive molecule under conditions that closely resemble those found in the SPAP assay. The isomerisation of spiropyran in media has previously been shown by Shiraishi, *et al*, and was further confirmed by our experimental results to successfully control which isomer is present in solution.³⁶⁶

Figure 4-17 shows the UV/Vis spectra obtained for **15** in media (0.05 mM) exposed to different wavelengths of light and temperatures. All samples were pre-conditioned with white light for 10 minutes, before being subject to 4-hours of: UV light (365 nm), white light, ambient light/temperature or 37 °C, 5% CO₂. The latter was also enclosed in foil and under constant irradiation of white light to determine whether isomeration could be controlled within an incubator (37 °C, 5% CO₂). Measurements were made immediately after removing the sample from the specified condition.

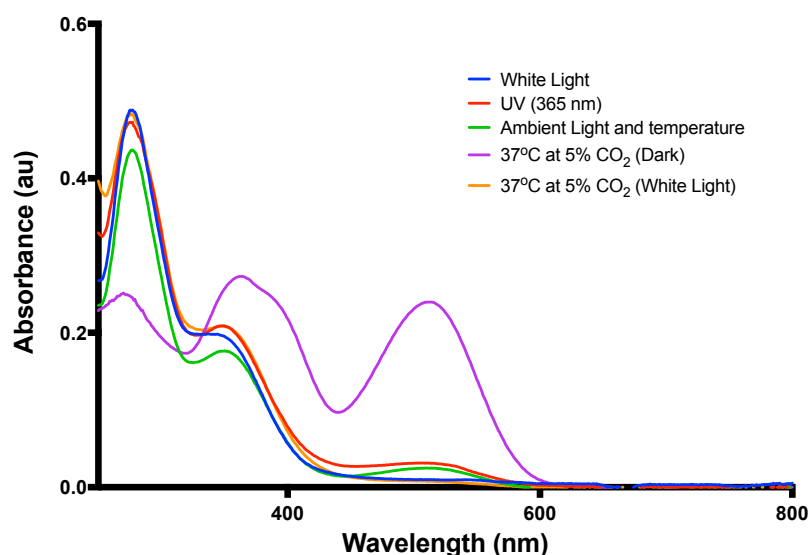


Figure 4-17 - UV/Vis spectra shown for 8-(azidomethyl)-1',3',3'-trimethyl-6-nitrospiro[chromene-2,2'-indoline]-5'-carboxylic acid (**15**) in DMEM-F12 media (conditioned for 10 minutes under white light) and exposed constantly for 4 hours under conditions found in the legend

As indicated by previous literature, an absorption band present between 500 to 600 nm indicates the formation of the MC isomer.⁸⁸ This is present within our spectra at 507 nm, when the spiropyran derivative is subject to ambient light and temperature, UV light (365 nm), and also in incubator conditions (37 °C, 5% CO₂) whilst kept in the dark over 4-hours.

In comparison to UV induced isomerisation, incubation of the sample in the dark resulted in a higher absorbance value at 507 nm. Spiropyran can undergo two isomerisation pathways, the heterolytic cleavage of the C-O bond by UV light resulting in a zwitterionic structure, and a mechanism involving a 6 π electrocyclic ring opening step, leading to the formation of a quinoidal resonance form, which may play a role in efficiently converting the SP to the MC isomer. Interestingly and in relevance to controlling specific isomers under incubatory conditions, we can confirm that when compound **15** is constantly irradiated with white light, it remains in its SP form, as evidenced by the lack of a band at 507 nm.

The peak areas of each spectra were determined to calculate changes in specific bands when SP→MC isomerisation occurred and to determine whether a single isomer at 100% conversion could be achieved. These are shown in Table 4-1 and Figure 4-18. Using wavelength band limits of 250 – 317 nm (indoline π - π^* transitions) and 435 – 601 nm (indoline and chromene conjugation) the areas were calculated using Spectragryph (Version 1.2.0).

A general trend for the peak areas shows that upon isomerisation, we see a reduction at the band representative of the indoline (250 to 317 nm), and an increase for the conjugated molecule (435 to 601 nm). Using the peak integrals it can be shown that thermal isomerisation is more effective than UV light induced isomerisation; however the SP isomer can be retained through constant application of white light.

Table 4-1 - Integrals of peak areas (UV/Vis) for 8-(azidomethyl)-1',3',3'-trimethyl-6-nitrospiro[chromene-2,2'-indoline]-5'-carboxylic acid (**15**) between 250-317 nm and 435-601 nm characteristic to the indoline fragment and MC isomer

Condition for SP-Azide	Peak Integral (au)	
	From 250 to 317 nm	From 435 to 601 nm
37 °C 5% CO ₂ (White Light) (a)	5.4	0.0
White Light (4Hr) (b)	8.2	0.0
Ambient (c)	7.6	1.3
UV (365 nm) 4 Hours (d)	6.7	1.0
37 °C 5% CO ₂ (4Hr) (Dark) (e)	1.2	14.5

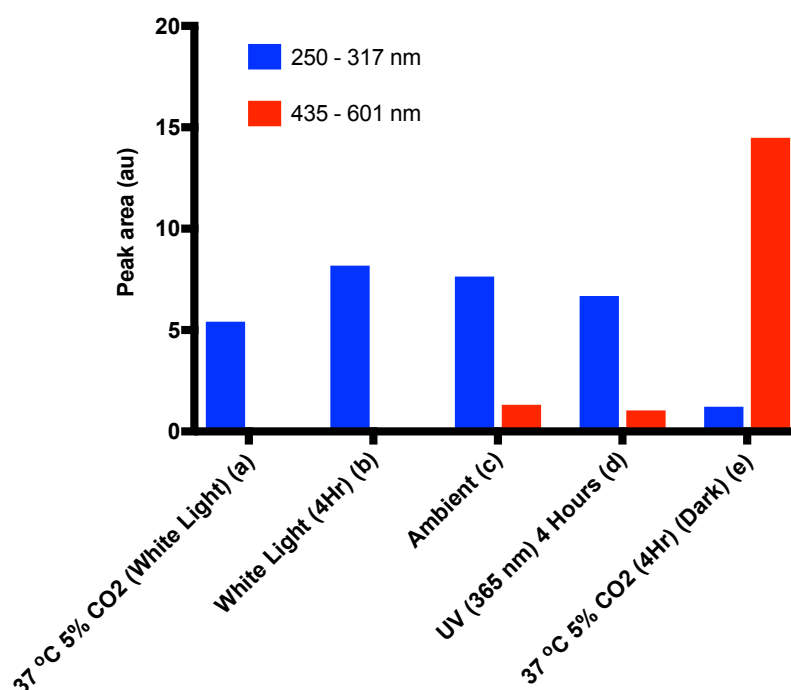


Figure 4-18 - Visual representation of peak area integrals for 8-(azidomethyl)-1',3',3'-trimethyl-6-nitrospiro[chromene-2,2'-indoline]-5'-carboxylic acid (**15**) between 250-317 nm and 435-601 nm characteristic to the indoline fragment and MC isomer

Synthesis and photo-physical characterisation of 8-(azidomethyl)-*N*-(3,4-dihydroxyphenethyl)-1',3',3'-trimethyl-6-nitrospiro[chromene-2,2'-indoline]-5'-carboxamide (16**)**

8-(azidomethyl)-*N*-(3,4-dihydroxyphenethyl)-1',3',3'-trimethyl-6-nitrospiro[chromene-2,2'-indoline]-5'-carboxamide (**16**) was successfully synthesised using **15** and dopamine hydrochloride (**1**), using HATU mediated amide formation (Figure 4-19).

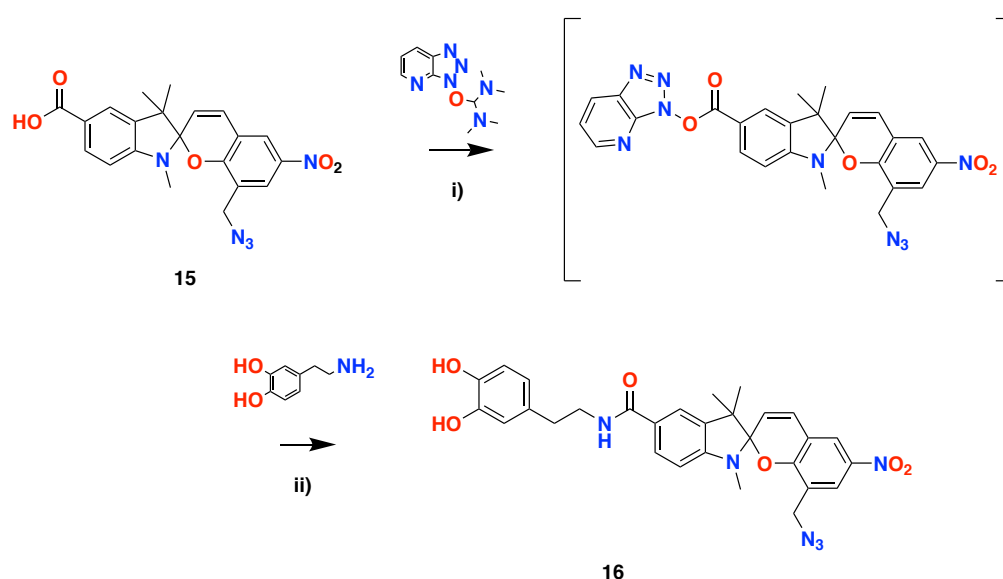


Figure 4-19 - Reaction scheme for the formation of 8-(azidomethyl)-*N*-(3,4-dihydroxyphenethyl)-1',3',3'-trimethyl-6-nitrospiro[chromene-2,2'-indoline]-5'-carboxamide (**16**) using HATU mediated coupling showing an in situ activated carboxylic acid (i) HATU, DIPEA, DMF, (ii) dopamine, DMF (42%)

Monitoring the reaction *via* thin layer chromatography and LCMS, two close bands were observed at an R_f of 0.33 and 0.35 with equal masses at m/z 557. As HATU coupling not only allows the formation of amide bonds, we assumed that modification had also occurred at the catechol, forming an ester with the dopamine. For this reason, purification of the material was conducted using preparative HPLC and the final compound was identified through ^1H NMR (Figure 4-20) and ^{13}C NMR (as shown in the experimental).

This loss of the carboxylic acid proton peak found at 12.36 ppm as well as the addition of the dopamine aromatic peaks in the 6.5 ppm region show that successful coupling has occurred. A shift in the CH₂ peaks confirms a reaction has taken place at the amino group of the dopamine and not at the catechol found on the molecule, as ester formation is possible using HATU mediated reactions. Although the aromatic linked CH₂ group found on the dopamine is not present in the NMR, it was confirmed with COSY that the peak is masked under the water peak found on the spectra (3.30 ppm).

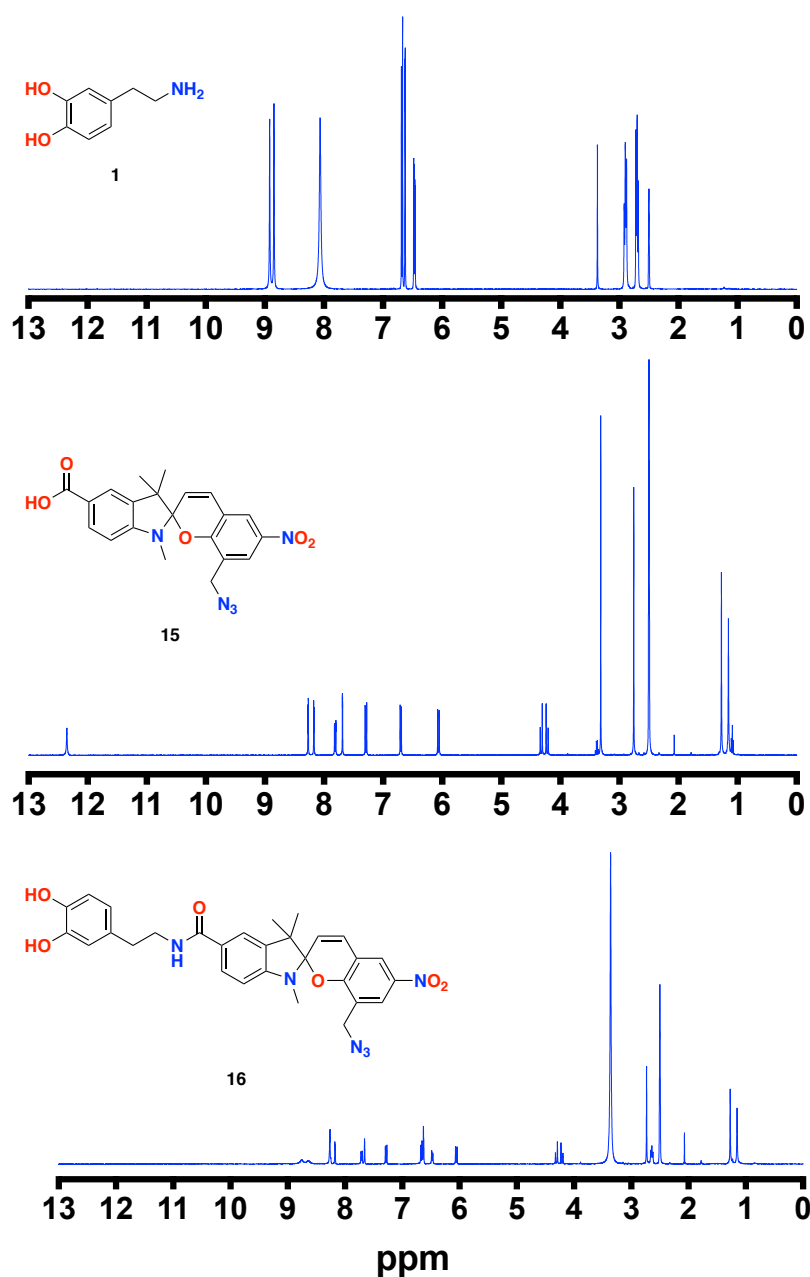


Figure 4-20 - ¹H NMR showing comparisons for compounds **1**, **15** and **16**

Photo-physical characterisation

Figure 4-21 shows the UV/Vis spectra obtained for compound **16** (0.05 mM). DMEM F12 media with L-glutamate (5mM) was used as the solvent and the solution was subject to a range of conditions as before. The solutions were pre-conditioned with 10 minutes of white light before being exposed to: UV light (365 nm), white light, ambient light and temperature, and incubator conditions (37 °C, 5% CO₂). Once again, the latter was kept in the dark and subjected to white light for the entire 4-hour period.

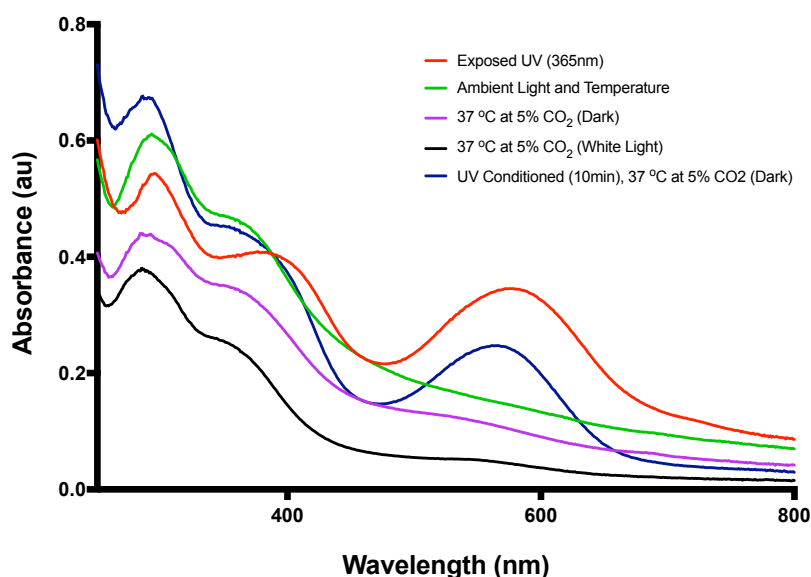


Figure 4-21 - UV/Vis spectra for 8-(azidomethyl)-N-(3,4-dihydroxyphenethyl)-1',3',3'-trimethyl-6-nitrospiro[chromene-2,2'-indoline]-5'-carboxamide (**16**) in DMEM-F12 media, all conditioned prior with white light and then exposed for 4 hours with conditions (final condition - conditioned with UV light rather than white light prior to incubation)

As before, controlling which isomer was present in different conditions was required, but in this case to determine whether we could eventually see a change in activity dependent of the isomer at the dopamine D₁ receptor. Due to this, an additional test was conducted, which included the pre-exposure of the solution to 10 minutes of UV light (365 nm) prior to incubation in the dark for 4-hours.

It was observed that the heterolytic cleavage of the C-O bond forming the zwitterionic form using UV light, showed a higher absorbance as to the thermal isomerisation leading to the quinoidal form. This indicated that the compound was favourable to isomerisation using UV light as opposed to thermal stimuli, contrary to compound **15**. Another observation which is pertinent to this thesis, shows that upon pre-exposure to UV light (365 nm), an increased absorbance is observed at 568 nm. This shows that isomerisation to the MC isomer is retained when compound **16** is kept in the dark and within the incubator. The importance of this was to avoid constant UV irradiation with our chosen cell assay. It has previously been shown by König, *et al*, that CHO cells exposure to ultraviolet-A (UVA) radiation, had resulted in significant modifications of nicotinamide adenine dinucleotide (NADH) attributed auto-fluorescence and inhibition of cell division.³⁶⁷

Determination of peak integrals between 264 to 334 nm (indoline) and 467 to 665 nm (characteristic of the MC isomer) were used to identify which conditions related to which isomer (Table 4-2 and Figure 4-22). A reduction in absorbance is observed in regard to the characteristic indoline peak, whilst the peak for the MC isomer increases when exposed to stimuli which converts SP→MC. It also shows that a brief 10-minute pre-exposure to UV light, we can retain and control which isomer is present over the 4-hour incubation period.

Table 4-2 - Integrals of peak areas (UV/Vis) for 8-(azidomethyl)-N-(3,4-dihydroxyphenethyl)-1',3',3'-trimethyl-6-nitrospiro[chromene-2,2'-indoline]-5'-carboxamide (**16**) between 264-334 nm and 467-665 nm characteristic to the indoline fragment and MC isomer

Condition for spiropyran-dopamine	Peak Integral (au)	
	From 264 to 334 nm	From 467 to 665 nm
Ambient (a)	5.2	0.0
Incubator (White Light) (b)	3.3	0.4
Incubator (Dark) (c)	3.4	0.4
UV Conditioned 10min (4hr) (d)	4.1	14.4
UV Exposed 365nm (e)	3.2	19.5

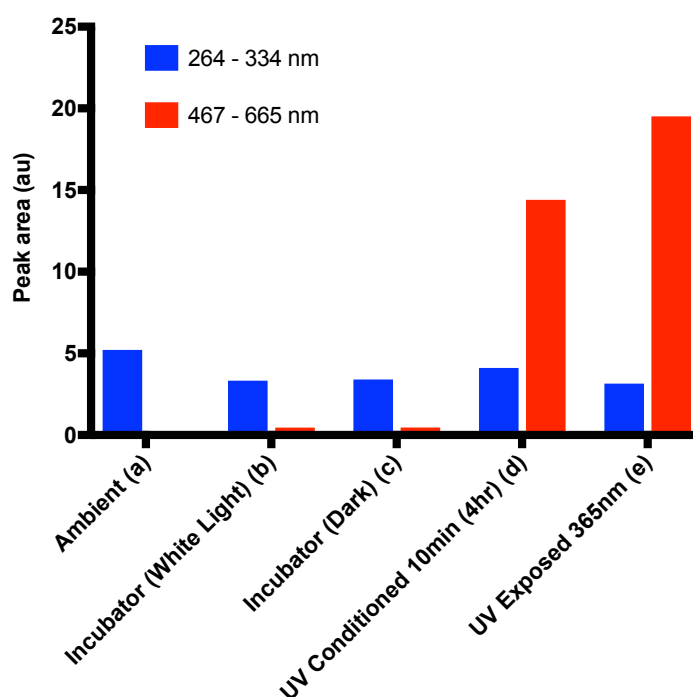


Figure 4-22 - Visual representation of peak area integrals for 8-(azidomethyl)-N-(3,4-dihydroxyphenethyl)-1',3',3'-trimethyl-6-nitrospiro[chromene-2,2'-indoline]-5'-carboxamide (**16**) between 264-334 nm and 467-665 nm characteristic to the indoline fragment and MC isomer

Reversible isomerisation between the SP and MC isomer was confirmed using UV/Vis spectroscopy, at a single wavelength. As identified, the peak at 568 nm (λ_{max}) was indicative of the MC isomer. When alternately exposing the sample to white (WL) and UV light (365 nm), we see the absorbance behaviour shown in Figure 4-23 is observed. Until the 5th conversion, where the absorbance increases rather than decrease as expected after WL exposure, we see effective reversible isomerisation occur. After this, we assume there is some evidence of photo-bleaching, or that our exposure to WL or UV light is not efficient enough (either in time or intensity or both).

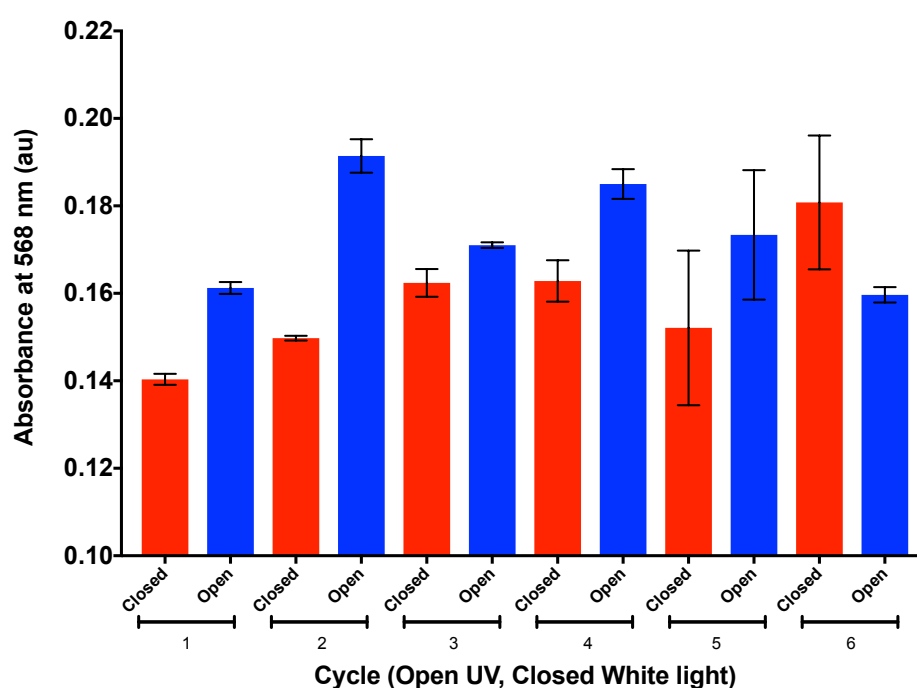


Figure 4-23 - Absorbance at 568 nm upon switching between white and UV light (365 nm) indicating the SP and MC isomer for 8-(azidomethyl)-N-(3,4-dihydroxyphenethyl)-1',3',3'-trimethyl-6-nitrospiro[chromene-2,2'-indoline]-5'-carboxamide (**16**, $n=3$ with error bars representing SD)

Biology - Structure activity relationship for spiropyran derivatives

The structure activity relationships were conducted for compounds **15** and **16** synthesised in this Chapter. Identifying the ability to control the isomer present of the spiropyran under elevated temperatures and under 5% CO₂ by using white light, we conducted three assays. These were run against basal and forskolin doped wells, to act as an internal standard to determine successful activation of the dopamine D₁ receptor.

The three conditions comprised of constant white light exposure, dark (foiled covered) and pre-conditioning of compound **16** with UV light (365 nm) prior to the 4-hour incubation period. The third condition was conducted due to UV/Vis measurements indicating that the compound was not as influenced by elevated temperatures, causing isomerisation from the SP to MC isomer. Pre-incubation of the molecule under UV light (365 nm) for 1 hour was conducted prior to the assay to ensure complete isomerisation had occurred.

Upon exposure to white light throughout the 4-hour incubation period, we observe no generation of SPAP above the baseline. Knowing from the UV/Vis measurements that this compound is in the SP isomer, we can initially say that in this closed form, activity at the D₁ receptor is suppressed (Figure 4-24). Figure 4-25 shows the dose response curve for compound **16** kept in the dark for the 4-hour incubation period. Pre-conditioned with white light for 1 hour prior to adding to the 96 well plates, we can see activity at high concentrations (10⁻⁵ M). Although the potency of the compound is low, we do observe activity at the D₁ receptor. Pre-incubation of compound **16** with UV light (365 nm) showed similar results within the SPAP assay, with low potency but retaining some activity at the D₁ receptor (Figure 4-26). This shows a clear indication that activation the D₁ receptor can be achieved dependent on isomeric conformations of the spiropyran.

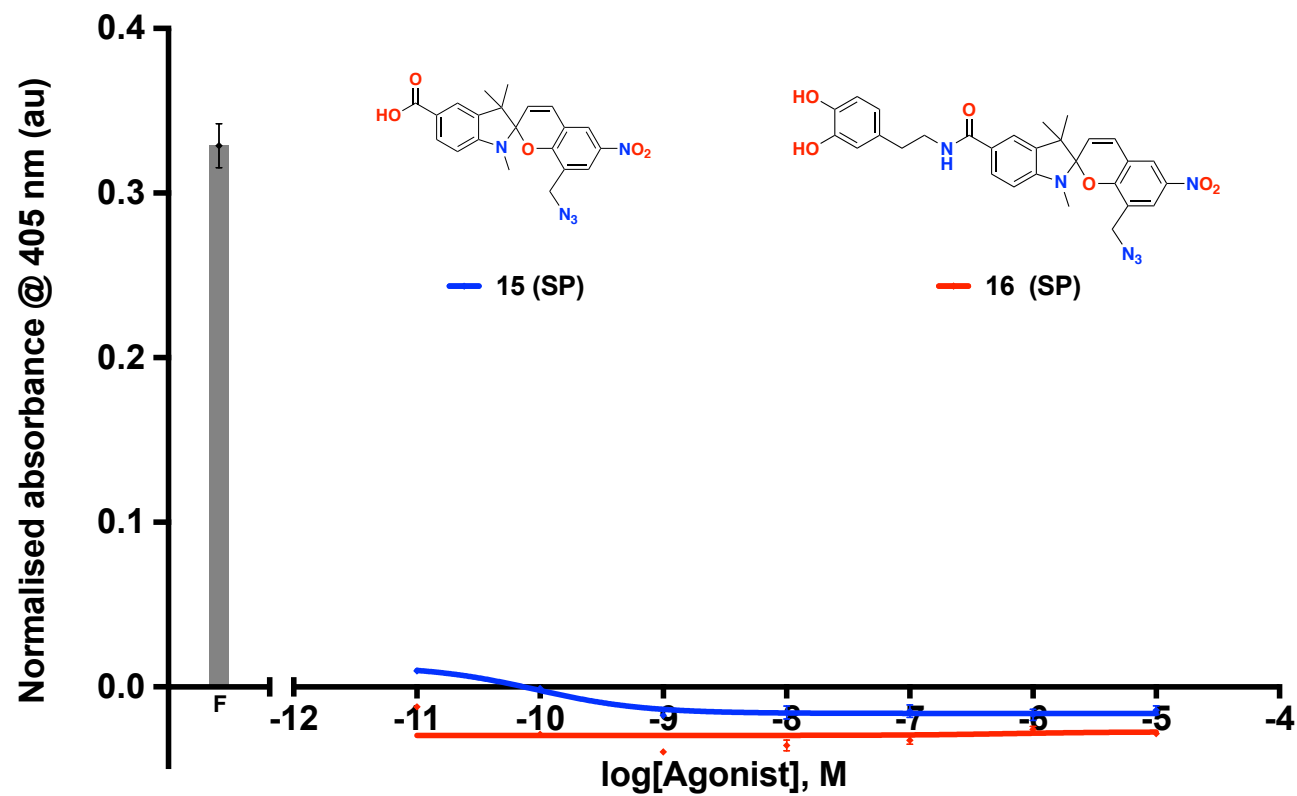


Figure 4-24 - Dose response of spiropyran derivatives with constant exposure to white light (compounds **15**, **16**) for the dopamine D_1 secreted placental alkaline phosphatase assay. Positive control of forskolin is shown as F ($n=3$, $N=5$, error bars represent SEM)

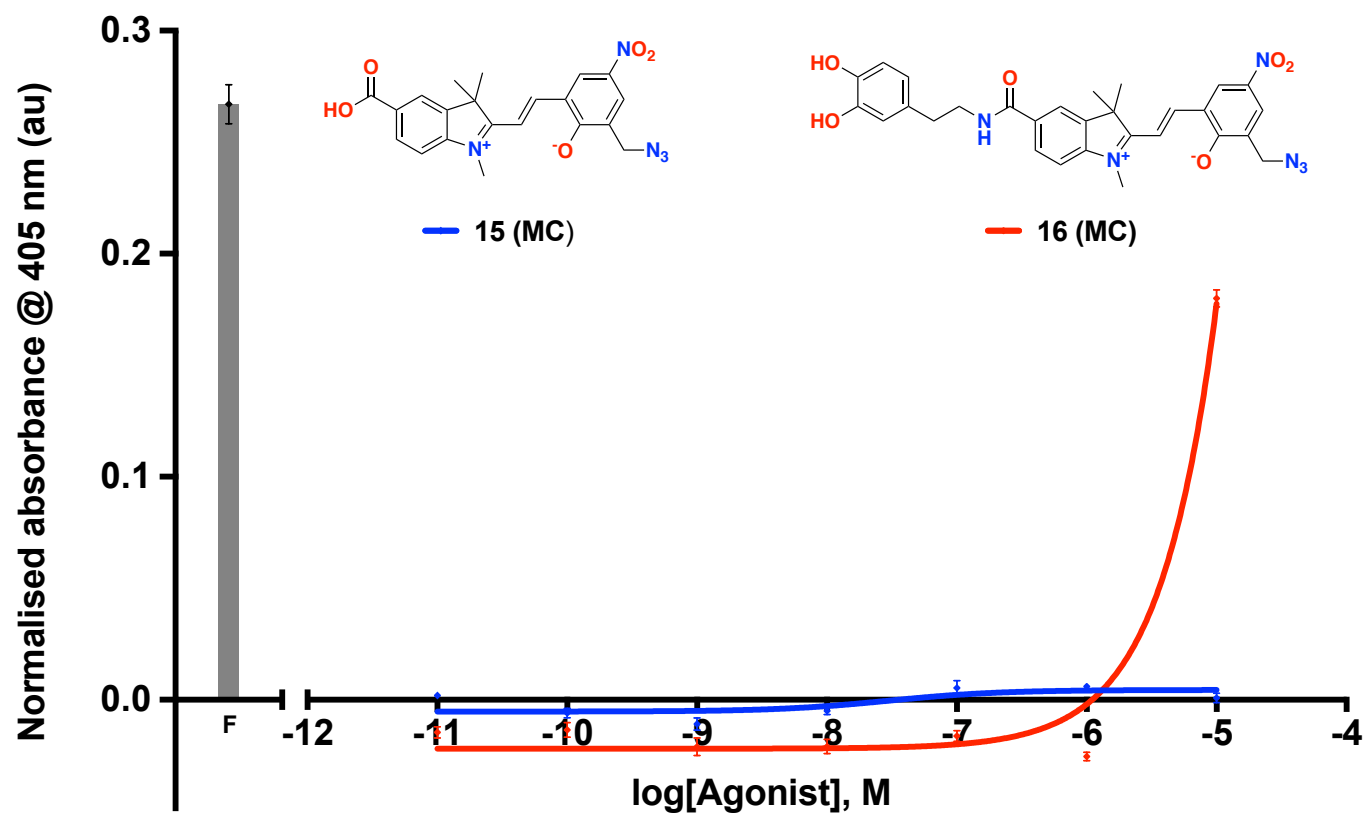


Figure 4-25 - Dose response of spiropyran derivatives kept in the dark (compounds **15**, **16**) for the dopamine D_1 secreted placental alkaline phosphatase assay. Positive control of forskolin is shown as F ($n=3$, $N=5$, error bars represent SEM)

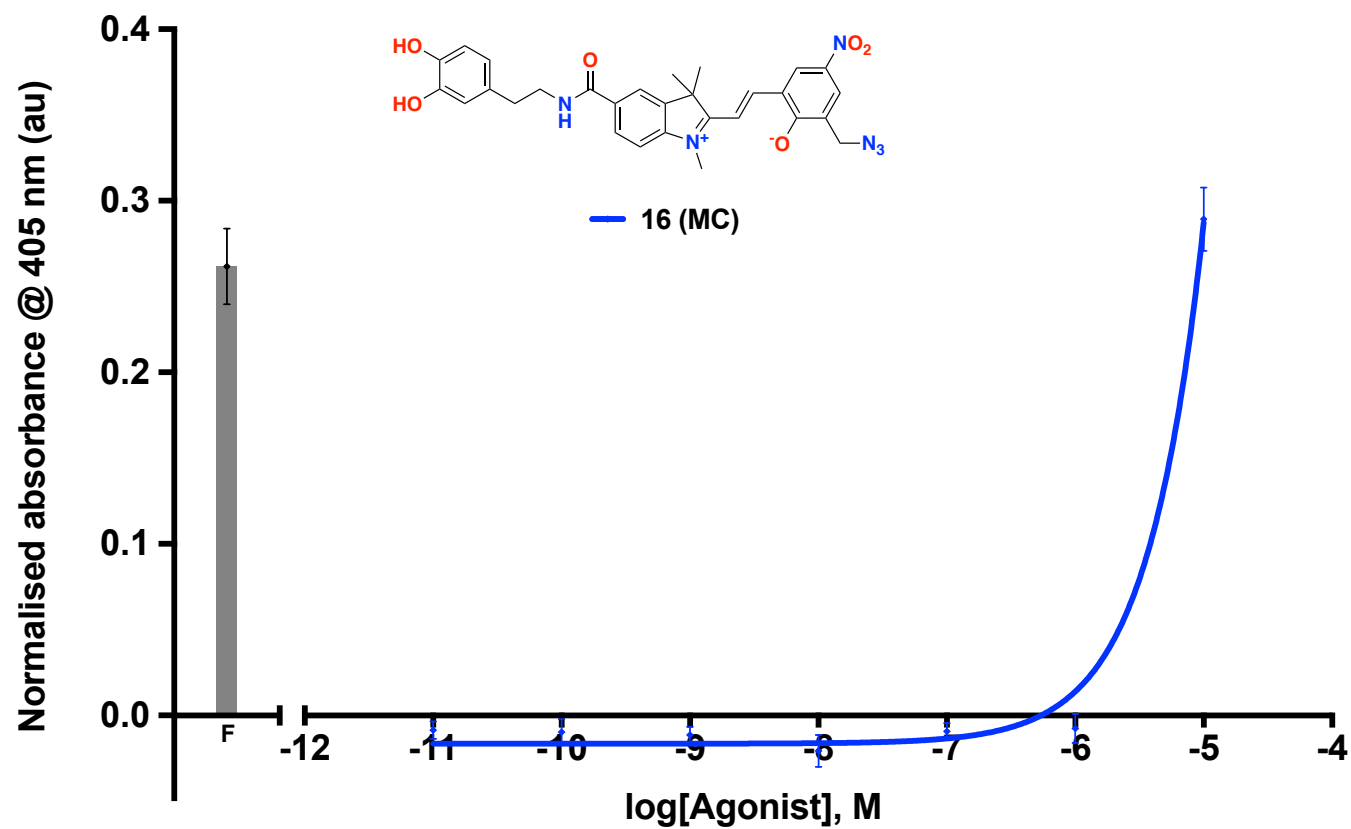


Figure 4-26 - Dose response of spiropyran derivatives pre-conditioned with UV light (365 nm, compounds **15**, **16**) for the dopamine D_1 secreted placental alkaline phosphatase assay. Positive control of forskolin is shown as F ($n=3$, $N=5$, error bars represent SEM)

Controlled activation for the D₁ receptor using the two isomers exhibited by the spiropyran-dopamine analogue follows similar results exhibited by Velema, *et al*, who showcased the controlled antibiotic properties of ciprofloxacin. It was shown that the active form was present as the MC isomer as we have identified.³¹² In contrast to this, it was shown by Zhang, *et al*, that the SP isomer is much more active than the polar MC isomer, in controlling and binding to α -chymotrypsin.¹¹⁹

The dynamic properties of spiropyran can be explained for both of these outcomes, with both polarity and steric conformations playing large parts in binding capabilities at membrane bound receptors, or large protein complexes. In our studies, we believe the binding of the compound occurs due to less steric hindrance and flexibility of dopamine when compound **16** is in its MC isomer. This is shown in Figure 4-26 where, upon isomerisation, the conjugated dopamine can interact with the D₁ binding pocket due to the extension of the molecule and its flexibility.

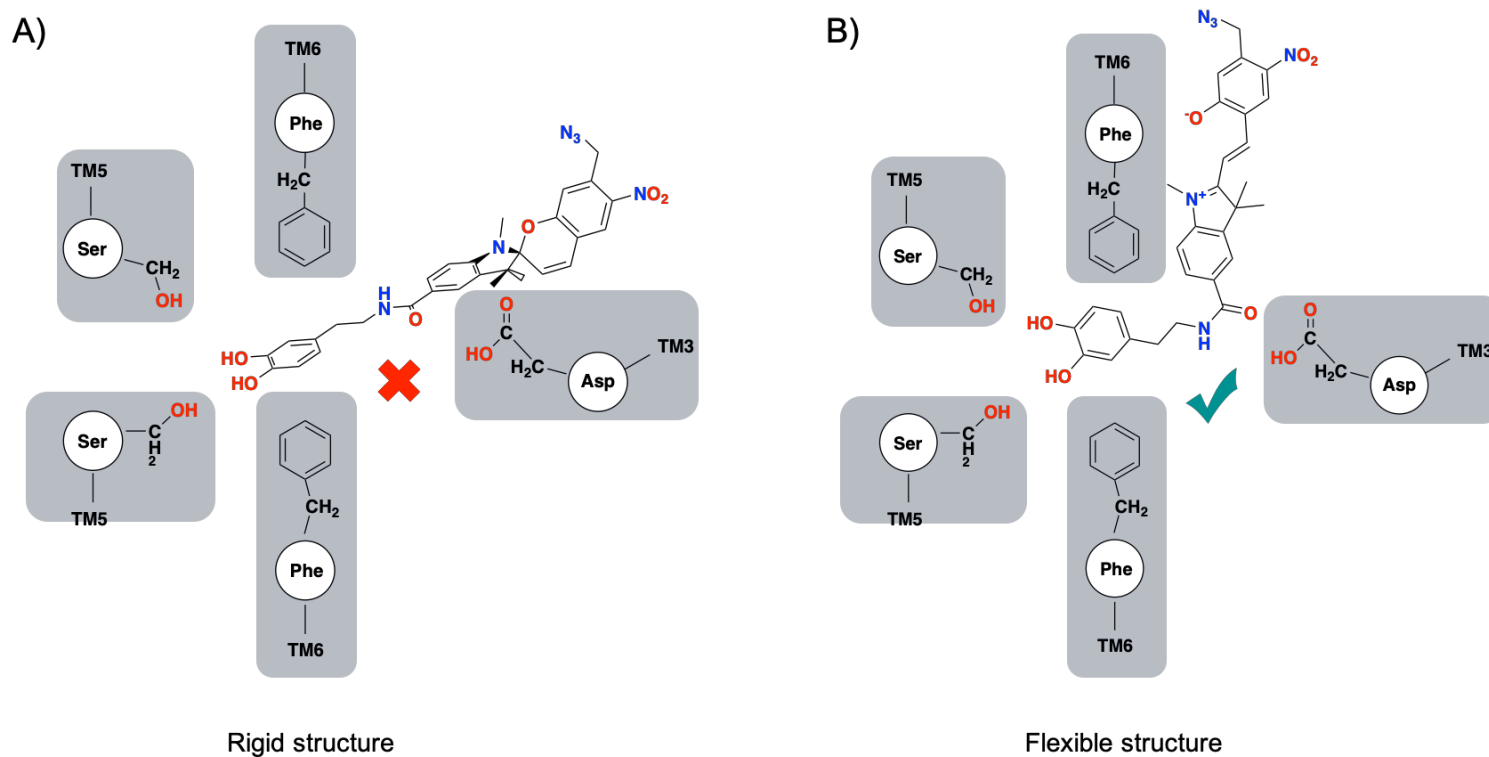


Figure 4-27 - Proposed binding interactions of compound **16** with the D₁ binding pocket in both SP and MC isomer. A) shows a rigid structure (SP), which does not allow the rearrangement of dopamine, to bind to key sites as identified for the D₁ receptor, B) shows a flexible structure (MC) which can orientate itself after isomerisation and interact with key binding sites as previously identified (green tick represents potency at the D₁ receptor, whilst red cross marks no activity above basal level in the SPAP assay)

Conclusion

This Chapter reports the successful syntheses and photo-physical characteristics of two dual functionalised spiropyran analogues 3-(3',3'-dimethyl-6-nitrospiro[chromene-2,2'-indolin]-1'-yl) propanoic acid (**11b**) and 8-(azidomethyl)-1',3',3'-trimethyl-6-nitrospiro[chromene-2,2'-indoline]-5'-carboxylic acid (**15**). It also reports the successful synthesis and pharmacological data for 8-(azidomethyl)-N-(3,4-dihydroxyphenethyl)-1',3',3'-trimethyl-6-nitrospiro[chromene-2,2'-indoline]-5'-carboxamide (**16**).

Initial synthesis of a dual functionalised spiropyran analogue (**11b**, 57%) showed limited use in our proposed system. This was due to the absence of reversible switching of isomers, using fixed wavelengths of light. Switching from the SP→MC isomer was observed when the compound was dissolved in water, resulting in a purple solution. In contrast, dissolution of the compound in organic solvents such methanol and DMSO yielded colourless solutions. Upon exposure to white light, we did not see conversion back to the SP isomer, hence an alternate approach was used.

Synthesis of a spiropyran analogue exhibiting an azide functionality located on the 'chromene' unit for surface tethering and a carboxylic acid functionality located on the aromatic ring of the indole moiety was successfully achieved using a multistep synthetic process involving the use of microwave chemistry. The spiropyran-azide derivative was obtained with 72% yield without the need for further purification. Photo-isomerism of **15** (0.05 mM) was shown to be successful in DMEM F12 media (+ L-glutamate (5 mM)) using both optical and thermal stimuli. Control over the isomerisation from the SP to MC isomer was achieved under UV light (365 nm), and at increased temperatures. Upon exposure to white light, reversion back to the SP isomer was achieved. Interestingly, under the constant exposure of white light, we could retain the SP isomer even at increased temperatures (37 °C).

Synthesis of the spiropyran-dopamine analogue (**16**) was successfully achieved using HATU mediated amide formation. Preparative HPLC was required to achieve isolation of the final compound, due to a side reaction forming an ester bond (from the carboxylic acid and catechol functionality of the dopamine). Switching from the SP→MC isomer was observed using media as a solvent. In contrast to compound **15**, isomerisation of **16** was more strongly influenced by UV light rather than temperature.

Using the CHO-D₁, CRE-SPAP assay, the activity and potency of compounds **15** and **16** were analysed in the SP and MC isomers. In the SP isomer, both compounds **15** and **16** showed no activity at the D₁ receptor (conducted under a constant irradiation of white light). This was also true for the MC isomer for compound **15**, showing no signs of non-specific activation of the receptor. At concentrations above 10⁻⁶ M, the generation of SPAP was observed when compound **16** was present in its MC isomer, both when kept in the dark, and when pre-conditioned with UV light. This is significant, as it shows that although a lower peak area for thermal isomerisation is observed in comparison to the UV light stimulated found within the UV/Vis spectra, both activate the receptor at 10⁻⁶ M. This indicates that both the heterolytic cleaved C-O and quinoidal form generated by the two stimuli, may have different absorbance values, but can successfully activate the D₁ receptor.

This Chapter successfully shows the controlled activation of the D₁ receptor using compound **16** in the SP and MC isomers present in solution. The next stage was to immobilise this onto a solid substrate, and by using surface bound ligands, control activity at the D₁ receptor.

Experimental

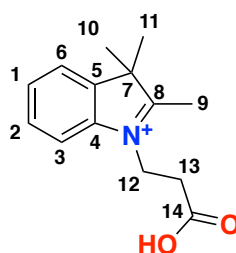
NMR, LCMS and chromatography were conducted as described in the experimental section in Chapter 3.

Preparative HPLC was conducted on a Waters Prep HPLC, using a Waters 2525 binary gradient module pump, 2487 UV detector, and 2767 autosampler/fraction collector for the purification of the spiropyran dopamine. The solvent system used was acetonitrile (solvent A) and water (solvent B) with 0.06% TFA. Gradient started at 5% (A) to 95% (B), and over 10 minutes to 90: 10%. A Gemini® 5 μm NX-C18 110 Å LC column (250 x 21.2 mm) was used for separation.

UV/Vis Spectroscopy - was conducted using a Varian Cary50 UV-Vis Spectrophotometer between 800-200 nm. Equipment was zeroed and baseline corrected for each solvent. Data was collected every 100 milliseconds, at a scan rate of 600 nm. A Hellma analytics quartz Suprasil® cuvette was used (10x10 mm) for all analysis using 3500 μl of the required solution. Photo-responsive materials were all pre-conditioned, subject to 10 minutes of white light before being incubated or placed under UV light for 4 hours. UV light at 365 nm (8W) was used for pre-incubation for isomerisation studies. Spectroscopic grade water was used for the characterisation in pH experiment, and pH was monitored with a Hanna instruments –pH 209 meter.

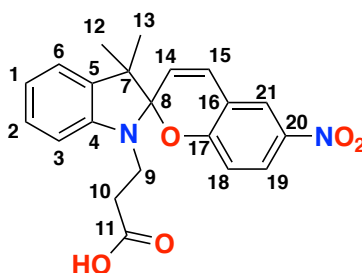
Spiropyran analogues

Synthesis of 1-(2-carboxyethyl)-2,3,3-trimethyl-3*H*-indol-1-ium iodide (10)³⁵⁵ - In a two-necked round bottom flask (RBF), equipped with a reflux condenser and rubber septum, 2,3,3-trimethylindolenine (10.1 g) and 3-iodopropionic acid (12.6 g) were dissolved in methyl ethyl ketone (10 ml) and heated under N₂/reflux at 100 °C for 12 hours. After cooling, the solid material was then filtered and dried under vacuum at 40 °C overnight to yield an orange solid. (18.6 g, 98%)



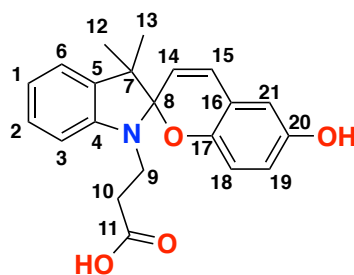
M.P. 177-179 °C; **¹H NMR** (DMSO-*d*₆) δ 12.73 (s, COOH, 1H), 8.06 – 7.93 (m, Ar-H (C3), 1H), 7.91 – 7.80 (m, Ar-H (C6), 1H), 7.63 (m, Ar-H (C1,2), 2H), 4.67 (t, *J* = 7.1 Hz, CH₂ (C12), 2H), 3.00 (t, *J* = 7.0 Hz, CH₂ (C13), 2H), 2.88 (s, CH₃ (C9), 3H), 1.54 (s, CH₃ (C10, 11), 6H); **¹³C NMR** (DMSO-*d*₆) δ 197.9 (C8), 171.5 (C14), 141.7 (C5), 140.8 (C4), 129.3 (C1), 128.9 (C2), 123.5 (C6), 115.6 (C3), 54.3 (C12), 43.6 (C7), 31.1 (C13), 21.9 (C10, 11), 14.4 (C9); **LC-MS, Eluted at:** 0.621 min: *m/z* = C₁₄H₁₈NO₂ [M]⁺ calcd 232.13; found 232.14.

Synthesis of 3-(3',3'-dimethyl-6-nitrospiro[chromene-2,2'-indolin]-1'-yl) propanoic acid (11a)³⁵⁵ - A dry round-bottom flask was wrapped with aluminum foil and then charged with the iodide salt (**10**, 2.13 g) in methyl ethyl ketone (20 ml). Piperidine (0.71 ml) and 2-hydro-5-nitrobenzaldehyde (1.01 mg) were added in one portion. The reaction mixture was heated to 80 °C, stirred and refluxed for 3 hours. Reaction mixture was then cooled to room temperature and refrigerated for 12 hours without stirring. The precipitated material was isolated and washed with cold methyl ethyl ketone. Product was then recrystallized in methanol and precipitated out in methyl ethyl ketone to obtain the desired product as a bright yellow solid. (1.63 g, 71%)



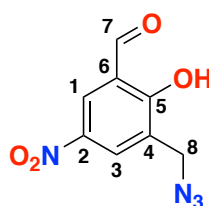
M.P. 111-113 °C; ¹H NMR (DMSO-*d*₆) δ 12.22 (s, COOH, 1H), 8.21 (d, *J* = 2.8 Hz, Ar-H (**C15**), 1H), 8.00 (dd, *J* = 9.0, 2.8 Hz, Ar-H (**C19**), 1H), 7.21 (d, *J* = 10.4 Hz, Ar-H (**C15**), 1H), 7.17 – 7.08 (m, Ar-H (**C2**, **3**), 2H), 6.86 (d, *J* = 9.0 Hz, Ar-H (**C18**), 1H), 6.80 (t, *J* = 7.4 Hz, Ar-H (**C1**), 1H), 6.66 (d, *J* = 7.7 Hz, Ar-H (**C6**), 1H), 6.00 (d, *J* = 10.4 Hz, Ar-H (**C14**), 1H), 3.60 – 3.22 (m, CH₂ (**C9**), 2H), 2.65 – 2.37 (m, CH₂ (**C10**), 2H), 1.19 (s, CH₃ (**C13**), 3H), 1.07 (s, CH₃ (**C12**), 3H); ¹³C NMR (DMSO-*d*₆) δ 172.8 (**C11**), 159.1 (**C20**), 146.1 (**C4**), 140.5 (**C17**), 135.6 (**C5**), 128.1 (**C6**), 127.6 (**C14**), 125.7 (**C2**), 122.8 (**C16**), 121.8 (**C18**, **19**), 119.2 (**C15**), 118.8 (**C1**), 115.5 (**C8**), 106.6 (**C21**), 106.5 (**C3**), 52.4 (**C7**), 33.2 (**C9**), 25.6 (**C10**), 19.5 (**C12**, **13**); LC-MS, Eluted at: 3.05 min: *m/z* = C₂₁H₂₁N₂O₅ [M+H]⁺ calcd 381.14; found 381.22.

Synthesis of 3-(6-hydroxy-3',3'-dimethylspiro[chromene-2,2'-indolin]-1'-yl) propanoic acid (11b) - A dry round-bottom flask was wrapped with aluminum foil and then charged with the iodide salt (**10**, 2 g) in methyl ethyl ketone (20 ml). Piperidine (1.09 ml) and 2,5-Dihydroxybenzaldehyde (760 mg) were added in one portion. The reaction mixture was heated to 80 °C, stirred and refluxed for 3 hours. Reaction mixture cooled to room temperature and refrigerated for 12 hours without stirring. The precipitated material was isolated and washed with cold methyl ethyl ketone. Product was then recrystallized in methanol and precipitated out in methyl ethyl ketone. After drying at 40 °C overnight in the vacuum oven, the product was obtained as a light pink solid. (1.13 g, 57%)



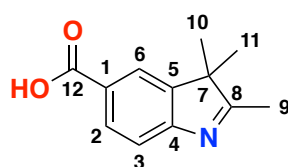
¹H NMR (DMSO-*d*₆) δ 12.19 (s, COOH, 1H), 8.90 (s, OH, 1H), 7.11 – 7.03 (m, Ar-CH (**C2**, **6**), 2H), 6.89 (d, *J* = 10.2 Hz, Ar-CH (**C15**), 1H), 6.73 (td, *J* = 7.4, 0.9 Hz, Ar-CH (**C1**), 1H), 6.59 – 6.54 (m, Ar-CH (**C18**, **19**), 2H), 6.52 – 6.43 (m, Ar-CH (**C3**, **21**), 2H), 5.73 (d, *J* = 10.2 Hz, Ar-CH (**C14**), 1H), 3.53 – 3.21 (m, CH₂ (**C9**), 2H), 2.62 – 2.35 (m, CH₂ (**C10**), 2H), 1.17 (s, CH₃ (**C12**), 3H), 1.03 (s, CH₃ (**C13**), 3H); **¹³C NMR** (DMSO-*d*₆) δ 173.3 (**C11**), 159.6 (**C20**), 146.6 (**C4**), 141.03 (**C17**), 136.1 (**C5**), 128.6 (**C6**), 128.1 (**C14**), 126.2 (**C2**), 123.3 (**C16**), 122.2 (**C18**, **19**), 119.7 (**C15**), 119.3 (**C1**), 116.0 (**C8**), 107.1 (**C21**), 107.0 (**C3**), 52.9 (**C7**), 33.7 (**C9**), 26.1 (**C10**), 19.9 (**C12**, **13**); **LC-MS**, Eluted at: 1.84 min: *m/z* = C₂₁H₂₂NO₄ [**M+H**]⁺ calcd 352.15; found 351.95, C₂₁H₁₉NO₄ [**M-H**]⁻ calcd 350.14; found 349.95.

3-Azidomethyl-2-hydroxy-5-nitrobenzaldehyde (12)¹¹⁹ - To a solution of sodium azide (166 mg, 2.60 mmol) in anhydrous DMSO (4.6 ml) was added 3-(chloromethyl)-2-hydroxy-5-nitrobenzaldehyde (500 mg, 2.30 mmol) in one portion. The mixture was stirred at room temperature under N₂ for 18 hours. H₂O (5 ml) was added dropwise and the mixture was extracted with ethyl acetate (2 x 20 ml). The combined organic phase was washed with H₂O (20 ml), dried over Na₂SO₄ and concentrated in vacuo to yield a dark yellow solid. Separation using a silica plug with toluene as a solvent resulted in a pure light-yellow product (421 mg, 78%).



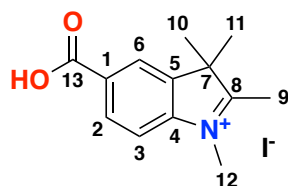
¹H NMR (DMSO-*d*₆) δ 10.21 (s, CHO-(C7), 1H), 8.64 (d, *J* = 2.9 Hz, Ar-H (C1), 1H), 8.50 (d, *J* = 2.9 Hz, Ar-H (C3), 1H), 4.62 (s, CH₂, 2H); ¹³C NMR (DMSO-*d*₆) δ 193.9 (C7), 163.4 (C5), 139.5 (C3), 130.5 (C6), 127.7 (C1), 126.7 (C4), 121.4 (C2), 47.9 (C8); LC-MS, Eluted at: 2.90 min: *m/z* = C₈H₆N₄O₄ [M+H]⁺ calcd 223.04; found 223.90, C₈H₅N₄O₄ [M-H]⁻ calcd 221.03; found 220.85; R_f = 0.7 (C).

Synthesis of 3,3-dimethyl-3*H*-indole-5-carboxylic acid (13**)**¹¹⁹ – To a solution of 4-hydrazinobenzoic acid (2 g) and 3-methyl-2-butanone in anhydrous ethanol (10 ml), H₂SO₄ (98%, 0.4 ml) was added slowly. Solution refluxed under N₂ for 18 hours, the mixture was allowed to cool to room temperature, and the resulting precipitate was removed by vacuum filtration. The filtrate was then basified, using saturated NaHCO₃ and washed with CH₂Cl₂ (2 x 30 ml). Combining the organic extracts, dried using Na₂SO₄, solvent was removed under reduced pressure to yield a dark red solid. (1.43 g, 53%)



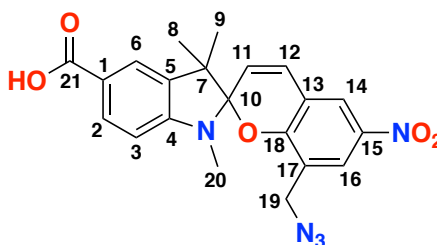
M.P. 193-195 °C; ¹H NMR (DMSO-*d*₆) δ 12.77 (s, OH, 1H), 7.99 (dd, *J* = 1.7, 0.6 Hz, CH-Ar-(**C6**), 1H), 7.91 (dd, *J* = 8.0, 1.7 Hz, CH-Ar-(**C2**), 1H), 7.50 (d, *J* = 8.0 Hz, CH-Ar-(**C3**), 1H), 2.25 (s, CH₃-(**C9**), 3H), 1.27 (s, CH₃-(**C10**, **11**), 6H); ¹³C NMR (DMSO-*d*₆) δ 191.7 (**C12**), 167.5 (**C8**), 157.4 (**C4**), 146.1 (**C5**), 129.6 (**C2**), 127.3 (**C6**), 122.7 (**C1**), 119.1 (**C3**), 53.5 (**C7**), 22.3 (**C10**, **11**), 15.4 (**C9**); **LC-MS, Eluted at:** 2.24 min: *m/z* = C₁₂H₁₄NO₂ [**M+H**]⁺ calcd 204.90; found 204.00; R_f = 0.81 (**B**, 20:80:0)

Synthesis of 1,3,3-trimethyl-2-methyleneindoline-5-carboxylic acid iodide (14)¹¹⁹ – 3,3-dimethyl-3*H*-indole-5-carboxylic acid (0.95 g) was added to a mixture of anhydrous toluene and acetonitrile (2:1, 30 ml). Methyl iodide (670 mg) was added dropwise and the mixture was refluxed for 18 hours. After cooling to room temperature, a red precipitate developed and was collected through vacuum filtration. Washed with ethanol (5 ml) and hexane (30 ml) to yield a light pink solid (1.024 g, 62%).



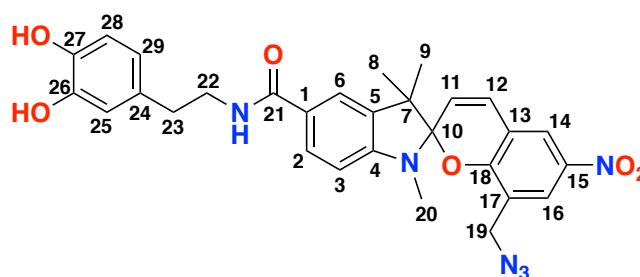
¹H NMR (DMSO-*d*₆) δ 8.39 (s, Ar-H (**C3**), 1H), 8.20 (dd, *J* = 8.4, 1.6 Hz, Ar-H (**C6**), 1H), 8.04 (d, *J* = 8.4 Hz, Ar-H (**C2**), 1H), 4.01 (s, CH₃ (**C12**), 3H), 2.83 (s, CH₃ (**C9**) 3H), 1.58 (s, CH₃ (**C10**, **C11**), 6H); ¹³C NMR (DMSO-*d*₆) δ 199.0 (**C8**), 166.5 (**C13**), 145.2 (**C5**), 141.9 (**C4**), 131.6 (**C1**), 130.3 (**C2**), 124.2 (**C6**), 115.4 (**C3**), 54.2 (**C7**), 35.0 (**C12**), 21.5 (**C10**, **C11**), 14.6 (**C9**); LC-MS, Eluted at: 0.43 min: *m/z* = C₁₃H₁₆NO₂ [M]⁺ calcd 218.11; found 218.06.

Synthesis of 8-(azidomethyl)-1',3',3'-trimethyl-6-nitrospiro[chromene-2,2'-indoline]-5'-carboxylic acid (15)¹¹⁹ – 3-Azidomethyl-2-hydroxy-5-nitrobenzaldehyde (**12**, 30 mg) and 5-Carboxy-1,2,3,3-tetramethyl-3*H*-indolium iodide (**14**, 46 mg) were added to a microwave vial (5 ml) equipped with a stirrer bar. Dry acetonitrile (3 ml) was added to the vial, followed by triethylamine (18.42 μ l) and was microwaved at 40 °C for 10 minutes at 250 W. Vial cooled to room temperature and yellow/green solid collected and washed with acetonitrile (5 ml). Compound was dried under vacuum to yield a pale yellow solid. (41 mg, 72%)



¹H NMR (DMSO-*d*₆) δ 12.35 (s, OH, 1H), 8.27 (d, *J* = 2.8 Hz, Ar-H (**C14**), 1H), 8.17 (d, *J* = 2.8 Hz, Ar-H (**C16**) 1H), 7.81 (dd, *J* = 8.2, 1.7 Hz, Ar-H (**C2**) 1H), 7.69 (d, *J* = 1.7 Hz, Ar-H (**C6**), 1H), 7.29 (t, *J* = 10.5 Hz, (**C12**), 1H), 6.70 (d, *J* = 8.3 Hz, Ar-H (**C3**), 1H), 6.06 (d, *J* = 10.4 Hz, Ar-H (**C11**), 1H), 4.34 – 4.21 (m, CH₂-N₃ (**C19**) 2H), 2.76 (s, CH₃ (**C20**), 3H), 1.27 (s, CH₃ (**C8**), 3H), 1.16 (s, CH₃ (**C9**), 3H); ¹³C NMR (DMSO-*d*₆) δ 167.4 (**C21**), 156.7 (**C18**), 151.0 (**C4**), 140.0 (**C5**), 135.7 (**C15**), 130.8 (**C2**), 128.4 (**C12**), 125.7 (**C16**), 122.9 (**C6**, **14**), 122.7 (**C1**), 121.7 (**C11**), 120.9 (**C17**), 118.9 (**C13**), 106.5 (**C3**), 106.3 (**C10**), 51.4 (**C7**), 48.0 (**C19**), 28.3 (**C20**), 19.5 (**C9**), 15.1 (**C8**); LC-MS, Eluted at: 3.36 min: *m/z* = C₂₁H₂₀N₅O₅ [M+H]⁺ calcd 422.14; found 422.05, C₂₁H₁₈N₅O₅ [M-H]⁻ calcd 420.13; found 420.00, C₄₂H₃₇N₁₀O₁₀ [2M-H]⁻ calcd 841.27; found 841.20.

Synthesis of 8-(azidomethyl)-*N*-(3,4-dihydroxyphenethyl)-1',3',3'-trimethyl-6-nitrospiro[chromene-2,2'-indoline]-5'-carboxamide (16) - 8-(azidomethyl)-1',3',3'-trimethyl-6-nitrospiro[chromene-2,2'-indoline]-5'-carboxylic acid (15, 300 mg) was added to a round bottom flask equipped with a magnetic stirrer bar. To this dopamine hydrochloride (1, 148 mg), HATU (270 mg), and dry DMF (1 ml) was added through a subaseal® whilst under N₂. DIPEA (372 µl) was added slowly to the stirring solution and left for 18 hours. The solution was then washed with 1 M HCl (2.37 ml), and extracted three times using EtOAc (12 ml). The extract was then washed with saturated sodium bicarbonate solution (5 ml) and then with brine (5 ml). Organic phase was dried using magnesium sulfate, and dried to form a purple solid. Preparative HPLC was then used to purify (35 mg) the compound to yield a yellow solid. (17 mg, 42%).



¹H NMR (DMSO-*d*₆) δ 8.76 (s, OH, 1H), 8.64 (s, OH, 1H), 8.29 – 8.23 (m, NH and Ar-H (C14), 2H), 8.17 (d, *J* = 2.8 Hz, Ar-H (C6), 1H), 7.71 (dd, *J* = 8.2, 1.7 Hz, Ar-H (C2), 1H), 7.66 (s, Ar-H (C16), 1H), 7.28 (d, *J* = 10.4 Hz, Ar-H (C11), 1H), 6.67 (d, *J* = 8.3 Hz, Ar-H (C3), 1H), 6.65 – 6.60 (m, Ar-H (C25, 28), 2H), 6.48 (dd, *J* = 8.0, 2.0 Hz, Ar-H (C29), 1H), 6.06 (d, *J* = 10.4 Hz, Ar-H (C12), 1H), 4.39 – 4.13 (m, CH₂-(C19), 2H), 3.36 (m, CH₂-(C22), 2H), 2.73 (s, CH₃-(C20), 3H), 2.68 – 2.56 (m, CH₂-(C23), 2H), 1.27 (s, CH₃-(C9), 3H), 1.15 (s, CH₃-(C8), 3H); ¹³C NMR (DMSO-*d*₆) δ 166.5 (C21), 157.4 (C24), 150.0 (C4), 145.5 (C27), 144.0 (C26), 140.5 (C5), 135.9 (C24), 130.9 (C2), 128.8 (C11), 128.4 (C6), 126.4 (C16), 126.2 (C17), 123.4 (C15), 123.2 (C1), 121.6 (C29), 121.4 (C14), 119.7 (C12), 119.4 (C25), 116.4 (C10), 116.0 (C13), 107.1 (C28), 106.6 (C3), 52.1 (C7), 48.4 (C19), 41.8 (C22), 35.4 (C23), 28.8 (C20), 26.3 (C8), 20.1 (C9); LC-MS, Eluted at: 2.91 min: *m/z* = C₂₉H₂₉N₆O₆ [M+H]⁺ calcd 557.21; found 557.30; R_f = 0.33 (B, 10:90:0)

Biological procedures

The experimental procedure for growing, maintaining and seeding the cells remained the same (Chapter 3 – Experimental). The SPAP assay also remained the same, although some modifications were made in relation to sample preparation and how the compounds were stored in the incubator for the 4-hour incubation period.

SPAP assay

Dilutions of the synthesised compounds were made at 10 mM in DMSO resulting in less than 0.01% of DMSO for the final concentrations in the assay and with a concentration range of 10^{-5} to 10^{-11} moles once again. All compounds in their diluted form were initially irradiated with white light for 1 hour prior to the cell assay. The following conditions were then used to test the activity dependant of photo-isomer:

- i) Constant white light irradiation throughout the 4-hour incubation period
- ii) 96 well plate wrapped in foil (dark conditions) throughout the 4-hour incubation period
- iii) Pre-irradiation of compounds in diluted form for 1 hour under UV light (365 nm)

Chapter 5 - Immobilisation and pharmacology of photo-responsive bio-ligands

Introduction

This Chapter focuses on the immobilisation of dopamine (**1**), spiropyran (**15**) and the spiropyran-dopamine analogue (**16**) on to solid support systems, and their interactions with the dopamine D₁ receptor. This introduction gives a brief outline of work conducted using immobilised neurotransmitters and their specific uses, as well as general surface functionalisation techniques.

Neurotransmitter immobilisation

There has been no reported literature of neurotransmitters bound to solid supports which have been used to activate cell bound receptors. It has been shown that when specific neurotransmitters are attached to solid supports they can still bind recombinant protein receptors. Vaish, *et al*, showed that recombinant membrane receptors bind to gold surfaces modified with serotonin and its precursor 5-hydroxytryptophan (5-HTP) (Figure 5-1).⁵⁴

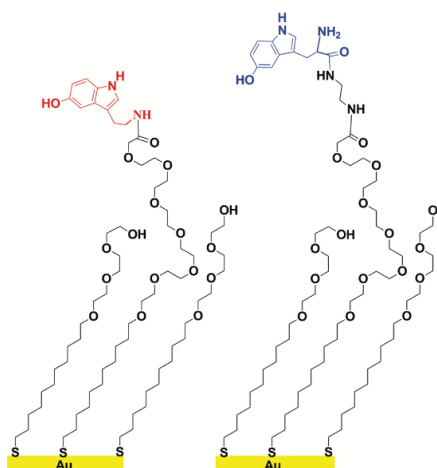


Figure 5-1 - Thiol based self-assembled monolayers on gold surfaces showing serotonin (left) and 5-hydroxytryptophan (right) functionalised surfaces (reproduced from Vaish, *et al*)⁵⁴

Through the use of a quartz crystal microbalance (QCM), binding of 5-hydroxytryptophan antibodies, 5HT₇, 5HT_{1A}, D₁, and GABA_{B1b} recombinant receptors on 5-HTP derived surfaces were studied (Figure 5-2). It was shown that binding was readily observed with the antibodies on the functionalised surface. Interestingly, the 5HT₇ recombinant receptor showed greater binding to the surface which exhibited the natural precursor of serotonin, 5-HTP. This suggests that the unaltered free amino group on the 5-HTP surfaces can play a role in interacting with the serotonin binding pocket when adhered to a surface. Evidence of non-specific binding was also observed to both the D₁ and GABA_{B1b} recombinant receptors. This is the first example of recombinant receptors binding to surfaces with both receptor selectivity and enantiomeric specificity, which is promising in the development of solid support systems in aid for the activation of cell surface receptors.

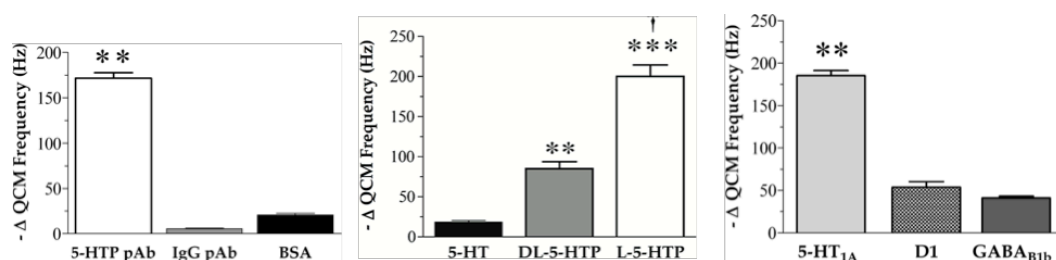


Figure 5-2 - Frequency of binding as determined by QCM for serotonin specific antibodies and recombinant receptors to serotonin and 5-HTP derivatised surfaces showing specific binding as well as preferential binding to 5-HTP surfaces (** $p < 0.01$, *** $p < 0.001$)⁵⁴

Dopamine immobilisation

Dopamine immobilisation in particular has been shown for a number of applications, but not for the specific activation of dopamine receptors. Examples exist where dopamine has been attached to a solid substrate through its aromatic, amine and catechol functionality. This includes the study conducted by Mannironi, *et al*,³⁶⁸ in an attempt to isolate RNA aptamers by *in vitro* selection from a pool of 3.4×10^{14} different RNA molecules. It was shown that dopamine attached through its amino group to a Sepharose bead (via amide coupling) was used as an affinity column to successfully bind to and isolate RNA aptamers specific to dopamine.

Modification at the aromatic core of dopamine was demonstrated by Funder, *et al*.²⁵³ It was shown using the Sonogashira reaction that a precursor of dopamine could be utilised to develop a nanoparticle comprised of a short PEG chain, terminating in dopamine (as shown in Figure 5-3). Critically, the article only describes the synthesis of the compound and not its biological relevance; the material was synthesised to select specific aptamers using the systematic evolution of ligands by exponential enrichment (SELEX).²⁵³

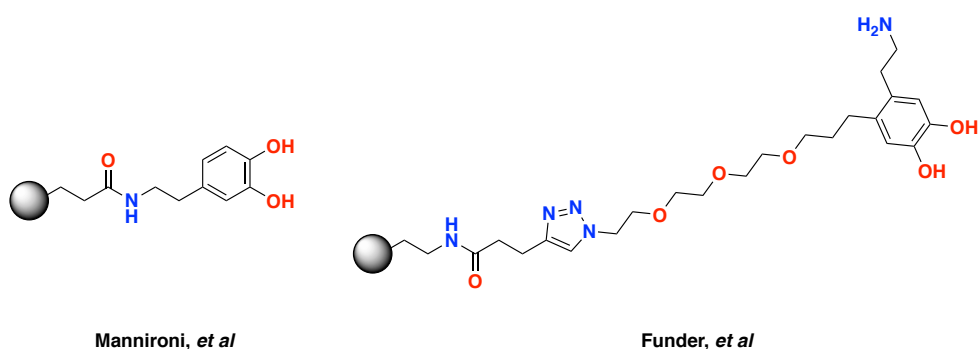


Figure 5-3 - Dopamine attached to solid supports using two methods, amide attachment (Mannironi, *et al*)³⁶⁸ and through azide coupling with a PEG chain attached directly to the aromatic core of dopamine (Funder, *et al*)²⁵³

The use of dopamine in the development of a multifunctional polymer coating has also been shown. Conducted by Messersmith, *et al*, the work focuses on using dopamine as a binding agent, inspired by adhesive proteins secreted by mussels to wet surfaces which typically consist of the dopamine precursor, DOPA.³⁶⁹ Successful deposition of dopamine in a thin layer was conducted by using a dip coating method as a base for further modification. A homogenous, thin adherent polymer film on a number of organic and inorganic substrates, using an aqueous solution of dopamine (2 mg of dopamine per 1 ml of 10 mM tris, pH 8.5) was achieved. This dopamine deposition strategy utilises the oxidative properties of the molecule to create a melanin based surface which forms a poly-dopamine film. As we aim to keep dopamine intact on our surfaces, it is important to understand how to prevent dopamine deposition in this way, avoiding oxidation of the molecule and retaining the ability to activate surface receptors.

Surface functionalisation

A number of surface types have been used for chemical and biological applications; these include silicon based materials (e.g. glass, mica), gold surfaces, hydrogels and also nanoparticles (e.g. silica, titania, cadmium selenide and iron oxide).^{87–89} These materials have been covalently modified or coated to achieve a desired surface functionality. As our work focuses on glass substrates and silica nanoparticles, we discuss surface preparation, fabrication and functionalisation below.

Surface activation for glass substrates

Glass substrates are typically cleaned before use as a biomaterial. A number of methods exist to conduct this, which eventually leads to an activated surface ready for modification. Plasma cleaning or etching is one such technique, where a plasma is generated under high pressure using oxygen, hydrogen, argon or other gasses.³⁷⁰ The plasma generated from each of these gasses can provide specific characteristics for the cleaned surfaces. For example, oxygen is typically used to oxidise the surface of glass, resulting in a super hydrophilic surface. These surfaces can then in turn react with a silane in creating functionalised surfaces. Piranha solution (a combination of sulfuric acid and hydrogen peroxide) to form a highly corrosive, oxidising agent is also regularly used. It is typically used in the chemical and microelectronics industry to remove any organic residues from silicon substrates, and to promote the generation of hydroxyl terminated surfaces.³⁷¹

Nanoparticle synthesis

Synthesis of nanoparticles can be achieved in a number of ways dependent on size³⁷², shape³⁷² and composition.³⁷³ As we focus on the use of silica nanoparticles in this project, we explore methods of nanoparticle fabrication in relation to these. Typically, the formation of silica nanoparticles is achieved using the sol-gel process, which is a wet chemistry technique following the steps shown in chronological order: hydrolysis and polycondensation, gelation, aging, drying, densification, and crystallization.³⁷⁴ This is known as a 'bottom up' approach to nanoparticle synthesis, whilst a 'top down' approach can also be used. This involves the formation of nanoparticles from a bulk surface, employing the use of lithography³⁷⁵ or milling techniques.^{376,377} These are usually used to generate soft metal nanoparticles, but have been used for the synthesis of silica nanoparticles as well.^{375,378}

Surface modification approaches

Functionalisation of the surface can be approached in two ways, using a 'bottom-up' or 'top-down' method. A 'bottom-up' synthetic approach is commonly used to generate an interface by covalently modifying the surface in a number of reaction steps to generate the desired functionality. The 'top-down' method, uses a series of compounds, such as a protein sequence generated in bulk, which is then attached to a surface in one go. This can be disadvantageous due to steric hindrance of large molecules, and cause issues such as non-homogenous surface chemistry.³⁷⁹ As we are synthesising short chains, the bottom up approach is the most relevant for this project.

Self-assembled monolayers

Self-assembled monolayers (SAMs) are molecular assemblies formed spontaneously on surfaces, in a highly ordered manner composed of organic constituents (Figure 5-4).³⁸⁰ Typically, alkylsilanes are used on oxidised surfaces, and sulphur containing compounds on gold. A number of terminal functional groups including but not limited to -NH_2 , -COOH , -SH , and -Br groups can be achieved.

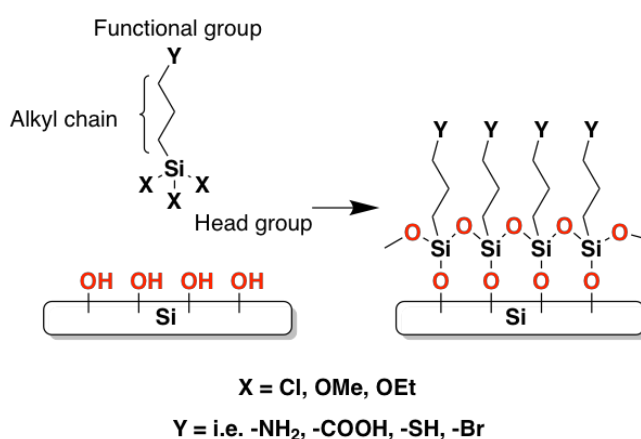


Figure 5-4 - Functionalisation of silicon based surfaces using silane based self-assembled monolayers

Chemistry - Synthetic approaches

Surface functionalisation of the biomaterials in this Chapter utilise the general chemistry of amide synthesis as described in the introduction for Chapter 4 (utilizing the DIC coupling method on a solid substrate). A small introduction is provided on click chemistry in this Chapter to explain the coupling method used to attach compound **15** to the solid supports systems to create a stimuli-responsive surface.

'Click' chemistry

'Click' chemistry is a term that was introduced in 2001 by Sharpless, *et al*, to describe reactions that are high yielding, stereospecific, simple to perform, and can be conducted in easily removable or benign solvents (such as water).³⁶² It has been commonly employed in wet chemistry and surface modification. Several reactions exist which fulfil the prerequisites to be classed as a 'click' reaction with one particular example of interest, the azide-alkyne interaction, or the Huisgen 1,3-dipolar cycloaddition using a copper catalyst (CuAAC, Figure 5-5).

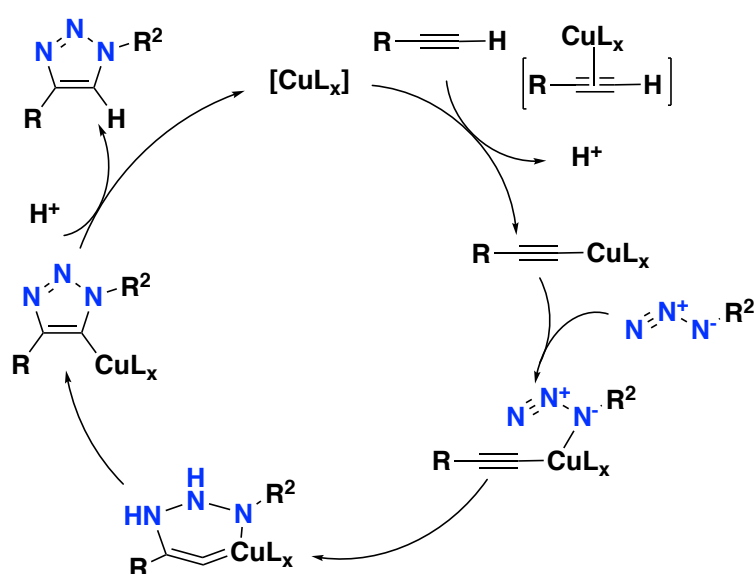
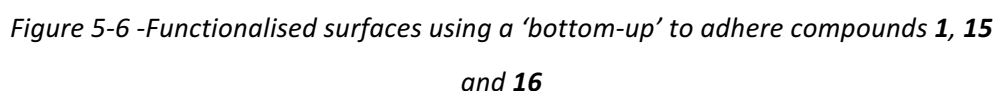


Figure 5-5 - Copper-catalysed azide-alkyne cycloaddition (CuAAC) cycle

The classical Huisgen 1,3-dipolar cycloaddition conditions which were first described in the 1960s did not fall into the above definition of a 'click' reaction as harsh conditions were required, producing a mixture of 1,4 and 1,5-regio-isomers.³⁸¹ It was not until the discovery by Tornøe, *et al* that copper (I) salts aid in the catalysis of the reaction that it was defined as a 'click chemistry' type reaction.^{382,383} This copper mediated reaction occurs in a regio-selective manner at room temperature with much shorter reaction times.

Establishing that spiropyran, whilst conjugated to dopamine in solution, could be used to control activity at the dopamine D₁ receptor (dependent on the photo-isomer), we aim to achieve translate this functionality to surface immobilised systems. This Chapter focuses on the immobilisation of compounds developed and tested in Chapter 3 and 4, which include **1**, **15** and **16** to flat surfaces and nanoparticles using amide and CuAAC chemistry (Figure 5-6). Using silanes, silica based substrates were modified in a bottom up approach, leading to the termination of dopamine, spiropyran and spiropyran-dopamine functionalised surfaces.



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Analysis of the solid supports were conducted using both chemical and physiological methods: WCA and ToF-SIMS for the glass coverslips; FTIR-ATR, dynamic light scattering (DLS), and UV/Vis spectroscopy for the nanoparticles.

Results and Discussion

Substrate preparation

Glass substrates

Glass substrates were successfully cleaned and prepared using oxygen plasma, confirmed by surface wettability measurements. It was shown that an angle below 5° was achieved after oxidation of the surface, leading to a very hydrophilic surface (Figure 5-7) in comparison to control samples (uncleaned) that display contact angles larger than 20° .

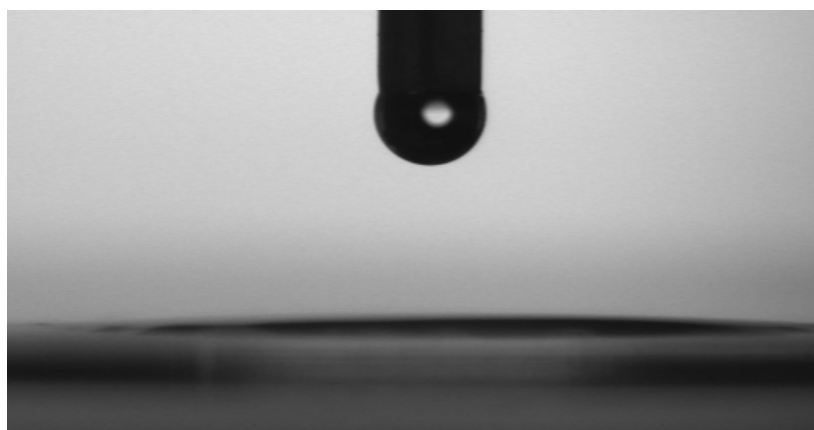


Figure 5-7 - Surface wettability of an oxygen plasma cleaned coverslip at 100 W for 1 minute (angle at 1.6°)

Nanoparticles

Synthesis of nanoparticles was conducted using the Stöber process.³⁸⁴ Developed in 1968, it was the first method used to develop monodisperse silica nanoparticles in solution, using triethoxyorthosilicate (TEOS) as a source. The equation derived by Bogush, *et al*, could be used to calculate the approximate amount of TEOS in combination with ammonium hydroxide, water and ethanol to produce monodisperse nanoparticles of a specific size within 20% of the specific targeted size.³⁸⁵

The expression (Equation 7) was derived to fit the experimental data from over 100 sample preparations (of silica nanoparticles) synthesised at 25 °C (between 40-180 minutes) with concentration ranges of 0.1-0.5 M TEOS, 0.5-17 M H₂O and 0.5-3 M NH₃ in ethanol. It was shown that an increase in water concentration would lead to an increase in particle diameter, before plateauing (at approximately 8 M) then decreasing. An increase in the concentration of ammonia, resulted in larger particles being formed, whilst elevated levels of TEOS concentrations, affected particle size distribution, making it less reproducible. It was also shown by Bogush, *et al*, that an increase in temperature resulted in a narrow size distribution, for all TEOS concentrations (studies were carried out with temperature ranges between 9-55 °C).

$$d = A[H_2O]^2 \exp(-B[H_2O]^{1/2}) \quad \text{Equation 7}$$

where

$$A = [TEOS]^{1/2} (82 + 151 [NH_3] + 1200 [NH_3] - 366[NH_3]^3) \quad \text{Equation 8}$$

$$B = 1.05 + 0.523[NH_3] - 0.128[NH_3]^2 \quad \text{Equation 9}$$

Where d - is the average diameter in nanometres

To ensure diffusion through the extracellular matrix, limit rates of cellular uptake and negate toxicity as well as to be large enough and have a sufficient surface area to be functionalised with our photo-responsive ligand, a mono-disperse particle size of 60 nm was aimed for. Through the use of the Stöber process, successful synthesis of mono-disperse nanoparticles was achieved, using TEOS (0.138 M), H₂O (5.3 M) and NH₃ (0.18 M) in ethanol.

Particle morphology and size was visualized by transmission electron microscopy (TEM, Figure 5-8). This indicated a particle size of 50.4 ± 10.5 nm (size dispersity was calculated using image J Ver.1.51m9) which was used throughout this Chapter.

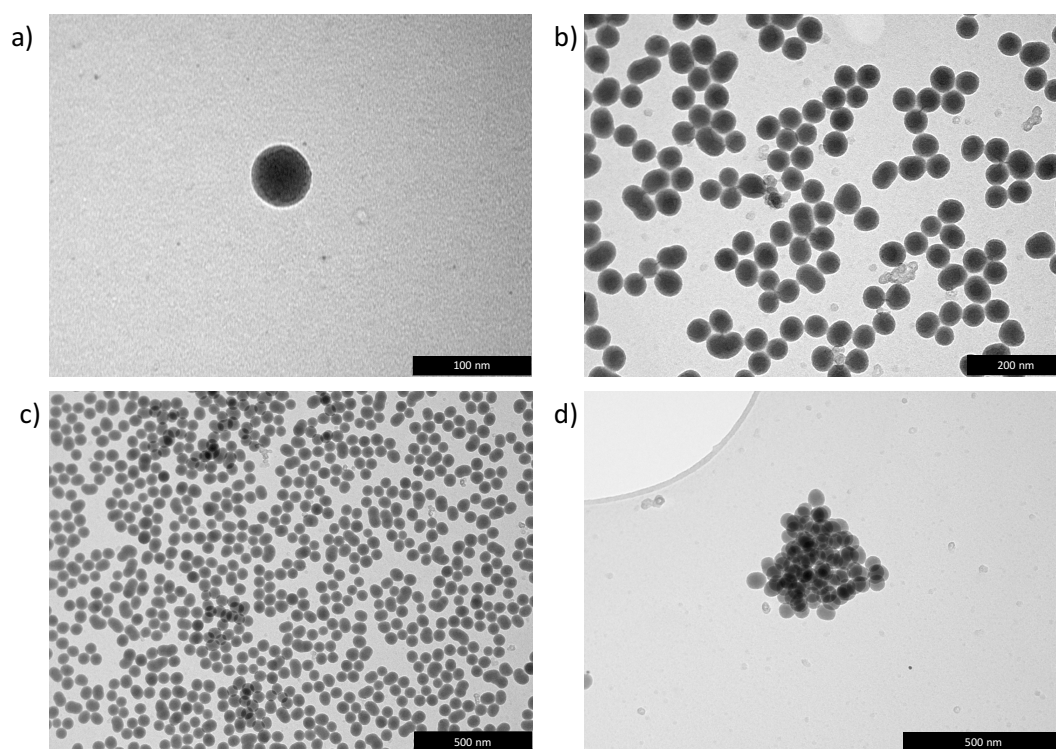


Figure 5-8 - TEM Images of single batch synthesized silica nanoparticles (a) 100 nm scale showing single nanoparticle, (b) 200 nm scale, (c) 500 nm scale, (d) 500 nm scale showing aggregation of the silica particles

After substrate preparation, surface functionalisation of the glass substrates and nanoparticles with dopamine and spiropyran was then conducted using a bottom up approach.

Dopamine surface modification series

Dopamine immobilised substrates were generated through carboxyethylsilane triol (CBEST) monolayers to generate a carboxylic acid terminated surface, which was then further modified using the *N,N'*-di-isopropylcarbodiimide coupling method to yield a dopamine functionalised surface (Figure 5-9).

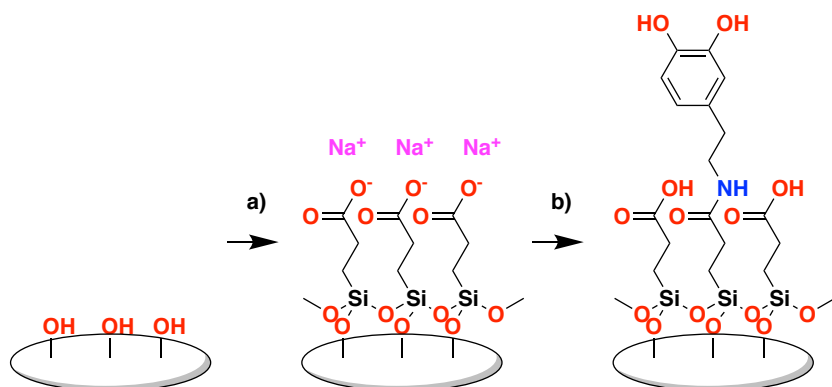


Figure 5-9 - General modification of silica based substrates to generate dopamine modified surfaces (a - carboxyethylsilane triol, PBS, 24h; b - DIC, DMF, dopamine, Et_3N)

Glass substrate

Following the procedure as shown in Figure 5-9, surface functionalisation of the glass coverslips was conducted. Changes in surface chemistry were initially monitored through surface wettability measurements as shown in Figure 5-10. Low contact angles were observed for the plasma cleaned and carboxylic acid terminated surfaces, whilst a large increase in angle was shown upon dopamine functionalisation. A minimal increase of water contact angle from 1.6° to 4.4° was observed after functionalisation using CBEST, but upon the addition of the dopamine, the angle increased to $58 \pm 3^\circ$.

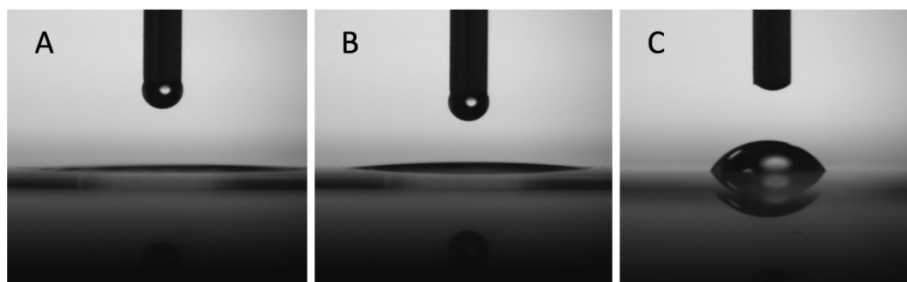


Figure 5-10 - Images showing surface wettability for unmodified plasma cleaned (A), carboxylic acid terminated (B), and dopamine functionalised surfaces (C)

In accordance with literature, low water contact angle values are typically observed with oxygen plasma cleaned surfaces, whilst contact angles in the range $15 - 25^\circ$, with one such example of $18.3 \pm 3.9^\circ$ have been observed for carboxylic acid terminated surfaces.³⁸⁶ As for dopamine modified surfaces, Messersmith, *et al*, reported water contact angles between 43 and 54° for various types of poly-dopamine modified surfaces, with a value of 54° for silicon based substrates, a close match to our experimental data.³⁶⁹ This large increase in contact angle from the carboxylic acid terminated surface to dopamine functionalised surfaces was understood to be caused by the orientation of dopamine on the surface. This was either the result of the multiple rotameric conformers exhibited by dopamine causing exposure of the aromatic moiety^{387,388} or, if the catechol binds to the carboxylic acid on the surface to form an ester due to the generation of a less hydrophilic surface due to the introduction of amino-terminated alkyl group.

Due to the limited information about chemical surface composition provided by surface wettability measurements, chemical analysis of the surfaces was conducted using ToF-SIMS, to confirm surface modification and homogeneity. The functionalised surfaces were analysed ($500 \times 500 \mu\text{m}$) in both positive (Figure 5-11) and negative (Figure 5-12) polarity modes. Subsequently, $150 \times 150 \mu\text{m}$ regions of interests were used to determine surface homogeneity within the individual samples.

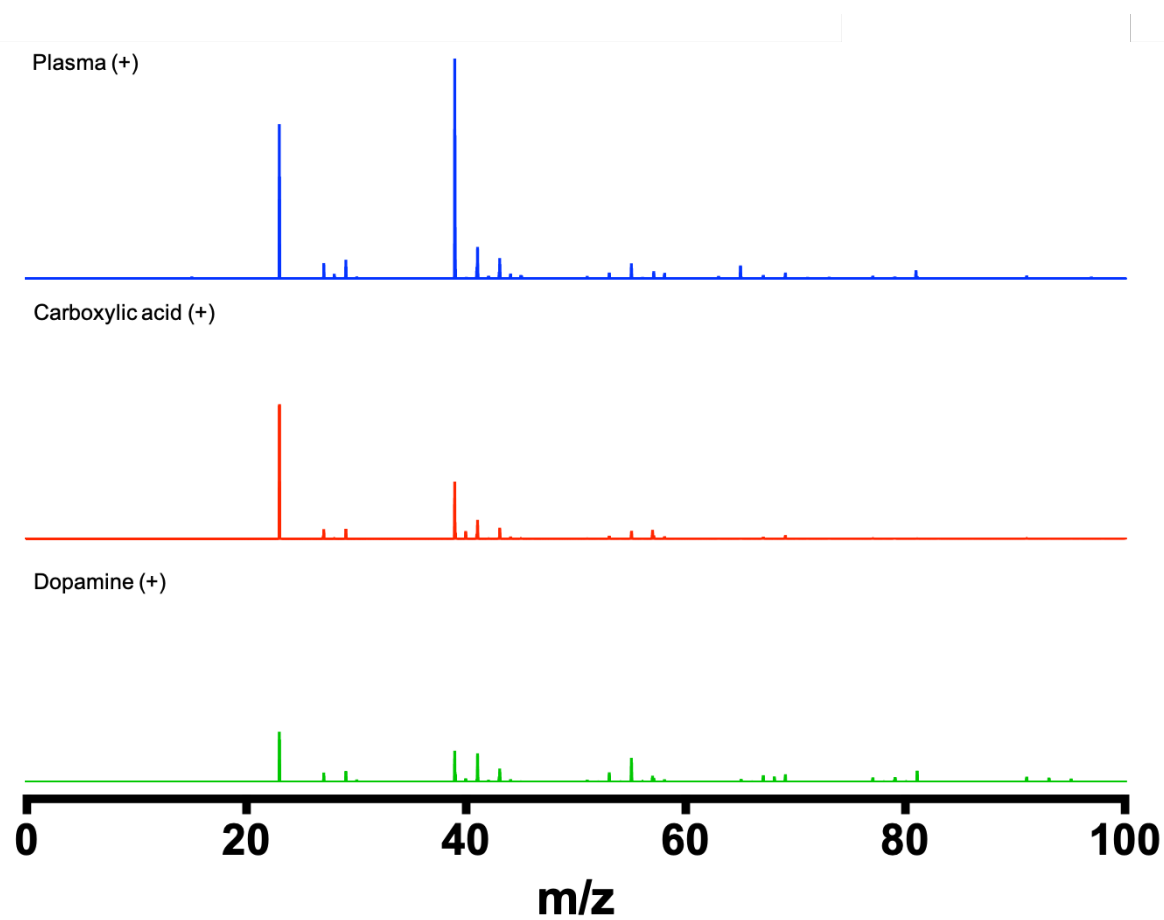


Figure 5-11 - Positive ion ToF-SIMS spectra for plasma cleaned glass, carboxylic acid and dopamine functionalised glass coverslips (m/z 0-100)

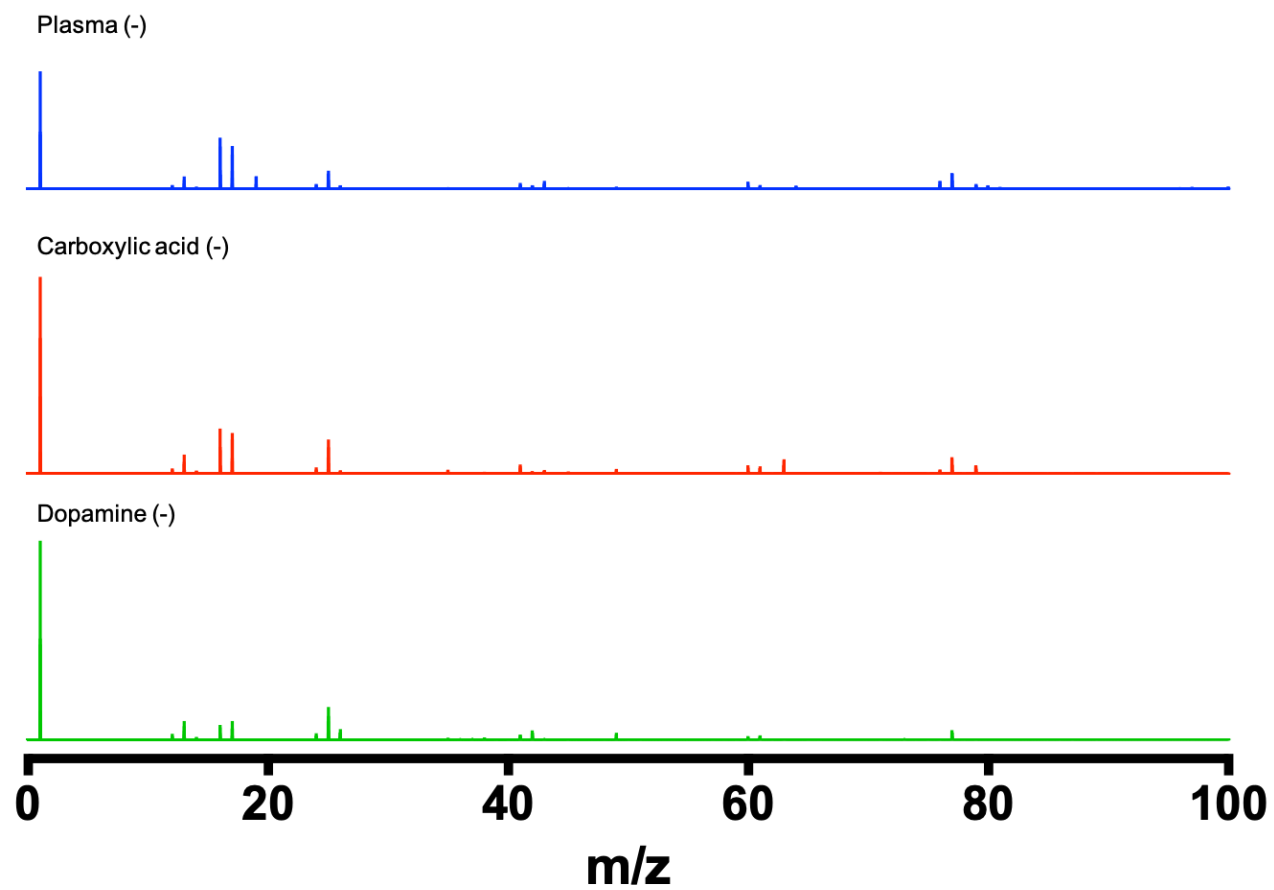


Figure 5-12 - Negative ion ToF-SIMS spectra for plasma cleaned glass, carboxylic acid and dopamine functionalised glass coverslips (m/z 0-100)

Principle component analysis (PCA) can be used to sift through large data sets between a variety of samples, which makes it ideal to determine changes and variables in the functionalised surfaces generated within this Chapter. PCA was conducted for both positive and negative spectra, as each polarity gives us distinct masses which relate to specific fragment ion patterns associated with particular surface chemistry.

Figure 5-13 shows the scores plot obtained for the positive ion spectra for the plasma cleaned, carboxylic acid and dopamine functionalised glass coverslips. This plot shows the components which account for most of the variance in the data, and can be used to assess the data structure and detect clusters, outliers, and trends. Using PC1 vs PC2, we can identify three distinct clusters, representing fragment ions which are lineally unrelated, associated with the plasma, carboxylic acid and dopamine functionalised surfaces. Within each cluster, we can identify homogeneity within samples.

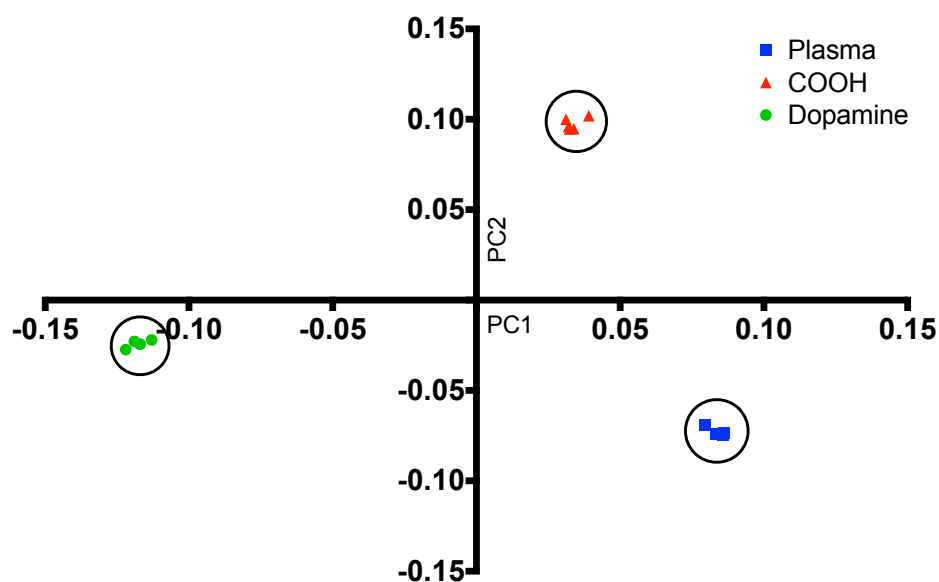


Figure 5-13 - Positive ion scores plot showing three distinct clusters of variance (n=5) for plasma, carboxylic acid and dopamine surfaces (PC1 vs PC2)

As each component assess variability in the data set, we can use a loadings plot to determine which ion fragments have the largest effect on different principle components. Loadings close to -1 or 1 indicate that the variable strongly influences the component. Loadings close to 0 indicate that the variable has a weak influence on the component. Figure 5-14 and Figure 5-15, shows mass ion fragments responsible for influencing PC1 and PC2.

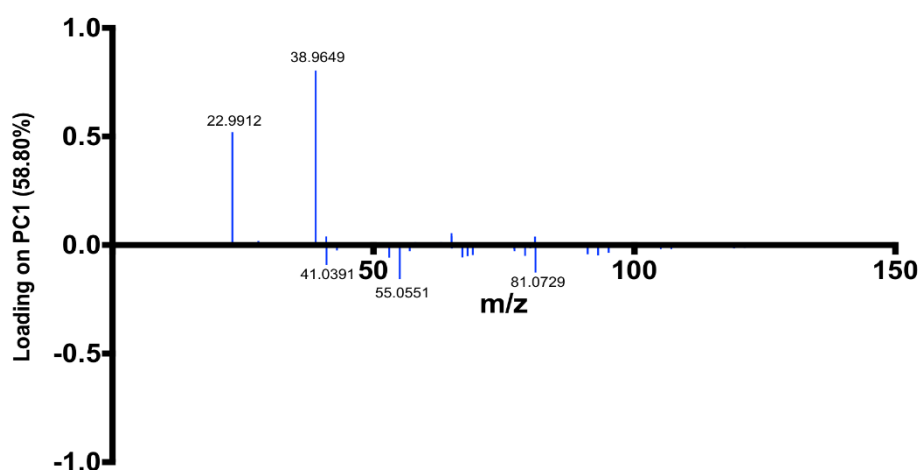


Figure 5-14 - Loadings plot to identify which ion fragments contribute to PC1 for the positive ion spectra for the plasma cleaned, carboxy- terminated and dopamine functionalised surfaces

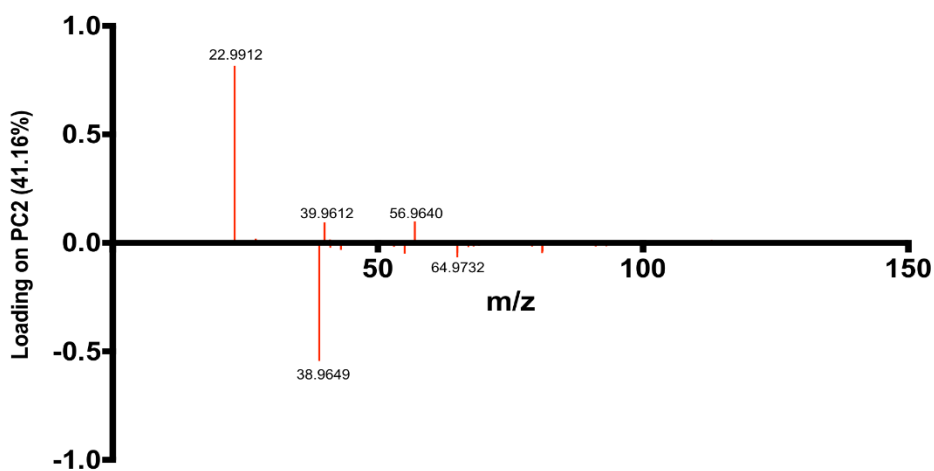


Figure 5-15 - Loadings plot to identify which ion fragments contribute to PC2 for the positive ion spectra for the plasma cleaned, carboxy- terminated and dopamine functionalised surfaces

Using the scores and loadings plot, a list of which mass fragments associated with each of the functionalised surfaces was generated. A selection of these are shown in Table 5-1, Table 5-2 and Table 5-3, for the plasma cleaned, carboxylic acid and dopamine terminated surfaces, respectively. The mass values were referenced with the Ion ToF software, to generate the most likely ion fragments associated with the m/z values, which had a maximum deviation of 150 ppm. From the data obtained, we can see the prominence of potassium and sodium ions present on the plasma cleaned surface, which is indicative of a non-modified surface. The presence in the top 5 generated ions of the Si⁺ ion also confirms this due to the composition of the glass.

Table 5-1 - Positive mass fragments associated with plasma cleaned surface as identified from PCA

m/z	Fragment	Deviation (ppm)
38.96	K ⁺	30.62
22.99	Na ⁺	62.22
27.98	Si ⁺	43.84
62.99	SiH ₃ O ₂ ⁺	83.04
58.07	C ₃ H ₈ N ⁺	22.83

The carboxylic acid terminated surface also shows the presence of a Na⁺ ion which distinguishes it from the other surfaces. This may be due to the increase in sodium on the surface as the carboxyethylsilane triol is a di-sodium salt, and even with repetitive washing the salt still may be present. Upon the addition of CBEST, we start to see the addition of carbon species. C₂H₃⁺ and C₂H₅⁺ fragments, relate to the carbon core of the silane, whilst the C₂HO⁺ gives us an indication of the presence of a carboxylic acid.

Table 5-2 - Positive mass fragments associated with carboxylic acid functionalised surfaces as identified from PCA

m/z	Fragment	Deviation (ppm)
22.99	Na ⁺	62.22
26.98	Al ⁺	19.96
27.02	C ₂ H ₃ ⁺	19.42
41.01	C ₂ HO ⁺	67.31
29.04	C ₂ H ₅ ⁺	47.34

On addition of the dopamine, the functionalised surface starts to demonstrate ion fragments with the core structure of C_xH_y⁺. This is indicative of fragmentation patterns associated with the aromatic structure of dopamine.

Table 5-3 - Positive mass fragments associated with dopamine functionalised surfaces as identified from PCA

m/z	Fragment	Deviation (ppm)
55.06	C ₄ H ₇ ⁺	5.89
81.07	C ₆ H ₉ ⁺	30.52
41.04	C ₃ H ₅ ⁺	0.61
53.04	C ₄ H ₅ ⁺	34.41
67.05	C ₅ H ₇ ⁺	22

Ion fragments from above were then compared for each functionalised surface. Si⁺ (plasma cleaned), C₂HO⁺ (carboxylic acid) and C₆H₉⁺ (dopamine) were chosen for their respective functionalised surfaces due to structural relevance. Relative peak intensities were compared (Figure 5-16) and ion map images (Figure 5-17) were shown to demonstrate the homogeneity of the functionalised surfaces.

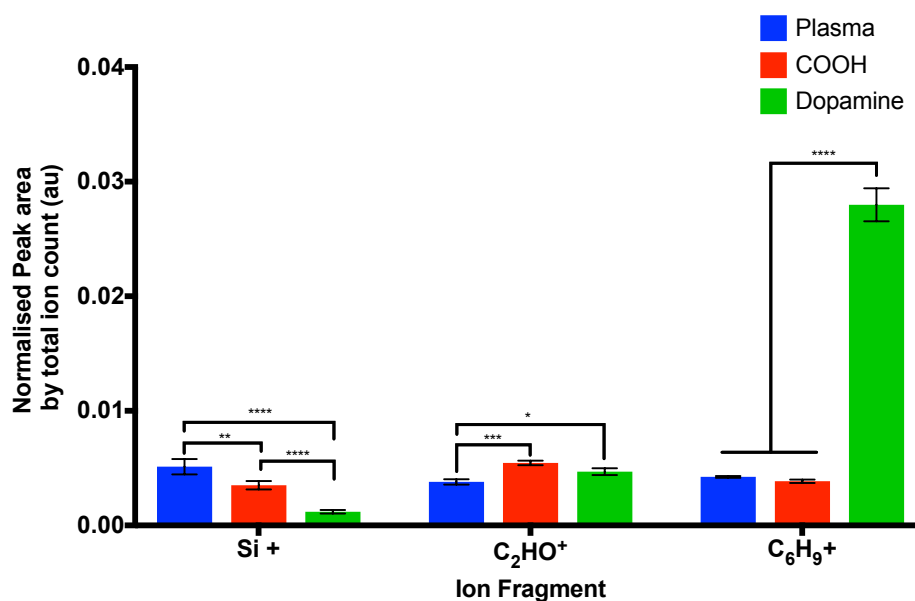


Figure 5-16 – ToF-SIMS normalised peak area by total ion count for Si⁺, C₂HO⁺, and C₆H₉⁺ ions for plasma cleaned, carboxylic acid and dopamine functionalised surfaces (Tukey's multiple comparisons test indicating significant differences **** $p < 0.0001$, *** $p = 0.0001$, ** $p = 0.0002$, * $p = 0.0437$, $n=5$, error bars represent SD)

The build-up of surface functionality is evident from the reduction of the Si⁺ ion fragment from glass. As ToF-SIMS measures the top layer of chemistry, subsequent surface functionalisation masks ions from the glass substrate, causing a reduction on the Si⁺ ion intensity. The C₂HO⁺ fragment ion was shown to be more prominent on the COOH terminated surface than the plasma cleaned and dopamine functionalised surface. This was confirmed statistically using Tukey's multiple comparison test where a significant difference was shown between the plasma cleaned and COOH surface ($p = 0.0001$), and the plasma cleaned and dopamine surface ($p = 0.0437$). No significant difference was seen between the COOH and dopamine functionalised surface. Observance of carbon based species on the plasma cleaned surfaces can be explained by adventitious carbon, possibly introduced by contaminations present in the sample chamber during oxygen plasma treatment.³⁸⁹ We also see an increase in the peak area for the C₂HO⁺ on the dopamine functionalised surface. In addition to the fragmentation of the amide bond formed, fragmentation can also occur in this manner from the catechol present.³⁹⁰

Finally, on dopamine modified surfaces the peak intensity of the $C_6H_9^+$ ion, indicative of the aromatic groups, is significantly higher compared to other surfaces ($p < 0.0001$). This is in line with the expected increase in aromatic groups after introduction of dopamine on the surface.

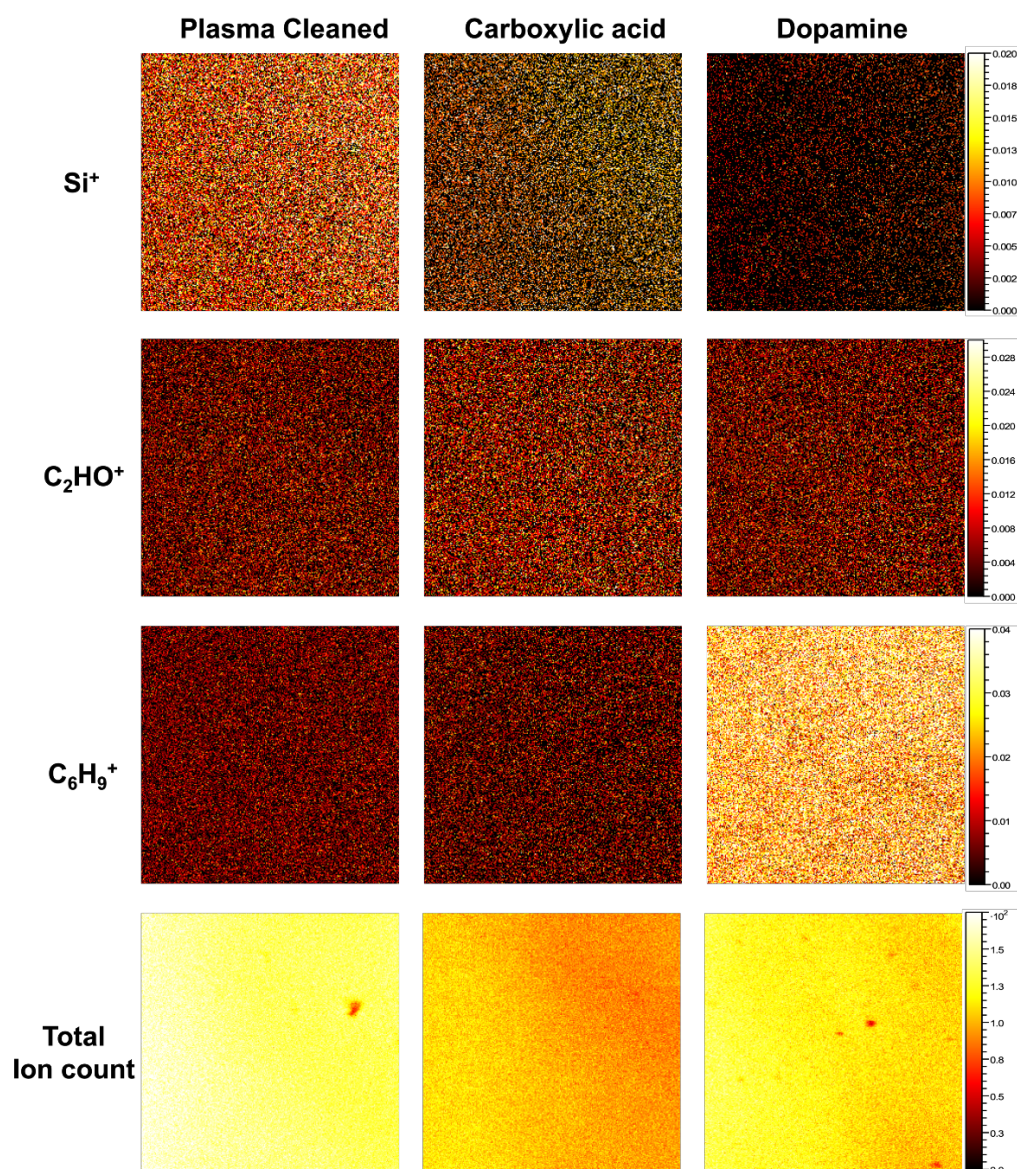


Figure 5-17 - Ion map images normalised by total ion count ($500 \times 500 \mu m$) for Si^+ , C_2HO^+ , and $C_6H_9^+$ for plasma cleaned, carboxylic acid terminated and dopamine functionalised surfaces and maps of total ion counts for each sample. (scale bar represents relative ion intensities of specified ions, brighter indicated more of the ion present in the sample)

Comparisons of the ion map images for the above fragments were shown in Figure 5-17. This allowed us to visualise ion distribution between samples, as well as within the sample, giving us an idea of homogeneity. A clear distinction can be made for the ion map images for both Si^+ and the C_6H_9^+ fragments. Statistically we have shown that the C_2HO^+ ion is more prominent on the carboxylic acid surface and can be seen with an increased presence from the ion map images. Equal distribution of the ions for all three samples allowed us to confirm surface homogeneity upon functionalisation was successfully achieved.

As above, PCA was conducted on the negative spectra giving us the scores plot as shown in Figure 5-18. As we can see, separation of the oxygen plasma cleaned and functionalized surfaces was mainly dependent on principle component 1. Three distinct clusters which represent each surface modification can be seen, although the distribution within each cluster shows some variability within the sample, especially for the dopamine functionalised surface.

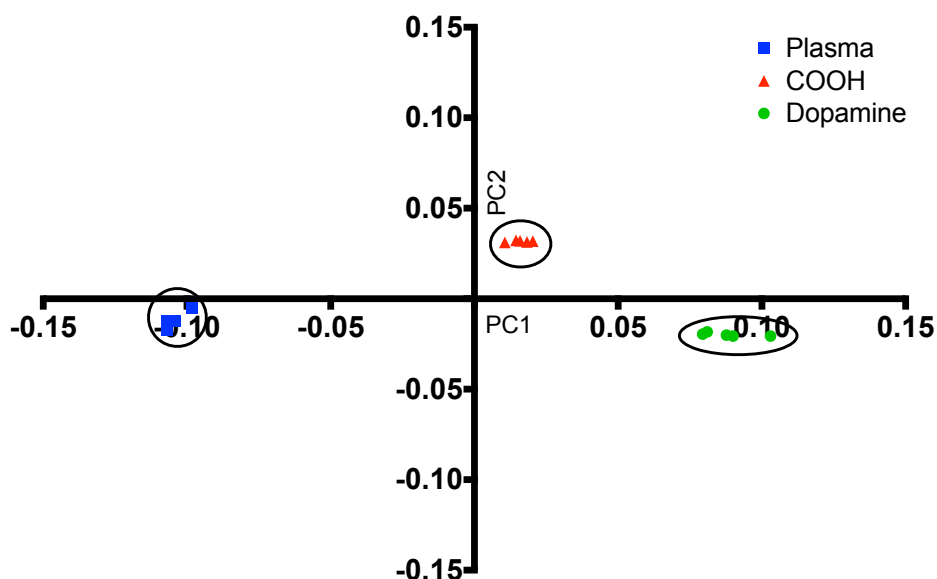


Figure 5-18 - Negative ion scores plot showing three clusters of variance (n=5) for plasma, carboxylic acid and dopamine surfaces (PC1 vs PC2)

Loadings for each component were then analysed to determine key ion fragments responsible for cluster separation, as shown in Figure 5-19 and Figure 5-20.

The negative ions associated with the plasma cleaned surface show groups which contain oxygen and silicon, representative of a non-functionalised, plasma cleaned surface. We can see some evidence of adventitious carbon in a few of the peaks assigned to the m/z values.

Table 5-4, Table 5-5 and Table 5-6 shows the key fragments identified for each of the plasma cleaned, carboxylic acid and dopamine functionalised surfaces, respectively.

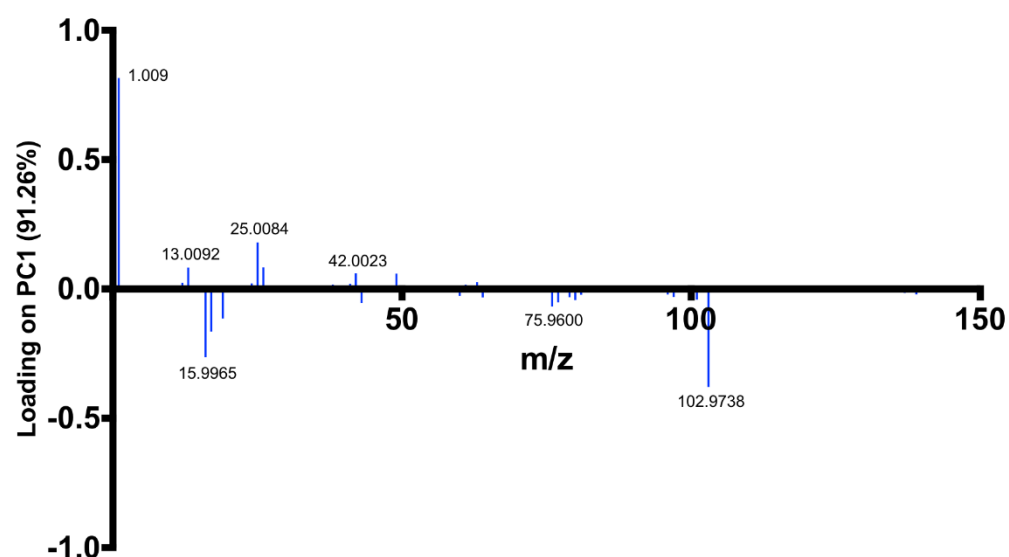


Figure 5-19 - Loadings plot to identify which ion fragments contribute to PC1 for the negative ion spectra for the plasma cleaned, carboxy- terminated and dopamine functionalised surfaces

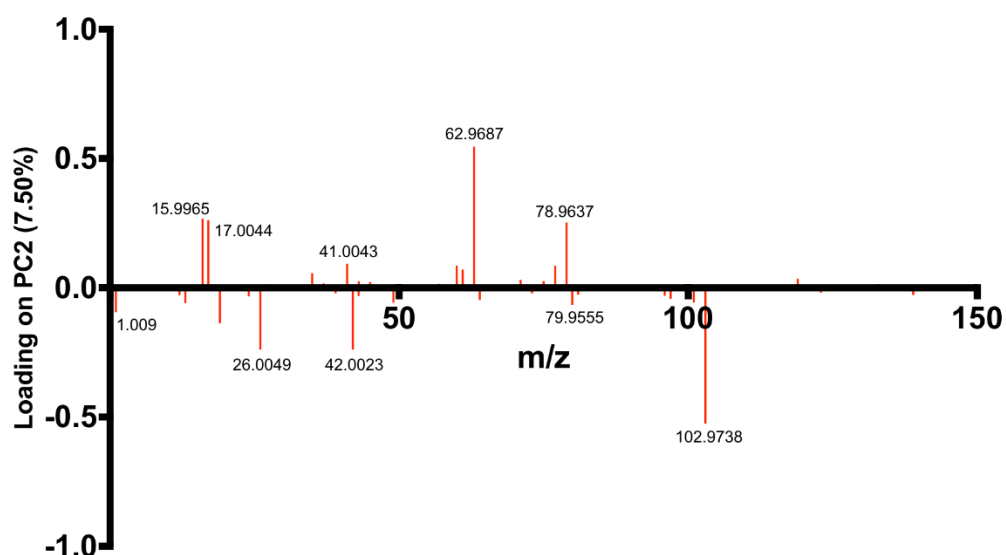


Figure 5-20 - Loadings plot to identify which ion fragments contribute to PC2 for the for the negative ion spectra for the plasma cleaned, carboxy- terminated and dopamine functionalised surfaces

The negative ions associated with the plasma cleaned surface show groups which contain oxygen and silicon, representative of a non-functionalised, plasma cleaned surface. We can see some evidence of adventitious carbon in a few of the peaks assigned to the m/z values.

Table 5-4 - Negative mass fragments associated with plasma cleaned surface as identified from PCA

m/z	Fragment	Deviation (ppm)
102.97	SiCHNO_3^-	11.95
15.99	O^-	99.1
17.01	HO^-	97.64
75.96	SiO_3^-	21.99
43.00	SiCH_3^-	11.58

As the PCA shows minimal variance in the second principle component, we can account for fragmentation ions present in both the plasma cleaned and carboxylic acid terminated surfaces. Not only does an oxygen plasma cleaned

surface contain a large amount of hydroxyl groups, so does the carboxylic acid. In particular, the CHO_2^- is representative of the tip of the carboxylic acid functional group. This was used as a marker to distinguish changes in surface chemistry upon the addition of dopamine.

Table 5-5 - Negative mass fragments associated with carboxylic acid functionalised surfaces as identified from PCA

m/z	Fragment	Deviation (ppm)
15.99	O^-	99.1
17.00	HO^-	97.64
78.96	PO_3^-	72.18
41.00	CHO_2^-	38.05
59.97	SiO_2^-	5.74

Carbon based species are shown to be associated with the dopamine functionalised surface, including the presence of nitrogen as shown from the CN^- ion fragment. This is indicative that dopamine has been successfully been functionalised on the surface but does not confirm orientation.

Table 5-6 - Negative mass fragments associated with dopamine functionalised surfaces as identified from PCA

m/z	Fragment	Deviation (ppm)
25.01	C_2H^-	22.99
26.01	CN^-	70.21
13.01	CH^-	105.69
42.00	CNO^-	102.64
49.01	C_4H^-	7.65

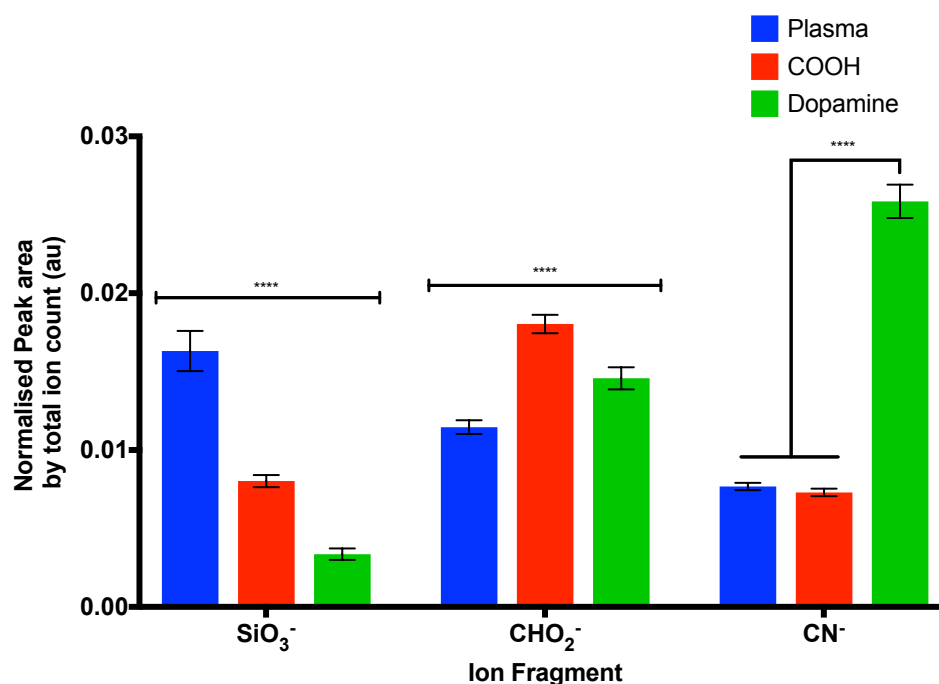


Figure 5-21 - ToF-SIMS normalised peak area by total ion count for SiO_3^- , CHO_2^- , and CN^- ions for plasma cleaned, carboxylic acid and dopamine functionalised surfaces (Tukey's multiple comparisons test indicating significant differences **** $p < 0.0001$, $n=5$, error bars represent SD)

Figure 5-21 shows the selected ion fragments (SiO_3^- , CHO_2^- , and CN^-) and their normalised peak area by total ion count. In line with the positive spectra, we see a decrease in the SiO_3^- fragment ion as surface chemistry is increased. CHO_2^- and CN^- were respectively identified for the carboxylic acid and dopamine functionalised surfaces in relation to their structure. In line with the ion map image (Figure 5-22), we see a significant difference in ion intensity for the CHO_2^- ion ($p < 0.0001$) between all the samples. The presence and large increase of the CN^- ion intensity confirms dopamine has been functionalised to the surface.

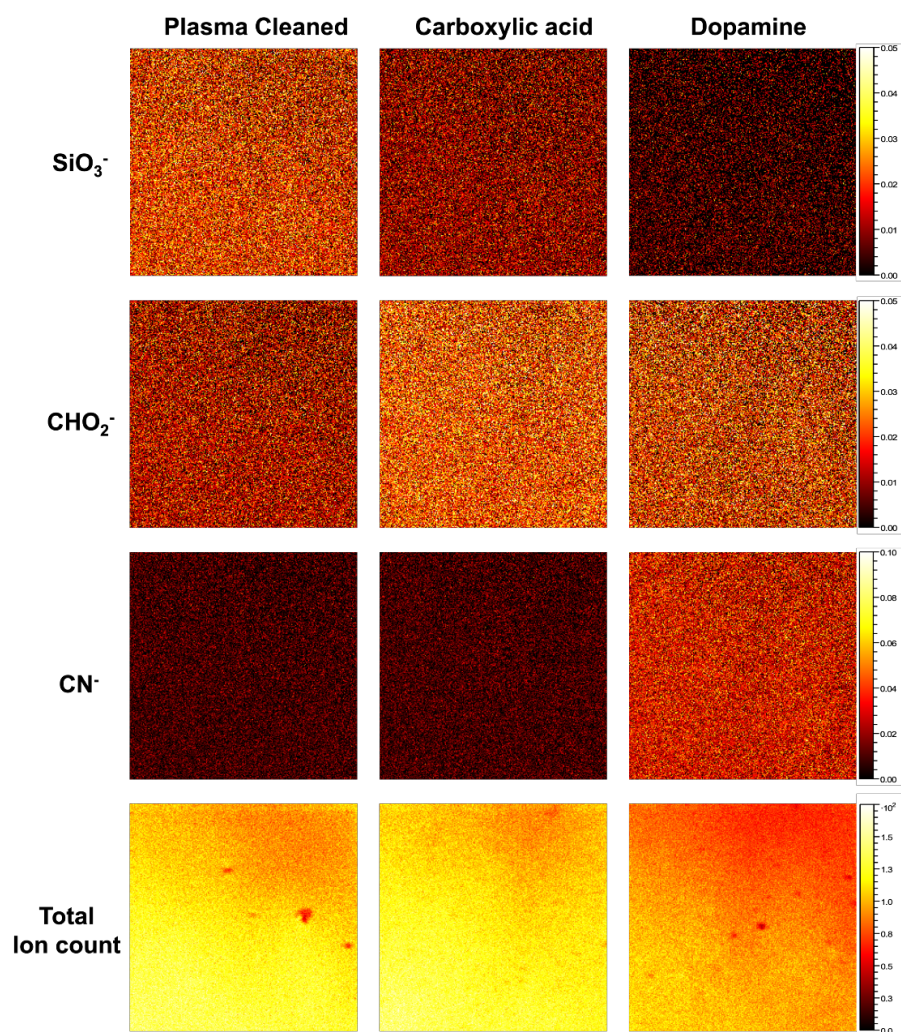


Figure 5-22 - Ion map images normalised by total ion count (500 x 500 μm) for SiO_3^- , CHO_2^- , and CN^- for plasma cleaned, carboxylic acid terminated and dopamine functionalised surfaces and maps of total ion counts for all samples (scale bar represents relative ion intensities of specified ions, brighter indicated more of the ion present in the sample)

Messersmith, *et al*, previously reported ToF-SIMS data from poly-dopamine surfaces in 2007.³⁶⁹ Using ToF-SIMS it was identified that specific masses correlate to a poly-dopamine surface, and in specific a trimer of 5,6-dihydroxyindole. The data obtained by Messersmith, *et al*, can be seen in Figure 5-23. A distinct pattern of $\alpha > \alpha > \beta$ (m/z 16 for α , and 26 for β) is observed, which suggests liberation of two hydroxyl groups and a portion of the aromatic group, identifying each subunit derived from the polymerised dopamine with a mass of 445.

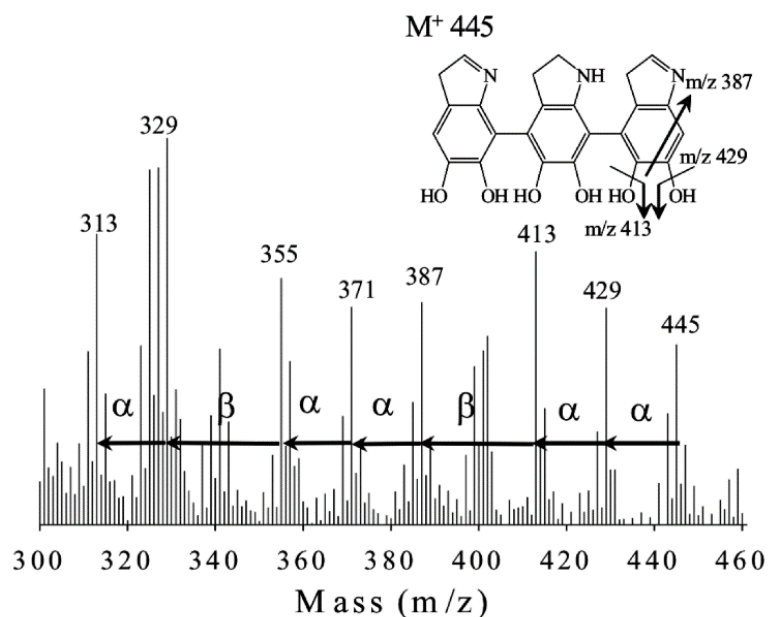


Figure 5-23 - Mass fragmentation pattern of poly-dopamine as determined by ToF-SIMS (reproduced from Messersmith, et al)³⁶⁹

In comparison to our data, the pattern indicating a trimeric poly-dopamine structure being formed is not observed. A peak is observed at a very low intensity at m/z 445 in our data, although there is no distinguishable pattern in mass fragmentation as shown in Figure 5-24. This allows us to confirm that dopamine has not been polymerised onto the surface.

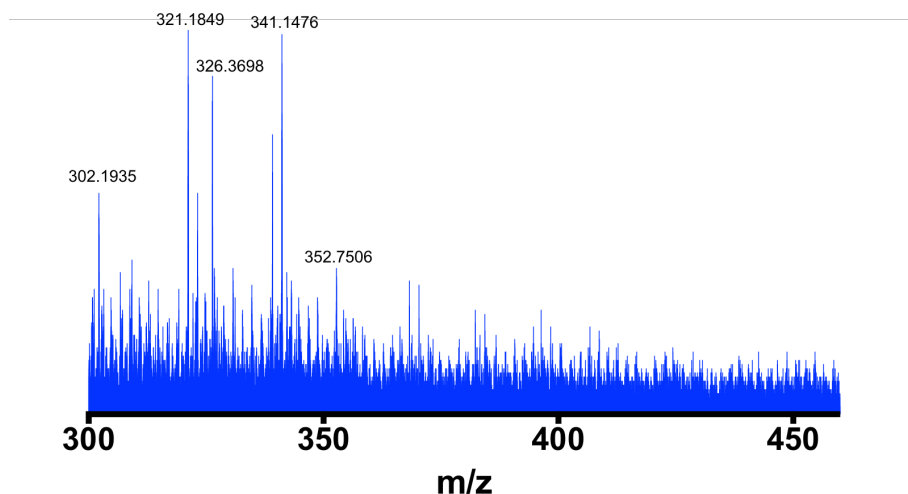


Figure 5-24 - ToF-SIMS mass spectrum between m/z 300-460 for dopamine functionalised surfaces. This shows no distinct pattern which suggest polymerisation has occurred on the surface as previously identified by Messersmith, et al³⁶⁹

In conclusion, from both the positive and negative spectra obtained by ToF-SIMS, we can confirm the successful oxidation, modification and functionalisation of the glass coverslips with plasma, carboxylic acid and dopamine respectively, in a homogenous manner.

Nanoparticles

Nanoparticle synthesis and functionalisation was successfully achieved, characterised by DLS and FTIR measurements. Zeta potential (ζ) measurements were conducted to measure the surface charge of the nanoparticles after each functionalisation step, using 1 mg of sample in a 1 in 10 ml PBS: H₂O solution at room temperature (n=3).

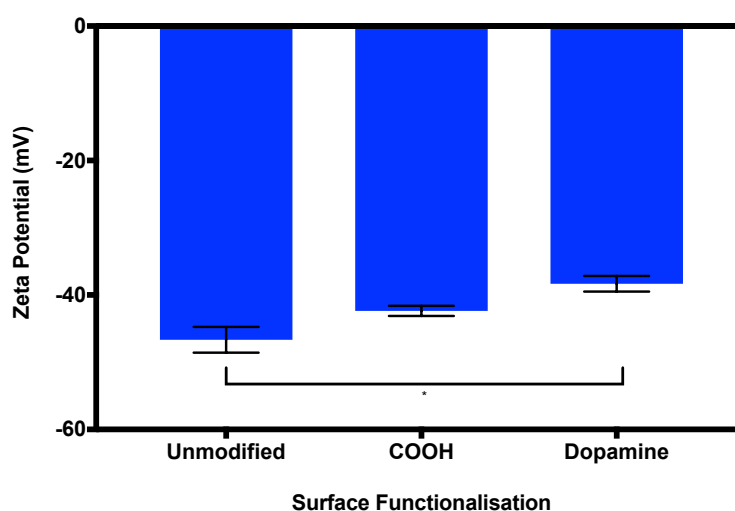


Figure 5-25 - ζ -potential measurements of unmodified, COOH functionalised and dopamine modified nanoparticles (n=3, error bars represent SD, * significant difference of samples unmodified vs. dopamine was shown at $p < 0.015$)

Figure 5-25 shows the experimental data obtained for the ζ -potential measurements. Values obtained for the unmodified silica and carboxylic acid terminated particles were consistent with literature values as reported by Feifel, *et al*, and An, *et al*, at -41.44 mV and -42.21 mV for unmodified and COOH modified particles, respectively.^{391,392}

There have been no reported values associated with the ζ -potential of dopamine functionalised nanoparticles to support our findings. We can assume, a low ζ -potential for dopamine indicates the presence of a surface which exhibits a negative functional group, in our case, the catechol. A statistical significance was identified through ANOVA, using Tukey's multiple

comparison test. A significant difference was seen between the unmodified vs. dopamine nanoparticles ($p < 0.015$) but no significant difference was seen between the other samples.

Figure 5-26 shows the FTIR-ATR data obtained for the unmodified, carboxy terminated and dopamine functionalised nanoparticles. Table 5-7 shows peaks identified in each spectrum, their wavenumber and also the associated functional group. This was used to identify and confirm changes in the surface chemistry after each modification was made.

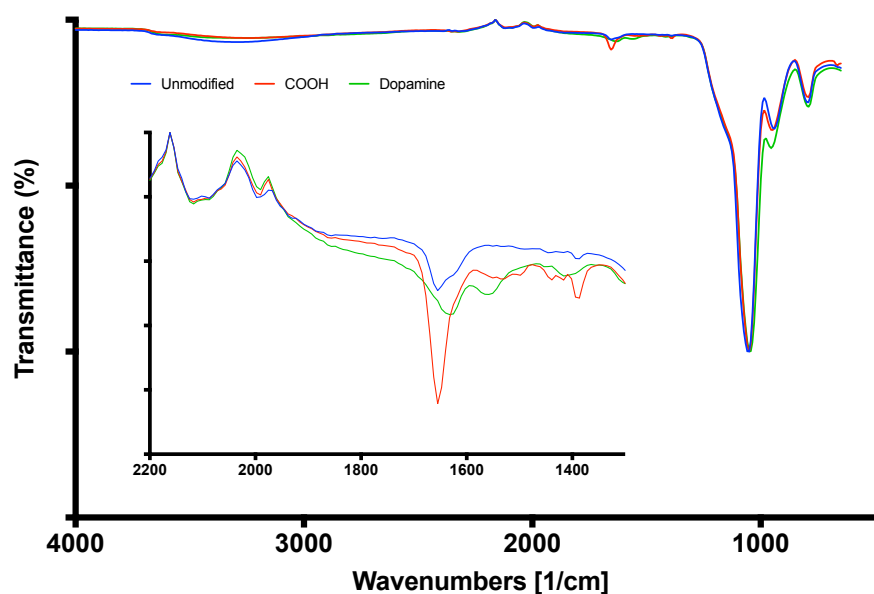


Figure 5-26 - FTIR showing unmodified, carboxylic acid and dopamine modified nanoparticles with a focus in the range of 2200 to 1300 cm^{-1}

The unmodified silica particles exhibited bands for Si-O, Si-OH and O-H groups, found at 960, 1058, and 3300 cm^{-1} respectively, which are subsequently found on the other samples. We see the generation of peaks at 1408 and at 1632 cm^{-1} which are representative of C-O and C=O stretching vibrations for the carboxylic acid functionalised nanoparticles. Further modification of the nanoparticles with dopamine was shown by the evidence of amide peaks (1528 (N-H), 1640 (C=O) cm^{-1}), as well as aromatic O-H deformation (1386 cm^{-1}) and C-H stretching (2847 cm^{-1}) bands.

Table 5-7 - FTIR-ATR peak assignments of unmodified, carboxylic acid and dopamine modified nanoparticles (type, s- strong, m- medium, w-weak signal)

Surface Modification	Functional group	Wavenumber (cm ⁻¹)	Type
Unmodified	Si-OH	960	s; Si-O stretching vibration
	Si-OH	1058	w-m; Si-OH deformation vibration
	O-H	3300	m; O-H stretching vibration
COOH	C-O (acid)	1408	s; stretching vibration
	C=O (acid)	1654	s; stretching vibration
Dopamine	O-H (aromatic)	1386	m; O-H deformation and C-O stretching vibration interaction
	N-H (amide)	1528	m; N-H bending
	C=O (amide)	1640	s; amide I (C=O stretching vibration)
	C-H (aromatic)	2847	w; broad, C-H stretching vibration

Spiropyran surface modification series

Spiropyran-dopamine functionalised surfaces were successfully generated using the procedure shown in Figure 5-27. Substrates were initially functionalized with 3-aminotrimethoxysilane (APTMS) to generate an amine terminated surface. 4-pentynoic acid was then reacted with the amine (*via* DIC amide coupling) resulting in an alkyne terminated surface. CuAAc was utilised to generate spiropyran functionalised surfaces, to which dopamine was then added (*via* DIC amide coupling).

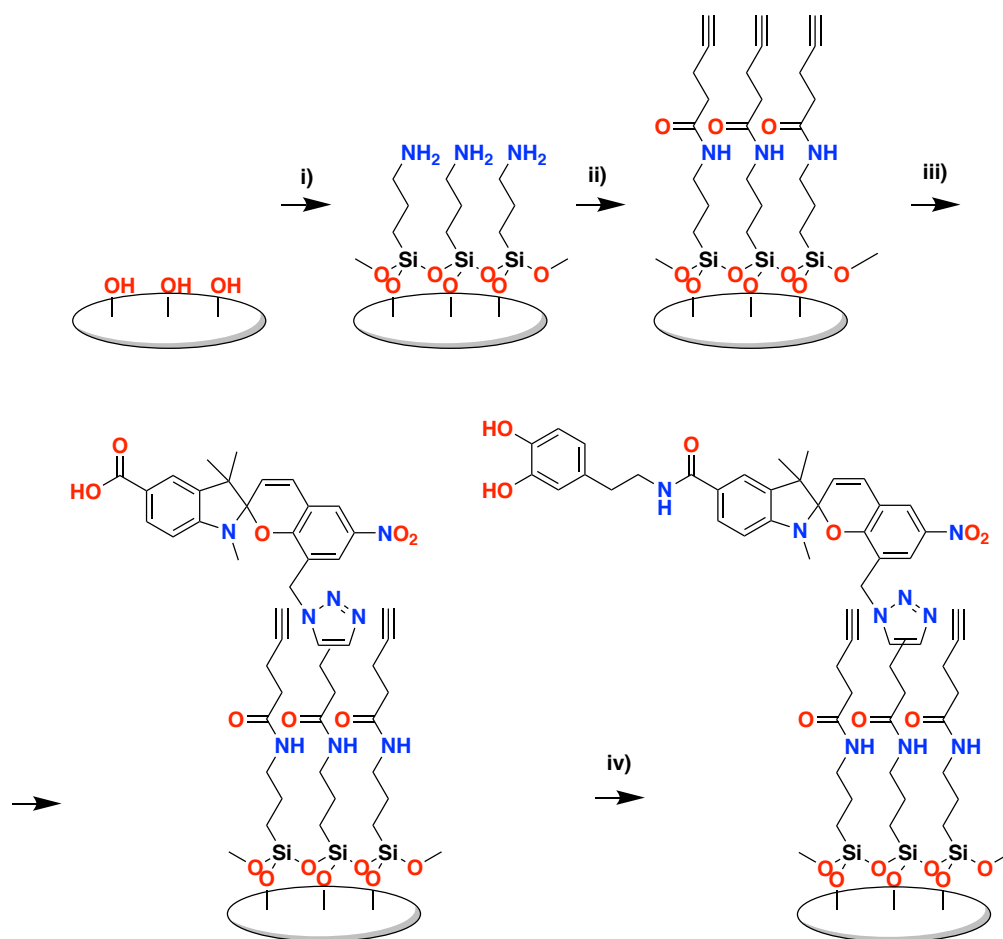


Figure 5-27 - General surface modification to generate spiropyran-dopamine surfaces (a- APTMS, RT, 1Hr; b- DIC, propionic acid, Et₃N, 24Hr; c- sodium ascorbate, copper sulfate, DMSO: H₂O, 24Hr; d-DIC, Et₃N, DMF)

Glass substrate

As a control, the spiropyran-dopamine conjugate was immobilised on to glass coverslips. This was to identify and confirm the surface modification procedure, before applying the same method to nanoparticles. Surface functionalisation was initially identified through surface wettability measurements. Figure 5-28 shows the contact angles obtained for the modified surfaces which are concurrent with what is expected with the particular functional groups. An increase of water contact angle is observed as the hydrophobicity of the functional groups increases.

It was shown that upon functionalisation resulting in an amino terminated surface, wettability increased from a low hydrophilic surface after plasma oxidation (4°) to contact angles of $36 \pm 3^\circ$. In comparison to literature values where APTMS surfaces with a water contact angle of approximately 41° Kyaw, *et al.* were reported,³⁹³ the value measured here is slightly higher. This can be explained by variations in surface densities of the silane or other methodological differences in sample preparation, storage or measurement. As the variability in water contact angle is dependent on the concentrations and time to which the silane and subsequent reactions take place, literature values can vary. For example, it has been shown that an increase in time and concentration for APTMS, can result in the formation of multilayers, which shows a higher variability in surface wettability to a monolayer.³⁹⁴ This is also true for the alkyne terminated surface, where literature values of around 59° as shown by Palomaki, *et al.*, was higher than our experimental value of $53 \pm 4^\circ$.³⁹⁵

Literature values of the SP/MC isomer have been shown around 73 and 60° respectively.¹³¹ Data for the modified surfaces we have obtained show a value of $71 \pm 3^\circ$ for the SP isomer, whilst the MC isomer at $70 \pm 4^\circ$. As our compound expresses a terminal carboxylic acid, we assume drastic changes in contact angle would not be observed due to its hydrophilic nature. Another explanation for our reduced change in angle could be due to the efficiency in switching of the surface from the SP→MC isomer, as well as a difference in surface densities compared to materials reported in literature.

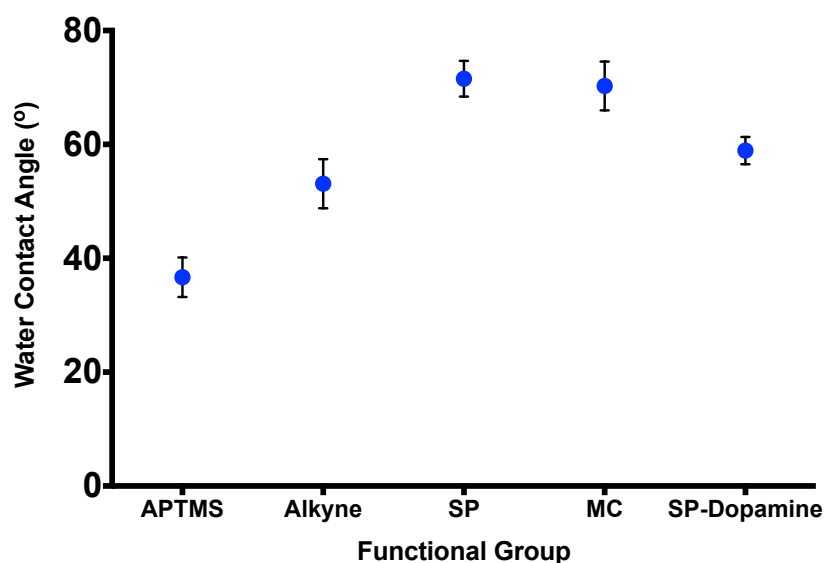


Figure 5-28 - Water contact angle data for plasma cleaned, amine, alkyne, spiropyran/merocyanine and spiropyran dopamine functionalised surfaces (n=3)

Comparing our experimental results from both the dopamine and spiropyran-dopamine functionalised glass coverslips, we observe similar contact angle values of $58 \pm 2^\circ$ and $59 \pm 3^\circ$, respectively. This gives an indication that both surfaces demonstrate similar chemical features at the surface.

As before, ToF-SIMS was utilised to determine surface chemistry of the functionalised surfaces. Figure 5-29 and Figure 5-30 show the positive spectra obtained for the plasma cleaned, amine, alkyne, spiropyran and spiropyran-dopamine functionalised surfaces, whilst Figure 5-31 and Figure 5-32 the negative ions for each (m/z 0-100, 200-400 respectively).

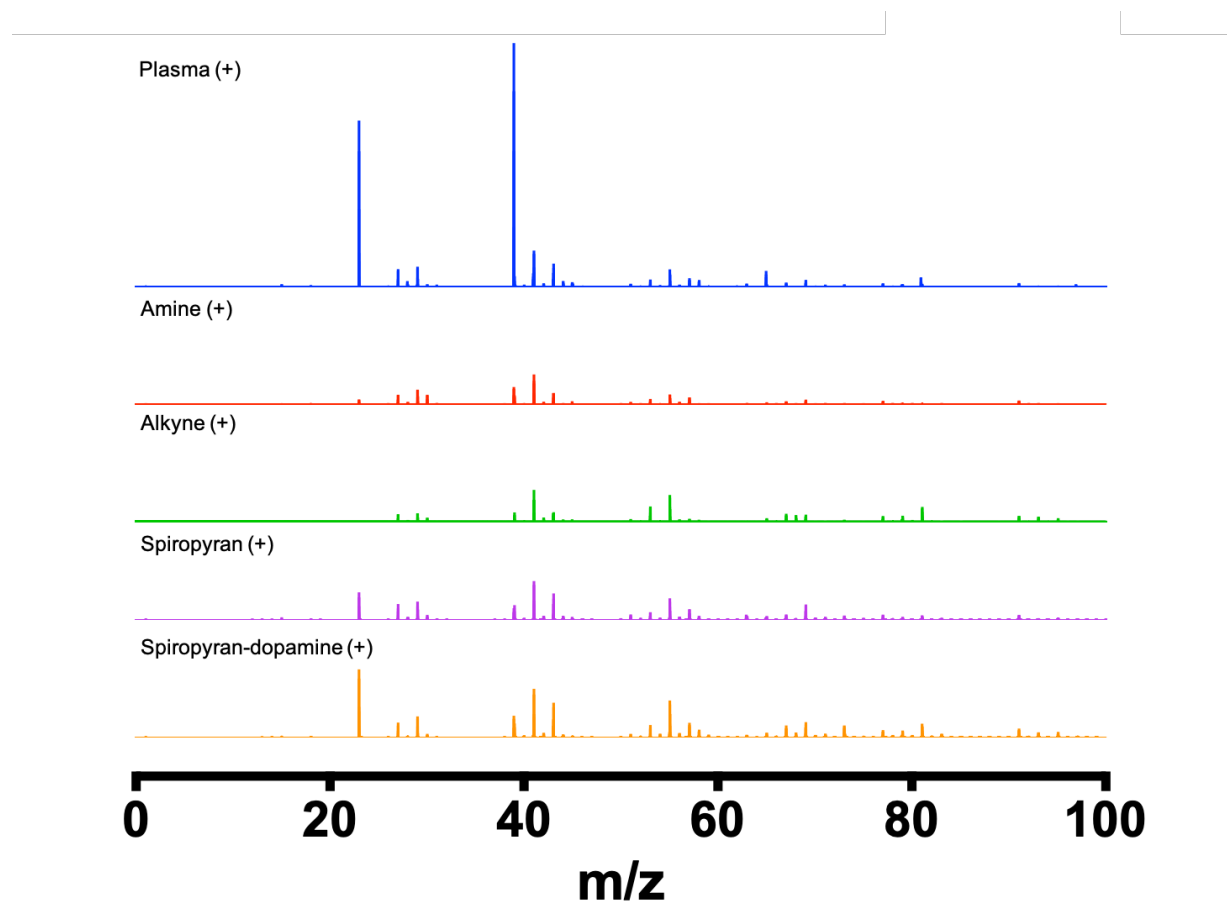


Figure 5-29 - Positive ion spectra for plasma cleaned glass, amine, alkyne, spiropyran and spiropyran-dopamine functionalised surfaces (m/z 0-100)

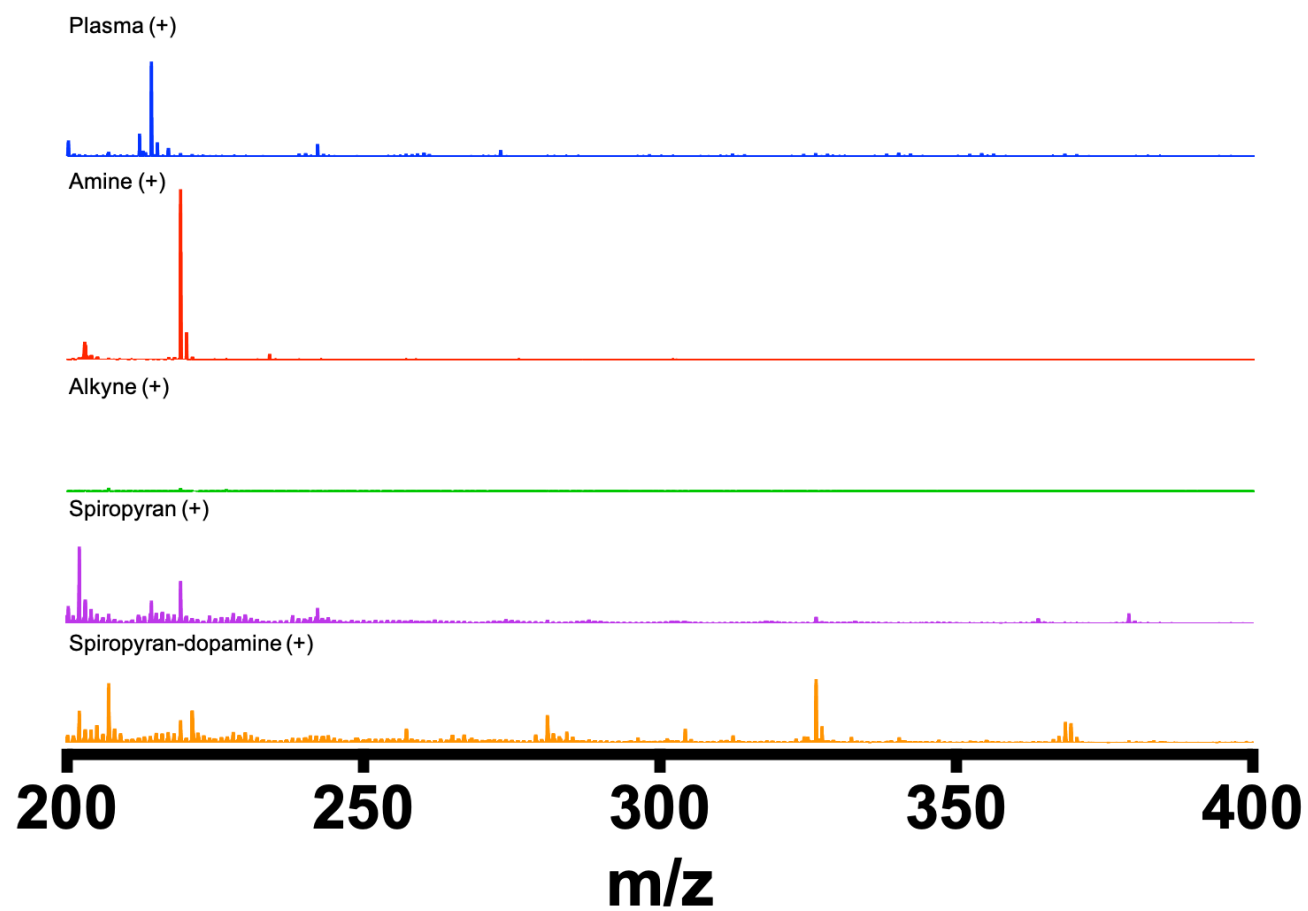


Figure 5-30 - Positive ion spectra for plasma cleaned glass, amine, alkyne, spiropyran and spiropyran-dopamine functionalised surfaces (m/z 200-400)

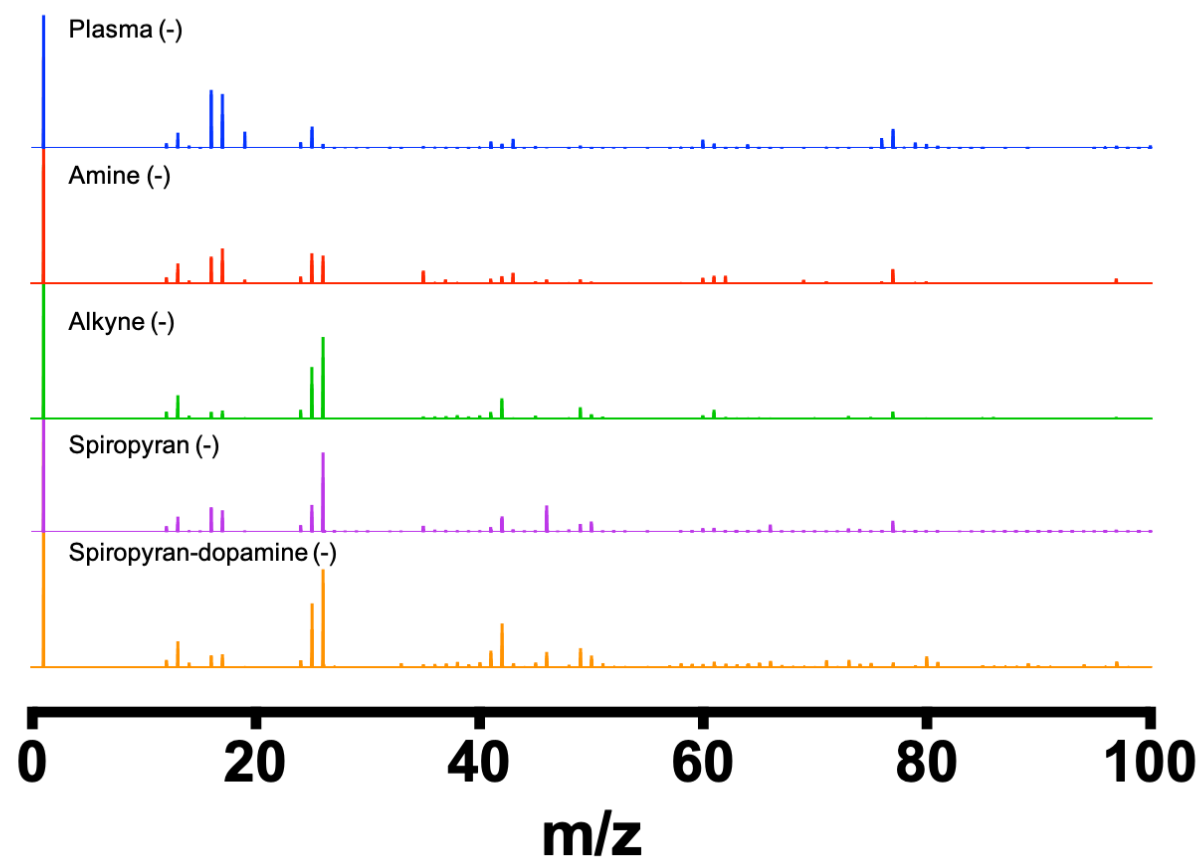


Figure 5-31 - Negative ion spectra for plasma cleaned glass, amine, alkyne, spiropyran and spiropyran-dopamine functionalised surfaces (m/z 0-100)

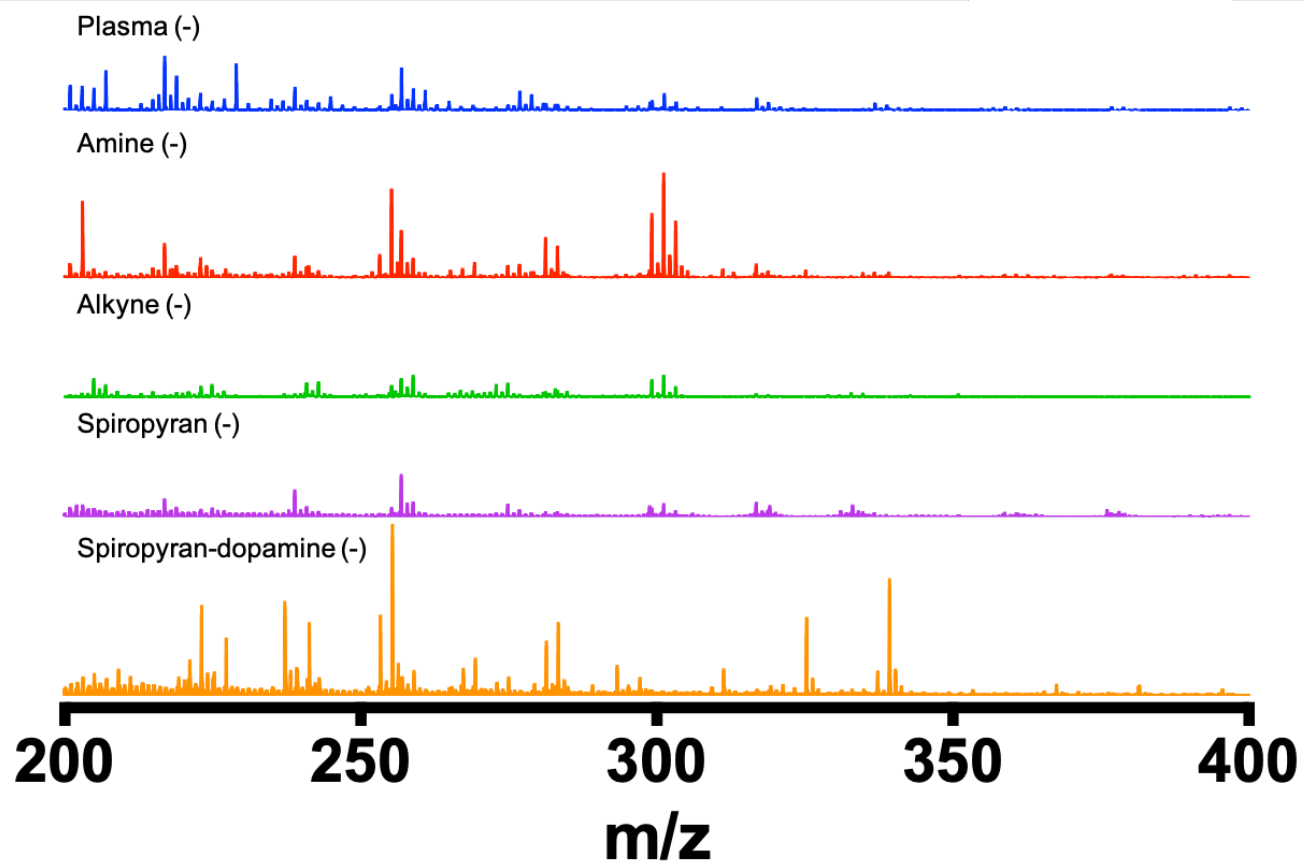


Figure 5-32 - Negative ion spectra for plasma cleaned glass, amine, alkyne, spiropyran and spiropyran-dopamine functionalised surfaces (m/z 200-400)

PCA was utilised to identify and explain any differences in surface chemistry. Three principle components were required to identify key fragments associated with each modified surface.

PC1 explains 89.73% variance between the samples but is not as informative as PC2 and PC3, which explain 5.51% and 4.11% of variance, respectively. This is clear from the comparisons made from PC1 vs PC2 and PC1 vs PC3, where the plasma cleaned surfaces have the most variance on the x-axis (PC1). This can be explained due to the different ion fragments generated by a functionalised and non-functionalised surface. For example, the plasma cleaned sample could have a much larger ion intensity for fragments which include sodium and potassium, in comparison to organic functional groups on modified surfaces. Using the scores plot, we can identify specific clusters associated with each functional surface, as shown in Figure 5-33 and Figure 5-34. In addition to this, to identify which fragments are associated with each cluster, we can use the loadings plot for the positive spectra, which are shown from Figure 5-35 to Figure 5-37.

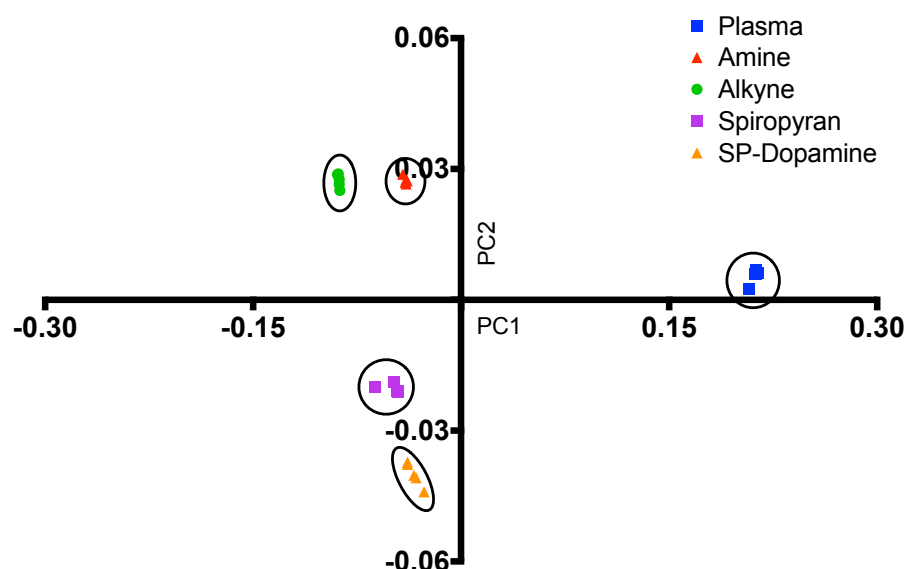


Figure 5-33 - Positive ion scores plot showing five distinct clusters of variance (n=5) for plasma cleaned, amine, alkyne, spiropyran and spiropyran-dopamine functionalised surface (PC1 vs PC2)

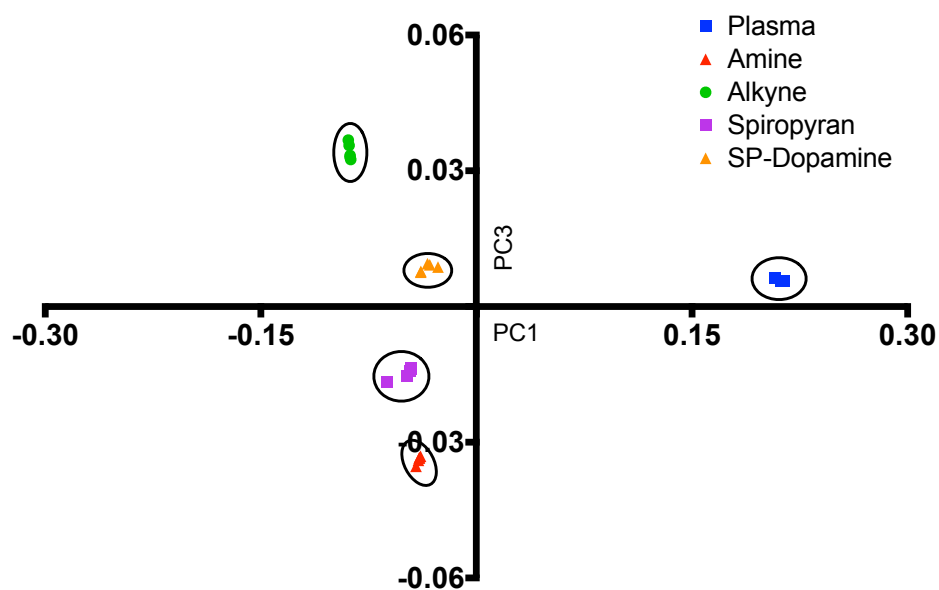


Figure 5-34 - Positive ion scores plot showing five distinct clusters of variance ($n=5$) for plasma cleaned, amine, alkyne, spiropyran and spiropyran-dopamine functionalised surface (PC1 vs PC3)

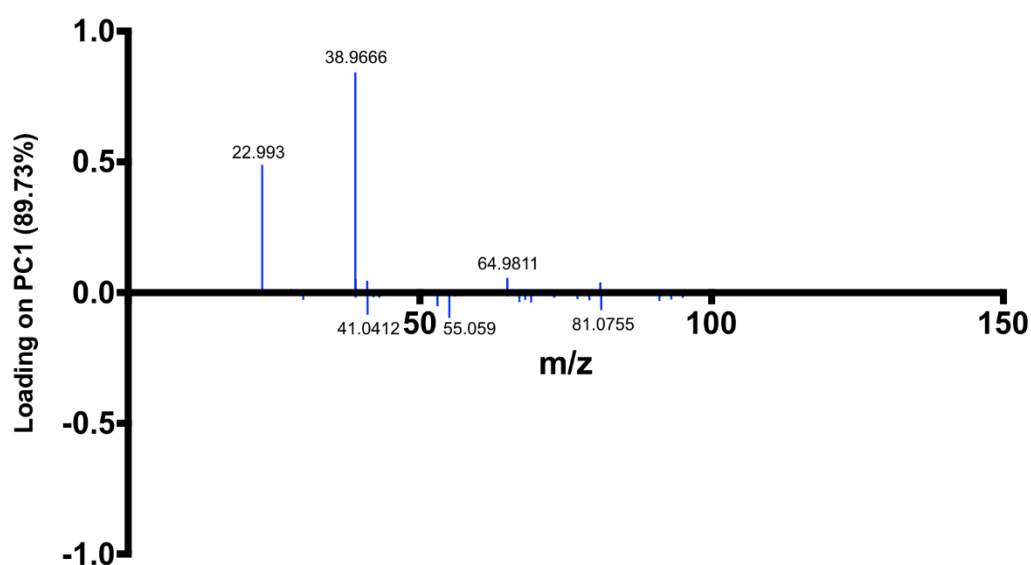


Figure 5-35 - Loadings plot to identify which ion fragments contribute to PC1 for the positive ion spectra for the plasma cleaned, amine, alkyne, spiropyran and spiropyran-dopamine functionalised surfaces

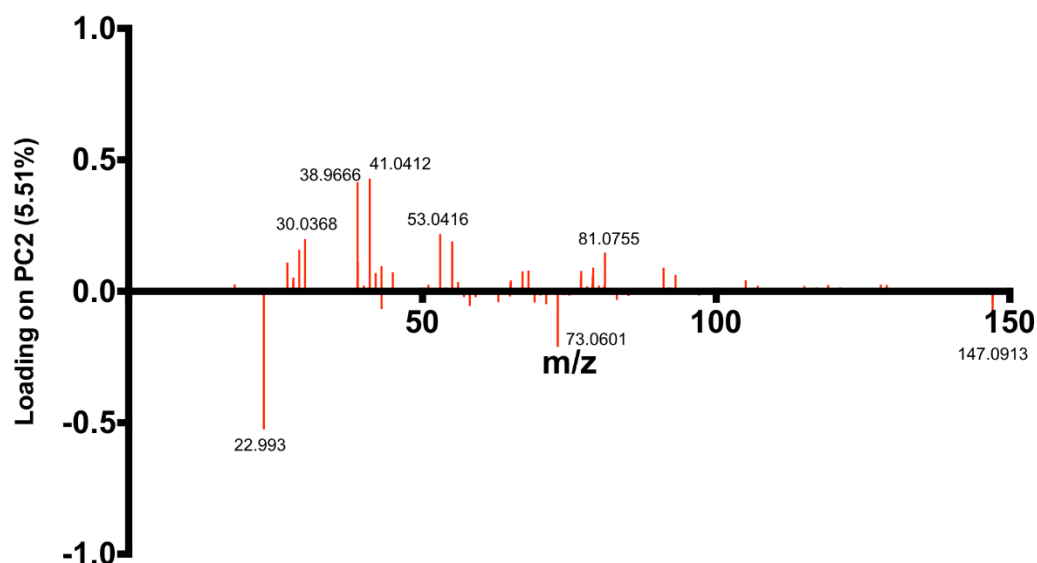


Figure 5-36 - Loadings plot to identify which ion fragments contribute to PC2 for the positive ion spectra for the plasma cleaned, amine, alkyne, spiropyran and spiropyran-dopamine functionalised surfaces

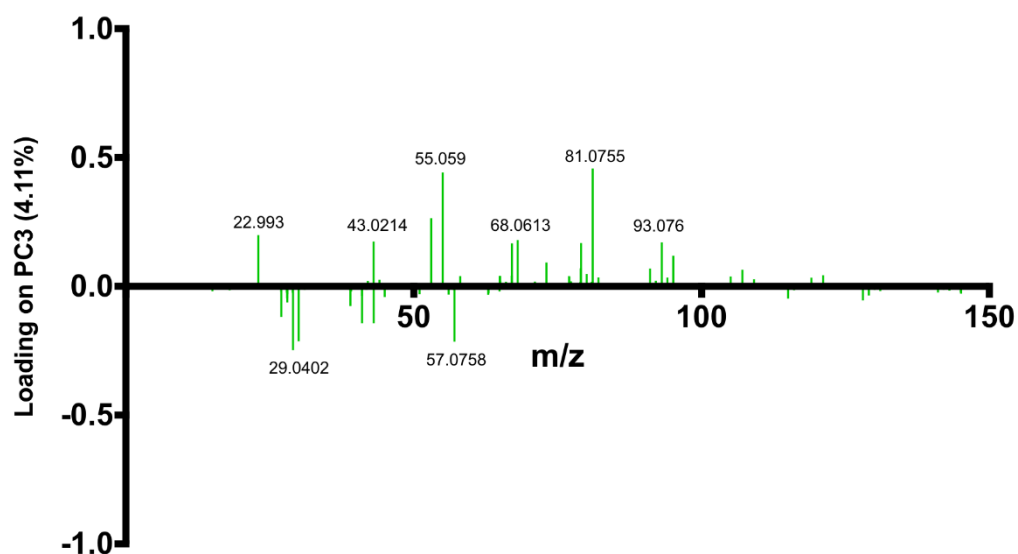


Figure 5-37 - Loadings plot to identify which ion fragments contribute to PC3 for the positive ion spectra for the plasma cleaned, amine, alkyne, spiropyran and spiropyran-dopamine functionalised surfaces

Using the scores and loadings plots generated for the positive ion spectra, we identified key fragment ions responsible for separation in each of the three principle components. Table 5-8 shows the mass fragments identified using PCA for the plasma cleaned surface. As we have shown before (Table 5-1), the presence of high levels of salt indicate a clean surface, whilst minimal and small carbon fragments can be associated with adventitious carbon.

Table 5-8 - Positive mass fragments associated with plasma cleaned surfaces as identified from PCA

m/z	Fragment	Deviation (ppm)
38.96	K^+	74.25
22.99	Na^+	140.5
64.98	$SiCH_3^+$	56.19
64.99	HO_4^+	32.56
27.98	Si^+	97.46

Upon silanisation of the surface using APTMS, we start to generate a larger number of carbon species. These are shown in Table 5-10, where we can also see nitrogen containing species (CH_4N^+) representing the terminal amino group.

Table 5-9 - Positive mass fragments associated with amine terminated surfaces as identified from PCA

m/z	Fragment	Deviation (ppm)
29.04	$C_2H_5^+$	37.01
57.08	$C_4H_9^+$	94.16
30.04	CH_4N^+	80.76
43.06	$C_3H_7^+$	79.53
41.04	$C_3H_5^+$	50.55

The alkyne terminated surface once again demonstrated a large amount of carbon species associated with the surface, in particular with fragments containing the structure $C_xH_y^+$. For example, the ion fragment $C_4H_5^+$ can be associate with a but-1-yne like fragment.

Table 5-10 - Positive mass fragments associated with alkyne surfaces as identified from PCA

m/z	Fragment	Deviation (ppm)
55.06	$C_4H_7^+$	76.73
41.04	$C_3H_5^+$	50.55
81.08	$C_6H_9^+$	62.59
53.04	$C_4H_5^+$	46.65
69.07	$C_5H_9^+$	44.65

Larger ion fragments are observed for both the spiropyran (Table 5-11) and spiropyran-dopamine functionalised surfaces (Table 5-12). Fragments containing a larger quantity of nitrogen are observed and can be explained by the 1,3-dipolar cycloaddition between the alkyne and azide. A large number of fragments associated with the spiropyran was also associated with the spiropyran-dopamine analogue surface, which was not surprising as they share the same structure, with similar fragmentation patterns.

Table 5-11 - Positive mass fragments associated with spiropyran surfaces as identified from PCA

m/z	Fragment	Deviation (ppm)
43.06	$C_3H_7^+$	79.53
71.09	$C_5H_{11}^+$	101.622
69.07	$C_5H_9^+$	44.51
202.08	$C_{11}H_{10}N_2O_2^+$	9.26
83.10	$C_6H_{11}^+$	113.41

Table 5-12 - Positive mass fragments associated with spiropyran-dopamine surfaces as identified from PCA

m/z	Fragment	Deviation (ppm)
22.99	Na^+	140.50
73.06	$\text{C}_2\text{H}_7\text{N}_3^+$	53.34
147.09	$\text{C}_9\text{H}_{11}\text{N}_2^+$	6.27
43.06	C_3H_7^+	79.53
58.07	$\text{C}_3\text{H}_8\text{N}^+$	88.26

Upon manual inspection of the positive spectra, large mass fragments which were not identified using PCA included fragments found at m/z values of 379.12 ($\text{C}_{21}\text{H}_{19}\text{N}_2\text{O}_5^+$) and 326.34 ($\text{C}_{19}\text{H}_{22}\text{N}_2\text{O}_3^+$). These fragments were shown to be associated with the spiropyran modified and spiropyran-dopamine functionalised surfaces respectively (Figure 5-38). From selecting 3 principle components, we have identified 99.35% of variance across all the samples. This can explain why we do not observe these higher mass values in our PCA. It may be possible to account for these fragments by increasing the number of principle components, but as the three components used are sufficient to make a clear distinction between our samples, this route has not been explored further.

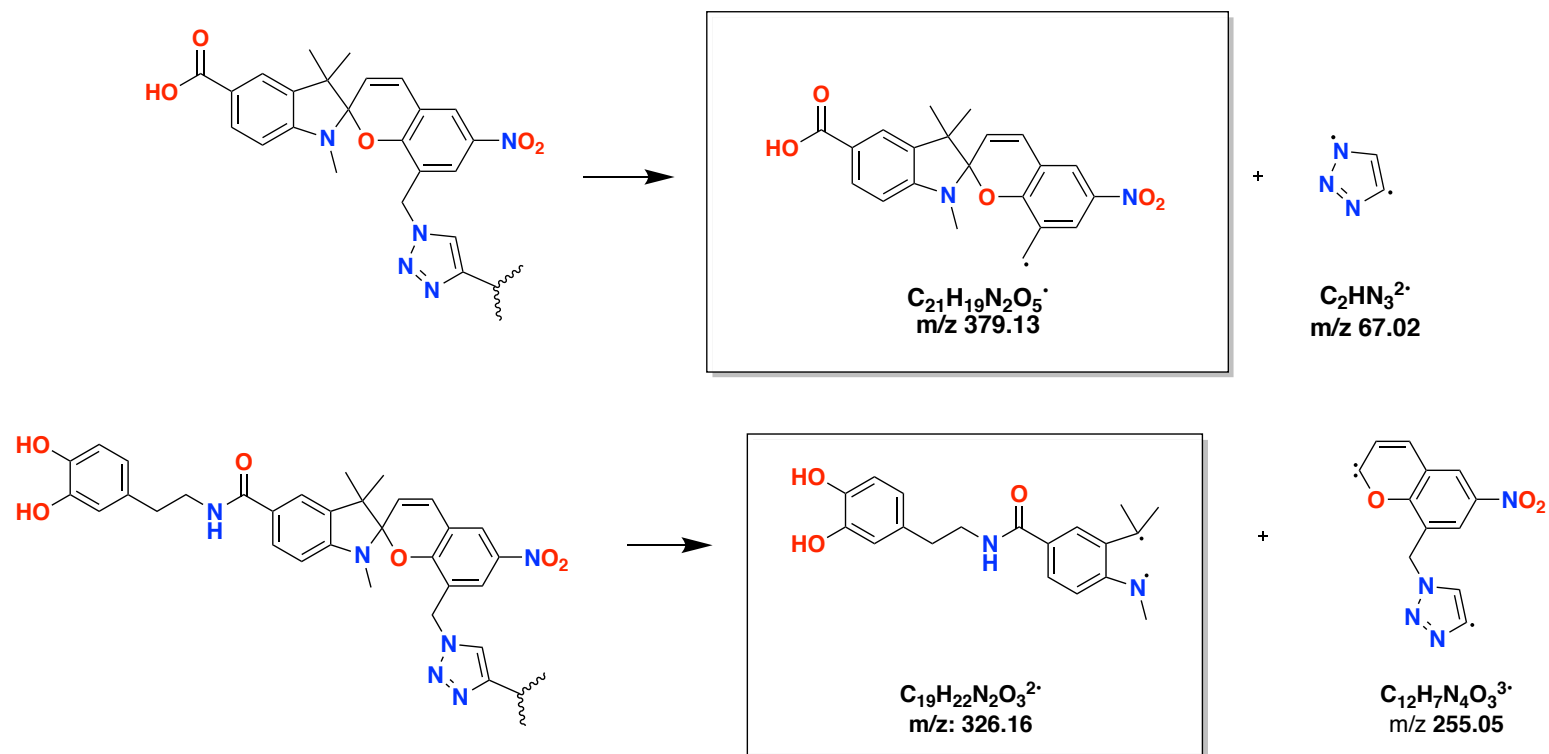


Figure 5-38 - Fragmentation of spiropyran and spiropyran dopamine surfaces into large ion fragments

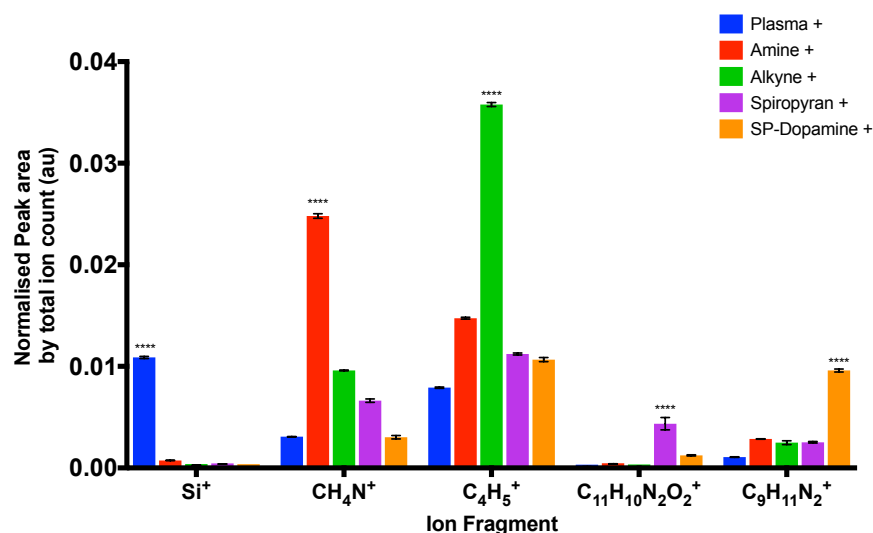


Figure 5-39 - ToF-SIMS normalised peak area by total ion count for Si^+ , CH_4N^+ , C_4H_5^+ , $\text{C}_{11}\text{H}_{10}\text{N}_2\text{O}_2^+$ and $\text{C}_9\text{H}_{11}\text{N}_2^+$ ions for plasma cleaned, amine, alkyne, spiropyran and spiropyran-dopamine functionalised surfaces (Tukey's multiple comparisons test was used to determine significant differences between stages of functionalisation **** $p < 0.0001$, $n=5$, error bars represent SD)

Select ions which represent the functionalised surfaces are shown in Figure 5-39. Whilst Si^+ is observed in all the samples, it is most prominent in the plasma cleaned sample as subsequent surface modification masks the underlying glass surface (relating to the depth sensitivity of ToF-SIMS). The peak area for the CH_4N^+ ion fragment is most prominent on the amine terminated surface, although peaks associated with the other functionalised surfaces can be accounted for due to fragmentation patterns. C_4H_5^+ was established as a key fragment for the alkyne terminated surface for its 'but-1-yne' fragment. As C_4H_5^+ is non-specific due to its common C_xH_y^+ structure we observe this again on our other surfaces. The mass spectra and ion fragmentations for the spiropyran and spiropyran-dopamine surfaces in comparison to the plasma, amine and importantly the alkyne surfaces, allow us to confirm that functionalisation has successfully occurred. All samples were found to be significantly different using Tukey's multiple comparison test ($p < 0.0001$) from their previous functionalisation. We can visualise the intensity of each mass fragment ions for each surface, and homogeneity using the ion map images shown in Figure 5-40.

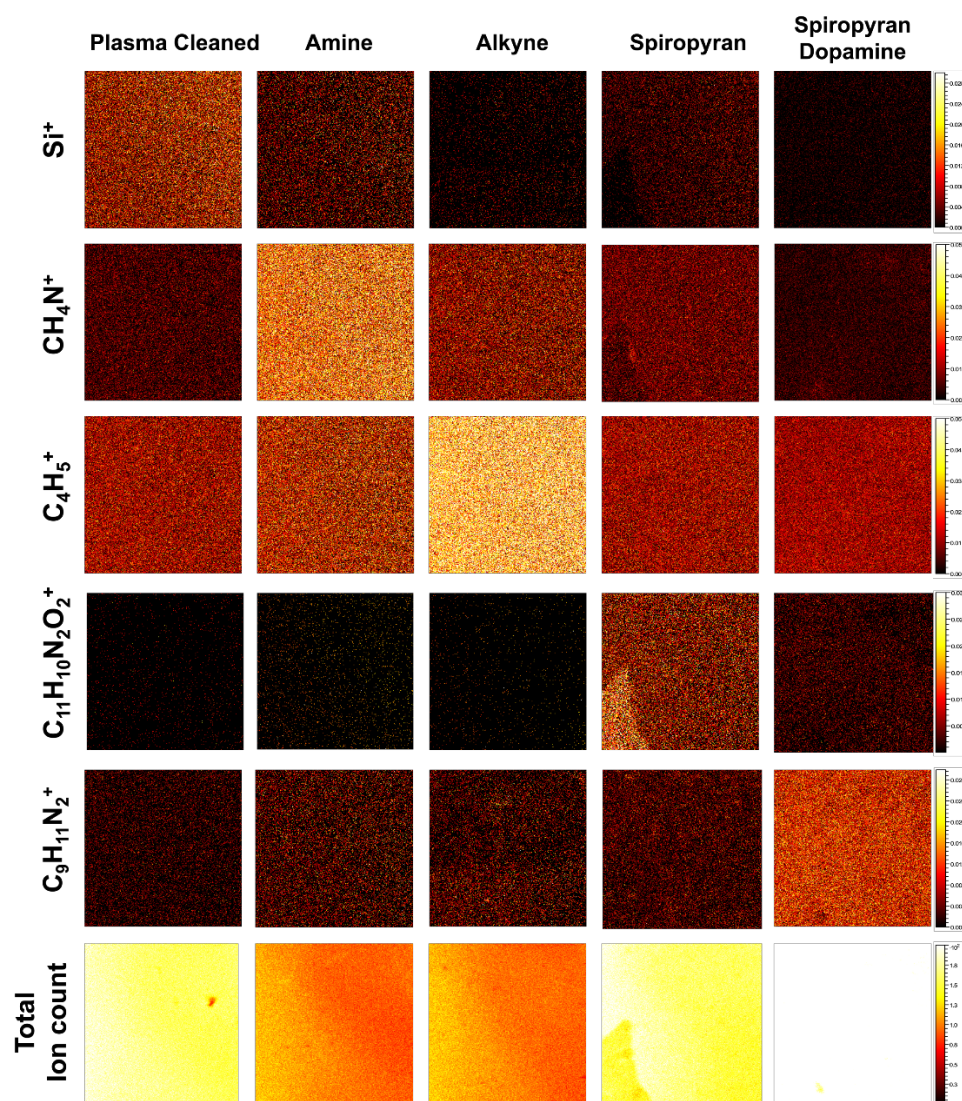


Figure 5-40 - Ion map images normalised by total ion count ($500 \times 500 \mu\text{m}$) for Si^+ , CH_4N^+ , C_4H_5^+ , $\text{C}_{11}\text{H}_{10}\text{N}_2\text{O}_2^+$ and $\text{C}_9\text{H}_{11}\text{N}_2^+$ ions for plasma cleaned, amine, alkyne, spiropyran and spiropyran-dopamine functionalised surfaces and maps of total ion counts for these samples (scale bar represents relative ion intensities of specified ions, brighter indicated more of the ion present in the sample)

Apart from the spiropyran functionalised surface, we observe homogeneity of the selected ions across the different samples. A slight inflection on the bottom left hand corner is observed in the spiropyran sample, where the intensity for some fragments is higher than the rest. Factors such as mishandling of the sample or experimental errors (glass coverslips were overlapping upon chemical functionalisation) may have caused an inhomogeneous chemical deposition.

Similar analysis was then conducted for the negative ion fragments. In this case, we required 4 principle components to establish key differences between the functionalised surfaces.

As shown, PC1 vs PC2 (Figure 5-41) allows us to make a clear distinction between the plasma and alkyne terminated surfaces along the first principle component. PC1 vs PC3 (Figure 5-42) then allows us to identify uncorrelated linear values for the amine terminated surface, whilst PC1 vs PC4 (Figure 5-43) accounts for differences in the spiropyran and spiropyran-dopamine surfaces.

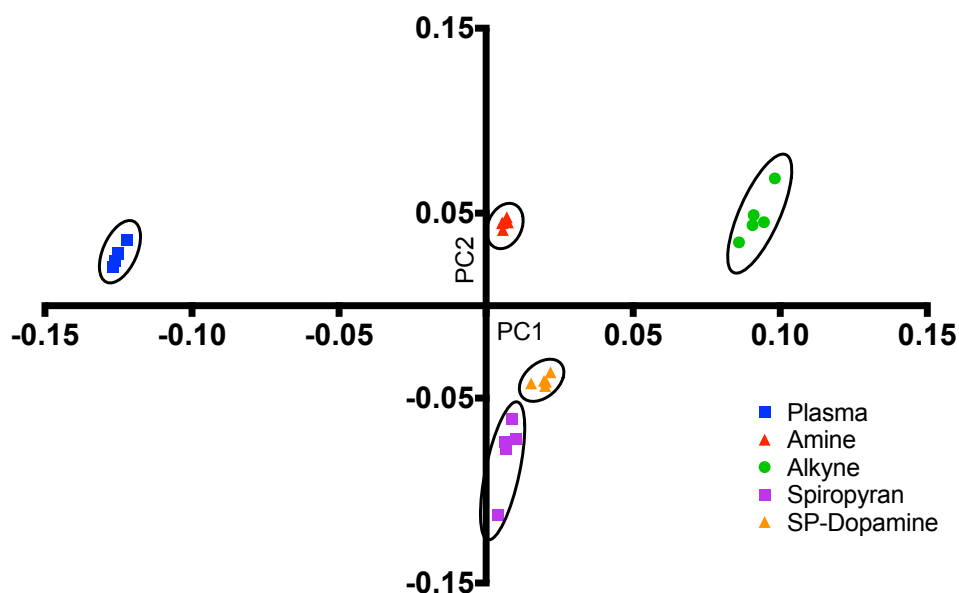


Figure 5-41 - Negative ion scores plot showing five distinct clusters of variance (n=5) for plasma cleaned, amine, alkyne, spiropyran and spiropyran-dopamine functionalised surface (PC1 vs PC2)

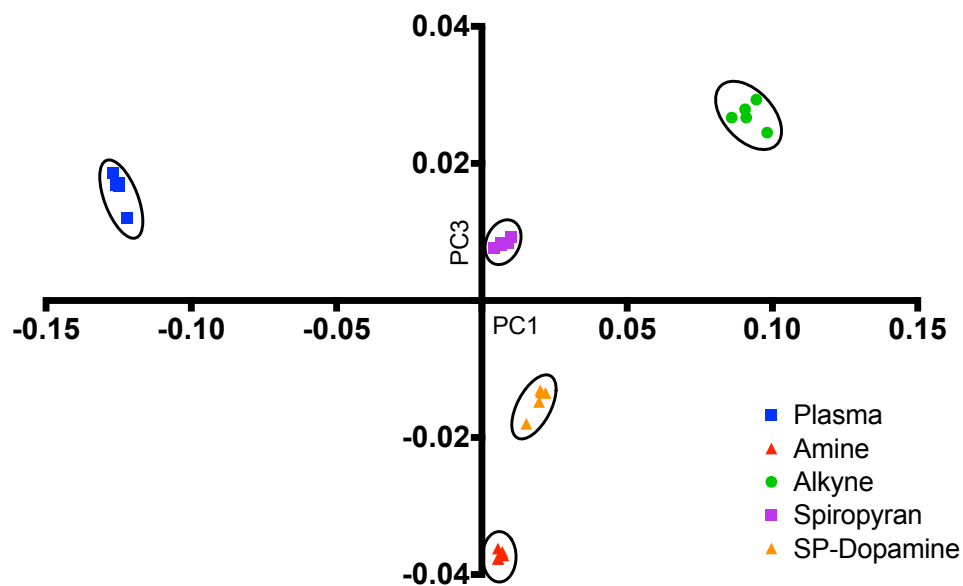


Figure 5-42 - Negative ion scores plot showing five distinct clusters of variance ($n=5$) for plasma cleaned, amine, alkyne, spiropyran and spiropyran-dopamine functionalised surface (PC1 vs PC3)

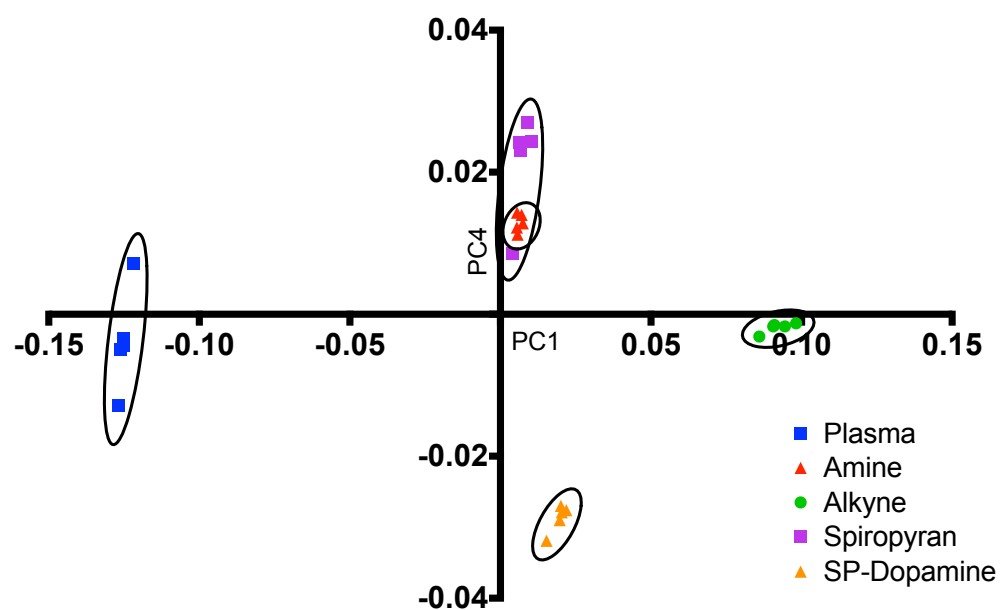


Figure 5-43 - Negative ion scores plot showing five distinct clusters of variance ($n=5$) and some inhomogeneity for the spiropyran functionalised surface for plasma cleaned, amine, alkyne, spiropyran and spiropyran-dopamine functionalised surface (PC1 vs PC4)

PCA then allowed us to generate the loadings plot for each component, and this was used to identify the variables (m/z) responsible for separation of the functionalised surfaces in the scores plots.

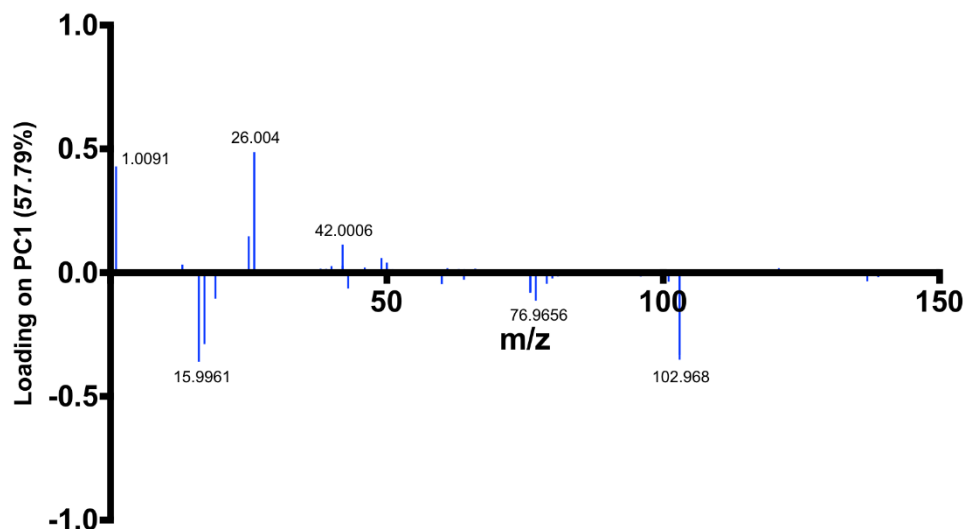


Figure 5-44 - Loadings plot to identify which ion fragments contribute to PC1 for the negative ion spectra for the plasma cleaned, amine, alkyne, spiropyran and spiropyran-dopamine functionalised surfaces

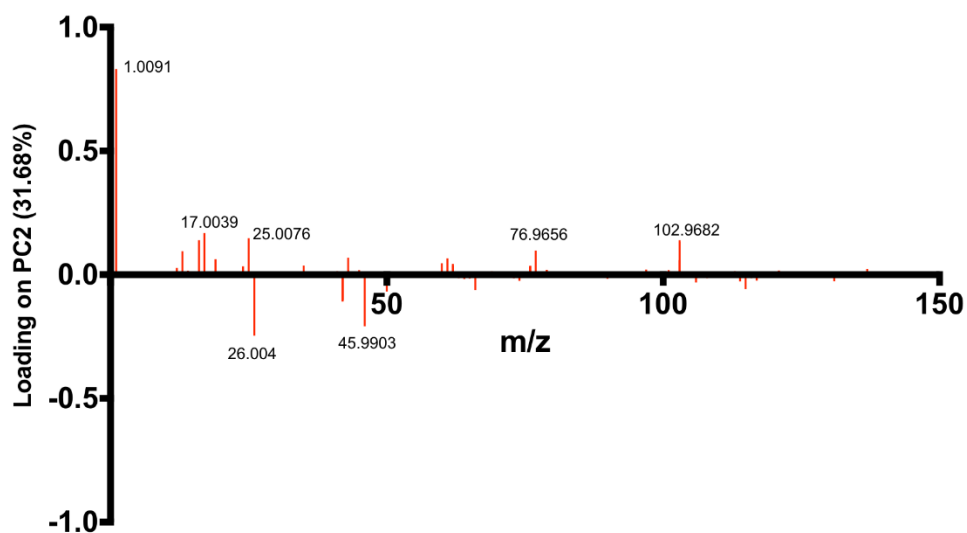


Figure 5-45 - Loadings plot to identify which ion fragments contribute to PC2 for the negative ion spectra for the plasma cleaned, amine, alkyne, spiropyran and spiropyran-dopamine functionalised surfaces

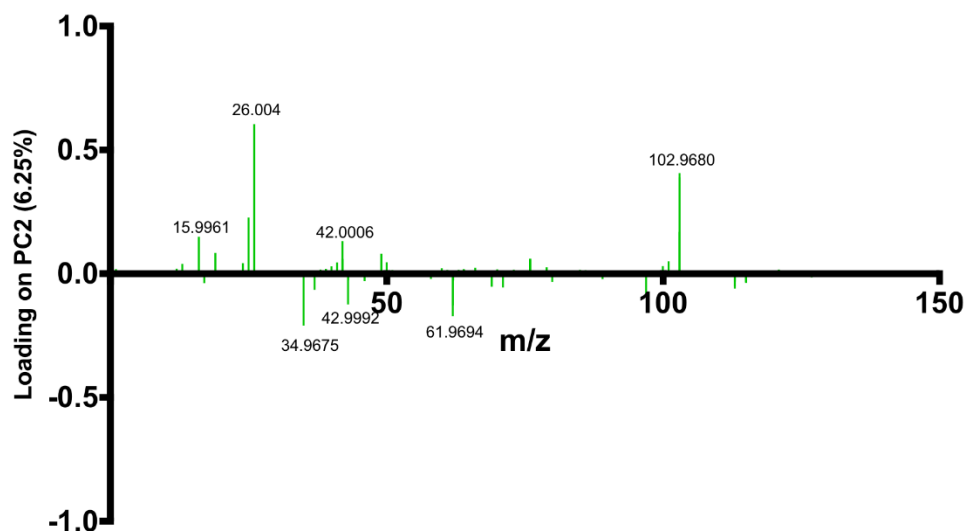


Figure 5-46 - Loadings plot to identify which ion fragments contribute to PC1 for the for the negative ion spectra for the plasma cleaned, amine, alkyne, spiropyran and spiropyran-dopamine functionalised surfaces

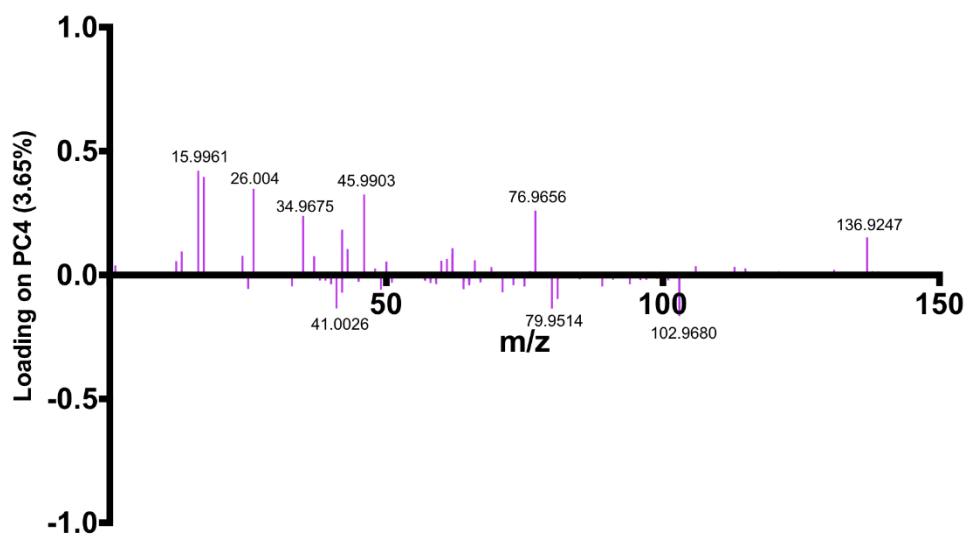


Figure 5-47 - Loadings plot to identify which ion fragments contribute to PC4 for the for the negative ion spectra for the plasma cleaned, amine, alkyne, spiropyran and spiropyran-dopamine functionalised surfaces

From using the scores and loadings plot, the negative ion data was processed to determine key ion fragments associated with each of the surfaces. The negative ion fragments shown in Table 5-13 are associated with the plasma cleaned surface. We observe an oxygen rich surface, which corresponds to a hydroxyl terminated surface. The prominence of the SiO_3^- and silicon based ions allows us to confirm successful cleaning and oxidation of the glass coverslip.

Table 5-13 - Negative mass fragments associated with plasma cleaned surfaces as identified from PCA

m/z	Fragment	Deviation (ppm)
15.99	O^-	74.1
102.97	SiCHNO_3^-	44.37
17.00	HO^-	68.24
76.97	SiHO_3^-	50.61
75.96	SiO_3^-	50.61

Table 5-14 gives us the negative ions for the amine terminated surface. It can be seen that although Si-based ion fragments are present, the addition of carbon based species show a change in surface chemistry has occurred. Observation of a chloride peak may be due to a variety of contamination sources such as an unclean sample chamber or mount, experimental errors (salts/biologics) and chlorine contaminants originating from the borosilicate glass.

Table 5-14 - Negative mass fragments associated with amine terminated surfaces as identified from PCA

m/z	Fragment	Deviation (ppm)
34.97	Cl ⁻	38.69
42.99	SiCH ₃ ⁻	27.94
71.01	SiC ₂ H ₅ N ⁻	115.83
68.99	SiC ₂ H ₃ N ⁻	105.44
112.98	SiC ₇ H ⁻	52.67

As determined from PCA, mass fragments showing an increase in the quantity of carbon, nitrogen and oxygen species, relative to the addition of the 4-pentynoic acid to generate the alkyne surfaces is shown in Table 5-15. The presence of CN⁻ and CNO⁻ ions gives us an indication of the formation of an amide bond.

Table 5-15 - Negative mass fragments associated with alkyne terminated surfaces as identified from PCA

m/z	Fragment	Deviation (ppm)
26.00	CN ⁻	35.6
25.01	C ₂ H ⁻	8.99
42.00	CNO ⁻	62.17
49.01	C ₄ H ⁻	37.24
50.01	C ₃ N ⁻	100.5

The prominence of the ion fragment NO_2^- is a distinct marker used to identify the successful immobilisation of spiropyran with the alkyne terminated surface (using CuAAC) as shown in Table 5-16.

Table 5-17, which represents the spiropyran-dopamine terminated surface, typically showed the presence of similar ion fragments compared to the spiropyran surface as expected. The increased presence of species which contain carbon and oxygen fragments is related to the introduction of the catechol moiety upon the addition of the dopamine to the surface.

Table 5-16 - Negative mass fragments associated with spiropyran surfaces as identified from PCA

m/z	Fragment	Deviation (ppm)
26.00	CN^-	35.6
45.99	NO_2^-	56.6
42.00	CNO^-	62.14
42.01	$\text{C}_2\text{H}_2\text{O}^-$	70.57
50.01	C_3N^-	100.5

Table 5-17 - Negative mass fragments associated with spiropyran-dopamine surfaces as identified from PCA

m/z	Fragment	Deviation (ppm)
102.97	SiCHNO_3^-	44.37
41.00	C_2HO^-	3.4
42.00	CNO^-	62.17
71.01	CHN_3O^-	14.95
49.01	C_4H^-	37.24

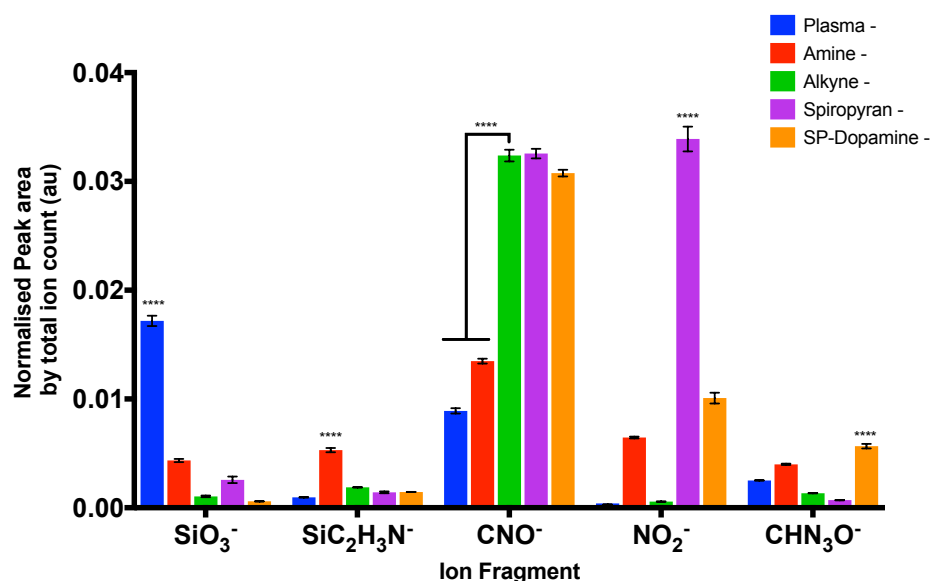


Figure 5-48 - ToF-SIMS normalised peak area by total ion count for SiO_3^- , $\text{SiC}_2\text{H}_3\text{N}^-$, CNO^- , NO_2^- and CHN_3O^- ions for Plasma cleaned, amine, alkyne, spiropyran and spiropyran-dopamine functionalised surfaces (Tukey's multiple comparisons test was used to determine significant differences between stages of functionalisation **** $p < 0.0001$, $n=5$, error bars represent SD)

Figure 5-48 shows the peak area normalised by total ion count for specific negative ion fragments associated with each of the modified surfaces. As before, we see a large reduction in the SiO_3^- ion fragment for the functionalised surfaces in comparison to the plasma cleaned sample. For the amine terminated sample, we see an increase in the $\text{SiC}_2\text{H}_3\text{N}^-$ ion in comparison to the rest. The CNO^- fragment is a very common fragment produced by compounds which contain amide bonds. Here, we see a large increase in CNO^- intensity for the alkyne terminated sample as well as the spiropyran and spiropyran-dopamine modified surfaces, confirming successful modification of the surfaces. A very large difference between the alkyne and spiropyran surfaces can be identified through the presence of the NO_2^- ion. This is also present on the spiropyran-dopamine, but at a reduced intensity. This may be due to the presence of the dopamine masking the fragmentation of the nitro component. Finally, the CHN_3O^- ion was shown to be more prominent on the spiropyran-dopamine sample, which may be due to the presence and reorientation of fragments caused by dopamine addition.

Figure 5-49 shows the ion map images for each of the fragments identified for each surface. As we can see, homogenous coverage has been achieved at every stage for all the ions except for the NO_2^- ion for the spiropyran-dopamine surface. This shows small patches with low ion intensities.

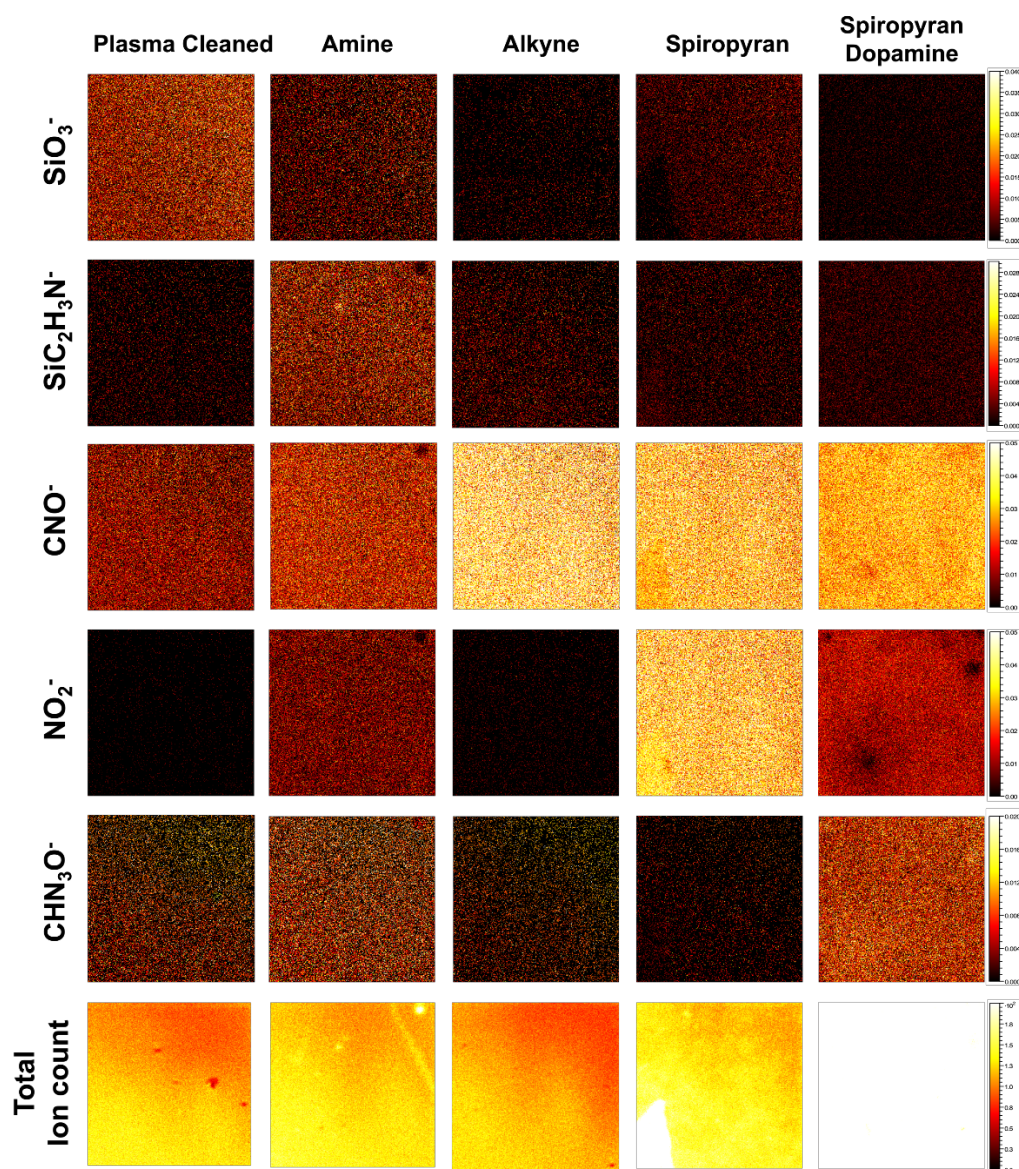


Figure 5-49 - Ion map images normalised by total ion count ($500 \times 500 \mu\text{m}$) for SiO_3^- , $\text{SiC}_2\text{H}_3\text{N}^-$, CNO^- , NO_2^- and CHN_3O^- ions for plasma cleaned, amine, alkyne, spiropyran and spiropyran-dopamine functionalised surfaces and maps of total ion counts for these samples (scale bar represents relative ion intensities of specified ions, brighter indicated more of the ion present in the sample)

Nanoparticles

ζ -potential measurements were performed at every stage of functionalisation to indicated changes in surface chemistry via changes in surface charge. Figure 5-50 shows the ζ -potential measurements conducted in a 1 in 10 ml PBS:H₂O solution at room temperature at 1 mg/ml for each modified step.

As we can see there is a large increase from the negative to the positive ζ -potential measurements upon functionalisation with APTMS. This is consistent with values observed by Feifel, *et al*, showing values of +39.63 mV.³⁹¹ In contrast to the positive values obtained for the alkyne terminated surface, it has been shown by Fazzini, *et al*, that a terminated silica nanoparticle exhibiting an alkyne has a potential value of -46.2 ± 5.8 mV.³⁹⁶ On addition of the spiropyran, we see a large shift from a positive to negative potential occurring. As no reported ζ -potential values in literature could be used to confirm the binding of the spiropyran and spiropyran-dopamine analogue to the nanoparticle surface, confirmation of the surface chemistry was obtained by FTIR spectroscopy.

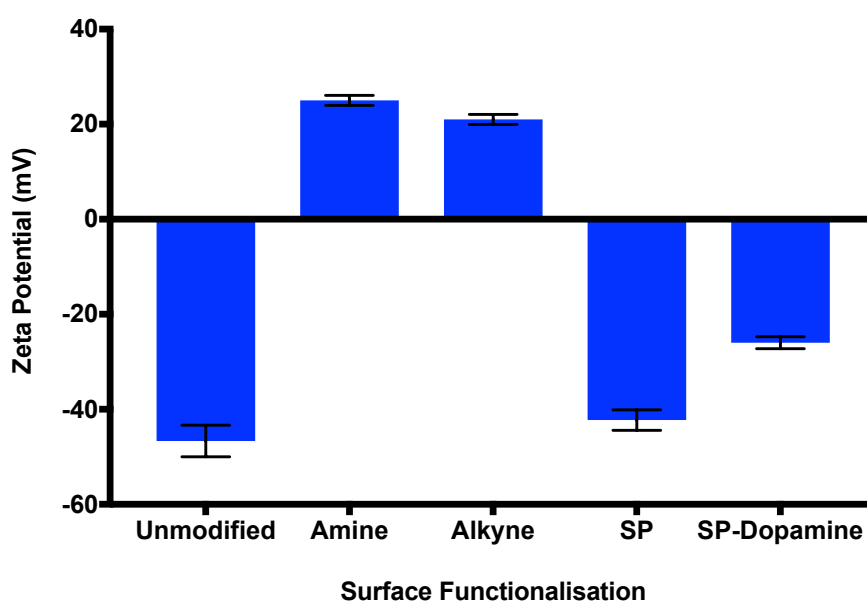


Figure 5-50 - ζ -potential measurements for spiropyran modified nanoparticles ($n=3$, error bars represent SD)

Figure 5-51 shows FTIR spectra obtained for each functionalised surface with the peak assignments in

Table 5-18. Peaks associated with the silica nanoparticle, Si-OH and Si-O-Si at 960 and 1058 cm^{-1} , were present in all samples. After functionalisation with APTMS a new peak at 1654 cm^{-1} appears due to introduction of the primary amine. A shift in wavenumber from 1654 to 1640 cm^{-1} is shown when the surface is modified with 4-pentynoic acid, representative of the formation of an amide (C=O). This reduction in wavenumber can be explained by the weakening of the carbonyl bond, specific to resonance effects caused by the conjugation of an amine. We also observe peaks at 2338 cm^{-1} and 3288 cm^{-1} which are associated with the carbon triple bonds and alkyl C-H stretches. The peak found at 1528 cm^{-1} is representative of the nitro group found on the photo responsive unit, confirming successful modification of the particle using CuAAc. Finally, an increased transmittance at 1640 cm^{-1} indicates an additional amide bond is formed when dopamine is conjugated to the spiropyran.

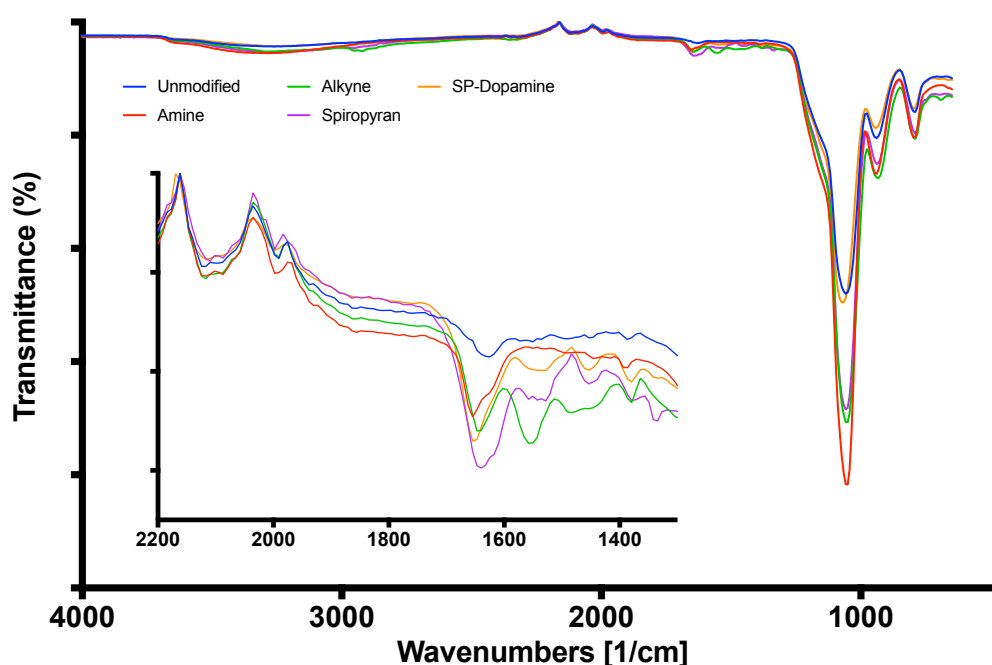


Figure 5-51 - FTIR showing spiropyran series terminated nanoparticles with focus in the range of 2200 to 1300 cm^{-1}

Table 5-18 - FTIR-ATR peak assignments for spiropyran nanoparticle modification
(s-sharp, m- medium, w-weak)

Surface Modification	Functional group	Wavenumber (cm ⁻¹)	Type
Unmodified	Si-OH	960	s; Si-O stretching vibration
	Si-O-Si	1058	w-m; Si-OH deformation vibration
Amine	NH ₂	1654	s-m; N-H deformation vibrations
Alkyne	C=O (amide)	1640	m-w; Amide (I) (N-H deformation and C-N stretching vibration)/ s; Amide I (C=O stretching vibration)
	C≡C	2338	w, triple carbon bond
	-C≡C-H	3288	w, C-H asymmetric stretch
Spiropyran	C-H	1446	R-CH ₃ (asymmetric deformation vibration), R-CH ₂ -R (CH scissor stretch)
	Ar-NO ₂	1528	Aromatic nitro compounds
	C=O (amide)	1640	m-w; Amide (I) (N-H deformation and C-N stretching vibration)/ s; Amide I (C=O stretching vibration)
	-COOH	3250	m-w; O-H stretching vibration
Spiropyran Dopamine	C-H	1148	R-CH ₃ (asymmetric deformation vibration), R-CH ₂ -R (CH scissor stretch)
	Ar-NO ₂	1528	Aromatic nitro compounds
	Amide	1647	m-w; Amide (I) (N-H deformation and C-N stretching vibration)/ s; Amide I (C=O stretching vibration)

UV/Vis spectroscopy was used to identify the parameters required to manipulate the structural isomers from SP→MC immobilised on the nanoparticles. The nanoparticles were exposed to white light, UV light (365 nm) and kept at 37 °C, 5% CO₂ (cell incubation and SPAP assay conditions) according to the procedures used in Chapter 4 to identify and manipulate the isomers present in each condition.

Spiropyran functionalised nanoparticles were shown to act similarly to spiropyran in solution. The peak areas observed between 488-600 nm (characteristic of the MC isomer) show that SP→MC isomerisation was more prevalent at higher temperatures compared to exposure to UV light (shown in Figure 5-52 and Table 5-19). Upon constant exposure to white light, the spiropyran on the nanoparticle remained in the closed SP isomer. This is in line with the observations made with the soluble molecule in Chapter 4.

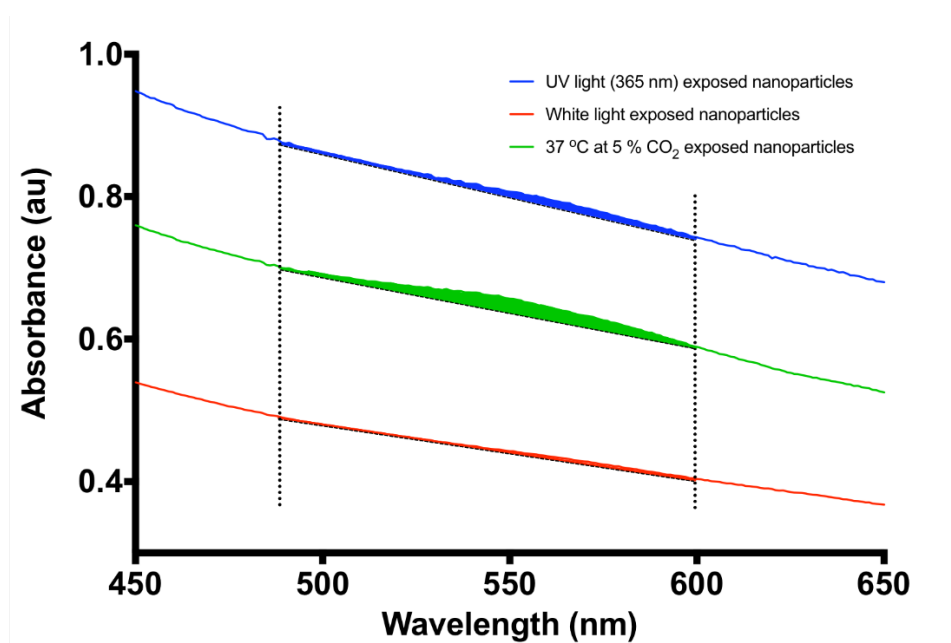


Figure 5-52 - UV/Vis analysis for spiropyran modified nanoparticles pre-exposed to white light, UV light (365 nm) and kept at 37 °C, 5% CO₂ for 4 hours before measurement (488 to 600 nm boundaries indicating merocyanine isomer)

Table 5-19 - Integrals of peak areas (UV/Vis) for spiropyran modified nanoparticles between 488-600 nm characteristic to the MC isomer

Condition imposed on spiropyran nanoparticles	Integrated peak area (au)
	Between 488 and 600 nm
White light	0.1
UV light (365 nm)	0.3
37 °C, 5% CO₂	0.9

Photo-physical characteristics of the spiropyran-dopamine functionalised nanoparticles once again mimicked the characteristics of the soluble molecule (**16**) as we observe a larger peak integral value for the UV stimulated in comparison to the thermal stimuli conversion (Figure 5-53 and Table 5-20). The peak area of each condition was obtained between 489 and 647 nm. An integral of 1.4 was obtained for the UV light (365 nm) exposed molecule (indicating the presence of the MC isomer), whilst values of 0 and 0.1 were obtained for white light exposed and 37 °C, 5% CO₂ conditions respectively (indicating the SP isomer). These low values may be explained by the presence of very low amounts of the merocyanine form or noise.

Pre-exposure of the nanoparticles to 10 minutes of UV light prior to incubation conditions was also conducted. This was to determine whether we could control isomerisation and maintain the MC isomer under the SPAP assay. A smaller integral value than the UV exposed at 1.4 was observed. This showed that we could maintain the open MC isomer with 10 minutes of pre-exposure and use this method to determine potency at the D₁ receptor with both the open and closed isomers when immobilised onto a solid substrate.

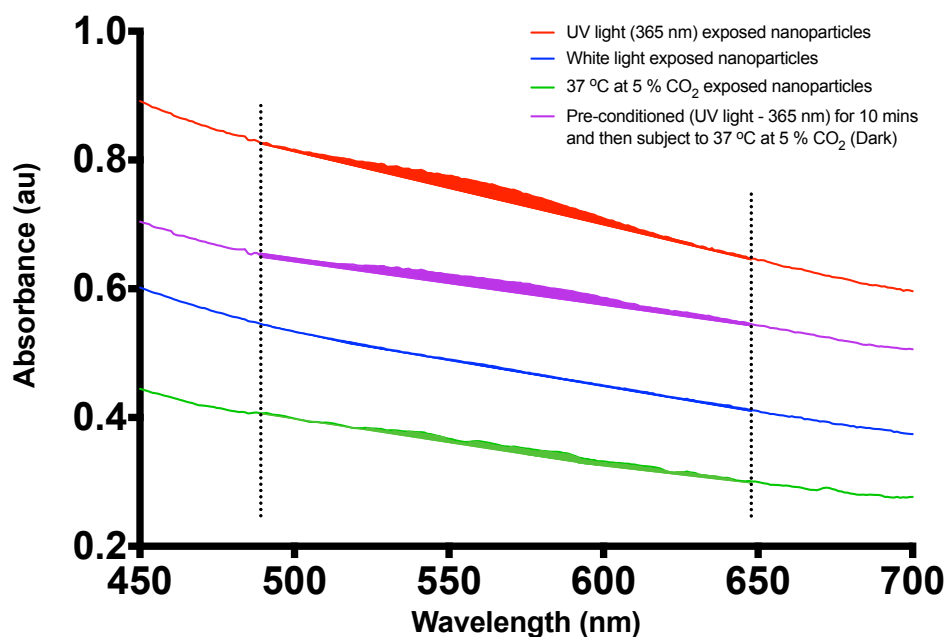


Figure 5-53 - UV/Vis analysis for spiropyran-dopamine nanoparticles when exposed to different wavelengths of light and environments (489 to 647 nm boundaries indicating merocyanine isomer)

Table 5-20 - Integrals of peak areas (UV/Vis) for spiropyran-dopamine modified nanoparticles between 489-647 nm characteristic to the MC isomer

Condition imposed on spiropyran-dopamine	Integrated peak area (au)
	Between 489 and 647 nm
White Light	0.0
UV Light (365 nm)	1.4
37 °C, 5% CO ₂	0.1
Pre-conditioned (UV light – 365 nm) for 10 mins then subject to 37 °C 5% CO ₂ (dark)	1.4

With the photo-physical characterisation conducted and after identification of the conditions required to keep the spiropyran-dopamine in its open and closed form when attached to a nanoparticle, we conducted the SPAP assay to determine potency at the D₁ receptor.

Biology – Structure activity relationship for ligands attached to solid supports

To investigate the ability to activate the dopamine D₁ receptor with an immobilised ligand, dopamine functionalised nanoparticles were used at a range of 10 pg/ml to 0.1 mg/ml. Control samples of non-modified and carboxylic acid terminated nanoparticles were also used to identify any non-specific activity. Forskolin at 10⁻⁴ M was used to confirm the production of SPAP from the cells whilst running the experiments as a positive control.

It was shown that no activity was exhibited by the unmodified, and carboxylic acid terminated silica nanoparticles, whereas activation of the receptor was shown to be successful at concentrations above 10⁻⁵ mg/ml for the dopamine functionalised particles. In contrast to soluble chemicals, nanoparticles have a tendency to diffuse, settle and agglomerate in dispersion media.³⁹⁷ The extent of these processes depends on nanoparticle size, shape, charge, and density as well as the viscosity and density of solution.³⁹⁸ These properties influence the transportation rate of nanoparticles to adherent cells on the culture plate and therefore affect the effective dose within cells. In our experiment, this may be why a much higher dose of the nanoparticles may be required to activate the cells. To determine an EC₅₀ value for the dopamine functionalised nanoparticles, a revision of the protocol was made, using a minimal concentration of 10⁻⁵ mg/ml.

Figure 5-55 shows the dose response curve obtained at the new concentrations. An EC₅₀ value of 0.0014 mg/ml (± 0.00029) was obtained and confirmed that we could successfully activate the dopamine D₁ receptor using 50 nm nanoparticles functionalised with dopamine.

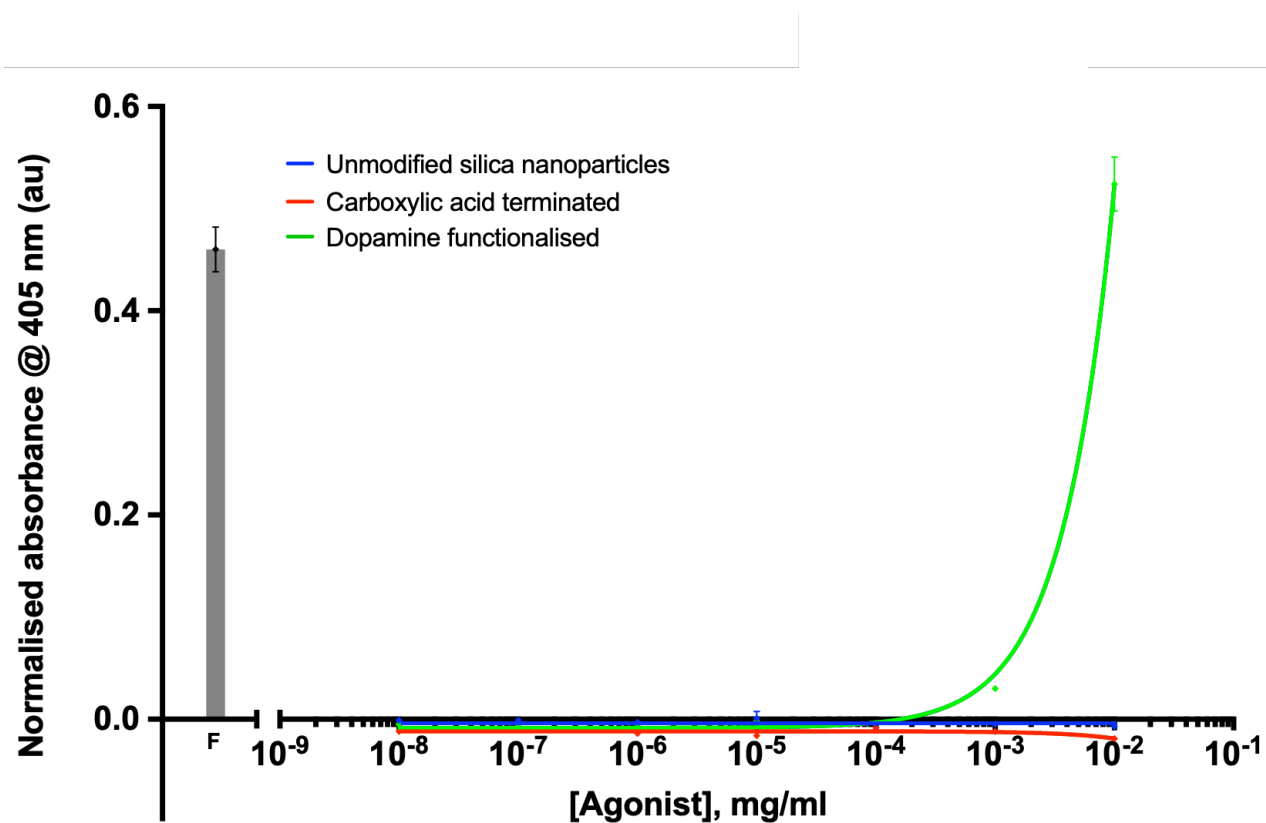


Figure 5-54 - Dose response curve for unmodified, carboxy terminated and dopamine functionalised nanoparticles for the dopamine D_1 secreted placental alkaline phosphatase assay. Positive control of forskolin is shown as F ($n=3$, $N=5$, error bars represent SEM, where error bars are not visible variation is very slight in the reported data)

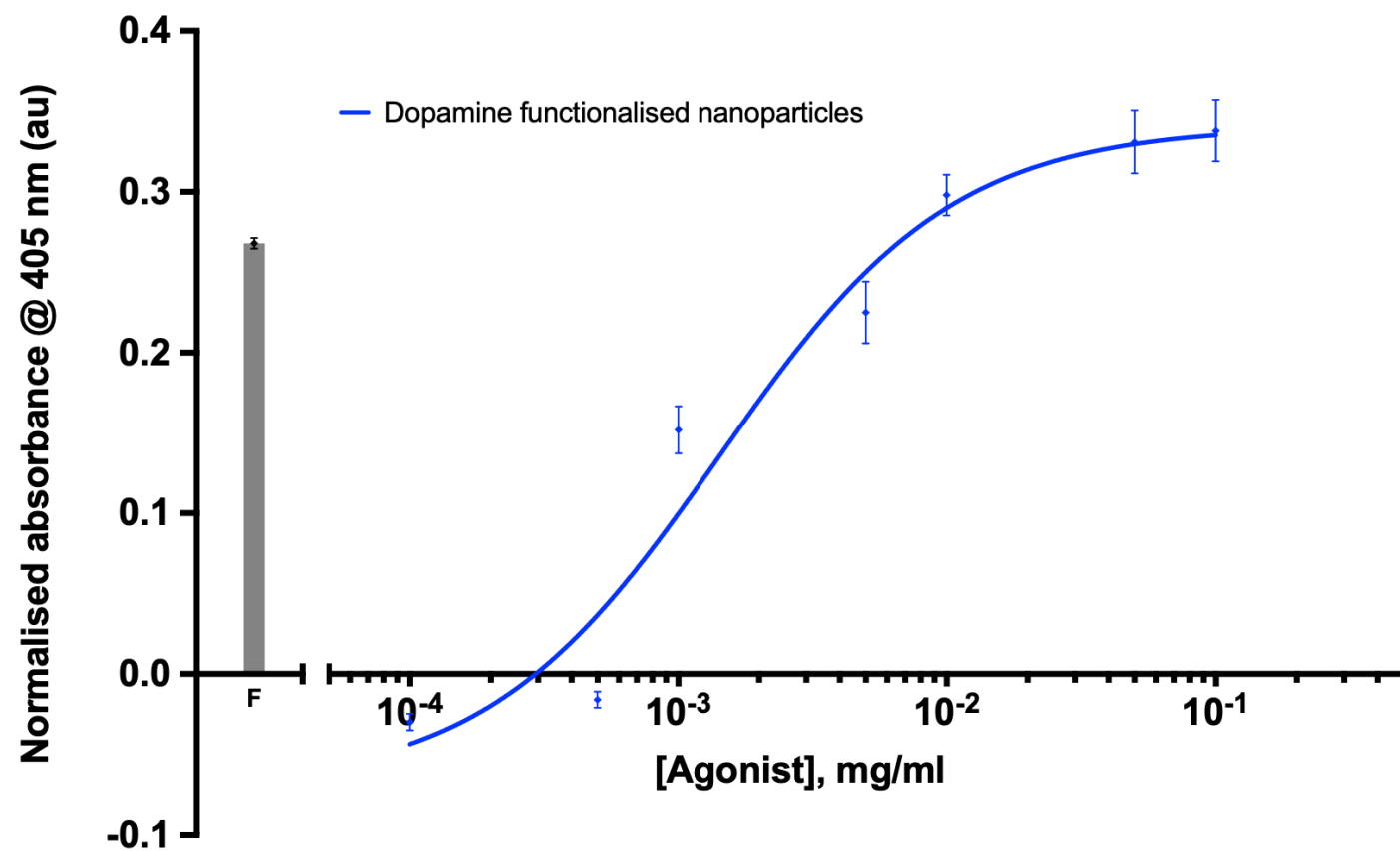


Figure 5-55 - Dose response curve for dopamine functionalised nanoparticles for the dopamine D_1 secreted placental alkaline phosphatase assay. Positive control of forskolin is shown as F ($n=3$, $N=5$, error bars represent SEM)

Activation of the D₁ receptor with the spiropyran-dopamine analogue attached to the nanoparticle was then conducted. Dependent on the photo-isomer, we aimed to activate the receptor with the open MC isomer and suppress activation using the closed SP isomer as demonstrated by the soluble molecules. Pre-conditioning of the nanoparticles with white light for 1-hour was done prior to incubation with the CHO-D₁ cells. The SPAP assay was then run under two conditions with constant exposure to white light (maintaining the SP isomer) and also in the dark (foil-wrapped, to identify thermal isomerisation to the MC isomer) on the generation of SPAP (shown in Figure 5-56 and Figure 5-57).

Similar results to those obtained for the soluble molecules in Chapter 4 were observed for the nanoparticle immobilised molecules. Upon illumination throughout the 4-hour incubation conditions (SP isomer) the spiropyran-dopamine bound analogue showed an EC₅₀ value of 0.08 ± 0.05 mg/ml, whilst the compound kept in the dark a value of 0.0047 ± 0.001 mg/ml was observed. This shift in the EC₅₀ value, from light to dark conditions, shows approximately a 17-fold increase in activity when the molecule is in its open MC isomer. From this we can say that there is a clear indication that the photo-isomer of the spiropyran has an effect on the activation of the dopamine D₁ receptor when immobilized to a solid support system.

An increased absorbance is shown for spiropyran functionalised nanoparticles in both the SP and MC isomer at high concentrations. As no activation of the receptor is observed using the soluble molecules, this result can be explained by absorbance caused by internalised nanoparticles at 405 nm.³⁹⁹

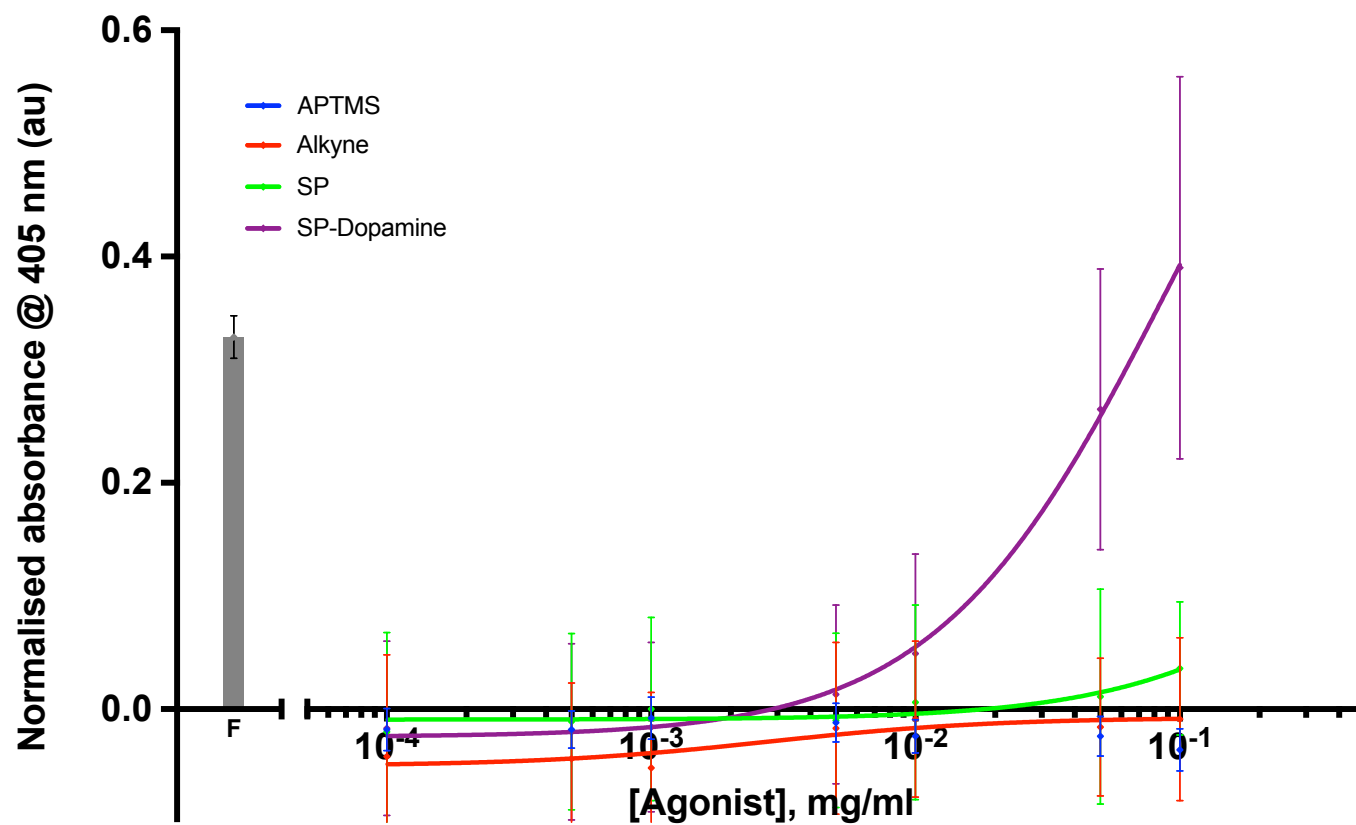


Figure 5-56 - Dose response curve for amine, alkyne, spiropyran and spiropyran-dopamine functionalised nanoparticles (under white light) for the dopamine D_1 secreted placental alkaline phosphatase assay. Positive control of forskolin is shown as F (n=3, N=5, error bars represent SEM)

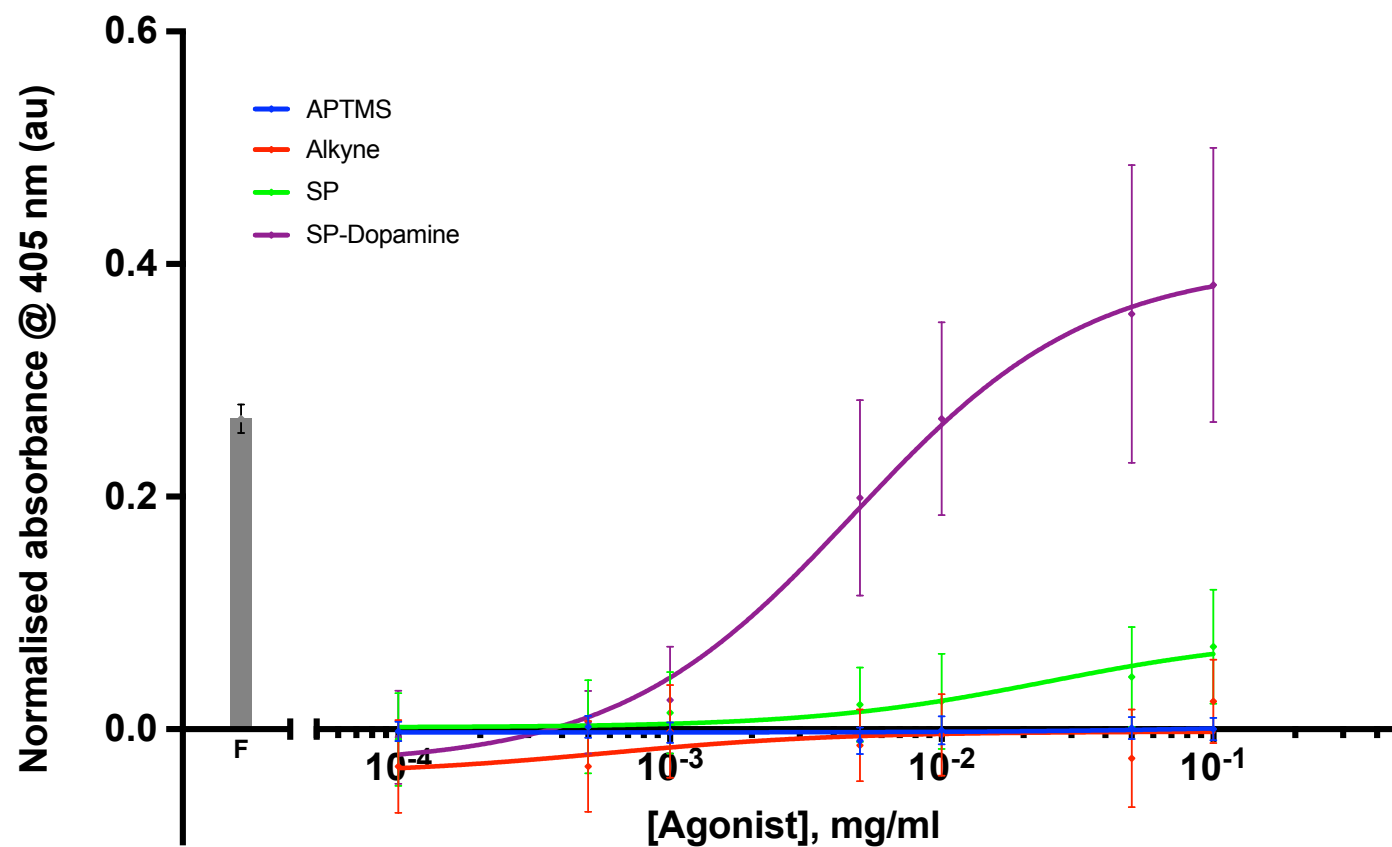


Figure 5-57 - Dose response curve for amine, alkyne, spiropyran and spiropyran-dopamine functionalised nanoparticles for the dopamine D_1 secreted placental alkaline phosphatase assay. Positive control of forskolin is shown as F ($n=3$, $N=5$, error bars represent SEM)

In our experimental conditions, internalisation of the nanoparticles may occur as the SPAP assay undertakes a 4-hour incubation period. As the surface receptor is activated, receptor mediated endocytosis is possible. With an average size of 50 nm and a ζ -potential of -40 mV, the particles are in the optimum range for internalisation.^{202,203} In addition to this, toxicity for silica nanoparticles of concentrations above 25 $\mu\text{g}/\text{ml}$ has been shown in literature for a number of mammalian cell lines.^{400,401}

As we did not measure or identify nanoparticle intake, we cannot confirm internalisation has occurred. Nevertheless, the current data demonstrates that the presence of silica nanoparticles does not adversely affect the results of our assays because the SPAP assay controls displayed no absorbance in our dose response curves for silica, carboxylic acid, amine and alkyne terminated nanoparticles. This is further confirmed with the addition of the *p*-nitrophenol phosphate to our 96-well plates at the end of the assay to determine SPAP generation. Enzymatically induced generation of *p*-nitrophenol from PNPP was only observed in the wells which contain the spiropyran-dopamine analogues and forskolin control (measured at 405 nm) Demonstrating that the absorbance measured was specifically to the presence of SPAP generated.

To determine if the nanoparticles have any detrimental effects on the CHO-D₁ cells, we must use assays which assess cell death, membrane damage, metabolic abnormalities and inflammatory responses.^{402–405} Selection of the appropriate cytotoxicity assay is vital to the accurate assessment of nanoparticle toxicity, such as using lactate dehydrogenase (LDH) leakage, or 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays.^{406,407} To detect sub-lethal cellular changes that do not result in cell death, genomic and proteomic array tests need to be identified.³⁹⁷

Conclusion

Successful immobilisation of dopamine, spiropyran and the spiropyran-dopamine analogue on both nanoparticles and glass coverslips was achieved, confirmed by FTIR and ToF-SIMS respectively.

Manipulation of each substrate was conducted using UV light and thermal stimuli to isomerise the photo-responsive unit into the SP and MC state. The photo-physical properties of the spiropyran and spiropyran-dopamine analogue adhered to the solid substrates were measured using UV/Vis (nanoparticles) and WCA (glass substrates). It was evident that conversion was successfully achieved by the characteristic MC band between 400 and 600 nm wavelength as shown for the nanoparticles. Changes in surface wettability confirmed isomerisation for the glass coverslips, where the open MC isomer was more hydrophilic than its closed counterpart. Similar observations were made for the bound compounds to their soluble counterparts using UV/Vis spectroscopy. Whilst **15** showed a higher integral peak area in response to thermal stimuli to convert from the SP→MC state, compound **16** was shown to be more responsive to optical stimulation.

Stimulation of the dopamine D₁ receptor was successfully shown using dopamine functionalised nanoparticles. This was significant as it allowed us to confirm that an immobilised ligand could successfully activate a receptor found at the cell surface. Confirming this, controlling the activation was conducted next using the two photo-isomers exhibited using compound **16**. SPAP was generated with both isomers of the spiropyran-dopamine conjugate but it was evident that, depending on the SP or MC isomer, a large shift in EC₅₀ value was observed. EC₅₀ values of 0.0047 and 0.08 mg/ml were observed at the D₁ receptor, for the MC and SP isomer of the spiropyran, respectively. This is approximately a 17-fold shift in the EC₅₀, which shows that we can successfully control activity at the D₁ surface receptor, dependant on isomer.

Experimental

Speciality chemicals including 3-chloro-5-nitrobenzaldehyde were purchased from fluorochem and 13 mm glass coverslips were obtained from Scientific Lab Supplies.

ToF-SIMS - measurements were conducted on an ToF-SIMS IV (IONTOF GmbH) instrument with the parameters of, 500 x 500 μm in both positive and negative modes using a Bi^{3+} source, at 25000 eV. Charging of the sample was compensated with low-energetic electrons from a flood gun. IonTOF SurfaceLab 6.8 was used for analysis of the mass spectra, using five, 150 x 150 μm regions of interest. Surfaces were then further analysed using principle component analysis (PCA) using Eigenvectors PLS-Toolbox 8.5 (R5) with MATLAB R2017a.

FTIR-ATR was conducted using an Agilent Cary 630 spectrometer attached with an attenuated total reflectance unit. Samples were background corrected, with a resolution of 16cm^{-1} , and with 32 scans per sample.

Zeta potential measurements were conducted using a Malvern Zetasizer Nano-ZS. 1 mg/ml of sample in 1:10 PBS in water solution was used as the solvent at 25 °C.

Statistical analysis was conducted using Graphpad Prism (Ver. 7.0c) for zeta potential measurements and ToF-SIMS ion intensities. Two-way analysis of variance (ANOVA) was conducted using Tukey's multiple comparison test with a 95% confidence interval.

Nanoparticle synthesis

Nanoparticles were synthesized using the Stöber method. A solution of TEOS (1.5 ml) in ethanol (48.5 ml) was added dropwise over a period of 10 to 15 minutes at room temperature, to a stirring solution of ethanol (46.75 ml), ammonium hydroxide (0.5 ml, 35% aqueous solution) and water (2.75 ml). This was then left for 6 hours generating a light grey/blue cloudy suspension. Solution was then centrifuged at 4800 RPM into a pellet and washed with ethanol (25 ml), isolated and then washed with water (25 ml). Nanoparticles were then dried under vacuum overnight ready for analysis and functionalisation. After drying, 420 mg of silica nanoparticles were isolated.

Oxygen plasma cleaning

Glass coverslips were sonicated in a glass rack with methanol: chloroform (1:1 ratio) for 5 minutes. Coverslips were then dried and placed in a clean glass petri dish, prior to oxygen plasma treatment. The plasma was generated using oxygen and in a vacuum chamber with a pressure of 9.0×10^{-2} psi, forming a bright purple/blue plasma (100 W). Coverslips were exposed to the plasma for 1 minute and used immediately for functionalisation.

General procedure for dopamine surface modification

Carboxylic acid

SiO₂ nanoparticles (100 mg) and plasma cleaned coverslips (n=15) were submerged in a solution of carboxyethylsilane triol di-sodium salt (2%) in phosphate buffered saline (PBS) solution (15 ml) for 24 hours. Substrates were then washed with PBS, acetone and then distilled water. Nanoparticles were centrifuged after each wash, and dried under vacuum; coverslips were dried under a constant stream of nitrogen.

Dopamine

Carboxylic acid terminated nanoparticles (80 mg) and glass substrates (in a dry petri dish) was added to a solution of dopamine hydrochloride (20 mM) in dry DMF. To this solution two equivalents of *N,N'*-diisopropylcarbodiimide and one equivalence of Et₃N was added. Glass coverslips were gently stirred on an orbital shaker, whilst nanoparticles were stirred using a magnetic stirrer plate overnight. The samples were washed sequentially with DMF, acetone, and then water. Nanoparticles were isolated from solution using a centrifuge at 4800 RPM for 5 minutes after each wash. These were then dried under vacuum after the final isolation step. Coverslips were dried under a constant stream of nitrogen.

General procedure for spiropyran surface modification

Amine

SiO₂ nanoparticles (100 mg) in a falcon tube and plasma cleaned coverslips (in a petri dish) were submerged in a solution of APTMS (2%) in toluene (15 ml) for 1 hour. Substrates were then washed with acetone and then distilled water. Nanoparticles were centrifuged after each wash, and dried under vacuum; coverslips were dried under a constant stream of nitrogen.

Alkyne

Amine functionalised nanoparticles (80 mg) in a falcon tube and glass coverslips (in a petri dish) was added to a solution of 4-pentynoic acid (20 mM) in dry DMF. To this solution 2 equivalents of *N,N'* di-isopropylcarbodiimide was added. The reaction was gently stirred on an orbital shaker, whilst nanoparticles were stirred using a magnetic stirrer plate overnight. The samples were then washed in DMF, acetone, and water. Nanoparticles were centrifuged after each wash, and dried under vacuum. Coverslips were dried under a constant stream of nitrogen.

Spiropyran

Alkyne terminated nanoparticles (50 mg) in a falcon tube and glass coverslips were placed individually into glass scintillation vials, backfilled with nitrogen. Compound **15** was prepared as a 20 mM solution in DMSO: H₂O (2:1) and added to each vessel. Copper sulfate (0.1 eq) and sodium ascorbate (0.5 eq) was added under a flow of nitrogen and promptly closed. Gently stirred overnight, samples were then washed in DMF, acetone, and water sequentially. Nanoparticles were centrifuged after each wash, and dried under vacuum, whilst coverslips were dried under a constant stream of nitrogen.

Spiropyran dopamine

Spiropyran functionalised nanoparticles (50 mg) in a falcon tube, and glass coverslips (in a dry glass petri dish) was added to a solution of dopamine hydrochloride (20 mM) in dry DMF. To this solution 2 equivalents of *N,N'*-diisopropylcarbodiimide and 1 equivalence of Et₃N was added. Gently stirred overnight, samples were then washed in DMF, acetone, and water sequentially. Nanoparticles were centrifuged after each wash, and dried under vacuum, whilst coverslips were dried under a constant stream of nitrogen.

Biological procedures

The experimental procedure for growing, maintaining and seeding the cells remained the same as mentioned in the previous Chapters. The SPAP also remained the same, although some modifications were made in relation to sample preparation and how the compounds were stored in the incubator for the 4-hour incubation period. Nanoparticles were used at a stock concentration of 1 mg/ml, which in turn were diluted to give a range between 0.1 mg/ml to 0.0001 mg/ml for the SPAP assay. As with the photo-responsive soluble molecules, the nanoparticles were subject to white light for 1 hour prior to the 4-hour incubation.

Chapter 6 - Conclusions

Synthesis of dopamine analogues which exhibit modifications at both the catechol and amino group has been successfully achieved and their potency at the dopamine D₁ receptor was explored.

It was observed that modifications at both the catechol and amino groups reduced or suppressed activity at the dopamine D₁ receptor. Reduced activity was observed with modifications made at the amino terminus (**2**, **9b-c**), giving the indication that the catechol can still interact with the two amino acid residues, Ser¹⁹⁸ and Ser²⁰², as well as the amino group with Asp¹⁰³ with hydrogen bonding. Complete suppression of activity as indicated by the SPAP assay, was evident in compounds which exhibited protection groups on the catechol (**3**, **4**, **7a-c**). The generation of SPAP was interestingly observed with the catechol protected *N*-alkylated analogues (**8a-c**), exhibiting a primary amine terminal group. Although our conclusions may suggest rearrangement of the molecule, allowing binding of the terminal amine/secondary amine formed within the reaction, with the Asp¹⁰³ residue, we cannot confirm this without further investigation. To understand the critical role the catechol plays on the endogenous dopamine ligand to activate the D₁ receptor, modifications were made at the amino moiety using amide chemistry to further this work.

A dual functional photo-responsive element which was able to be adhered to a solid support, 8-(azidomethyl)-1',3',3'-trimethyl-6-nitrospiro[chromene-2,2'-indoline]-5'-carboxylic acid (**15**, 72%) and to dopamine through its amino group was synthesised, 8-(azidomethyl)-*N*-(3,4-dihydroxyphenethyl)-1',3',3'-trimethyl-6-nitrospiro[chromene-2,2'-indoline]-5'-carboxamide (**16**, 42%). Analysis of the compounds to determine their photo-physical characteristics and the ability to switch between its two conformational isomers were successfully conducted. It was shown that for compound **15** the formation of the merocyanine state occurred at a considerably higher intensity upon

thermal isomerisation at 37 °C compared to UV activation. In contrast, increased merocyanine formation by UV activation compared to thermal activation was observed for **16**.

Conducting the CRE-SPAP assay, it was shown that activation of the D₁ receptor could successfully be achieved dependent on the photo-isomer of compound **16** present in solution. In the MC isomer, we see activation of the receptor, with the presence of SPAP at high concentrations. This can be explained by the extension and flexibility of compound **16** allowing the catechol to interact with the serine residues found in the TM5 region, and the amide with hydrogen bonding at the Asp¹⁰³. The SP isomer, showed no activity at the receptor, which can be explained by the rigidity of the compound.

In controlling the activity at the D₁ receptor in solution, the successful immobilisation of compounds **1**, **15** and **16** onto solid substrates was achieved for both nanoparticles and glass coverslips (identified by FTIR and ToF-SIMS respectively). The ability to control which isomer was present as a function of the external stimulus applied was demonstrated using surface wettability measurements, whilst photo-physical characteristic of the nanoparticles were measured via UV/Vis spectroscopy. The spiropyran-dopamine analogue showed similar results to the soluble molecules where isomerisation to the MC isomer was achieved efficiently using UV light and dark conditions whilst exposure to white light throughout a 4-hour incubation period kept the compound in its closed SP isomer under elevated temperatures.

Activation of the D₁ receptor using dopamine immobilised nanoparticles was achieved, playing a significant role in understanding that ligands can be immobilised to solid substrates to activate cell surface receptors. The ability to control activity at the D₁ receptor using the SP and MC isomer of compound **16** while immobilised on a nanoparticle was also shown. The resulting EC₅₀ value was 17 times higher for the MC compared to the SP isomer, with the former activating the receptor at a lower concentration.

From the work conducted within this thesis we can start to understand that the control of GPCRs can be achieved using ligands both attached to photo-responsive elements as well as solid support systems. This paves the way for the development of methods to stimulate different and multiple cell surface receptors and can lead to new applications such as biomimetic surfaces, acting like artificial synapses in the future. The impact of developing artificial synapses, can lead to application which may be able to replace or interact to compensate for damaged neuronal tissue, as a type of drug delivery mechanism which may aid in therapeutic use for neurodegenerative diseases.

One of the aims of SLAB is to generate a method to simultaneously stimulate and monitor neurons. This thesis, indicates clearly that using dopamine bound to solid substrates with a photo-responsive unit (depending on photo-isomer) can control activity at the dopamine D₁ receptor.

Chapter 7 - Future work

This project proves the concept of controlling activity at the dopamine D₁ receptor as it set out to using a surface bound photo-responsive coupled neurotransmitter in different structural isomers. Further work must be conducted to achieve the goals of the SLAB system and a brief overview of the experiments required to progress this study to its full potential as well as to advance the SLAB system is shown below.

The synthesis of the dopamine library was successful in parts, but due to the failure of a few reactions, namely the removal of benzyl group, whilst retaining Boc protection on the *N*-alkylated dopamine analogues as well as the synthesis of a small *N*-alkylated dopamine analogue, a complete library was not obtained. Due to this, we must consider alternative synthetic strategies which may include further modifications to the endogenous dopamine ligand, or the use of alternative agonists potent at the D₁ receptor. The use of alternative starting materials, as explored by Ginos, *et al*,²⁴⁷ may also contribute to higher yields than what has been expressed within this project, and may allow a number different *N*-alkylated dopamine analogues to be synthesised. To determine alternative analogues which may contribute to a higher potency, we should consider and delve deeper into molecular modelling and simulations, allowing us to determine the ideal size and structure which may interact with key binding sites identified for the D₁ receptor.

As this project aimed to stimulate receptors which have been known to affect synaptic plasticity, alternate ligands which cause this effect would also need to be explored. Compounds such as pramipexole, a D₂ dopamine agonist or serotonin may be easier to modify as there is no catechol present for oxidation. Antagonists are also required to progress this study, weakening the connections between synapse.

Whilst we have shown conversion of the spiropyran to both the SP and MC isomer using UV/Vis spectroscopy, we have not specifically determined the different rotamers present in each form when switching occurs using UV light/thermal stimuli. When attached to dopamine, these structures can differ in a number of ways, which may cause the activation/suppression of activity at the D₁ receptor. Identification of these would allow us to simulate and further investigate binding of the synthesised analogues to the dopamine D₁ receptor, and allow us to determine future analogues which may be able to bind to different GPCRs.

General surface experiments such as the identification of concentration of dopamine and spiropyran-dopamine on nanoparticles and glass substrates need to be conducted. This would allow us to compare activity at the D₁ receptor for both solution and bound ligands. In addition to this, stability studies which address oxidation and hydrolysis/photo bleaching of the dopamine and photo-responsive surfaces respectively need to be conducted. This allows us to determine whether the surfaces would be able to withstand biological conditions for long periods of time. Focusing on the glass substrates, we must be able activate the photo responsive element in a spatial and temporal manner using equipment such as a spatial light modulator, and be able to characterise this change in real time. The importance of this is to monitor how long it takes for the pharmacophore to be revealed and retracted in comparison to activation of the receptor. Experiments which address biological aspects such as cell adhesion and toxicity studies for neuronal binding to glass substrates needs to also be addressed. This may require additional surface modification to allow cells to adhere and provide a structural support for our method of stimulation.

Finally, the collaborative attempt to combine this system with others who have developed long term single cell monitoring, the virtual environment and also a way to understand neural linguistics. Communication between cells, to understand how memory and learning works.

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