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“But then you can't expect a monolith to love you back.”

– Professor River Song

Doctor Who, ‘The Husbands of River Song’
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Studies on Unmodified Silica Monolith Production, Encapsulation and Separation

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ABSTRACT

Described herein is a series of investigations on the design and preparation of a microfluidic device for the chemical analysis, including separation and detection of target analytes. Different components of the device are explored and optimised in order to be suitable for a microfluidic device application.

Silica monolith columns were utilised as the stationary phase media for sample separation, however optimisation of preparation techniques was required in order to produce monoliths with porosities suitable for chromatographic applications. Preparation times, incubation periods and treatment methods were optimised in order to prepare desirable pore characteristics within the monoliths. A novel encapsulation technique was explored in order to produce a column whose encapsulation media was enmeshed with the outer monolith layer, so as to prevent solution from flowing around the monolith column instead of through it, a phenomenon known as the “wall-effect”, which impedes proper separation.

Methods of functionalising the monoliths were explored in order to produce columns capable of separating out samples of importance. Different preparation methods of the common C18 stationary phase were explored, both pre- and post-encapsulation, and compared with a bare silica column and a commercial column to determine their efficacy as functionalisation methods. A novel stationary phase functionality was developed and characterised using the Tanaka Test to evaluate retention characteristics. Monolith sol-gels were modified with the chemiluminescence reagent tris(2,2′-bipyridyl)ruthenium(II) in an attempt to create a flow-through monolith rod for on-column or post-column chemiluminescence detection.
Chemiluminescence detection zone channels were modified and optimised to be more suited to a microfluidic device; to allow for smaller volumes than are normally required in post-column chemiluminescence detection or flow injection analysis, without compromising sensitivity. Smaller channel dimensions and an easier-to-produce channel configuration were compared with commonly utilised flow-cells, such as spiral or serpentine flow-cells, and limits of detection determined with different chemiluminescence reagents and model analytes of importance. Various material colours, relating to the emission wavelengths of commonly utilised chemiluminescence reactions, were explored in order to determine which colour would reflect the greatest amount of light back to a photodetector, lowering the limits of detection achievable with the smaller channel dimensions.

Blister pack reagent storage was examined as a means of on-device long-term chemiluminescence reagent storage, with a variety of chemiluminescence reagents and storage conditions studied. Microfluidic device prototype designs were prepared in SolidWorks for a range of different applications, including one-dimension, and multi-dimension separations; as well as utilising single or dual reagent streams for chemiluminescence detection.
ACKNOWLEDGEMENTS

Firstly, I would like to thank my Supervisors, both those still with me and those who have left for to pursue other avenues: Professor Neil Barnett, Professor Paul Francis, Professor Stephen Haswell, Doctor Zoe Smith, Associate Professor Xavier Conlan, and Doctor Paul Stevenson. Through the years you have offered your knowledge, wisdom, and advice, without which I probably would not have made it through this endeavour. I would also like to extend my gratitude to Doctor Egan Doeven and Richard Alexander, though not listed supervisors, you’ve acted as such on many occasions: proofreading, educating, and guiding me through my work in unfamiliar areas of research, especially when it came to CAD designs.

To my colleagues and friends at Deakin University, I would like to thank you for the memories, the advice, and help over these years. To Jessica Learey and Kim Quayle: you’ve helped me stay sane, and kept me going when I wanted to give in. You’ve been my travel buddies, and have (sometimes literally) held my hand when things got rough. Your presence made this challenge enjoyable on even the toughest days.

To my Mum and Dad: though you’re convinced I’ve only done this to get the ‘funny hat’, you’ve supported me through my 20+ years of education, and have always encouraged me to get out and do greater things. Thank you for the love and support you have always given me. To all my family: you have always shown support, and have nodded along caringly as I explain things that at times I don’t even fully understand. Finally, to the Laird-iest Laird of them All, my dearest Cameron. You’ve been there for me the most out of everyone, especially in these final months, and no words can describe how much your love and support has meant to me over these years. Thank You.
LIST OF PUBLICATIONS

The following is a list of publications resulting from the work presented in this thesis:

Peer-Reviewed Journal Articles:


Patents:

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Term</th>
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<tr>
<td>[Ru(bpy)₃]²⁺</td>
<td>Tris(2,2'‑bipyridyl)ruthenium(II)</td>
</tr>
<tr>
<td>[Ru(bpy)₃]³⁺</td>
<td>Tris(2,2'‑bipyridyl)ruthenium(III)</td>
</tr>
<tr>
<td>[Ru(bpy)₃]Cl₂.₆H₂O</td>
<td>Tris(2,2'‑bipyridyl)ruthenium(II) chloride hexahydrate</td>
</tr>
<tr>
<td>µTAS</td>
<td>Miniaturised/micro total analysis system</td>
</tr>
<tr>
<td>3D</td>
<td>Three dimensional</td>
</tr>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>BET</td>
<td>Brunauer‑Emmett‑Teller model</td>
</tr>
<tr>
<td>BJH</td>
<td>Barrett‑Joiner‑Halenda model</td>
</tr>
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<td>CAD</td>
<td>Computer aided design</td>
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<tr>
<td>CE</td>
<td>Capillary electrophoresis</td>
</tr>
<tr>
<td>CL</td>
<td>Chemiluminescence</td>
</tr>
<tr>
<td>COC</td>
<td>Cyclic olefin copolymer</td>
</tr>
<tr>
<td>DFM</td>
<td>Design for Manufacture</td>
</tr>
<tr>
<td>DFMA</td>
<td>Design for Manufacture and Assembly</td>
</tr>
<tr>
<td>DFT</td>
<td>Density functional theory</td>
</tr>
<tr>
<td>FEP</td>
<td>Fluorinated ethylene propylene</td>
</tr>
<tr>
<td>FIA</td>
<td>Flow injection analysis</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>H₂SO₄</td>
<td>Sulfuric acid</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HILIC</td>
<td>Hydrophilic interaction chromatography</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>i.d.</td>
<td>Internal diameter</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium chloride</td>
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<tr>
<td>KMnO₄</td>
<td>Potassium permanganate</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid chromatography</td>
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<tr>
<td>LOC</td>
<td>Lab‑on‑a‑chip</td>
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<tr>
<td>LOD</td>
<td>Limit of Detection</td>
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<tr>
<td>MEKP</td>
<td>Methylethylketone peroxide</td>
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<td>Methanol</td>
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<tr>
<td>ODS</td>
<td>Chloro(dimethyl)octodecylsilane</td>
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<tr>
<td>PAH</td>
<td>Polycyclic aromatic hydrocarbon/s</td>
</tr>
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<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>Polycarbonate</td>
</tr>
<tr>
<td>PDMS</td>
<td>Poly(dimethylsiloxane)</td>
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<tr>
<td>PEEK</td>
<td>Poly ethyl ethylene ketone</td>
</tr>
<tr>
<td>PEO</td>
<td>Polyethylene oxide</td>
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<td>PET</td>
<td>Polyethylene terephthalate</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>PFA</td>
<td>Poly(fluoroalkoxy)</td>
</tr>
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<td>PMMA</td>
<td>Poly methyl methacrylate</td>
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<tr>
<td>PMT</td>
<td>Photomultiplier tube</td>
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<tr>
<td>POC</td>
<td>Point-of-care diagnostics</td>
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<tr>
<td>Polymer</td>
<td>Pluronic F127 polymer</td>
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<tr>
<td>PTFE</td>
<td>Poly tetra fluoro ethylene</td>
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<tr>
<td>RSD</td>
<td>Relative standard deviation</td>
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<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
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<tr>
<td>SiPM</td>
<td>Silicon photomultiplier</td>
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<td>TEOS</td>
<td>Tetraethyl orthosilicate</td>
</tr>
<tr>
<td>TMOS</td>
<td>Tetramethyl orthosilicate</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
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CHAPTER ONE

INTRODUCTION

1. Microfluidic Devices and the Need for Portable, Sensitive Detection
2. Silica Monolith Columns
3. Chemiluminescence Detection
4. Microfluidic Device Design
5. Project Aims
1 Microfluidic Devices and the Need for Portable, Sensitive Detection

Miniaturised Total Chemical Analysis Systems (µTAS) were first defined by Manz et al. in 1990, describing all processes of chemical analysis, from sample preparation through separation and detection being performed on a single, miniaturised device. The concept emerged from the scaling down of hydrodynamics and diffusion principles, which had previously been demonstrated by Poiseuille in 1840 using glass capillaries around 10 µm in diameter.

Through the use of microfluidic systems, high resolution separations and sensitive detection can be achieved whilst decreasing the required volumes of samples and consumption of reagents, making it promising for applications in areas such as medical analysis, chemical synthesis and microanalytical systems (in particular lab-on-a-chip systems). Other advantages include increased automation of processes, reduced manufacturing costs and increased complexity of systems. The lab-on-a-chip (LOC) system generally miniaturizes an analysis that would normally take an entire laboratory to complete, and shrinks it to fit onto platforms of the millimetre and centimetre range, whilst also cutting analysis times down from days or hours to just minutes.

Since the early 1990s research in the area of microfluidics and microfabrication has increased exponentially and continues to develop technologies capable of meeting the requirements of microfluidics devices today.

Performing all of these chemical analysis processes on miniaturised devices enables greater portability and at-scene detection in fields such as medicine (point of care diagnostics) and forensics for quantitative results.
A lot of LOC and µTAS devices have focussed on point of care diagnostics, and have branched into forensics to perform DNA extraction and amplification on-chip for forensic analysis.\textsuperscript{18-28} Few devices have been published which focus on critical forensic detection for use by law enforcement to analyse complex samples such as seizure samples, and more research in this area is required.\textsuperscript{29-30}

In order to analyse complex samples, the device needs to perform several functions. Separation is important in order to separate the components of the complex sample to obtain accurate results. Traditional particle packed columns require relatively high pressures in order to perform efficient separations, and the particles are not ideal for incorporation into a chip. Continuous bed columns, such as monolithic columns, require lower pressures than particle packed columns, and can easily be integrated into a microfluidic chip without blocking channels.\textsuperscript{31-32}

Chemiluminescence detection offers selective and sensitive detection of forensically, clinically and environmentally important samples,\textsuperscript{33-34} utilising robust, inexpensive and potentially portable equipment,\textsuperscript{35} and has often been utilised as post-column detection for high performance liquid chromatography (HPLC).\textsuperscript{33, 36} Chemiluminescence reagents have often been utilised in the literature for the detection of illicit drugs such as heroin (and its metabolites),\textsuperscript{37-38} or methamphetamine,\textsuperscript{33, 36, 39} and so are ideal for sensitive detection of illicit substances in a microfluidic device.

\section{Silica Monolith Columns}

\subsection{Preparation}

Monolithic columns, or continuous bed columns, offer many advantages over the conventional particle packed columns used in liquid chromatography (LC), with
greater total porosity and permeability. These characteristics lead to lower back pressures and the ability to operate at higher flow rates, without causing damage to the column itself. The first continuous bed columns utilised for liquid chromatography consisted of polyurethane foams, but they offered low performance and had limited pressure stability, making them less than ideal candidates for chromatography systems, especially high performance liquid chromatography (HPLC). Hjerten and co-workers introduced polymer-based monolithic materials in the 1980s, but it was the pioneering work of Soga and Nakanishi that led to what many research groups now use for the basis of their monolith compositions. The authors noted that it was the combination of sol-gel chemistry of silica with the phase separation mechanism from the polycondensation of the alkoxy silanes in the presence of water soluble polymers that caused the phase-separation of monoliths, producing the porous silica framework (see Equation 1.1). Silica monolith columns consist of a continuous porous rod of silica which has bimodal porosity, consisting of pores in the micrometre and nanometre ranges (see Figure 1.1). This bimodal porosity produces ideal parameters for monolithic applications in separation science.

\[
\text{Si(RO)}_4 + \text{H}_2\text{O} \rightarrow \text{Si(OH)(RO)}_3 + \text{ROH}
\]

\[
\text{(RO)}_3\text{Si} - \text{OH} + \text{(RO)}_3\text{Si} - \text{OH} \rightarrow \text{(RO)}_3\text{Si} - \text{O} - \text{Si(RO)}_3 + \text{H}_2\text{O}
\]

\[
\text{(RO)}_3\text{Si} - \text{OH} + \text{(RO)}_3\text{Si} - \text{OR} \rightarrow \text{(RO)}_3\text{Si} - \text{O} - \text{Si(RO)}_3 + \text{ROH}
\]

Equation 1.1: Condensation of silicon alkoxide to form siloxane oligomer, which then links together to form a gel network, the basis of phase-separated monolith formation.
A number of methods for the control of pore properties of silica monoliths have now been developed and reported. In the case of Fletcher and co-workers’ method, for example, the alkoxy silane is tetramethyl orthosilicate (TMOS), which reacts with the water within the polymer solution (through the diluted acid) to produce the silica framework of the column, with the acid acting as a catalyst to promote the reaction. The treatment processes, including ammonium hydroxide treatment, that the wet-gel monoliths undergo following phase separation and incubation, etch the silica surface of the column creating nanometre-sized pores, and giving the monolith a higher surface area, a characteristic which is favourable for their use in chromatography.

*Figure 1.1: Silica monolith skeleton demonstrating bimodal pore structure. Monolith shown is 270215 monolith.*

Commercial monolithic HPLC columns first became available in 1999, with the introduction of the Chromolith® column (Merck, Darmstadt, Germany). Little investment had been made into commercial competition due to intellectual property protection, but a few varieties of commercial monolithic columns are now available.
on the market. Whilst a few other companies have produced commercially available columns, their potential has yet to be fully realised for chromatography in place of particle packed columns, partly due to the lack of viable encapsulation methods.56

2.2 Monolithic encapsulation

Earlier encapsulation techniques utilised for monolith applications in separation sciences were based around those used for particle packed columns – which involved filling a plastic or metal tube with silica particles, and then compressing the tube by applying and maintaining an external pressure.57 This cartridge system, based on radial compression of the column which was developed by the Waters Corporation,58 was not a practical solution for monolith encasement as it was far more suited to a particle packed column model than a continuous bed column, like silica monoliths. The method, when used with monolithic columns, can result in high external flow rates, or wall-effects, where large volumes of mobile phase travel around the outside of the column, with minimal stationary phase interaction, resulting in poor separations.59 To use this method for column encapsulation also required constant pressure to be applied to the external surfaces of the column which is impractical.31 Researchers at Merck KGaA developed an encasement method using polyethyl ether ketone (PEEK) cladding around the column, which was claimed to be both solvent and pressure durable by the authors.50 This technique is now one of the most common encasement methods available today. However, as indicated by Neue and co-workers,60 the backpressure that can be applied to a commercial monolith clad in PEEK tubing is limited by this encasement material. Furthermore, the authors state that the pressure limit also restricts the speed of analysis, which is undesirable for efficient separations. The authors noted in this
2007 publication\cite{15} that commercial monoliths were still limited to single column diameters, approximately 4.2 mm i.d., whereas technological advances since the time of publication have seen varied column diameters become available.\cite{15} The MonoClad\textsuperscript{®} column, developed by GL Sciences, Inc. (Torrance, CA, USA), surrounds a monolith with two layers of polymer, which is then encased in a stainless steel tube.\cite{15,16} The first polymer layer is deposited around the monolith and is chemically inert to many reversed-phase HPLC organic solvents. The clad monolith is then placed inside the steel tube and embedded with a second polymer to bind it with the tube. This encapsulation method also allows the fabrication of longer monoliths, with a reported maximum length of 25 cm;\cite{16} however, it is a time consuming process requiring multiple encapsulation steps. Miyazaki \textit{et al.} concluded that the cladding procedure of MonoClad columns also required improvement to correct peak fronting.\cite{16}

The most commonly employed technique used by many research laboratories involves thermoshrinking (or heat shrink) polytetrafluoroethylene (PTFE) or PEEK tubing, which as the name suggests, shrinks when exposed to temperatures in excess of approximately 300\textdegree{}C, and wraps around the monolith and any tubing attached.\cite{51,56,64,70} While the heat shrink tubing appears to form a tight seal around the monolith, it does not properly bind to the outer walls of a monolith, and can therefore detach during operation.\cite{15} If detachment occurs, a phenomena known as the ‘wall-effect’ can occur, where the mobile phase solution flows around the monolith column faster than through the column, with minimal stationary phase interaction, resulting in a poor separation. This is illustrated in Figure 1.2.
Figure 1.2: (a) Proper solution flow through encapsulated monolith column. (b) Solution flow through a column whose encapsulation has been compromised. Mobile phase flows faster around the column than through the column, with minimal stationary phase interaction, causing wall-effects.

The accepted requirement of monolith encasement is that the cladding needs to be able to withstand high pressures and should not interfere with the adsorption/desorption characteristics of the column.\textsuperscript{31, 56} None of these techniques, however, appear to address the aforementioned common problem of high flow rates around the outside of the monolith, where it meets the casing, and as such is an issue which is required to be overcome in order to utilise house-made monolithic columns for separation.

### 2.3 Characterisation of silica monoliths

Characterisation of monoliths is important for their application. Many methods of monolith preparation are published,\textsuperscript{56} and with each different method, there is potential to alter pore structure. It is established that starting composition, solution mixing, incubation conditions, treatment and calcination can affect the pore structure achievable of silica monoliths.\textsuperscript{71-72} Careful monitoring of preparation processes can ensure desired pore structures are achievable for the chosen application. Commonly used methods in the literature for characterisation are scanning electron microscopy.
to observe pore structure and measure macropore size; and utilising porosity analysers (nitrogen or mercury adsorption/desorption) to determine surface area, nanopore sizes and pore volumes. Liquid permeability is also used to determine approximate percentage porosity of a monolith, however it should be noted that none of these methods take into account pores trapped within monolith structures which are inaccessible.

Scanning electron microscopy is used to measure the macropore diameters of monoliths. In-built software on the instrument/computer interface is useful in accurately measuring these diameters, but can also be measured by hand using callipers and scaling factors. Electron microscopy is also useful in observing macropore homogeneity throughout the monolith structure. It has been established for particle packed columns that radial heterogeneity can severely impact separations, causing the mobile phase to flow faster along the walls of the column, also contributing to wall effects. This phenomenon can also occur in monoliths, and several studies have attempted to explore and overcome the issue.

Nitrogen adsorption/desorption is one of the most commonly used methods for determining surface areas and nanopore characteristics, however, mercury porosimetry has also been reported. The Brunauer, Emmett, Teller (BET) and Barrett, Joyner, Halenda (BJH) models were first used to analyse monolith porosity in the 1990s, and are still widely used in the literature today. However, more accurate models are available, such as Density Functional Theory (DFT), but are not widely used, as they are only available on more modern, compatible instruments.
2.4 Monolith Functionalisation

Surface functionalisation of silica monoliths enables the separation of a greater variety of analytes compared to unmodified bare silica. Many stationary phases have been described for the functionalisation of silica particles for particle packed columns, and some of these methods have been applied to silica monolith columns.\(^8\) Recent papers have demonstrated a wide range of applicable stationary phases, with reversed phase being the most commonly described stationary phase.\(^5\)\(^0\)\^-\(^9\)\(^3\)\(^-\)\(^9\) Hydrophilic-Interaction Chromatography (HILIC),\(^9\)\(^4\)\^-\(^9\)\(^9\) Ion-Exchange\(^1\)\(^0\)\(^0\)\^-\(^1\)\(^0\)\(^2\) and chiral separation\(^1\)\(^0\)\(^3\)\^-\(^1\)\(^0\)\(^6\) stationary phases are also among those described in the literature.

Many of these published methods have been adapted from particle functionalisation methods, and so are performed in batch methods prior to encapsulation. A few in-situ modification methods have been reported, particularly for reversed phase functionalisation using chloro(dimethyl)octodecylsilane (ODS).\(^5\)\(^2\)\^-\(^5\)\(^3\)\(^,\)\(^8\)\(^5\) Homogenous carbon loadings and good separation have been achieved using these methods.\(^5\)\(^2\)\^-\(^5\)\(^3\)

Recently, monolithic columns have been identified as a favourable option for multidimensional liquid chromatography. This is due to their versatility, superior permeability and efficiency at high linear velocity,\(^5\)\(^7\)\^-\(^7\)\(^0\)\^-\(^7\)\(^8\) and the increasing variety of stationary phases available.\(^8\)\(^4\) Two dimensional chromatography (2DLC), or multidimensional chromatography, paves the way for comprehensive analysis of complex samples,\(^1\)\(^0\)\^-\(^1\)\(^1\)\(^7\) however the application of silica monolith columns are not often utilised in multidimensional chromatography,\(^1\)\(^1\)\(^2\)\^-\(^1\)\(^2\)\(^3\) with some papers only featuring columns of the same functionality.\(^1\)\(^1\)\(^8\),\(^1\)\(^2\)\(^1\) There are two types of 2DLC which can be performed: comprehensive 2DLC, where all eluted peaks from the first dimension are separated on the second dimension,\(^1\)\(^1\)\(^2\),\(^1\)\(^2\)\(^4\)\^-\(^1\)\(^2\)\(^7\) and heart-cut 2DLC,
where selected eluent fractions from the first dimension are separated on the second dimension.\textsuperscript{112}

It is desirable to utilise two columns of different separation mechanisms to increase the resolving power of complex samples, however, characterisation of the stationary phase needs to be performed in order to ensure orthogonality between the stationary phases can be achieved.

Characterisation of the applied stationary phase is also an important part of functionalisation, in order to determine stationary phase properties, and therefore appropriate analytes and conditions which can be applied; as well as ensuring efficient separations will be achievable.\textsuperscript{128} Over the years, many methods have been developed to evaluate column characteristics such as kinetic properties;\textsuperscript{129} efficiency (plate number) and silanol activity;\textsuperscript{130} and reversed-phase behaviour.\textsuperscript{131}

Tanaka \textit{et al.} developed a characterisation method where solutions of various analytes were separated under specific conditions, which was indicative of properties such as number of alkyl chains, hydrophobicity, steric selectivity, hydrogen bonding capacity, anion exchange capacity and cation exchange capacity.\textsuperscript{132} This formally became known as the Tanaka Test, and is utilised in industry as well as in research to characterise column retention characteristics.\textsuperscript{133-137}

\section{Chemiluminescence Detection}

\subsection{General Principles}

Chemiluminescence, the production of light from a chemical reaction, is an increasingly useful tool in analytical chemistry,\textsuperscript{138} and has been applied to many techniques such as high performance liquid chromatography,\textsuperscript{139} capillary
electrophoresis\textsuperscript{,140} and flow injection analysis\textsuperscript{141} to afford sensitive and selective detection of target analytes.

\[ A + B \rightarrow C^* + \text{products} \]

\[ C^* \rightarrow C + h\nu \]

Equation 1.2: General mechanism for direct chemiluminescence

Direct chemiluminescence is the result of two or more species reacting and creating an excited state product, which then releases light when relaxing back to the ground state (Equation 1.2).\textsuperscript{138, 142-143} Chemiluminescence reactions are analytically useful as the intensity of the emitted light is proportional to the concentrations of the chemical species involved. These reactions are also a selective method of detection, due to the relatively small number of analytes which are capable of eliciting the emission from a chemiluminescence reagent,\textsuperscript{138, 143-144} and since no external excitation source is required for this technique, there is an absence of background emissions from such a source, and greater sensitivity is achievable compared to other methods.\textsuperscript{138, 142-143, 145-146}

### 3.2 Chemiluminescence Reagents

#### 3.2.1 Acidic Potassium Permanganate

Acidic potassium permanganate is one of the most widely used chemiluminescence reagents in analytical chemistry.\textsuperscript{147} As a detection method, acidic potassium permanganate chemiluminescence exhibits some selectivity towards a variety of analytes, in applications such as pharmaceutical and clinical analysis, agriculture and environment, food, consumer products and materials.\textsuperscript{147} In particular, acidic potassium permanganate is useful in the detection of phenolic and polyphenolic
compounds, such as morphine and other opiate alkaloids derived from Papaver somniferum,\textsuperscript{38, 148-150} as well as those found in food and beverages, such as wines.\textsuperscript{151}

### 3.2.2 Tris(2,2′-bipyridyl)ruthenium(III)

Tris(2,2′-bipyridyl)ruthenium(III) \([\text{Ru(bpy)}_3]^{3+}\) chemiluminescence was first observed by Hercules and Lytle in 1966,\textsuperscript{152} and has been used for several decades for sensitive and selective detection of a variety of compounds, including tertiary amines,\textsuperscript{153} antibiotics,\textsuperscript{154-157} non-phenolic opiate alkaloids,\textsuperscript{38, 158} and a variety of other illicit drugs.\textsuperscript{39, 159} In order to elicit the typical orange emission from the chemiluminescence reaction, the \([\text{Ru(bpy)}_3]^{3+}\) species needs to be generated via chemical or electrochemical oxidation of tris(2,2′-bipyridyl)ruthenium(II) \([\text{Ru(bpy)}_3]^{2+}\).\textsuperscript{33, 153, 160-161} Chemical oxidation methods include adding solid lead(IV) dioxide to a \([\text{Ru(bpy)}_3]^{2+}\) solution,\textsuperscript{161} and filtering the green oxidised solution prior to use; or mixing the ruthenium reagent with an oxidising solution (on-line or off-line), such as cerium(IV) sulfate or potassium permanganate.\textsuperscript{161} Introduction of a suitable analyte causes reduction of \([\text{Ru(bpy)}_3]^{3+}\) to the excited state species \([\text{Ru(bpy)}_3]^{2+*}\), which releases light around 610 nm.

### 3.2.3 Luminol

Luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) chemiluminescence was first reported in 1928 by Albrecht.\textsuperscript{162} The oxidation of luminol produces a bright blue emission, around 425 nm, although can shift depending on reaction parameters.\textsuperscript{163-165} Commonly, luminol has been applied in forensic science as a visualization tool for blood; however it can be used to detect a wide range of analytes, including pharmaceuticals,\textsuperscript{166} polyphenolic compounds,\textsuperscript{167} pesticides,\textsuperscript{168} and bleaches.\textsuperscript{169}
Flow Injection Analysis Instrumentation for Chemiluminescence Detection

Flow injection analysis (FIA) has been utilised in chemiluminescence detection for several decades now. FIA was first described in 1975 by Ruzicka and Hansen, and was adapted from existing continuous flow analysis systems. In this technique, an aqueous sample is injected into a continuous flowing liquid carrier stream, which merges with a reagent in front of a detector. FIA can be utilised with a variety of detection techniques, including optical and electrochemical techniques, but is especially useful in chemiluminescence detection. FIA allows for the reproducible mixing of solutions, providing fast, reliable chemiluminescence signal intensity results, using quite simple instrumentation.

The instrumentation consists of a sample injection port, often with an injection holding loop, which introduces a precise aliquot of sample into a flowing carrier stream. The carrier stream merges with the reagent stream at a confluence point prior to, or within, a flow-cell or reaction chamber, which is placed flush against the window of a photodetector, such as a photomultiplier tube (PMT).

Chemiluminescence Detection Flow-Cells

There are several influencing factors on chemiluminescence reactions by the design of the detector, which can alter the intensity produced or the detectability of the emission: (1) confluence point distance, the distance between solution merging and the detector; (2) channel dimensions, the deeper the channel, the larger amount of analyte there will be within the channel, and therefore there will be more light produced directly in front of the detector; and (3) channel configuration and
solution mixing, which influences the kinetics of the reaction, and therefore the intensity of the emission. 174-177

Over the past 40 years, flow-cell designs for chemiluminescence detection have been reported in the literature. The simplest form of flow-cells are reaction vessels or chambers, where a void is machined into a piece of polymer or glass, 178 with inlets and an outlet, in which solutions are merged and reacted. A clear cover for the vessel is placed against a photodetector, to allow light transfer to the detector. Reaction vessels include colorimeter flow-cells, similar to those used in spectrometers and colorimeters, 179 as well as the bubbler reaction vessel, 180-181 and fountain flow-cells. 182-183

Tubing based reactors are among the most common flow-cells used in chemiluminescence today. Most often, a transparent or translucent piece of tubing, polymer or glass, 184-190 is coiled into a spiral shape and placed in front of the photodetector (Figure 1.3 a-b). Coiled tubing was first reported for use in flow injection analysis by Ruzicka and Hansen in 1975, 170 and in 1979, Rule and Seitz reported the first use of this cell for chemiluminescence detection. 191 A variety of polymers can be utilised for a coiled flow-cell, depending on the needs of the reaction. Polytetrafluoroethylene (PTFE or Teflon) tubing is most often utilised, due to its solvent durability and robustness, 139, 175-176, 186, 192-198 however other polymers have been reported in the literature, including poly(fluoroalkoxy) (PFA), 199 fluorinated ethylene propylene (FEP), 200 and Tygon® tubing.

Polymer tubing flow-cells are advantageous in that they can easily be modified to accommodate different reactions. For example, for longer lasting chemiluminescence emissions, such as that from luminol, a longer piece of tubing can be coiled in order
to allow most of the reaction to occur in front of the photodetector, however, it is limited by the size of the photomultiplier window.

Despite spiral tubing flow-cells being the most popular design utilised, other tubing configurations have appeared in the literature. Campins-Falco et al. have explored several different tubing configurations to determine the most efficient design. A conventional quartz flow-through cell was compared with a mixing coil configuration, spiral configuration, and a bundle of tubing within a plastic cuvette. Other variations include adding an inner tube which extends to the centre of the flow-cell, to allow solutions to merge right in the centre of the photodetector, such as the one published by Hu and co-workers.
Figure 1.3: Common flow-cell designs: (a) glass coiled tubing flow-cell;\textsuperscript{139} (b) PTFE tubing coil flow-cell;\textsuperscript{139} (c) machined spiral flow-cell;\textsuperscript{174} (d) machined serpentine flow-cell.\textsuperscript{139}

Modifications are often made to tubing based flow cells to enhance signal intensities. As most tubing is translucent, and is often not flat against the surface of the detector, light from the chemiluminescence reaction can be lost, decreasing the signal intensity and sensitivity. Backing the tubing with a reflective material, such as a plate of aluminium, aluminium foil or a mirror, is a common practice to enhance the light reflected back to the PMT.\textsuperscript{148, 185, 201-203} Glass tubing offers an advantage over plastic tubing due to its transparency, but it is much harder to coil glass tubing in-house, as the glass needs to be heated to a malleable temperature first, and then coiled into the correct shape.
Machined flow-cells have been growing in popularity for use in chemiluminescence detection. The use of computer aided design allows for more efficient designs to be produced, which enable better mixing of reagent and analyte solutions within the cell to maximise emission intensity.\textsuperscript{174-175} Spirals similar to coiled tubing can be machined (Figure 1.3c),\textsuperscript{177} however other designs such as more complex serpentine designs can also be produced (Figure 1.3d).\textsuperscript{174-177}

A variety of commercially available machined flow-cells and detectors are available,\textsuperscript{175, 204-205} which incorporate complex serpentine designs, with single inlet and dual inlets in front of the detector, to maximise detectability of faster chemiluminescence reactions.\textsuperscript{175, 204}

Optimal confluence point distance is dependent on the reaction being utilised. For fast chemiluminescence reactions, such as potassium permanganate, a short confluence point distance is favourable, allowing the entire emission to occur in front of the detector.\textsuperscript{176} If the confluence point distance is too great, most or all of the reaction may occur before the solution reaches the detector. For slower reactions, such as pyrogallol,\textsuperscript{206} a long mixing coil prior to the detection flow-cell is often required to ensure the maximum intensity of emission occurs in front of the detector, and not in the waste. The confluence point is often easily adjustable for reactions within a flow-cell, as it is usually a matter of merging the two solutions at a Y- or T-piece attached to a length of tubing or a mixing coil, prior to the flow-cell (shown in Figure 1.4), however depending on the reaction being used, experimentation may be required to ensure the confluence point is precisely the correct distance prior to the detector to allow the most intense portion of the emission to be detected.\textsuperscript{176}

Confluence point distance is not as easy to adjust within a manufactured chip though, as the point is already machined into the plastic, and cannot be adjusted. Terry and
co-workers examined confluence points within machined flow-cells, similar to what would appear within a microfluidic device, for the fast ruthenium reagent reaction, and determined that the confluence point should be as close to the detector as possible, in order for most of the reaction to occur in front of the detector.\textsuperscript{176-177} If slower chemiluminescence reactions were to be used for the detection of analytes of interest, considerations for confluence point distance would need to be incorporated into the device, in order to allow for the reactants to fully mix prior to the detector.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Illustration of confluence point distance. If the length of tubing is too long or short, the reaction will occur external to the flow-cell and detector.}
\end{figure}

Channel dimensions also need to be considered when designing a detection zone within a device. Commonly used tubing coils have standard internal diameters (i.d.), around 0.8 mm.\textsuperscript{148, 175, 177, 191, 202, 207} Machined flow-cell channels are often modelled on these dimensions, around 0.7 \times 0.7 mm, or 0.8 \times 0.8 mm.\textsuperscript{174} Depending on the channel design, channel width can usually be altered easily in Computer Aided Design (CAD) software, and channel depth is easily modified by changing the depth of cut when machining, usually in the machining G-Code. However, such deep channels may be impractical for use within a microfluidic device, due to the volume required to fill the channels, and therefore the volume of waste that would be produced. For a device to be used in the field, large volumes of waste are impractical to handle, and so would be detrimental to the portability of the device.
Channel configurations can alter the rate of mixing of reactions.\textsuperscript{174, 176-177} There are many publications exploring different chemiluminescence reactor and flow-cell designs, from reaction chambers,\textsuperscript{178, 180-182, 200, 208-210} which include fountain cells,\textsuperscript{182-183, 200} to tubing based reactors, such as coiled tubing or knitted/knotted tubing,\textsuperscript{148, 170, 184, 191, 202, 207, 211-214} to machined and manufactured designs, such as 3D-printed flow-cells,\textsuperscript{215} spiral cells,\textsuperscript{139, 174-177, 216-218} or more complex serpentine and sinusoidal cells.\textsuperscript{174-177} These more complex machined designs tend to offer more reproducible emissions (cell-to-cell), and better mixing efficiency than other coiled tubing designs, and therefore better emission intensities, especially when combined with highly reflective materials such as white polymers, or mirror-backed clear polymers.\textsuperscript{177}

Some of the channel configuration designs explored in the literature, while highly efficient, do pose an issue if attempting to integrate into a microfluidic device. Designs where the solutions merge in the centre of the cell, where a PMT is most sensitive, often have channels along the back face of the material to allow this.\textsuperscript{174-175, 177} This can complicate matters when designing and manufacturing a device, as extra channels, and machining on multiple faces of the material are required in order to achieve this, which increases the cost of the device, and the time to produce it. As a result, simpler, yet still as efficient designs need to be explored in order to produce a chemiluminescence reaction and detection zone suitable for a microfluidic device.

### 3.5 Model analytes

Model analytes were chosen for this work to be explored as representatives of forensically, environmentally and pharmaceutically important analytes, which could potentially be detected by the microfluidic device which will be designed. Chosen
analytes would also need to be useful in examining reaction kinetics of a device design. Analytes were chosen based on the following criteria:

1. Analytes of interest and/or importance; It is advantageous to study compounds which would likely be encountered in an applied device, across several scientific fields.
2. Extensively studied analytes; particularly those who have been utilised in flow-cell comparisons in previous research, as more direct comparisons can be made as to the advantages and disadvantages of the chosen designs.
3. Sensitivity; as minor changes may be made in flow-cell/device designs, analytes with low limits of detection are favourable to comprehensively determine which design is ultimately more advantageous.

3.5.1 Morphine

Morphine is an opiate alkaloid used in pain relief. As a metabolite of heroin, it can be used forensically to determine heroin use.\textsuperscript{38, 219-220} However, detection of morphine is critical in several other areas, including pharmaceutical industry process streams,\textsuperscript{150, 221} to ensure a pure product is found, as well as in clinical toxicology.\textsuperscript{222}

The literature reports that potassium permanganate is extremely sensitive for detecting morphine, due to the compound containing both a phenol and a furan bridge, as can be seen in Figure 1.5.\textsuperscript{147} It has been reported that compounds which possess these structural features produce the greatest emission intensities with the permanganate reagent.\textsuperscript{147, 223} This reaction has also previously been used for the comparison of flow-cells,\textsuperscript{174-177} due to its high reproducibility and simple instrument setup.
Ofloxacin is a fluoroquinolone broad spectrum antibiotic (Figure 1.6), which has been shown to have decreasing effectiveness on a number of bacteria over the past few decades. Most of the antibiotics ingested by patients are excreted during urination, as they are not utilised by the body, spreading into waterways and allowing antibiotic-resistant bacteria to thrive. With few new antibiotics being developed and approved for medicinal use in recent years, and toxic effects and growing antibiotic resistance in bacteria (partially due to the misuse of antibiotics, and partially due to over-prescription by general practitioners) it is growing more important to be able to selectively detect minute concentrations of this and other antibiotics in the environment, especially waterways, to help combat release and the growth of antibiotic resistant bacteria. It has previously been shown that ofloxacin, among other antibiotics, can be sensitively detected using the ruthenium chemiluminescence reagent ([$\text{Ru(bpy)}_3]^{3+}$).
Sodium hypochlorite is used in a variety of applications, including commercial cleaning agents, water treatment, and in dentistry, however despite these uses, sodium hypochlorite can pose health issues, including aiding in the formation of carcinogens, as well as causing irritation and allergic reactions for some people. Sodium hypochlorite’s reaction with luminol is also very bright and long lasting, which is useful in visually exploring the mixing of a chemiluminescence reaction within flow-cells, and observing channel morphologies.

4 Microfluidic Device Design

Often researchers focus on producing a chip that performs too many functions, or only serves a single purpose, which cannot be easily adapted to mass manufacturing – the downfall of a lot of devices. This can sometimes result in a ‘chip-in-a-lab’ scenario, where a device is miniaturised, and can perform all of its functions,
but only within a laboratory, and with many cables and tubing still required for ancillary equipment, such as detectors or fluidic pumps, as shown in Figure 1.7.

Figure 1.7: (a) Cancer monitoring chip, requiring external pumping of solutions. (b) Chip-in-a-lab (Scale bars 1 cm (inset) and 10 cm), from Streets and Huang.

A principle was developed in the 1970s called Design for Manufacture (DFM), or Design for Manufacturability and Assembly (DFMA), which instructs designers to design their device with consideration for easy mass manufacturing in the future, but can be applied to all science disciplines employed in a microfluidic device.

4.1 Design for Manufacture Methodology

The Design for Manufacture (DFM) or Design for Manufacture and Assembly (DFMA) methodology has two principles, to:

1. Reduce the number of assembly operations by reducing the number of parts
2. Make assembly operations easier to perform

Employing the DFM methodology can help in avoiding adding unnecessary manufacture costs from as early as the design phase. In the 1970s, it was found that designers were focussing on assembly as the cost-reducing part of production, causing more waste of materials. Boothroyd et al. comment that greater savings could be made in relation to both the cost of parts manufacture and assembly,
through understanding manufacturing processes based on product design information.249

In terms of the design, manufacture, and assembly of a microfluidic device, the creator should consider what “back-end processes” can be incorporated into the design and production stages of device preparation, so as to reduce time and cost during assembly.242 This is sometimes dependent on the manufacture method being utilised, as well as the function of the device itself.242 Becker uses ‘fluidic access holes’ or inlet holes, among others, as an example of a back-end process which can be incorporated into the manufacture step. For example, if laser cutting or injection moulding, inlet holes can be incorporated into the design, and produced during the initial production of the layer, instead of completing post-production.242 In terms of designing for assembly, a designer should consider complete rotational symmetry of a part in order to allow the part to be assembled any way, regardless of orientation. Conversely, asymmetry can be incorporated into the design to ensure that a part cannot be inserted or assembled any other way (see Figure 1.8).242, 249
Incorporating these modifications can complicate the design somewhat, but will also eliminate waste during assembly of parts caused by parts being misassembled. Becker comments that issues arising from poor design and assembly, which can drive up the costs of manufacture, have been hurdles in mass commercialisation of some microfluidic devices, as researchers consider commercial viability an afterthought to their research objectives. It is therefore important to incorporate the Design for Manufacture principles throughout device design, prototyping and manufacture.
Manufacturing Methods

Microfluidics fabrication developments have expanded into using a wide range of materials. Laser etching,\textsuperscript{250-251} microcontact printing,\textsuperscript{252-253} and moulding\textsuperscript{18, 254-256} for devices were among the first advances made from the mid-1990s. Materials used for microfluidic chip fabrication are comprehensively reviewed by Ren \textit{et al.}\textsuperscript{257, 258} and include silicon, glass, hydrogels, thermoplastics, elastomers and paper.\textsuperscript{257, 259} Thermoplastics, however, are the most commonly used material, with a wide range of plastics used to fabricate disposable microfluidics chips, including cyclic olefin copolymer (COC),\textsuperscript{256, 260-261} polycarbonate (PC),\textsuperscript{262} poly(dimethylsiloxane) (PDMS),\textsuperscript{263-264} polyethylene terephthalate (PET)\textsuperscript{265} and polytetrafluoroethylene (PTFE).\textsuperscript{266} These plastics can be manipulated in a variety of different ways to form the microfluidics chip, such as injection moulding, embossing, casting and etching,\textsuperscript{266} as described above. Novel innovations on old techniques, such as origami, have also led to microfluidic device fabrication from paper, which can be folded by hand, and when unfolded reveals the results of the analysis.\textsuperscript{267}

Microfluidics devices are becoming more prominently used in a range of biological and chemical analyses,\textsuperscript{268} and their applications range from in-lab chemical processing devices to medical,\textsuperscript{15, 269-274} and environmental diagnostics tools.\textsuperscript{275-282} However the application of microfluidics analysis and all its potential has seldom been used in detecting forensically important seizure samples,\textsuperscript{283-284} and most of the devices are non-disposable,\textsuperscript{285} meaning this field needs further exploration to reach its true potential.
5 Project Aims

The work presented in this thesis explores the various components required within this microfluidic device for the separation and detection of analytes of importance, and the optimisation of each feature in order to transition into a combined device.

Specific project objectives include:

- To prepare, characterise, and optimise monolithic columns with desirable pore characteristics for use in liquid chromatography
- To explore and prepare different functionalisation procedures for monoliths for efficient chromatographic separations
- To develop and optimise flow-cell channel designs and materials for on-chip chemiluminescence detection, compatible with microfluidic devices
- Use the information collected to inform microfluidic device design
CHAPTER TWO

STUDIES ON UNMODIFIED SILICA MONOLITH PRODUCTION, ENCAPSULATION AND SEPARATION

1. Introduction
2. Experimental
3. Results and Discussion
4. Conclusion
1 Introduction

Silica monoliths, in analytical chemistry terms, are defined as “a continuous porous object, whose morphology and pore structure can be varied in a wide range”. In recent years, monoliths have been used in a wide variety of applications, especially in chromatographic separations. Pioneering research by Nakanishi and Soga focussed on optimising the sol-gel chemistry to produce monoliths with desired pore structure and characteristics, particularly for application as high performance liquid chromatography (HPLC) column stationary phases. The careful control of starting compounds, phase separation, gelation, aging and treatment can enable the preparation of monoliths whose characteristics are suitable for fast chromatographic separations, but with the wide variety of starting materials available, optimisation and porosity studies are required to ensure desired pore structures are achieved.

However, while efficient columns for chromatography can be prepared; encapsulation and interfacing with instrumentation can be troublesome. A number of encapsulation methods for monolith columns have been described, though many require expensive materials, or numerous steps in order to properly encase a monolith column for efficient HPLC separations. One of the most commonly employed encapsulation methods for silica monolith columns is heat shrink tubing, made of either polyether ether ketone (PEEK) or polytetrafluoroethylene (PTFE), which shrinks around the column when heated to set temperatures. Most methods utilised heat the tubing in such a way to form a tight seal around the monolith itself, but due to the monolith’s porosity, do not properly adhere to the surface of the monolith, and wall-effects (fast external flow rates) can occur.
This chapter details the production, optimisation, and characterisation of unmodified silica monoliths, and subsequent research into better encapsulation media for in-house prepared monoliths. The porosity characteristics of the monoliths produced will then be compared to the desirable characteristics of chromatography columns reported in the literature. In addition, the new method of encapsulation will be compared to a conventional heat-shrink tubing encapsulation method to determine its efficacy as an encapsulation method.
2 Experimental

2.1 Chemicals

Unless otherwise stated, Milli-Q deionised water was used in all experiments. Acetonitrile, ammonium hydroxide, heptane, Pluronic F127 Polymer and tetramethyl orthosilicate (TMOS) were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Isopropanol, methanol and acetone were obtained from Chem Supply (Gillman, SA, Australia). Acetic acid was purchased from Ajax Finechem (Sydney, NSW, Australia). Thiourea was obtained from BDH Chemicals (USA).

Polystyrene casting resin and methyl-ethyl ketone peroxide catalyst (Recochem, Epping, VIC, Australia) were purchased from a local hardware store. PTFE heat shrink tubing (4.8 mm × 1.2 m) was purchased from Element14 (Chester Hill, NSW, Australia).

2.2 General instrumentation

Microscope images were obtained using a Nikon Eclipse Ni-U Microscope, equipped with a DS-Qi2 16.25 Megapixel Monochrome Digital Camera (Scientific Equipment Pty Ltd, Huntingdale, VIC, Australia). NIS Elements BR Basic Research Software (Scientific Equipment Pty Ltd, Huntingdale, VIC, Australia) was used to record resin penetration depth.

Unless otherwise stated, photographs were taken using a Huawei P8 13 megapixel mobile phone camera (model: HUAWEI GRA-UL00) with automatic ISO, f-stop and exposure settings.

Chromatographic analysis of the encapsulation methods was performed on an Agilent Technologies 1200 series liquid chromatography instrument (Agilent
Technologies, Mulgrave, VIC, Australia), equipped with a quaternary pump with solvent degasser, autosampler and diode array detection module to measure absorbance at 254 nm. Data was obtained and processed with Agilent ChemStation software. Injections were 1 µL and elution was performed at 1 mL min\(^{-1}\) under isocratic conditions. Plate heights were calculated using the Foley-Dorsey Equation using Wolfram Mathematica 10.1 (Hearn Scientific, South Yarra, Victoria, Australia).

The mobile phase for all HPLC analyses was a mixture of filtered (0.45 µm) deionised water (Continental Water Systems, Australia) and HPLC grade methanol at a ratio of 98:2 water:methanol. Thiourea (BDH Chemicals, USA) was used as the test analyte, which was prepared at a concentration of 0.4% in the mobile phase solution.

### 2.3 Monolith preparation

Monoliths were prepared in-house as described by Fletcher et al.\(^7\) using Pluronic F127 polymer, tetramethyl orthosilicate (TMOS) and 0.02 M acetic acid. Pluronic F127 Polymer (0.432 g) was added to 0.02 M acetic acid (4 mL), and stirred in an ice bath for 45 minutes. Tetramethyl orthosilicate (2 mL) was then added to the mixture, and stirred for a further 45 minutes in an ice bath. The clear, homogenous solution was then transferred to a pre-fabricated acrylic mould (see Figure 2.1) via syringe and needle, and placed in an oven at 40°C for 72 hours.

Monoliths were removed from the mould using deionised water after the 72 hour incubation period in the oven and then rinsed in deionised water for 24 hours. To increase the number and volume of nanopores, monoliths were submerged in a solution of 1.0 M ammonium hydroxide, and heated to 90°C (set temperature) in an
oil bath overnight. Monoliths were finally rinsed in deionised water to remove any remaining solution, until a neutral pH was reached. To harden the monoliths and remove any remaining organic material, they were calcined in a furnace at 600°C, unless otherwise stated.

Figure 2.1: (a) Computer aided design (CAD) image of one half of the prefabricated mould for monolith production. Mould dimensions measure 100 × 100 × 10 mm per half. (b) Prefabricated mould for monolith production.

2.4 Characterisation of Monoliths

Two methods were used to characterise the monoliths produced: scanning electron microscopy (SEM) for a visual analysis of the through pores (macropores); and nitrogen adsorption/desorption at 77 K to determine the porosity (mesopores) and surface area of monoliths.

Scanning electron microscopy was performed using a Zeiss Supra 55VP Scanning Electron Microscope. Monolith samples were affixed to metal SEM stubs using Electrodag 1415 silver paint (Agar Scientific, Emgrid, Parooka, SA, Australia), with the sides of the samples also coated. The samples were then degassed in a vacuum chamber for at least 48 hours, before being transferred to a Leica EM Gold Sputter Coater. Samples were coated with 4-10 nm of gold before being removed from the
chamber and transported to the SEM vacuum chamber immediately. Following imaging, printed images of monolith samples were examined and pore diameter (µm) was determined using the in-built measuring software of the SEM and recorded on the images.

Porosity analysis was performed using a Micromeritics Tristar 3000 Surface Area and Porosity Analyser. Samples were degassed using the Smartprep equipment at 150°C for 60 minutes prior to being transferred to the instrument. Later porosity analysis was performed on a Quantachrome Autosorb iQ3 Automated Gas Sorption Analyser (Quantachrome Instruments, Boynton Beach, Florida, Model: ASIQC0V00211-6), with samples degassed using the inbuilt degassing equipment. Nitrogen adsorption and desorption was measured at 77 K for both instruments. Specific surface area was determined according to the Brunauer-Emmet-Teller (BET) model\textsuperscript{79, 202}. Pore volume and pore size distributions were determined using the Barrett-Joyner-Halenda (BJH) model, with a 25 point analysis\textsuperscript{81}. Recent literature from the International Union of Pure and Applied Chemistry (IUPAC)\textsuperscript{83} has stated that both of these porosity characterisation models are now outdated, and although the Quantachrome Autosorb Analyser was capable of analysis using more accurate models (such as density functional theory (DFT)), monolith samples were still analysed using BET and BJH models for consistency with earlier work.

Overall monolith porosity (percentage porosity) was calculated using water weight difference and overall volume of the monolith. The monolith’s physical measurements were taken using calipers, and then dried and weighed, and placed in water and all air exuded, and then reweighed. The equation below (Equation) demonstrates the calculation for percentage porosity:
\[
\phi = \frac{m_w - m_d}{\pi \times r^2 \times h} \times 100
\]

*Equation 2.1: Percentage porosity of a monolith*

Where \( \phi \) is the porosity, \( m_w \) is mass of the wet sample, \( m_d \) is the mass of the dry sample, \( r \) is the radius of the sample in centimetres, and \( h \) is the height of the sample in centimetres. This method only accounts for accessible pores, *i.e.* does not include pores that are trapped within the superstructure of the monolith, and therefore also inaccessible by the nitrogen adsorption/desorption testing.

### 2.5 Optimisation

Monoliths underwent several variations in the production method described above to alter the pore structure of the monoliths for better HPLC performance. Firstly, mixing times were strictly controlled to 45 minutes per step to ensure continuity across batches. Base treatment temperature was altered from set 90°C (with an in-flask temperature of around 72°C), to 120°C, which resulted in an approximate 90°C in-flask temperature. Furthermore, calcination temperatures were altered, ranging from 400°C through 600°C at 50°C increments.

A magnetic stirrer/hotplate with a built in thermocouple was used to accurately determine and set the oil bath temperature. A standard laboratory thermometer was used to measure in-flask temperatures throughout experiments.

### 2.6 Monolith encapsulation

The choice of materials for the proposed encasement method considered various factors. Firstly, the material had to be solvent durable in order to withstand the various organic solvents (such as acetonitrile (ACN) and methanol (MeOH)) routinely used in HPLC. The second consideration came from availability and ease
of use. The use of thermoplastic materials such as cyclic olefin copolymer (COC) and poly(methyl methacrylate) (PMMA), used in injection moulding of plastics, for example, would require additional steps in the moulding/encapsulation process. Attempts to injection mould a thermoplastic around a monolith poses a number of physical problems associated not least with pressure and flow. Accordingly, thermoplastics would require heating until the polymer melts and becomes homogenous, then carefully pouring into a temperature-resistant mould containing the monolith. As soon as the thermoplastic is removed from the heat source, however, it would begin cooling and setting, resulting in a reduced working-time, and preventing the correction of any errors that may occur.

Having examined these facts, casting/polymer resins were investigated as the encasement material as these set at room temperature through the addition of a catalyst, instead of melting, pouring and then cooling. Experimentation has demonstrated that at room temperature the working time of the resin mixed with the catalyst is in excess of 30 minutes before the resin is no longer malleable.

Monoliths were encased using Diggers’ Casting and Embedding Resin (Recochem, Inc. Lytton, QLD, Australia), mixed with a methyl ethyl ketone peroxide (MEKP) catalyst to harden the resin, due to its availability and relatively low cost. The resin mixture was mixed with catalyst in a 20:1 ratio, 20 mL resin to ~1 mL catalyst (1:1 resin (mL): catalyst (droplet)).

The design of an encapsulation method required that it also be relatively simple, fast, and all materials readily available or easily obtained. As such, casting resin and catalyst were purchased at a local hardware store, with a cost of around AU$1.00 per
monolith for resin and catalyst. The original mould materials make use of readily available laboratory materials: Terumo® syringes and wax/baking paper.

Various methods were explored for mould design using the aforementioned materials, as outlined in Table 2.1, below. The design for method E1 was based on regular casting moulds. A cut-open syringe was lined with baking paper for the easy removal of the set resin. Two syringe plungers then had their rubber stoppers removed, and a small circular hole cut into the centre, for the HPLC fittings to slot into. These two stoppers comprised the ends of the mould. By slotting the HPLC fittings into the rubber stoppers, the mould is able to be resin-tight (no resin leakage occurs) and the monolith can be suspended above the bottom of the mould, for uniform surrounding of the monolith with resin (Figure 2.2).
Figure 2.2: Monolith encasement method (E1, E2, E4, E5, and E6). (a) Lined cut-open syringe with rubber plunger ends in place; (b) with fittings in place; (c) & (d) monolith inserted into mould between fittings.

Table 2.1: Monolith encasement method materials list

<table>
<thead>
<tr>
<th>Method</th>
<th>Materials</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1</td>
<td>Resin</td>
</tr>
<tr>
<td></td>
<td>20 mL syringe, cut open</td>
</tr>
<tr>
<td></td>
<td>Rubber stoppers</td>
</tr>
<tr>
<td></td>
<td>HPLC fittings (inserted into stoppers)</td>
</tr>
<tr>
<td>E2</td>
<td>Resin</td>
</tr>
<tr>
<td></td>
<td>20 mL syringe, cut open</td>
</tr>
<tr>
<td></td>
<td>Rubber stoppers</td>
</tr>
<tr>
<td></td>
<td>HPLC fittings (inserted into stoppers)</td>
</tr>
<tr>
<td></td>
<td>HPLC tubing (inserted into fittings)</td>
</tr>
<tr>
<td>E3</td>
<td>Resin</td>
</tr>
<tr>
<td></td>
<td>10 mL syringe, ends cut off</td>
</tr>
<tr>
<td></td>
<td>Rubber stoppers</td>
</tr>
<tr>
<td></td>
<td>HPLC fittings (inserted into stoppers)</td>
</tr>
<tr>
<td></td>
<td>HPLC tubing (inserted into fittings)</td>
</tr>
<tr>
<td></td>
<td>Vertical rig</td>
</tr>
</tbody>
</table>
For methods E1, E2, E4, E5 and E6, approximately 15 mL of resin was collected in a syringe and transferred to a medium weigh boat, followed by approximately 12-15 drops of the methyl ethyl ketone peroxide (MEKP) catalyst. The mixture was then stirred slowly with a flat spatula, ensuring no air bubbles were produced within the mixture. When the mixture was smooth and the catalyst evenly distributed, it was poured over and around the monolith until the fittings were completely covered in resin. The monolith, in resin, was then left in a fume hood overnight to allow the resin to set properly before the encased monolith was removed from the mould and the HPLC fittings were carefully extracted.
For method E3, approximately 10 mL of resin was collected in a 10 mL syringe and transferred to a medium weigh boat, where 10 drops of the MEKP catalyst were added. As above, the mixture was stirred slowly with a flat spatula until the mixture was homogenous, and then it was poured into the prepared vertically suspended 10 mL syringe.

A purpose-built aluminium mould was designed and fabricated for encapsulating monoliths, used in method E7 (see Figure 2.3). One end houses a long HPLC fitting which is securely fastened into the end of the mould. The opposite end includes a sliding piece of aluminium, holding a second long HPLC fitting, which can be adjusted along the length of the mould (using the bolt) to perfectly fit and securely hold the monolith. Resin is then prepared and poured over and around the monolith as previously described. The mould is sprayed with a mould release spray prior to encapsulation to aid in removal of encapsulated monoliths.

Method E8 utilised the same pre-fabricated aluminium mould described above, but uses regular HPLC fittings.

Following encasement, each monolith underwent testing to determine if flow through could be achieved. The HPLC fittings, with tubing attached, were reinserted into the monolith and a solution of coloured food dye and deionised water was pushed through using a syringe by hand. If the colour could be seen throughout the monolith, flow was achieved, and thus further testing with a HPLC manifold could be continued.
Figure 2.3: Encapsulation mould for method E7, with removed encapsulated monolith.

Figure 2.4: Pre-fabricated encapsulation mould. Monolith is inserted between two fittings, one placed in the sliding bar, the other in the fixed end of the mould. Resin was then poured around the monolith.

For comparison, some monoliths were encased in PTFE shrink tubing, the conventional encasement method for most laboratories \(^{51, 294}\). These monoliths were
slipped into heat shrink tubing sleeves approximately 5 mm in diameter. Steel chromatography tubing was then inserted at either end, and the monolith and tubing were passed over a heat source (heat gun) until shrinking occurred and a tight seal formed onto the monolith and the tubing. Shrink tubing encapsulated monoliths were then placed in an oven at around 150°C to allow the tubing to settle evenly, as can be seen below in Figure 2.5.

*Figure 2.5: Heat shrink tubing encapsulated monolith.*
3 Results and Discussion

3.1 Characterisation of monoliths

Scanning electron microscopy images of monolith samples were obtained and examined to determine macroporosity of the monoliths. For each sample, 10 macropores were selected and measured for their diameter. The results of these analyses are summarised in Table 2.2.

Porosity (mesopore sizes) and surface area analysis was conducted on samples from monolith batches using nitrogen adsorption/desorption; macropore sizes were determined using scanning Electron Microscopy, and overall monolith porosity was determined using water weight porosity calculations, as described in Equation 2.1.

![SEM micrographs of monolith 160514-1](image)

*Figure 2.6: SEM micrographs of monolith 160514-1. Images obtained using SE2 detector, at 1.00 kV and an aperture of 20 µm. (a) Pre calcination; (b) Post calcination. Calcination temperature: 600°C.*

As can be seen from Figure 2.6, there is little difference between the structure pre- and post-calcination, with median pore sizes around 4-6 µm. The results of all porosity measurements are detailed below in Table 2.2.
Table 2.2: Physical properties of the monoliths produced. Batch numbers relate to the date of preparation of the monoliths, i.e. 050514 was prepared on May 5, 2014. The hours listed beside some monolith batches relate to the incubation periods of the monolith solution, as discussed later in this chapter.

<table>
<thead>
<tr>
<th>Batch</th>
<th>NH$_4$OH Temp. (°C)</th>
<th>Calc. Temp. (°C)</th>
<th>BET/BJH Surface Area (m$^2$g$^{-1}$)</th>
<th>BET/BJH Pore Volume (cm$^3$g$^{-1}$)</th>
<th>BET/BJH Pore Size (nm)</th>
<th>SEM Pore Size (µm)</th>
<th>Water Weight Porosity (%)</th>
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<td>050514</td>
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<td>232</td>
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<td>9</td>
<td>6.3</td>
<td>94</td>
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</tr>
<tr>
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<td>15</td>
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<td>68</td>
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<td>8</td>
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<td>84</td>
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<tr>
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<td>233</td>
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<td>9</td>
<td>5.8</td>
<td>88</td>
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<tr>
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<tr>
<td>060215</td>
<td>90</td>
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<td>203</td>
<td>0.5</td>
<td>10</td>
<td>5.0</td>
<td>89</td>
</tr>
<tr>
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<td>10</td>
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<td>83</td>
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<tr>
<td>090215</td>
<td>90</td>
<td>550</td>
<td>234</td>
<td>0.7</td>
<td>11</td>
<td>5.8</td>
<td>82</td>
</tr>
<tr>
<td>130215</td>
<td>120</td>
<td>550</td>
<td>126</td>
<td>0.2</td>
<td>6</td>
<td>4.0</td>
<td>78</td>
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<tr>
<td>2 hours</td>
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<td></td>
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<td>130215</td>
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<td>6</td>
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<td>72 hours</td>
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</tbody>
</table>
The average specific surface area is approximately 240 m$^2$/g, which is similar to many of the literature values$^{51,294}$. With the exception of a few batches of monoliths, mesopore sizes are approximately 10 nm in diameter, and macropore sizes around 4-6 µm. Average nanopore volume was determined to be approximately 0.6 cm$^3$/g, and overall porosity was around 80-90%. The high percentage porosities, as determined by the water weight difference measurements (Equation 2.1), demonstrate the suitability of the monoliths for use in HPLC. This porosity will allow for low backpressures, and therefore higher flow rates can be used if necessary, without causing damage to the column or the instrument. The large variation in monolith characteristics, particularly those of batches 010714 and 140714, can be accounted for by the lack of strict control of the preparation parameters (i.e. mixing times, ammonium hydroxide treatment times and temperature, etc.) during early experimentation. After the observation of wide variations, more care was taken
during the preparation steps to ensure uniformity in monoliths across different batches, and optimisation was performed to ensure desirable porosity characteristics would be achieved for use as HPLC columns.

### 3.2 Monolith Optimisation

Prior to optimisation, the form and porosity of some batches of monoliths did not correspond to the reported characteristics in the literature\(^{51}\). Figure 2.7 demonstrates some of the monolith morphologies that were observed using a scanning electron microscope. The literature states that there are three possible monolithic structural types that can be produced during phase separation\(^{45, 51}\). These include (i) spinodal decomposition, or “silica-in-air”, where there are discontinuous silica particles within a continuous gaseous phase (Figure 2.7a); (ii) nucleation, or “air-in-silica”, where there are discontinuous air pockets within a silica skeleton, as pictured below in Figure 2.7b; and (iii) bicontinuous, the desired skeletal structure of silica monoliths, where both silica and gas phases are continuous, and flow-through can be achieved, as previously shown in Figure 2.6. This structure has been described as “sponge-like”\(^{51}\) and is the most common morphology for silica monoliths.
Figure 2.7: Monolith pore morphologies. (a) "silica-in-air" morphology; (b) "air-in-silica" morphology. Both images are of batch 200614.

The structures seen here in Figure 2.7 demonstrate how variable the synthetic process can be, and how easily the structure can be altered from the desired outcome, and therefore how important it is to strictly monitor and optimise production. Therefore, procedures were investigated to both explore and strictly control the monolith preparation in order to achieve the desired porosity characteristics for use in chromatography. These experiments are outlined below in the following sections.

3.2.1 Decreased Incubation Period

It was noted that upon the initial use of the prefabricated monolith mould, after approximately two hours, the monoliths looked ‘complete’, with visual characteristics of monoliths that had completed the 72 hour incubation period as described in the literature.\textsuperscript{48, 71, 294} Monoliths were white in appearance and had a small degree of shrinkage, but not to the same extent as monoliths that had completed 72 hours incubation.\textsuperscript{43} As such, a series of experiments were undertaken
to observe and compare the physical characteristics of monoliths incubated for the shorter 2 hours, versus those incubated for full 72 hours.

Porosity analysis using SEM and BET/BJH methods was performed on the monoliths, which other than their incubation periods underwent the same treatments as described by Fletcher and co-workers.\textsuperscript{51} All monoliths studied were calcined in a furnace at 600°C prior to being characterised.

Table 2.3: Comparison of physical properties of monoliths incubated for 2 hours versus 72 hours.

<table>
<thead>
<tr>
<th>Batch</th>
<th>Surface Area (m(^2)/g)</th>
<th>Pore Diam. ((\mu)m)</th>
<th>Pore Diam. (nm)</th>
<th>Pore Volume (cm(^3)/g)</th>
<th>Surface Area (m(^2)/g)</th>
<th>Pore Diam. ((\mu)m)</th>
<th>Pore Diam. (nm)</th>
<th>Pore Volume (cm(^3)/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>060614</td>
<td>193</td>
<td>6.2</td>
<td>10</td>
<td>0.5</td>
<td>232</td>
<td>6.3</td>
<td>10</td>
<td>0.6</td>
</tr>
<tr>
<td>180714</td>
<td>229</td>
<td>2.4</td>
<td>8</td>
<td>1.1</td>
<td>204</td>
<td>1.7</td>
<td>11</td>
<td>0.6</td>
</tr>
<tr>
<td>280714</td>
<td>226</td>
<td>2.6</td>
<td>9</td>
<td>0.4</td>
<td>309</td>
<td>2.2</td>
<td>10</td>
<td>0.8</td>
</tr>
<tr>
<td>060215</td>
<td>203</td>
<td>5.0</td>
<td>10</td>
<td>0.5</td>
<td>235</td>
<td>7.3</td>
<td>10</td>
<td>0.6</td>
</tr>
<tr>
<td>130215</td>
<td>126</td>
<td>4.0</td>
<td>6</td>
<td>0.2</td>
<td>148</td>
<td>5.0</td>
<td>6.0</td>
<td>0.2</td>
</tr>
<tr>
<td>181116</td>
<td>200</td>
<td>2.1</td>
<td>15</td>
<td>1.1</td>
<td>225</td>
<td>2.1</td>
<td>15</td>
<td>1.2</td>
</tr>
<tr>
<td>090117</td>
<td>238</td>
<td>1.5</td>
<td>9</td>
<td>1.1</td>
<td>247</td>
<td>2.4</td>
<td>12</td>
<td>1.3</td>
</tr>
<tr>
<td>100117</td>
<td>218</td>
<td>1.7</td>
<td>11</td>
<td>1.1</td>
<td>218</td>
<td>1.7</td>
<td>12</td>
<td>1.3</td>
</tr>
</tbody>
</table>

The lack of patterning of pore characteristics between the two incubation periods with the first three sets of monoliths (batches 060614, 180714 and 280714) listed in Table 2.3 can be attributed to the initial lack of strict control over the preparation processes, which were more meticulous in all later monoliths. With all later batches that underwent changes in the incubation period, a trend of slightly better porosities were seen with the regular 72 hour incubation period, than the 2 hour period. Though
it was visually evident that the monoliths had undergone full phase separation after
the 2 hour period, reviewing the literature demonstrates that these sol-gels, while
having undergone some phase separation, may not have stabilised macropore
structures or controlled pore-size distribution\textsuperscript{56}. The characterisation data for the two
incubation periods demonstrated little difference in surface areas, micrometer pore
diameters and pore volume, which are all influenced by the incubation period and
phase separation which occurs during this time. The possible instability of the
macropore structures led to all monoliths in future work undergoing the full 72 hour
incubation at 40°C.

It is important to also note that the mostly-similar nanometer pore-sizes between the
two incubation periods are attributed to the base-treatment solvent exchange the
monoliths underwent in ammonium hydroxide following rinsing, as opposed to
being greatly influenced by the incubation period itself\textsuperscript{295}. As this ammonium
hydroxide treatment was kept at relatively similar temperatures, little variation in the
degree of surface modification to the monoliths in the nanometre range was seen
between those undergoing different incubation periods.

\subsection{3.2.2 Adjusted ammonium hydroxide treatment temperature}

Ammonium hydroxide treatment temperatures were varied for different batches in
order to obtain desired pore sizes and surface area. The literature used for the
preparation of these monoliths indicated a base treatment temperature of 90°C\textsuperscript{51}, but
it was not clear if this was the set temperature of the hot plate/oil bath, or the in-flask
temperature (measured throughout treatment). The ammonium hydroxide treatment
was set up for monoliths using a magnetic stirrer/hotplate with a built in
thermocouple to accurately determine and set the oil bath temperature. A standard
laboratory thermometer was used to measure the in-flask temperature, and was monitored throughout the duration of the ammonium hydroxide treatment.

**Table 2.4: Modified ammonium hydroxide temperature porosity results.**

<table>
<thead>
<tr>
<th>Monolith Batch</th>
<th>Oil Bath Temp (°C)</th>
<th>In-Flask Temp (°C)</th>
<th>Surface Area (m²/g)</th>
<th>Pore Volume (cm³/g)</th>
<th>Pore Size (µm)</th>
<th>Pore Size (nm)</th>
<th>Porosity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>060315</td>
<td>90</td>
<td>72</td>
<td>503</td>
<td>1.3</td>
<td>10.2</td>
<td>5</td>
<td>81</td>
</tr>
<tr>
<td>090315</td>
<td>90</td>
<td>75</td>
<td>244</td>
<td>0.5</td>
<td>8.4</td>
<td>4</td>
<td>73</td>
</tr>
<tr>
<td>030315</td>
<td>115</td>
<td>90</td>
<td>153</td>
<td>0.3</td>
<td>7.8</td>
<td>4</td>
<td>81</td>
</tr>
<tr>
<td>240215</td>
<td>120</td>
<td>92</td>
<td>148</td>
<td>0.2</td>
<td>5.2</td>
<td>4</td>
<td>78</td>
</tr>
</tbody>
</table>

It was discovered that the set temperature of 90°C resulted in an in-flask temperature of around 72°C. Bringing the temperature up to 120°C, a point discussed in some literature,\(^{45, 51}\) raised the in-flask temperature closer to 90°C, with 115°C being the closest hot plate set temperature required to keep the in-flask temperature at 90°C, in laboratory conditions. Porosity analysis demonstrated an increase in temperature to 120°C resulted in dramatically decreased surface areas (50% decrease) with an associated decrease in pore volumes and sizes (30% decrease), despite the in-flask temperature only being increased 2°C above the desired set point. The variation in actual in-flask temperatures between 060315 and 090315 monolith batches can be accounted for by a difference in ambient temperature, affecting the temperature of the water in the reflux system and the temperature of the glassware. These variations demonstrate the need for the ammonium hydroxide treatment to be performed in a temperature-controlled environment, to increase uniformity across batches.

Overall, it can be observed that increasing the oil bath temperature above 90°C set temperature has detrimental effects on the surface area and all pore properties of the
silica monoliths, in regards to the desired properties, excluding overall determined percentage porosity, suggesting that the optimal temperature for ammonium hydroxide treatment of monoliths is an in-flask temperature of 90°C or below.

3.2.3 Calcination Temperature Optimisation

Fletcher et al. have reported on the effect of a handful of calcination temperatures (550°C-650°C) on the porosity properties of silica monoliths, and approximately 43% of the reported monoliths did not form, or monoliths broke before analysis could be performed. As such, calcination temperatures between 400°C and 600°C at 50°C increments were explored as part of this work for their effect on overall pore properties and porosity.

Table 2.5: Calcination temperature optimisation results. Results are an average of three measurements at each temperature.

<table>
<thead>
<tr>
<th>Calcination Temperature (°C)</th>
<th>Surface area (m²/g)</th>
<th>Pore volume (cm³/g)</th>
<th>Pore Size (nm)</th>
<th>Pore Size (µm)</th>
<th>Porosity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>400</td>
<td>179</td>
<td>0.3</td>
<td>12</td>
<td>2.0</td>
<td>75</td>
</tr>
<tr>
<td>450</td>
<td>123</td>
<td>0.3</td>
<td>11</td>
<td>1.4</td>
<td>82</td>
</tr>
<tr>
<td>500</td>
<td>128</td>
<td>0.3</td>
<td>10</td>
<td>1.8</td>
<td>78</td>
</tr>
<tr>
<td>550</td>
<td>181</td>
<td>0.5</td>
<td>10</td>
<td>1.7</td>
<td>88</td>
</tr>
<tr>
<td>600</td>
<td>178</td>
<td>0.5</td>
<td>10</td>
<td>1.5</td>
<td>87</td>
</tr>
</tbody>
</table>

Higher percentage porosity, large surface areas and up to 25 nm mesopores are desirable characteristics of monoliths for use in HPLC. The altered calcination temperatures created a range of effects on the porosity characteristics of the monoliths. Higher temperatures, such as 550°C and 600°C produced the greatest
surface area and overall porosity, but slightly smaller pore volumes and sizes than some of the other temperatures. It was decided that a calcination temperature of 550°C would be used for future monolith preparation, as it produced the largest surface areas, which will allow greater degrees of functionalisation for HPLC application.

3.3 Monolith Encapsulation

3.3.1 Solvent Durability Testing

Prior to using the chosen resin with monolith encapsulation for HPLC, the solvent durability of the resin was tested. HPLC often involves the use of solvents in a range of concentrations in the mobile phase. For the encapsulation method to be successful, the resin needed to be able to withstand some of the more common solvents.

Acetonitrile, ethanol, heptane, isopropanol, methanol, tetrahydrofuran and toluene were tested with the resin. Some of the selected solvents are commonly used in stationary phase preparation, so it was crucial that the resin could endure them in order to modify the stationary phase of the monoliths. Resin was poured into a small mould, and allowed to set. The hardened resin was then submerged in the given solvent for 24 hours. Mass readings and visual observations were taken, these are summarised below in Table 2.6.
Figure 2.8: Impact of solvents on resin samples. The only major damage occurred to samples exposed to acetonitrile and tetrahydrofuran.

Table 2.6: Resin solvent durability testing results with selected common HPLC solvents.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Initial Mass (mg)</th>
<th>Final Mass (mg)</th>
<th>Difference (mg)</th>
<th>% Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetonitrile</td>
<td>5842</td>
<td>5821</td>
<td>-21</td>
<td>-0.4</td>
</tr>
<tr>
<td>Ethanol</td>
<td>7151</td>
<td>7206</td>
<td>55</td>
<td>+0.8</td>
</tr>
<tr>
<td>Heptane</td>
<td>7679</td>
<td>7681</td>
<td>3</td>
<td>+0.0</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>7075</td>
<td>7076</td>
<td>1</td>
<td>+0.0</td>
</tr>
<tr>
<td>Methanol</td>
<td>6898</td>
<td>7074</td>
<td>176</td>
<td>+2.6</td>
</tr>
<tr>
<td>Tetrahydrofuran</td>
<td>6252</td>
<td>6608</td>
<td>357</td>
<td>+5.7</td>
</tr>
<tr>
<td>Toluene</td>
<td>7144</td>
<td>7254</td>
<td>110</td>
<td>+1.5</td>
</tr>
</tbody>
</table>
Acetonitrile and tetrahydrofuran had the greatest physical effect on the resin, as seen in Figure 2.8. Both solvents caused breakages to the resin within 24 hours, with tetrahydrofuran reducing most of the resin to smaller crystal-shaped pieces (Figure 2.9a), compared to the splintering of resin caused by the acetonitrile (Figure 2.9b).

Figure 2.9: (a) Effect of tetrahydrofuran on casting resin. (b) Effect of acetonitrile on casting resin.

In most cases, the mass of the resin had an overall increase, even after 72 hours of drying. No investigation was conducted into the cause of the mass increase, as the changes were insignificant, with less than 3% increase in all cases except for tetrahydrofuran. After observing the devastating effects of the acetonitrile and tetrahydrofuran to the resin, seen in Figure 2.8 and 2.9, it was initially decided that use of these solvents in the mobile phase should be avoided due to the damage that could be caused to the encapsulation media by extended use. However, as acetonitrile is a more commonly used solvent in HPLC than tetrahydrofuran, the acetonitrile durability of the resin was further tested to examine what percentage composition of acetonitrile could potentially be in the mobile phase before causing damage to the encapsulation material.
Table 2.7: Resin mass changes caused by differed acetonitrile content of mobile phase. * denotes resin samples which were damaged by the acetonitrile.

<table>
<thead>
<tr>
<th>Acetonitrile Content (%)</th>
<th>Change in Mass (mg)</th>
<th>% Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.2</td>
<td>0.1</td>
</tr>
<tr>
<td>2</td>
<td>4.6</td>
<td>0.6</td>
</tr>
<tr>
<td>10</td>
<td>12.4</td>
<td>0.2</td>
</tr>
<tr>
<td>20</td>
<td>100.6</td>
<td>1.6</td>
</tr>
<tr>
<td>50*</td>
<td>337.4</td>
<td>5.5</td>
</tr>
<tr>
<td>80*</td>
<td>260.3</td>
<td>4.1</td>
</tr>
<tr>
<td>100*</td>
<td>194.7</td>
<td>3.2</td>
</tr>
</tbody>
</table>

Figure 2.10: Acetonitrile concentration durability of resin, submerged for 24 hours.

As can be seen in Table 2.7 and Figure 2.10, the damage to the resin began occurring around 50% acetonitrile concentration, with small pieces of resin cracking and breaking off from the bulk of the resin, as observed previously with the 100% acetonitrile concentration. Further increases in acetonitrile concentration resulted in
increasing damage, with large pieces of resin splintering off from the resin sample at 100% concentration. The minor damage at 50% acetonitrile after 24 hours of treatment indicates that the resin could likely withstand a 50% acetonitrile mobile phase for separations in general, but not for extended periods of use.

3.3.2 Binding of resin to outer monolith surface

The first monolith encased in resin was split and observed both by the naked eye and under magnification to demonstrate the binding of the resin to the outer surface of the monolith itself.

![Scanning Electron Micrograph of a monolith (Batch 300514F) encased in resin. Sample charging is seen in the top left corner of the image.](image.png)

Figure 2.11: Scanning Electron Micrograph of a monolith (Batch 300514F) encased in resin. Sample charging is seen in the top left corner of the image.

Using method E2 (see Table 2.1), multiple monoliths were encased in resin and their flow-through properties evaluated. As outlined in Figure 2.11 above, the resin
leeched into the outer surface of the monoliths during setting. It was hypothesised that by doing so, the resin encasement method would prevent high flow rates along the outer surfaces of monoliths (between the monolith and the casing, such as PTFE heat shrink tubing), therefore improving HPLC separations.

\[\text{Figure 2.12: (a) A resin encased monolith, magnified; (b) a shrink tubing encased monolith, magnified.}\]

Figure 2.12a in particular, demonstrates the seepage of resin, as the colour and texture gradients can be seen to change across the monolith surface. The grey middle band is the area of the monolith that has been engulfed by resin during encapsulation, and presents a surface with a slight sheen (from the resin), but a grey-white colour from the monolith. Comparatively, in Figure 2.12b an apparently clear interface can be seen between the monolith’s outer edge and the heat-shrink tubing, suggesting that the heat shrink tubing does not bind to the outer surface of the monolith during heating and shrinkage. This was further explored using scanning electron microscopy (SEM) on the microscale (Figure 2.13). The green circle in Figure 2.13b focusses on the clearly definable line between the monolith’s edge and the shrink tubing wall, confirming that no attachment to the monolith is present, whereas a diffuse interface can be seen with the resin encapsulation method in Figure 2.13a. The definitive line in Figure 2.13b differs greatly from Figure 2.13a,
where the resin is enmeshed with the monolith’s outer wall, lending weight to the theory that high external flow rates will not be achievable with the resin encased monoliths.

![SEM images of encased monoliths with green circles highlighting the crossover sections between encasement and monolith. a) Resin encased; b) Conventional PTFE shrink tubing encased.]

Encasing with the resin could also potentially prevent ‘defective’ monoliths from not being used as columns in HPLC. When transferring monolith solution to a mould, air bubbles may become trapped on the surface of the mould, resulting in deformities along the length of the column (see Figure 2.14). Careful measures are often taken to prevent this, such as vigorously tapping the mould on a hard surface to dislodge the air bubbles; however, if not done correctly, air bubbles can result in large air pockets or dents visible on the outer surface after calcination.\textsuperscript{64, 107} When using PTFE or PEEK heat shrink tubing as the encasement method, the tubing does not properly fill these pockets during the shrinking stage, allowing for large volumes of the mobile phase solution to accumulate there, which can lead to high external flow rates and a poor separation (see Figure 2.15).
Figure 2.14: Monolith (from batch 140815) with damage from mishandling (L) and air bubble formation during incubation (R).

Figure 2.15: Heat shrink tubing encapsulated monolith (batch 140815). Deformities can still be seen through heat shrink tubing (within the green circle), indicating that the method has not properly bound to the monolith wall.

It was discovered during experimentation that the accumulation of solution in these pockets also caused the heat shrink tubing to detach from the wall of the monolith completely, causing high external flow rates. When using a resin to encapsulate the monolith, however, the liquid resin was able to fill the feature produced by the air pockets on the outer surface before setting, as was previously shown in Figure 2.13a. Once the resin had set, it was immovable from these gaps, therefore overcoming the issue of surface irregularities and ‘unusable’ monolithic columns.

3.3.3 Resin ingestion depth

The depth of the resin ingestion was measured across several different encapsulated monoliths to determine the extent and uniformity of the ingestion along a monolith,
with results summarised in Table 2.8. The encased monoliths were cut into six segments, and ingestion depths measured using calipers under a microscope.

*Table 2.8: Resin ingestion depth (in mm) measurements for three different encased monoliths, cut into six segments. A ‘-‘ denotes that no monolith was present in the segment.*

<table>
<thead>
<tr>
<th>Segment</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>20140626</td>
<td>-</td>
<td>1.2</td>
<td>0.5</td>
<td>0.4</td>
<td>1.1</td>
<td>-</td>
</tr>
<tr>
<td>20141020</td>
<td>1.3</td>
<td>1.3</td>
<td>0.4</td>
<td>0.3</td>
<td>1.1</td>
<td>1.2</td>
</tr>
<tr>
<td>20160208</td>
<td>-</td>
<td>0.9</td>
<td>0.5</td>
<td>0.4</td>
<td>0.8</td>
<td>-</td>
</tr>
<tr>
<td>Average</td>
<td>1.3</td>
<td>1.1</td>
<td>0.5</td>
<td>0.4</td>
<td>1.0</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Measurement on three separate encased monoliths demonstrated an average of 0.4 mm ingestion at the centre of the monoliths, and around 1.2 mm at the ends of the monoliths. Since approximately 0.3-1.2 mm of the outer surface of the monolith is penetrated by the resin, deeper surface irregularities are also covered by the resin, creating an even contact point between the mobile phase and the encased surface of the monolith, which will no longer interfere with the flow properties and potentials of the monolithic column. The resin ingestion does, however, impact the overall diameter of the monolith. For example, a 4.6 mm diameter monolith post-encapsulation will more likely be 2.2 - 4.0 mm in diameter, which will therefore require lower flow rates to achieve the optimal linear velocity, without increasing run time, and can therefore result in less reagent consumption.
3.3.4 Flow testing

Encapsulated column performance was initially tested using coloured food dye pumped through the monolith to determine if any defects were present, and if flow-through could be achieved. Due to the nature of the resin ingression into the monolith’s outer surface, the ends of the monolith contacting the HPLC fittings were also filled with resin, resulting in no flow-through being achieved. Modifications to the encapsulation process were made to overcome this issue, including covering the ends with cyanoacrylate, and small pieces of heat shrink tubing, but neither method was successful in overcoming the issue. As a result, the HPLC fittings were removed from the encapsulated column once the resin had fully set, and 1-2 mm drill bits were used to hand drill holes into both ends of the encapsulated monolith through the HPLC fitting hole, until the monolith surface was reached. This created a gap between the HPLC fitting and the raw monolith through which solution could then flow (see Figure 2.16).

![Encapsulated monolith with hole drilled through monolith end to enable solution flow through. Green arrow highlights drilled hole.](image)

*Figure 2.16: Encapsulated monolith with hole drilled through monolith end to enable solution flow through. Green arrow highlights drilled hole.*
Figure 2.17 shows the separation of a green food dye into its components (yellow and blue dyes) on the unmodified silica column post-encapsulation, following the aforementioned drilling through the inlet and outlet of the column. Flow through was achieved with yellow dye eluting from the column first, followed by the blue dye.

Figure 2.17: Flow testing of resin encapsulated monolith using coloured food dye in deionised water.

Preliminary testing of the monolith encapsulation method demonstrated the likely success of the method in practical applications, and therefore experimentation with HPLC separations began to determine the viability of the method in comparison with heat shrink tubing encapsulation, a commonly employed encapsulation method for in-house made monoliths.294

3.3.5 HPLC Testing

Matching pairs of monoliths from the same batches were prepared as columns for HPLC experimentation. The first monolith was encapsulated using the resin method described in this chapter, and the other was encapsulated using heat shrink tubing, with steel HPLC tubing and fittings used for connection to the HPLC instrumentation. Three batches of monoliths were used for HPLC experimentation to
check repeatability of the methods. An unretained analyte (thiourea) was used to examine the two described encapsulation methods. Chromatograms featured in Figure 2.18 were able to demonstrate the repeatability of the resin encapsulation method across different monolith batches.
Figure 2.18: Resin encapsulated (blue line) and heat shrink tubing encapsulated (orange line) monolith pair comparison using HPLC. (a) Monolith pair 1; (b) monolith pair 2 and (c) monolith pair 3 of resin encapsulated and heat shrink tubing encapsulated monoliths.
It is, however, apparent that the heat shrink tubing encapsulation method was not as repeatable, with peak shapes differing between monolith batches. The irregular peak shapes and peak fronting and tailing are common distortions in relation to wall-effects, seen with all three heat shrink tubing encapsulated monoliths. This can be accounted for by the poor binding of the heat shrink tubing to the outer surface of the monolithic column, allowing higher solutions flow rates along the walls of the monoliths, and therefore creating the wall-effects. Premature elution of the analyte observable with the heat shrink tubing encapsulated monolith of Pair 1 (Figure 2.18a) has been determined to be caused by partial or full detachment of the heat shrink tubing from the outer wall of the monolith, allowing large volumes of the mobile phase, and higher mobile phase flow rates with minimal stationary phase interaction to occur. This in turn caused the mobile phase and analyte to elute much faster.

Table 2.9: Comparison of peak information between three pairs of monoliths encapsulated in the two described methods.

<table>
<thead>
<tr>
<th>Monolith Pair</th>
<th>tr(min)/%RSD</th>
<th>Peak area/%RSD</th>
<th>Peak Height</th>
<th>Symmetry</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Shrink Tubing</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.4/16</td>
<td>6163/6.0</td>
<td>176</td>
<td>0.4</td>
</tr>
<tr>
<td>2</td>
<td>0.4/20</td>
<td>5813/2.0</td>
<td>275</td>
<td>0.5</td>
</tr>
<tr>
<td>3</td>
<td>0.4/12</td>
<td>5937/2.2</td>
<td>325</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Resin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.5/1.1</td>
<td>5713/1.2</td>
<td>514</td>
<td>0.7</td>
</tr>
<tr>
<td>2</td>
<td>0.2/2.9</td>
<td>5962/3.0</td>
<td>787</td>
<td>0.3</td>
</tr>
<tr>
<td>3</td>
<td>0.3/0.8</td>
<td>6083/1.5</td>
<td>834</td>
<td>0.4</td>
</tr>
</tbody>
</table>
Good reproducibility was able to be achieved within each resin encapsulated column, with peak area relative standard deviations (RSD) being 3.0% or lower (Table 2.9). Repeatability with the heat shrink tubing encased monoliths was somewhat worse, with a maximum RSD of 6.0% for peak area, and 12-20% variation in retention times, compared to less than 3.0% variation for the resin encapsulated columns, which can be accounted for by the detachment of the heat shrink tubing from the wall of the monolith.
4 Conclusions

Silica monoliths were successfully prepared according to literature methods, with physical properties desirable for their application in liquid chromatography being achieved. Further optimisation and monitoring of the preparation and treatment methods, such as ammonium hydroxide treatment and calcination, resulted in greater control of the phase separation and pore formation of the monoliths, allowing for specific chromatography-desirable physical properties to be more easily achieved.

The resin encapsulation method proved durable against most common liquid chromatography solvents at high concentrations, and was able to successfully overcome wall-effects when applied to liquid chromatography, through the engulfment of the outer monolith surface. HPLC experimentation indicated that the resin encapsulation method outperformed the commonly utilised heat shrink tubing encapsulation of monoliths. The findings from this work have resulted in a peer reviewed publication.296
CHAPTER THREE

EVALUATION OF FUNCTIONALISED SILICA MONOLITHS FOR SEPARATION AND DETECTION

1. Introduction
2. Experimental
3. Results and Discussion
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CHAPTER THREE

1 Introduction

In order to produce useful separations, monolithic columns need to be functionalised to form stationary phases with appropriate retention characteristics. The C18 stationary phase is the most widely used in the separation of important analytes.\textsuperscript{297-301} The application of useful stationary phases to house-made monoliths for integration into microfluidic devices is important. Almost the same method of C18 functionalisation used for silica particle modification can be applied to silica monolith modification.\textsuperscript{84} Methods of C18 functionalisation for monoliths have been published in the past, for both pre- and post-encapsulation functionalisation.\textsuperscript{50, 52, 64, 86-89, 91-93}

Alternative stationary phases are required in order to achieve efficient, orthogonal two-dimensional separations of complex samples.\textsuperscript{302} Lubricin, a glycoprotein derived from bovine synovial fluid, which aids in lubrication of joints, has previously been shown to readily adsorb to a variety of different substrates, with long term stability.\textsuperscript{303-305} Though known to have these anti-adhesive properties, the lubricin’s central domain is hydrophilic,\textsuperscript{305} and its ability to bond to most surfaces, including silica,\textsuperscript{306} means that it could have potential applications in chromatography.

The Tanaka Test, developed by Tanaka and co-workers\textsuperscript{132} and better described by Cruz et al.,\textsuperscript{135} explores the separation of selected compounds to assist in determining potential stationary phase properties.\textsuperscript{132, 135-136, 307-308} The Tanaka Test is an industry standard for evaluating HPLC column performance,\textsuperscript{133-134} and has been utilised to evaluate a number of different stationary phases.\textsuperscript{137, 309-310} The relative retention of specific compounds is used to describe retention capacity, hydrophobicity, steric selectivity and silanophilic properties, including indication of ion exchange
properties, and the data is often graphically represented to better illustrate differences or similarities between columns.

Sol-gels with compositions similar to that of the monolith sol-gel have previously been functionalised with chemiluminescence reagents for application in electrogenerated chemiluminescence (ECL) detection. The functionalisation of a monolithic column in a microfluidic device with a chemiluminescence reagent, such as \([\text{Ru(bpy)}_3]^{2+}\), could enable direct detection of target analytes on-column, or immediately post-column, without the need for further pumps or flow-cells, and could greatly reduce reagent consumption for post-column chemiluminescence detection.

This chapter aims to explore preparation methods of \(\text{C}_{18}\) stationary phases for monolithic columns, both pre- and post- encapsulation, which will be compared with a variety of commercially available \(\text{C}_{18}\) columns. Furthermore, the glycoprotein lubricin will be evaluated for its stationary phase properties when applied to a monolithic column. Finally, a variety of chemiluminescence reagent sol-gels containing \([\text{Ru(bpy)}_3]^{2+}\) will be evaluated for their potential as post-column online detectors for the detection of target analytes.
2 Experimental

2.1 Chemicals

Unless stated otherwise, all chemicals were of analytical grade. Deionised water was used throughout the following experiments.

Acetonitrile, ammonium hydroxide, butylbenzene, caffeine, chloro(dimethyl)octodecysilane (ODS), chlorotrimethylsilane, dichloromethane, heptane, ofloxacin, penty benzene, phenol, Pluronic F127 polymer, potassium chloride, o-terphenyl, tetramethyl orthosilicate, trifluoroacetic acid (TFA), and triphenylene were supplied by Sigma-Aldrich (Castle Hill, NSW, Australia). Acetone, ethanol, hydrochloric acid (32%), isopropanol, methanol, orthophosphoric acid (85%), sodium chloride, and toluene were purchased from Chem Supply (Gillman, SA, Australia). Glacial acetic acid, benzylamine, nitric acid (70%), sodium hydroxide, and sodium phosphate monobasic were purchased from Ajax FineChem (Sydney, NSW, Australia). Thiourea was purchased from BDH Chemicals (Poole, England). Sulfuric acid (98%) was acquired from RCI Labscan (Gillman, SA, Australia). Tris(2,2’-bipyridyl)ruthenium(II) chloride hexahydrate ([Ru(bpy)3]Cl2·6H2O) was purchased from Strem Chemicals (Newbury Port, MA, USA). A commercial bleach solution (42 g/L sodium hypochlorite, 9 g/L sodium hydroxide) was purchased from a local supermarket.

Codeine, morphine, oripavine and thebaine were supplied by SunPharma (Port Fairy, VIC, Australia).

The lubricin glycoprotein was purified from bovine synovial fluid at Deakin University using the method described by Greene and co-workers.304
2.2 Monolith Preparation

Monoliths were prepared according to the method described by Fletcher et al. and as prepared in Chapter 2. 0.432 g of Pluronic F127 polymer was dissolved in 0.02 M acetic acid (4.0 mL) over an ice bath over 40 min. 2.0 mL of tetramethyl orthosilicate (TMOS) was added to the solution and stirred for a further 40 min, before being transferred to moulds and incubated in an oven at 40°C for 72 hours. Unless otherwise stated, monoliths underwent base treatment in 1.0 M ammonium hydroxide at 90°C for 18 hours, and were then calcined in a furnace at 550°C overnight.

2.3 Column Functionalisation

The prepared monoliths were then functionalised for use as HPLC columns as described below.

2.3.1 C18 Batch Functionalisation

Monoliths were C18 functionalised using a modification of a method developed at the University of Hull. Monoliths were placed in a conical flask with 0.7 mL of chloro(dimethyl)octodecylsilane (ODS) and 15 mL of toluene. The flask was drowned in nitrogen before being attached to a nitrogen reflux system, and lowered into a spinning oil bath heated to 100°C for 24 hours. Monoliths were removed from the flask and rinsed twice with 10 mL washes of toluene for 3-4 hours each time. Following this, the monoliths were rinsed twice in 10 mL of ethanol for 3-4 hours. Monoliths were then dried and encapsulated using the resin encapsulation method described in Chapter 2.
2.3.2 C18 Post-Encapsulation Functionalisation

Encapsulated monoliths were functionalised according to the method described by Soliven and co-workers.52-53 To begin, 50 mL of dried heptane was flushed through the monolith at a flow rate of 4.0 mL/min to prepare the column. A 1% (v/v) solution of chloro(dimethyl)octadecylsilane (ODS) in dried heptane was used as the cyano silane solution. The cyano silane solution was pumped through the monolith at 4.0 mL/min until five column volumes had passed through. Following this, dried heptane was pumped through the column at the same flow rate for another 30 minutes. This process was repeated until a total of 100 mL had passed through the column. The endcapping solution consisted of a 1% (v/v) solution of chlorotrimethylsilane in dried heptane, pumped through the column at a rate of 4.0 mL/min until 100 mL had passed through the column.

2.3.3 Lubricin Post-Encapsulation Functionalisation

Monoliths were functionalised with the glycoprotein lubricin, purified from bovine synovial fluid.304 Monoliths were prepared and encapsulated according to the method described in Chapter 2. The column was thoroughly rinsed with deionised water and then flushed with phosphate buffered saline (PBS) solution (120 mM, pH 7.0) for 1 hour. Lubricin was diluted in the PBS solution (2 mL total) and then pumped through the monolith using a syringe pump (100 µL/min), and allowed to soak inside the monolith to achieve proper functionalisation for 24 hours. Following functionalisation, the lubricin column was rinsed with PBS, and stored in a refrigerator at 4°C until required for HPLC testing. Functionalisation properties were tested using the Tanaka Test.
2.4 Reagents

2.4.1 Phosphate buffered saline

Phosphate buffered saline (PBS) was prepared as described in Zappone et al.\textsuperscript{303} by dissolving 0.3506 g of sodium chloride (NaCl), 0.0101 g of potassium chloride (KCl) and 0.7801 g of sodium phosphate monobasic in 50 mL of deionised water. pH was adjusted to 7.0 using 1.0 M sodium hydroxide. Final solution concentrations were 120 mM NaCl, 10 mM sodium phosphate monobasic, and 2.7 mM KCl.

2.4.2 Tris(2,2'-bipyridyl)ruthenium(II)

1 mM [Ru(bpy)\textsubscript{3}]\textsuperscript{2+} was prepared by dissolution of [Ru(bpy)\textsubscript{3}]Cl\textsubscript{2}.6H\textsubscript{2}O crystals in either deionised water or 0.05 M sulfuric acid (H\textsubscript{2}SO\textsubscript{4}).

2.5 General instrumentation

2.5.1 High Performance Liquid Chromatography

All chromatography experiments were performed using an Agilent 1200 HPLC system (Agilent Technologies, Mulgrave, Victoria, Australia), consisting of a quaternary pump with solvent degasser, auto-sampler, and a variable wavelength detection module that monitored the absorbance at a chosen wavelength. Analysis was performed at room temperature, unless otherwise stated. Data was obtained and processed with Agilent ChemStation software.

For Tanaka Tests, an isocratic 2% methanol mobile phase was used with 1 µL injection volume, 1 mL/min flow rate, and UV detection at 254 nm for all columns. Further details of mobile phase composition for each of the test solutions is found below in Chapter 3, section 2.6.
For opiate alkaloid separation, an isocratic mobile phase consisting 3% methanol and 97% filtered deionised water (0.1% TFA) was used for all separations. $1 \times 10^{-6}$ M analytes were prepared in the mobile phase. A 5 µL injection volume, 1 mL/min flow rate and UV detection at 280 nm were used for the separation and detection of all four analytes and the test mixture.

Chemiluminescence detection of the opiate alkaloid separation was performed by Lachlan Soulsby, under the supervision of myself and Professor Paul Francis.\textsuperscript{315} For the combined HPLC/chemiluminescence detection system, an Agilent 1260 series HPLC system was used, equipped with a quaternary pump with solvent degasser, autosampler, and diode array detector. A PTFE coiled tubing flow-cell was used, placed against the window of a photomultiplier tube (PMT) (Electron Tubes model 9828SB, ETP, NSW, Australia), housed within a custom-made light tight box. Reagents were propelled using a Gilson Minipuls 3 peristaltic pump (John Morris Scientific, NSW, Australia), and the column eluent from the HPLC merged with the reagent line at a T-piece, immediately prior to the flow-cell.\textsuperscript{315}

2.5.2 Scanning Electron Microscopy

Scanning electron microscopy was performed using a Zeiss Supra 55VP Scanning Electron Microscope. Monolith samples were affixed to metal SEM stubs using Electrodag 1415 silver paint (Agar Scientific, Emgrid, Parooka, SA, Australia), with the sides of the samples also coated. The samples were then degassed in a vacuum chamber for at least 48 hours, before being transferred to a Leica EM Gold Sputter Coater. Samples were coated with 4-10 nm of gold before being removed from the chamber and transported to the SEM vacuum chamber immediately. Samples were imaged using an SE2 detector (Secondary electron), at 3kV accelerating voltage.
2.5.3 **Photography**

Photographs were taken using either a Huawei P8 13 megapixel mobile phone camera (model: HUAWEI GRA-UL00) with automatic ISO, f-stop and exposure settings, or using a Canon EOS 6D DSLR camera (Canon, Tokyo, Japan) (f-stop, ISO and exposure recorded with photographs throughout chapter). An orange filter was placed in front of the camera lens for photographs of monolith sol-gels excited by an ultra-violet light source (UV light) (CAMAG UV Lamp 4, 366 nm and 254 nm, CAMAG, Muttenz, Switzerland).

2.6 **Tanaka Testing**

Tanaka tests were performed as a preliminary test to determine possible surface modification functionality, as previously described. Four analyte solutions were prepared, which provide indications for 6 possible stationary phase properties. Test solutions were prepared according to Cruz *et al.*, with modifications to mobile phase and flow rates to suit the shorter monolithic columns used in this work.

Solution A was used to determine the amount of alkyl chains ($k_{AB}$), hydrophobicity ($a_{(CH2)}$), and steric selectivity ($a_{T/O}$). 0.1 mg/mL thiourea (unretained analyte), 0.6 mg/mL pentylbzene, 0.4 mg/mL butylbenzene, 0.5 mg/mL triphenylene and 0.5 mg/mL o-terphenyl were combined in the mobile phase of 98:2% water:methanol. The amount of alkyl chains ($k_{AB}$) is determined by the corrected retention factor for pentylbzene. Selectivity of pentylbzene to butylbenzene was calculated using adjusted retention times to determine hydrophobicity ($a_{(CH2)}$). Steric Selectivity ($a_{T/O}$) was determined using the selectivity of triphenylene to o-terphenyl using adjusted retention times.
Solution B was used to determine hydrogen bonding capacity. 0.1 mg/mL thiourea (unretained analyte), 0.5 mg/mL caffeine, 1.0 mg/mL phenol were combined in a mobile phase of 98:2% deionised water:methanol. The selectivity of caffeine to phenol using adjusted retention times determined the hydrogen bonding capacity ($\alpha_{C/P}$).

Solution C was used to determine ion exchange capacity at pH > 7. 0.1 mg/mL thiourea, 0.5 mg/mL phenol and 0.5 mg/mL benzylamine were combined in a mobile phase consisting of 98:2% buffer:methanol. The potassium monophosphate buffer was adjusted to pH 7.2 through dropwise addition of 1.0 M potassium hydroxide. The ion exchange capacity ($\alpha_{B/P}$) at pH > 7 was determined using the selectivity of benzylamine to phenol.

Solution D was used to determine ion exchange capacity at pH < 3. 0.1 mg/mL thiourea, 0.5 mg/mL phenol and 0.5 mg/mL benzylamine were combined in a mobile phase consisting 98:2% buffer:methanol. The potassium monophosphate buffer was adjusted to pH 2.7 through the dropwise addition of 3.0 M orthophosphoric acid. The selectivity of benzylamine to phenol determined ion exchange capacity ($\alpha_{B/P}$) at pH < 3.

All functionalised columns were compared to a bare silica encapsulated monolith column as a control, to determine if any changes in stationary phase properties occurred.

The C$_{18}$ batch functionalised monolith was also compared with a commercial C$_{18}$ particle packed column (XTerra MS C18 5 µm 4.6 × 50 mm, Waters Corporation, Rydalmere, NSW, Australia) and a commercial C$_{18}$ monolith column (Chromolith® SpeedROD RP-18e 50-4.6 mm, Merck KGaA, Darmstadt, Germany). The Tanaka
Test solutions were separated on each column, and selectivities calculated based on adjusted retention times.

### 2.7 Tris(2,2'′-bipyridyl)ruthenium (II) sol-gel functionalisation

Tris(2,2'′-bipyridyl)ruthenium(II) ([Ru(bpy)$_3$]$^{2+}$) functionalisation of sol-gels was performed in order to explore their application as post-column detection media for on-line detection; and as potential photoredox catalysis particles.

#### 2.7.1 Pre-monolith encapsulation batch functionalisation

For pre-encapsulation batch functionalisation, monoliths were prepared as described in Chapter 2, and then modified using the following methods:

**KS001**

Monoliths were soaked in a 1 mM [Ru(bpy)$_3$]$^{2+}$ solution (prepared in deionised water) for 30 mins. Monoliths were then rinsed until the water remained clear, and then dried in an oven at 40°C.

Monoliths had one end dipped into a 1 mM [Ru(bpy)$_3$]$^{2+}$ solution, allowing capillary action to penetrate the monolith to approximately 2.5 cm along its length. Monoliths were then rinsed in deionised water.

**KS011**

Monoliths were soaked in a 1.0 mM [Ru(bpy)$_3$]$^{2+}$ solution, prepared in 0.05 M H$_2$SO$_4$. The monolith in solution was sonicated for 30 mins to ensure full penetration of the [Ru(bpy)$_3$]$^{2+}$ solution, before drying for 24 hours. Monoliths were thoroughly rinsed with deionised water prior to undergoing various solvent extractions (ethanol, acetone, methanol, acetonitrile, dichloromethane, water) to determine if any solvent could remove all of the adsorbed [Ru(bpy)$_3$]$^{2+}$. 
2.7.2 Immobilisation

For $[\text{Ru(bpy)}_3]^{2+}$ immobilisation, $\text{Ru(bpy)}_3\text{Cl}_2\cdot 6\text{H}_2\text{O}$ was added to sol-gel solutions prior to gelation, in order to immobilise the $[\text{Ru(bpy)}_3]^{2+}$ within the monolith/sol-gel structure. The general preparation methods for parent and modified sol-gels can be found below in Table 3.1; and the full written methods found in Appendix 1. Weight fractions for each of the immobilised sol-gels are listed in Table 3.2.
Table 3.1: Immobilised tris(2,2’-bipyridyl)ruthenium(II) sol-gel preparation methods. Full written methods can be found in Appendix 1.

<table>
<thead>
<tr>
<th>Parent</th>
<th>Reference</th>
<th>Reactants</th>
<th>Treatment</th>
<th>Modified</th>
<th>Modification</th>
</tr>
</thead>
<tbody>
<tr>
<td>KS002</td>
<td>51</td>
<td>0.432g Pluronic F127 Polymer, 2 mL 0.02 M acetic acid, 4 mL TMOS, 40 mg [Ru(bpy)₃]Cl₂</td>
<td>Mixed in ice bath; Oven dried (40°C, 72 hrs); ammonium hydroxide (1 M, 90°C, 18 hrs); calcined in furnace (600°C, overnight)</td>
<td>KS014</td>
<td>4 mL of 0.1 M HCl as acid</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>KS015</td>
<td>No ice bath during mixing</td>
</tr>
<tr>
<td>KS003</td>
<td>314</td>
<td>2.5 mL TMOS, 5 mL HCl (0.1 M), 0.14 g [Ru(bpy)₃]Cl₂, 2 mL 10 mM NaOH</td>
<td>Mixed in ice bath; Oven dried (40°C, 72 hrs); Dried in desiccator (72 hrs, RT)</td>
<td>KS004</td>
<td>No NaOH added</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.432 g F127 polymer added</td>
</tr>
<tr>
<td>KS005</td>
<td>311-313</td>
<td>12 mL TMOS, 4.8 mL water, 1.6 mL 0.1 M HCl, 10 mg [Ru(bpy)₃]Cl₂, 9 mL of 10 mM PBS</td>
<td>Mixed at RT; dried in desiccator (RT)</td>
<td>KS006</td>
<td>NaOH gelation</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>KS010</td>
<td>0.432 g F127 polymer added</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Oven dried (40°C, 72 hrs); or dried in desiccator (RT, 72 hrs)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>KS016</td>
<td>Ice bath used during mixing</td>
</tr>
<tr>
<td>KS007</td>
<td>316</td>
<td>15 mL TMOS, 7.5 mL water, 2.1 mL 0.1 M HCl, 40 mg [Ru(bpy)₃]Cl₂, 5 mL PBS (pH 7.0)</td>
<td>Mixed at RT; Air dried (RT)</td>
<td>KS008</td>
<td>0.432 g F127 polymer added</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>KS009</td>
<td>0.432 g F127 polymer added, PBS added to initiate gelation</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>KS012</td>
<td>Higher polymer content (0.6 g)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>KS013</td>
<td>Lower water content (2.5 mL)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>KS017</td>
<td>Ice bath used during mixing</td>
</tr>
</tbody>
</table>
Table 3.2: Weight fractions for the preparation of tris(2,2’-bipyridyl)ruthenium(II) immobilised sol-gels.

<table>
<thead>
<tr>
<th>Sol-Gel</th>
<th>TMOS</th>
<th>Water</th>
<th>Polymer</th>
<th>0.01 M PBS</th>
<th>Ru(bpy)₃Cl₂</th>
<th>0.1 M HCl</th>
<th>0.02 M Acetic Acid</th>
<th>0.01 M NaOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>KS002</td>
<td>0.314</td>
<td>-</td>
<td>0.066</td>
<td>-</td>
<td>0.006</td>
<td>0.614</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>KS003</td>
<td>0.203</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.014</td>
<td>0.585</td>
<td>-</td>
<td>0.198</td>
</tr>
<tr>
<td>KS004</td>
<td>0.185</td>
<td>0.347</td>
<td>0.031</td>
<td>-</td>
<td>0.01</td>
<td>0.426</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>KS005</td>
<td>0.423</td>
<td>0.166</td>
<td>-</td>
<td>0.345</td>
<td>0.001</td>
<td>-</td>
<td>0.065</td>
<td>-</td>
</tr>
<tr>
<td>KS006</td>
<td>0.492</td>
<td>0.193</td>
<td>-</td>
<td>-</td>
<td>0.003</td>
<td>0.151</td>
<td>-</td>
<td>0.16</td>
</tr>
<tr>
<td>KS007</td>
<td>0.505</td>
<td>0.247</td>
<td>-</td>
<td>0.165</td>
<td>0.001</td>
<td>0.082</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>KS008</td>
<td>0.574</td>
<td>0.281</td>
<td>0.048</td>
<td>-</td>
<td>0.004</td>
<td>0.093</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>KS009</td>
<td>0.469</td>
<td>0.229</td>
<td>0.04</td>
<td>0.183</td>
<td>0.004</td>
<td>0.076</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>KS010</td>
<td>0.617</td>
<td>0.241</td>
<td>0.043</td>
<td>-</td>
<td>0.004</td>
<td>0.095</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>KS012</td>
<td>0.423</td>
<td>0.29</td>
<td>0.05</td>
<td>0.166</td>
<td>0.003</td>
<td>0.068</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>KS013</td>
<td>0.462</td>
<td>0.226</td>
<td>0.054</td>
<td>0.181</td>
<td>0.004</td>
<td>0.074</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>KS014</td>
<td>0.283</td>
<td>-</td>
<td>0.06</td>
<td>-</td>
<td>0.006</td>
<td>0.652</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>KS015</td>
<td>0.314</td>
<td>-</td>
<td>0.066</td>
<td>-</td>
<td>0.006</td>
<td>-</td>
<td>0.614</td>
<td>-</td>
</tr>
<tr>
<td>KS016</td>
<td>0.438</td>
<td>0.171</td>
<td>-</td>
<td>0.321</td>
<td>0.001</td>
<td>0.067</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>KS017</td>
<td>0.505</td>
<td>0.247</td>
<td>-</td>
<td>0.165</td>
<td>0.001</td>
<td>0.082</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
3 Results and Discussion

HPLC columns used throughout this section are colour coded according to the following table (Table 3.3) to enable easier identification, except where noted otherwise.

_Table 3.3: Abbreviations and colour co-ordination of different functionalised columns used throughout this work._

<table>
<thead>
<tr>
<th>Column</th>
<th>Abbreviation</th>
<th>Colour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmodified Silica Monolith</td>
<td>UMC</td>
<td>Blue</td>
</tr>
<tr>
<td>Commercial Particle Packed C18</td>
<td>PP-C18</td>
<td>Orange</td>
</tr>
<tr>
<td>Commercial Monolith C18 (Chromolith®)</td>
<td>Chrom C18</td>
<td>Grey</td>
</tr>
<tr>
<td>Post-encapsulation C18 Monolith</td>
<td>PE-C18</td>
<td>Purple</td>
</tr>
<tr>
<td>Batch functionalised C18 Monolith</td>
<td>BF-C18</td>
<td>Green</td>
</tr>
<tr>
<td>Lubricin functionalised Monolith</td>
<td>LUB</td>
<td>Yellow</td>
</tr>
</tbody>
</table>

3.1 C18 Modification

Initially, to confirm successful functionalisation, the unmodified monolith and the post encapsulation C18 monolith were compared using a series of alkylbenzene compounds. As seen in Figure 3.1, the unmodified column showed no retention of the alkylbenzene compounds prior to functionalisation, but a good separation post-functionalisation, demonstrating the application of the chloro(dimethyl)octodecylsilane (ODS) to the stationary phase, enabling the retention of the polycyclic aromatic hydrocarbons (PAH).
Figure 3.1: Separation of an alkylbenzene mix on an unmodified silica monolith column (blue line), and the same column after post-encapsulation C\textsubscript{18} functionalisation\textsuperscript{52} (purple line). 20 µL injection, 1 mL/min, 98:2% di water: methanol mobile phase. Analytes in order of elution: 1 toluene, 2 ethylbenzene, 3 propylbenzene, 4 butylbenzene, 5 hexylbenzene.

In order to gain more information about their retention characteristics, the Tanaka Test was used to compare the in-house functionalised monoliths with commercial columns. Unfortunately, due to column damage, the post-encapsulation C\textsubscript{18} modification column (PE-C18) was unable to undergo Tanaka Testing, and so a batch functionalised C\textsubscript{18} monolith column (BF-C18) was used for this comparison. The literature indicates that alkyl chain, hydrophobicity and steric selectivity factors relate to C\textsubscript{18} stationary phases\textsuperscript{135}. Table 3.4, below, reports the adjusted retention times of the compounds within each of the four solutions, for four different columns: an unmodified silica monolith column (UMC), a C\textsubscript{18} particle packed commercial column (PP C18), a commercial C\textsubscript{18} Chromolith\textsuperscript{®} monolithic column (Chrom C18), and the batch functionalised C\textsubscript{18} monolith column (BF-C18).
Table 3.4: Adjusted retention times of 50 mm HPLC columns, separating Solutions A, D, E and F for the Tanaka Tests. An unmodified silica monolith column (UMC), Commercial C\textsubscript{18} particle packed column (PP C\textsubscript{18}), Merck Chromolith\textsuperscript{®} C\textsubscript{18} monolithic column (Chrom. C\textsubscript{18}) and C\textsubscript{18} batch modified silica monolith column (BF- C\textsubscript{18}) were compared.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Butylbenzene</th>
<th>Pentylbenzene</th>
<th>Triphenylene</th>
<th>o-terphenyl</th>
<th>Caffeine</th>
<th>Phenol</th>
<th>Benzylamine</th>
<th>Phenol</th>
<th>Benzylamine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adjusted Retention Time (TR) (min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>UMC</td>
<td>PP-C\textsubscript{18}</td>
<td>Chrom C\textsubscript{18}</td>
<td>BF-C\textsubscript{18}</td>
<td>UMC</td>
<td>PP-C\textsubscript{18}</td>
<td>Chrom C\textsubscript{18}</td>
<td>BF-C\textsubscript{18}</td>
<td>UMC</td>
</tr>
<tr>
<td>A</td>
<td>0.069</td>
<td>0.794</td>
<td>3.05</td>
<td>0.85</td>
<td>1.494</td>
<td>0.768</td>
<td>0.683</td>
<td>0.519</td>
<td>0.161</td>
</tr>
<tr>
<td>B</td>
<td>0.077</td>
<td>0.802</td>
<td>3.812</td>
<td>0.91</td>
<td>0.071</td>
<td>0.8</td>
<td>4.293</td>
<td>0.85</td>
<td>0.064</td>
</tr>
<tr>
<td>C, pH 7.2</td>
<td>0.241</td>
<td>2.033</td>
<td>0.964</td>
<td>0.081</td>
<td>0.736</td>
<td>0.259</td>
<td>0.661</td>
<td>0.028</td>
<td></td>
</tr>
<tr>
<td>D, pH 2.7</td>
<td>0.234</td>
<td>1.812</td>
<td>0.976</td>
<td>0.088</td>
<td>0.071</td>
<td>0.14</td>
<td>0.099</td>
<td>0.034</td>
<td></td>
</tr>
</tbody>
</table>
As can be seen in Figure 3.2, the unmodified monolith column and C_{18} monolith column (BF-C18) exhibit different functionalities, confirming surface modification using the batch functionalisation method. It has been stated that the greater values of alkyl chains, hydrophobicity and steric selectivity indicate greater retention of hydrocarbons.\textsuperscript{128} The commercial particle packed C_{18} column (PP-C18) demonstrated the strongest C_{18} properties with a high number of alkyl chains, hydrophobicity and steric selectivity, and some hydrogen bonding capacity. The XTerra column used as the commercial particle packed column is endcapped, and so should show reduced hydrogen bonding capacity.\textsuperscript{317} The Chromolith® C_{18} column exhibited a great number of alkyl chains, but little of the other important C_{18} properties.
As discussed in Cruz et al.\textsuperscript{135}, variations in functionalisation can be readily identified using the Tanaka Test, as seen in Figure 3.2. The three C\textsubscript{18} columns analysed here exhibit slightly different functionality, even though all are C\textsubscript{18} columns. The functionality of the Chromolith\textsuperscript{®} and batch functionalised monolith columns are very similar, with the Chromolith\textsuperscript{®} demonstrating greater hydrogen bonding capacity, and the batch functionalised monolith showing slightly greater hydrophobicity and steric selectivity functionalities. The similarity between these two column functionality tests indicates that the batch functionalised column is well functionalised with the ODS. Guiochon has discussed in his 2007 review that it is likely that monolith columns and conventional silica particles have different surface chemistries, which can make them difficult to compare in terms of the retention patterns obtained.\textsuperscript{61}

In order to test the usefulness of the applied stationary phase, four opiate alkaloids of interest, morphine, codeine, oripavine and thebaine, were separated using the batch functionalised monolith.
**Figure 3.3**: Separation of opiate alkaloids of interest on the batch-functionalised C18 column using UV absorbance at 280 nm. Analytes in order of elution: morphine (M), codeine (C), oripavine (O), and thebaine (T).

Opiate compounds were separated using the batch functionalised column (Figure 3.3), in under 5 minutes, similar to previous works.\textsuperscript{198, 315} Although the isocratic separation on this particular column does not allow good resolution in such a short period of time, each analyte was able to be distinguished from the others. Through the use of chemiluminescence detection, more selective detection is achievable, as seen in Figure 3.4 below, as acidic potassium permanganate will only detect the phenolic opiates (morphine and oripavine), and [Ru(bpy)\textsubscript{3}]\textsuperscript{2+} will only detect the non-phenolic opiates (codeine and thebaine), providing greater resolution for each of the analytes of interest.\textsuperscript{198} The data obtained for Figure 3.4 was collected by Lachlan Soulsby at Deakin University using the same batch functionalised monolith under the supervision of myself and Professor Paul Francis and has been re-created with permission.
Figure 3.4: Opiate alkaloid separation of morphine (M), codeine (C), oripavine (O) and thebaine (T) on the house-made batch functionalised C18 column, detected with (a) UV detector (280 nm); (b) potassium permanganate chemiluminescence reagent; and (c) [Ru(bpy)$_3$]$^{3+}$ chemiluminescence reagent. Mobile phase: 97:3% deionised water: methanol; flow rate 1 mL/min; 5 µL injection volume. Separations performed by Lachlan Soulsby.$^{315}$
Combining these inexpensive house-made monoliths with selective detection systems (such as chemiluminescence) has the potential to allow for rapid, on-chip analysis of specific analytes, as can be seen in Figure 3.4.

Further optimisation of the batch functionalisation method and separation conditions could result in better resolution achieved with the house-made columns, potentially reaching the resolution and separation efficiency of commercial columns,\textsuperscript{198} such as the Merck Chromolith® which was previously explored in this chapter.

### 3.2 Lubricin Functionalisation

Our research group was interested to see if lubricin could be used as a novel stationary phase for liquid chromatography, as it has been shown that the lubricin compound readily binds to silica.\textsuperscript{306} The lubricin glycoprotein was applied to the monolith column, and then compared with an unmodified column from the same batch.
Figure 3.5: Separation of Tanaka Test mixtures on (a) Unmodified column, (b) Lubricin column. Solution A (blue line), Solution B (green line), Solution C (orange line) Solution D (grey line) were all analysed. 20 µL injection, 1.0 mL/min flow rate, with UV detection (254 nm). Mobile phases consisted of 2% methanol to 98% deionised water for solutions A & B, and 98% buffer for solutions C & D.

As can be seen in Figure 3.5, some degree of separation is seen with the unmodified column for each of the solutions, whereas most of the analytes are co-eluting on the
lubricin column, with only Solution C (Figure 3.5b, orange trace) showing any
degree of separation of phenol and benzylamine, and partial separation of Solution B
(Figure 3.5b, green trace).

Table 3.5: Adjusted retention times of analytes used in Tanaka Testing with the
unmodified column (UMC) and the lubricin column (LUB).

<table>
<thead>
<tr>
<th>Solution</th>
<th>Adjusted Retention Time (T&lt;sub&gt;R&lt;/sub&gt;) (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UMC</td>
</tr>
<tr>
<td>A</td>
<td></td>
</tr>
<tr>
<td>Pentylbenzene</td>
<td>0.077</td>
</tr>
<tr>
<td>Butylbenzene</td>
<td>0.069</td>
</tr>
<tr>
<td>Triphenylene</td>
<td>0.071</td>
</tr>
<tr>
<td>o-Terphenyl</td>
<td>0.064</td>
</tr>
<tr>
<td>B</td>
<td></td>
</tr>
<tr>
<td>Caffeine</td>
<td>1.494</td>
</tr>
<tr>
<td>Phenol</td>
<td>0.161</td>
</tr>
<tr>
<td>C, pH 7.2</td>
<td></td>
</tr>
<tr>
<td>Phenol</td>
<td>0.241</td>
</tr>
<tr>
<td>Benzylamine</td>
<td>0.736</td>
</tr>
<tr>
<td>D, pH 2.7</td>
<td></td>
</tr>
<tr>
<td>Phenol</td>
<td>0.234</td>
</tr>
<tr>
<td>Benzylamine</td>
<td>0.071</td>
</tr>
</tbody>
</table>

Based on the adjusted retention times of the analytes on both the unmodified column
and the lubricin column (Table 3.5), it was shown that the lubricin column eluted
analytes faster than the unmodified silica column, which confirmed the adherence of
the glycoprotein to the silica monolith structure. However, the faster elution also
indicated a lack of analyte interaction with the stationary phase. The Tanaka Test
results did show that the modification indicated a likely anion exchange property, as
demonstrated in Figure 3.6. It should be noted that the lubricin physico-chemically
adsorbs to surfaces as an end-grafted brush, which serves to coat and repel like-
covered surfaces via steric repulsion,<sup>303-304, 318-319</sup> and so acting as an anion
exchanger was not unexpected.
Figure 3.6: Tanaka test results for unmodified silica column and lubricin functionalised silica column. Unmodified column (blue), lubricin column (yellow).

The difference in retention times and general chromatogram between the two columns shows that the lubricin adhered to the surface of the monolith column, and by observing the plot in Figure 3.6, it is clear that the application of lubricin has modified the potential stationary phase properties of the monolith.

Further investigations into the potential anion exchange properties of the lubricin column are required, however it can be noted that if unsuccessful as a stationary phase (which would likely be the result of decreased retention times of suitable analytes), lubricin could be applied elsewhere within a microfluidic device as a lubricating agent to decrease adherence and analysis times.
3.3 Tris(2,2’-bipyridyl)ruthenium(II) functionalisation

Application of the chemiluminescence reagent $[\text{Ru(bpy)}_3]^{2+}$ to monolithic columns was pursued in the hopes of creating a combination separation-detection stationary phase. Sol-gel encapsulated $[\text{Ru(bpy)}_3]^{2+}$ also has potential applications in photoredox catalysis, so producing solid sol-gels containing the $[\text{Ru(bpy)}_3]^{2+}$ that are translucent and easily crushed into smaller particles was desirable. Two different approaches were taken to produce these sol-gels: pre-encapsulation functionalisation, where a $[\text{Ru(bpy)}_3]^{2+}$ solution was adsorbed onto the bare silica monolith; and pre-incubation immobilisation, where the tris(2,2’-bipyridyl)ruthenium(II)chloride hexahydrate ($\text{Ru(bpy)}_3\text{Cl}_2.6\text{H}_2\text{O}$) crystals were added to various sol-gel solutions in an attempt to incorporate it within a monolith structure. Some previously published immobilisation methods were used for this work as basis for further investigation into preparing sol-gels with desirable properties.

3.3.1 Pre-encapsulation functionalisation

KS001

Monoliths prepared according to the methods in Chapter 2 were soaked in a 1 mM $[\text{Ru(bpy)}_3]^{2+}$ solution for 30 mins. Following incubation, the monoliths were rinsed in deionised water, with a significant amount of the $[\text{Ru(bpy)}_3]^{2+}$ washing out of the monolith, as can be seen in Figure 3.7 below. A slight decrease in phosphorescence intensity when exposed to a UV light was seen after a full 2 hours of washing, when the rinse water remained clear.
Figure 3.7: KS001 [Ru(bpy)$_3$]$^{2+}$ soaked monoliths being rinsed following incubation (48 hours). Some of the [Ru(bpy)$_3$]$^{2+}$ has leached from the monoliths into the deionised water.

When the rinsing water remained clear after several washes, the monoliths underwent ammonium hydroxide treatment and washing. One of the KS001 monoliths was calcined at 550°C, and after the 18 hour treatment, it appeared that the high temperatures had broken down the [Ru(bpy)$_3$]$^{2+}$ within the monolith, turning the monolith a silver-grey colour (Figure 3.8). From this result, experimentation was undertaken to explore possible calcination temperatures that would leave the [Ru(bpy)$_3$]$^{2+}$ intact and luminescently active, while still removing unwanted organic material and monolith precursors.
Figure 3.8: KS001 monolith pre- and post-calcination (orange-yellow and silver-grey monoliths respectively), photographed under (a) normal light; and (b) a UV light at 366 nm, with an orange filter. No phosphorescence is seen from the calcined monolith when exposed to the UV light, and therefore the [Ru(bpy)$_3$]$^{2+}$ complex has been completely degraded by the calcination procedure.

With a melting point over 300°C for [Ru(bpy)$_3$]Cl$_2$, calcination temperatures around this point were explored, to maintain large enough through-pore sizes and hardening of the monoliths, without compromising the integrity of the [Ru(bpy)$_3$]$^{2+}$ within the monolith. Monoliths were calcined at temperatures between 250°C and 350°C.
Figure 3.9: Calcination drop tests: 1 – control, no calcination; 2 – 250°C; 3 – 300°C; 4 – 350°C. (a) drop tests under UV light, with an orange filter. (b) drop tests, post calcination, next to corresponding monoliths that were calcined at the same temperature.

Drop test images demonstrated a gradual degradation of the [Ru(bpy)$_3$]$^{2+}$ solution, and upon inspection under a UV light, exhibited no emission beyond 300°C (Figure 3.9a). The monoliths, however, continued to phosphoresce under the UV light beyond 300°C, but with a gradually fading strength, as seen in Figure 3.10b.

While the orange colour of the monolith remained after calcination at 250°C, light emitted from the calcined monoliths when exposed to UV light was significantly lower than an uncalcined KS001 monolith, and further decreased with increased calcination temperature, with a colour change from orange to green.

It has been established that [Ru(bpy)$_3$]$^{3+}$ has a greenish appearance, similar to the calcined monoliths shown in Figure 3.10a, and when exposed to a UV light does not emit, unlike the [Ru(bpy)$_3$]$^{2+}$ state.$^{316}$ However, as can be seen in Figure 3.10b,
emission does occur when exposed to the UV light, so at least some of the 
[Ru(bpy)$_3$]$^{3+}$ is still intact.

Figure 3.10: (a) KS001 tris(2,2’-bipyridyl)ruthenium(II) monoliths, which have been 
calcined at 250°C (left), 300°C (centre) and 350°C (right). (b) Calcined monoliths 
from (a) emitting once exposed to a UV light at 366 nm.

In order to confirm that degradation, not oxidation, had occurred, the KS001 
monolith calcined at 350°C was photographed in a dark room with the addition of 1 
mM ofloxacin. With a 10 s exposure, no emission was seen, and after-the-fact, no 
colour change back to orange had occurred, demonstrating that ([Ru(bpy)$_3$]$^{3+}$) was 
not present, and the compound was not reduced to [Ru(bpy)$_3$]$^{2+}$ upon the addition of 
the ofloxacin.

As discussed in Chapter 2, calcination is important in monolith preparation as it 
assists in removing any remaining volatile compounds or organic material from the 
preparation stages. However, if already calcined monoliths were to then undergo 
the [Ru(bpy)$_3$]$^{2+}$ treatment described in method KS001, then the loss of light 
intensity, and potential [Ru(bpy)$_3$]$^{2+}$ degradation could be avoided.

The ‘dip-dyed’ monoliths from method KS001 were exposed to a UV light, and 
photographed with an orange filter. As can be seen in Figure 3.11b, below, the dyed 
portion of the monolith exhibited bright emission when exposed to the UV light, and
the unmodified monolith did not emit. Though no further application of this method was explored, it is important to note that there is potential to have a dual-functionalised monolith, as shown here, with one end used for separation, and the other for detection, without needing to include a separate detection manifold. Some leeching did occur when rinsed with water, however much of the [Ru(bpy)$_3$]$^{2+}$ remained on the monolith.

Figure 3.11: Dip-dye [Ru(bpy)$_3$]$^{2+}$ monolith. (a) Under normal light (ISO-1600, f/2.8, 1/500 sec); (b) under UV light, 366 nm. (ISO-1600, f/2.8, 1/20 sec)

KS011

Monoliths that were soaked in a 1 mM solution of [Ru(bpy)$_3$]$^{2+}$, prepared in 0.05 M sulfuric acid, were dried and rinsed in several different solvents to see if further extraction of the [Ru(bpy)$_3$]$^{2+}$ would occur. Generally, when rinsing in deionised water after soaking and drying the monolith, some [Ru(bpy)$_3$]$^{2+}$ will wash out, as previously shown in Figure 3.7. For the soaking functionalisation methods to be further applied, especially for post-column detection, the monoliths need to retain [Ru(bpy)$_3$]$^{2+}$ after exposure to common HPLC solvents. Acetonitrile, acetone, dichloromethane, ethanol and methanol were used to attempt to desorb the [Ru(bpy)$_3$]$^{2+}$ from the monolith structure.

Monoliths were soaked in each solvent for 30 minutes before removal, and then rinsed with deionised water. The solvent was visually examined for any sign of
extraction, indicated by change in solvent colour. To ensure accuracy, the solvents were placed under a UV light and examined for any orange emission.

*Table 3.6: Results of the solvent extraction testing performed on the KS011 monoliths.*

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Extraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetonitrile</td>
<td>No</td>
</tr>
<tr>
<td>Acetone</td>
<td>Yes, Some</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>No</td>
</tr>
<tr>
<td>Ethanol</td>
<td>No</td>
</tr>
<tr>
<td>Methanol</td>
<td>No</td>
</tr>
</tbody>
</table>

The results demonstrated that only the acetone was able to extract any \([\text{Ru(bpy)}_3]^{2+}\) from the column, and only a very small amount, with a slight colour change to the rinse solution.

*Figure 3.12: KS011 monolith under normal light (ISO-800, f/2.8, 1/80 sec); and under UV light (ISO-12800, f/2.8, 1/25 sec) after washing with acetone*

After rinsing with water, the monoliths retained \([\text{Ru(bpy)}_3]^{2+}\), but were very pale orange in appearance (Figure 3.12a), indicating a majority of the complex had been washed away via deionised water or acetone. When exposed to the UV light, very little emission was seen (Figure 3.12b).
Adsorbing the [Ru(bpy)$_3$]$^{2+}$ onto the bare silica surface, without any surface modification, was somewhat successful. However, a majority of the complex was washed off with initial rinsing, resulting in pale colour and dull emissions when exposed to a UV light.

The longevity of this method is also unknown. Repeated use in chemiluminescence detection could result in removal of all of the complex over time.

Modification techniques have been explored where the [Ru(bpy)$_3$]$^{2+}$ is covalently bonded to a silica monolith through surface and/or complex modification, and have been applied to electrochemical detection$^{314}$, sequential injection analysis and flow injection analysis$^{322}$.

### 3.3.2 Pre-incubation Immobilised Tris(2,2´-bipyridyl)ruthenium(II)

Pre-incubation immobilised [Ru(bpy)$_3$]$^{2+}$ was achieved following some literature methods,$^{51,311-314,316}$ as well as modifications to the published methods, which could potentially form desirable characteristics for the sol-gels. Since we were looking to apply the sol-gels as photoredox catalysis particles, or for immobilised post-column detection media, desirable characteristics included: retention of the [Ru(bpy)$_3$]$^{2+}$ within the immobilised structure after washing with solvents; and flow-through ability through the formation of the bi-modal pore structure desirable for separation applications. Crushability was also desirable, in order to easily create smaller particles for photoredox catalysis applications.

The following tables show the resulting sol-gels, imaged both under normal light and under UV light with an orange filter placed in front of the camera.
Table 3.7: Results of the sol-gels prepared through the modification of Fletcher et al. monolith preparation methodology.\textsuperscript{51}

<table>
<thead>
<tr>
<th>Sol-gel</th>
<th>Normal light</th>
<th>UV light (and filter)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>KS002</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td>Prepared by adding Ru(bpy)_3Cl_2( \cdot )6H_2O to the monolith solution, and then following the monolith preparation procedure, including base treatment and calcination, as described in Chapter 2. The sol-gel exhibited a bright orange colour, and phosphoresced when exposed to an ultraviolet light source.</td>
</tr>
<tr>
<td>KS014</td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
<td>The hydrochloric acid used in this method was able to dissolve the polymer, and resulted in a product with a similar appearance to a monolith. The final product does resemble the KS002 monolith, however, is greatly prone to cracking making it difficult to prepare a long column for separation.</td>
</tr>
<tr>
<td>KS015</td>
<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
<td>A large degree of shrinkage was seen with the KS015 method, with the monoliths having an almost white outer layer, with a pale orange colour throughout. Little difference was seen between this method, and the ice bath stirred method of KS002,</td>
</tr>
</tbody>
</table>
meaning an ice bath would be unnecessary for preparation. The appearance is slightly more opaque compared to the KS002 sol-gel. The curvature of the monolith seen here is a result of the rinsing process, with the monolith having been placed in a container that did not have a flat base. While much shrinkage did occur, the column itself is able to remain as a single piece, and so could easily be encapsulated and applied in post-column detection.
Table 3.8: Results of the sol-gels prepared following Greenway et al. sol-gel preparation methodology.\textsuperscript{314}

<table>
<thead>
<tr>
<th>Sol-gel</th>
<th>Normal light</th>
<th>UV light (and filter)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>KS003</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td>The KS003 sol-gel required drying in a desiccator to solidify. The resulting sol-gel was easily cracked, with a large degree of shrinkage, but an intense orange colour remained. When rinsing, much of the $[\text{Ru(bpy)}_3]^{2+}$ was washed from the sol-gel, but a dark orange colour still remained. This darker orange/red colour was a result of the higher $[\text{Ru(bpy)}_3]^{2+}$ content, compared to the other sol-gels (1.4 wt% compared to 0.6 wt% for the KS002 sol-gel). When exposed to a UV lamp, some light emission was seen.</td>
</tr>
<tr>
<td>KS004</td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
<td>The addition of the polymer to the KS003 solution to produce these KS004 sol-gels resulted in a bright orange colour, with a translucent-opaque appearance. The monoliths were crushed into a fine powder with ease, and their intense orange colour still remained. Bright emission was seen when exposed to UV lamp.</td>
</tr>
</tbody>
</table>
Table 3.9: Results of the sol-gels prepared following Collinson et al. methodology.$^{311-313}$

<table>
<thead>
<tr>
<th>Sol-gel</th>
<th>Normal light</th>
<th>UV light (and filter)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>KS005</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td>Without the addition of a polymer, the sol-gel was unable to undergo phase separation, and therefore no porosity was present. When dried, the sol-gel easily cracked and broke into smaller pieces. The glassy appearance allowed easy penetration of the UV light, and so a bright emission was seen. This sol-gel could be applied for photoredox catalysis, as it did crush fairly easily into smaller particles due to its fragility.</td>
</tr>
<tr>
<td>KS006</td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
<td>A large degree of shrinkage was seen with this method, however the use of the NaOH as the base catalysed gelling agent was very effective, with gelation occurring very quickly. No polymer was added to this sol-gel solution, so would not be suitable as part of a flow-through detector, without crushing the sol-gel. Emission when exposed to a UV lamp is similar to that of the KS005 sol-gel.</td>
</tr>
<tr>
<td>KS010 (A-D)</td>
<td>See Figure 3.13</td>
<td>This sol-gel solution was treated with different gelation and incubation periods (see Section 3.3.3 below)</td>
<td></td>
</tr>
<tr>
<td>-------------</td>
<td>-----------------</td>
<td>-----------------------------------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>KS016</td>
<td><img src="image1" alt="Image" /></td>
<td>The KS016 gel formed a transparent orange outer layer, with a darker opaque inner body, giving the appearance of a rock candy. The preparation method is the same as that of KS005, but with an ice bath used during the mixing steps. The main difference seen is the two layer morphology, and the inner body’s opacity. Otherwise, colouring is very similar, and a similar degree of cracking was seen when compared to the KS005 sol-gel.</td>
<td></td>
</tr>
</tbody>
</table>
### Table 3.10: Results of the sol-gels prepared following Gorman’s methodology.\(^{316}\)

<table>
<thead>
<tr>
<th>Sol-gel</th>
<th>Normal light</th>
<th>UV light (and filter)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>KS007</td>
<td></td>
<td></td>
<td>The KS007 method produced a glassy sol-gel which readily cracked. The sol-gel had a darker orange-red appearance, and phosphorescence was slightly duller than other sol-gels produced.</td>
</tr>
<tr>
<td>KS008</td>
<td>N/A</td>
<td></td>
<td>This method was a modification of KS007, with the addition of the Pluronic F127 polymer. However, the solution was unable to gel without the addition of an alkaline initiator, such as sodium hydroxide or phosphate buffered saline.</td>
</tr>
<tr>
<td>KS009</td>
<td></td>
<td></td>
<td>The solution from KS008 was treated with the phosphate buffered saline, and was able to gel when placed in a desiccator. A pale orange, translucent sol-gel was produced, which was relatively fragile. Emission was seen when exposed to a UV lamp. SEM imaging showed that no through-pores were formed, which is likely due to the rapid gelation of the solution.</td>
</tr>
</tbody>
</table>
The higher polymer content in the KS012 sol-gel, compared to the KS009, increased opacity of the sol-gel, resulting in a more translucent product than the KS007 parent sol-gel. This product was less prone to cracking, and a relatively bright emission can be seen.

The lower water content of the KS013 sol-gel resulted in a more intense orange colour compared to KS012 monoliths. Crystals were again translucent, and exhibited a brighter emission when exposed to the UV light source.

Transparent solid chunks of sol-gel were achieved after gelation and drying, with a relatively deep orange colour. There is little difference between the KS007 and KS017 methods in terms of colour, strength (crushability) and cracking, hence the use of the ice bath makes little difference and is not required.
3.3.3 Modified gelation and incubation methods

As observed with previous iterations in Section 3.3.2, different incubation conditions can be used to solidify the sol-gels produced, including storage in a desiccator to dehydrate the gel, and incubation in an oven at 40°C. Addition of a basic solution can also induce gelation of the solution, such as sodium hydroxide or phosphate buffered saline. Subsequently, a sol-gel solution underwent different incubation conditions. For this study, a sol-gel solution, based on Collinson and co-workers’ methodology,311 and similar to KS005, with Pluronic F127 polymer added, was treated with different gelation and incubation periods, as described below in Table 3.11, to compare the resulting sol-gels.

*Table 3.11: Results of the modification of gelling and incubation conditions on the KS010 sol-gels.*

<table>
<thead>
<tr>
<th>Sol-Gel</th>
<th>Gelation Treatment</th>
<th>Incubation</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>KS010A</td>
<td>No treatment</td>
<td>Oven, 40°C</td>
<td>Solidified, glassy sol-gel, with cracking and flaky appearance</td>
</tr>
<tr>
<td>KS010B</td>
<td>PBS</td>
<td>Oven, 40°C</td>
<td>Solidified, glassy sol-gel, with a large degree of shrinkage</td>
</tr>
<tr>
<td>KS010C</td>
<td>PBS</td>
<td>Desiccator, RT</td>
<td>Solidified, translucent-opaque appearance, large degree of shrinkage</td>
</tr>
<tr>
<td>KS010D</td>
<td>No Treatment</td>
<td>Desiccator, RT</td>
<td>Did not gel/solidify without addition of base</td>
</tr>
</tbody>
</table>
As can be seen in Figure 3.13, the different methods of inducing gelation, and incubation, result in very different gel properties. KS010A and KS010C resulted in flaky, easily cracked products, whereas KS010B resulted in a sturdy sol-gel rod. The addition of the phosphate buffered saline (PBS) resulted in fairly rapid gelation of the solution, leading to a larger degree of shrinkage compared to the KS010A product. The use of the desiccator also produced a more opaque product, likely the result of more severe dehydration caused by the desiccant.
CHAPTER THREE

This study of the KS010 sol-gels and their gelation and incubation demonstrate the importance of selecting the right reaction parameters in order to produce a more desirable product. For example, a more translucent or transparent sol-gel is favourable in order to ensure an efficient transfer of photons to a detector for chemiluminescence detection; however, a more opaque product indicates the possibility of the sol-gel having undergone phase-separation, which would make it more useful as a monolith for HPLC applications.

3.3.4 Overall Discussion

Published methods were repeated in this work to gain a better understanding of the effect of several variables in order to immobilise the [Ru(bpy)_3]^{2+}. Through the addition of polymers to methods KS004, KS009, and KS010, which were imaged using scanning electron microscopy (SEM) to observe porosity, it became apparent that no phase separation occurred, even though prepared solutions were somewhat similar to monolith preparation methods reported in the literature. Due to the lack of phase separation, no macro pores were seen on any microscope images taken of selected methods, and therefore those methods would not be useful in preparation of a [Ru(bpy)_3]^{2+} monolithic column for both separation and detection. The exceptions to this were KS002, KS014 and KS015, which were prepared according to the monolith preparation procedure, and therefore could potentially undergo phase separation.

It also became apparent that the adsorption of [Ru(bpy)_3]^{2+} solutions onto a bare silica monolith may be an effective method of preparation for a detection column. The KS011 columns prepared using this method, though pale orange in colour and retaining little [Ru(bpy)_3]^{2+}, were solvent durable against common HPLC solvents.
such as acetonitrile and methanol, and were still phosphorescent when excited by the UV light.

Of the earlier sol-gel monoliths that were imaged using scanning electron microscopy, no macropores were seen when imaging. The lack of macropores suggests that those particular monolith sol-gels would be unsuitable as separation-detection combination columns. However, later batches of the sol-gels, such as KS012 and KS013, which contain the polymer, appear as though they may have the macropore structure desirable for use as a HPLC column. However, due to time and budget constraints, no further SEM imaging was able to be undertaken during this project to explore the possibility of macropore formation.

3.3.5 Potential Applications

The sol-gels created in this chapter have potential applications as on-column or post-column detectors, where the \([\text{Ru(bpy)}_3]^{3+}\) species could be directly reacted with analytes post separation, or the \([\text{Ru(bpy)}_3]^{2+}\) solution can be applied to a silica monolith column, such as in methods KS001 and KS011 as an on-column stationary/detection phase. The sol-gels could also be applied in photoredox catalysis, as tris(2,2'-bipyridyl)ruthenium(II) has been utilised in solution form for this application\textsuperscript{323-328} for a number of years.
3.4 Oxidation of immobilised tris(2,2′-bipyridyl)ruthenium(II) to tris(2,2′-bipyridyl)ruthenium(III)

In order for a chemiluminescence reaction to occur, the \([\text{Ru}(\text{bpy})_3]^{2+}\) must first be oxidised to \([\text{Ru}(\text{bpy})_3]^{3+}\) prior to reaction with an appropriate analyte.\(^{153}\) Commonly, in liquid phase chemiluminescence, the \([\text{Ru}(\text{bpy})_3]^{2+}\) is chemically oxidised either off-line using lead(IV) dioxide, or on-line with an oxidising solution such as cerium(IV) sulfate.\(^{153}\) On-line generation of \([\text{Ru}(\text{bpy})_3]^{3+}\) is favourable for the immobilised species as the reagent itself would be in a solid state, and so would require a liquid phase to oxidise.

Although the \([\text{Ru}(\text{bpy})_3]^{2+}\) was able to be encapsulated within the monolith and gel structures, in this work earlier sol-gels (KS002 and KS003) were unable to be oxidised by cerium(IV) sulfate. However, a previously published literature method\(^{153, 316, 329}\) stated heated nitric acid was able to oxidise \([\text{Ru}(\text{bpy})_3]^{2+}\) to \([\text{Ru}(\text{bpy})_3]^{3+}\), including \([\text{Ru}(\text{bpy})_3]^{2+}\) immobilised within a sol-gel structure.\(^{316}\) Sol-gel KS006 was chosen for oxidation, as several sol-gel rods remained in-tact, which would be desirable for a flow-through post-column detection application. A KS006 rod, and a sol-gel rod that had been crushed into both small and large particles, were oxidised using this nitric acid method, with the acid heated to around 50°C. After 5 minutes, the smaller sol-gel particles were fully oxidised, and had a bright green appearance. After 30 minutes, the larger particles and the full rod were fully oxidised using this method. After this process, the KS006 oxidised sol-gel rod and particles were left to sit to observe oxidation state longevity. After >6 months, the sol-gel rod had retained the green colour, as shown below in Figure 3.14a. Observation under a UV light next to an unoxidised sol-gel demonstrated a lack of \([\text{Ru}(\text{bpy})_3]^{2+}\) being present within the sol-gel, due to the sol-gel not emitting light (Figure 3.14b), but it
was not known whether this was caused by the oxidation, or degradation of the 
[Ru(bpy)$_3$]$^{3+}$ within the sol-gel.

Figure 3.14: (a) green oxidised sol-gel KS006, over 6 months after oxidation. (b) 
green 'oxidised' monolith and unoxidised [Ru(bpy)$_3$]$^{2+}$ sol-gel KS006 under a UV 
light.

To confirm that oxidation had occurred, the green sol-gel was photographed in a 
dark room following the addition of 1 mM ofloxacin. If the [Ru(bpy)$_3$]$^{3+}$ species was 
present, light would be emitted as the [Ru(bpy)$_3$]$^{3+}$ is reduced to an excited state 
[Ru(bpy)$_3$]$^{2+*}$, which would then release photons. Images were taken with 10 sec 
exposure to ensure the camera would be able to capture any light emitted.

As can be seen in Figure 3.15, over the course of 30 sec after the addition of the 1 
mM ofloxacin, the sol-gel began to emit light, with increasing intensity, 
demonstrating the presence of the oxidised species in the green sol-gel.
Figure 3.15: Evolution of the reaction of the oxidised KS006 sol-gel with 1 mM ofloxacin over the course of 30 seconds.

As previously discussed by Gorman\textsuperscript{316}, penetration of the sol-gel by the ofloxacin is slow, and therefore maximum emission is reached after an extended period of time. 2 minutes after addition of the 1 mM ofloxacin, the sol-gel was still emitting light, further demonstrating this slow penetration (Figure 3.16). Once the reaction was complete, the sol-gel returned to an orange appearance, and was able to be re-oxidised by the heated nitric acid.
Figure 3.16: Slow penetration of the sol-gel by ofloxacin (1 mM) after approximately 2 minutes of reaction.

The longevity of the oxidative state is an important part of the potential application of these sol-gels in chemiluminescence detection. By generating a stable oxidised species, the sol-gel could be stored in front of a photodetector for an extended period of time prior to being used for detection. Unfortunately, this sol-gel showed no flow-through properties, and so to be utilised in post-column detection would need to be crushed and inserted into tapered glass tubing for storage in front of a photodetector. Depending on the materials used in the manifold, online re-oxidation could potentially be achieved with hot nitric acid, without needing to remove the sol-gel, and so could be applied as a stable, regenerative oxidised chemiluminescence detection media.
CHAPTER THREE

4 Conclusions

This chapter explored the application of several stationary phases to the silica monolith columns prepared in Chapter 2. C_{18} functionalisation of the monoliths was achieved both pre- and post-encapsulation, and despite decreased resolution using the batch functionalised column when compared to a commercially available column, reasonable selectivity was achieved for the opiate alkaloid separation when combined with chemiluminescence detection.

The lubricin functionalisation was successful, and through the Tanaka Test showed possible anion exchange properties. Retention times were decreased compared to the unmodified column, however, and so may not be ideal for chromatographic separations.

Sol-gels incorporating the \([\text{Ru(bpy)}_3]^{2+}\) were successfully prepared, with a few methods (KS012 and KS013) showing potential for post-column chemiluminescence detection. Regenerative oxidation of the ruthenium complex within the sol-gels was able to be achieved using heated nitric acid, with the lifetime of the oxidised species being >6 months. Oxidised sol-gels could be applied as post-column detectors within a microfluidic device, due to the longevity and stability of the oxidised species.
CHAPTER FOUR

STUDIES IN ANALYTICAL CHEMILUMINESCENCE

MICROFLUIDIC DEVICE DESIGN

1. Introduction
2. Experimental
3. Results and Discussion
4. Conclusions
1 Introduction

Chemiluminescence detection has been widely used in analytical techniques such as flow injection analysis\textsuperscript{142, 145-146} and high performance liquid chromatography,\textsuperscript{33, 36, 139, 330} with sensitivity and selectivity the main drivers for its application in these disciplines. A number of applications for microfluidic devices have also been reported.\textsuperscript{331-335}

The basic instrumentation setup for chemiluminescence detection has remained mostly unchanged over the last 40 years, since methods such as flow injection analysis (FIA) were introduced.\textsuperscript{191, 336} The instrumentation used for chemiluminescence detection comprises a sample injection into a flowing carrier stream, merging with one or more reagents within a reaction chamber or zone, which is placed flush against the window of a photomultiplier tube, or similar detector. Technological advances have seen chemiluminescence detection setups miniaturised to fit within microfluidic devices,\textsuperscript{334, 337-338} however the setup remains mostly unchanged in these configurations as well.

Most commonly, solutions are merged at a T- or Y-shaped junction just prior to entering a reaction and detection coil, made of glass or polymer tubing.\textsuperscript{170, 191} However, recent advances in design and manufacturing capabilities have enabled the production of polymer flow-cells, which are able to incorporate both the solution merging points and mixing zones within the one cell.\textsuperscript{20, 215-217} Channel dimensions often utilised in chemiluminescence detection flow-cells are relatively large, usually $0.7 \times 0.7$ mm or $0.8 \times 0.8$ mm, which are incompatible with a microfluidic chip for several reasons: (1) depth of cut could compromise chip manufacturability, depending on thickness of material used; and (2) volumes of reagents required are...
large compared to most common devices, which have total volumes in the μL range. Common machined flow-cell channel configurations, such as spirals, or the serpentine with over 100 reversing turns, require machining on both sides of the flow-cell in order to enable solution merging within the centre of the detector. Ease of manufacture is a driving force for microfluidic devices, and multi-sided or multi-step manufacturing process can over-complicate a device and open it up to points of failure, hence many devices are single-layered, with very basic channel designs incorporated. Simple single-layer S-shaped serpentine mixers are commonly used in microfluidic devices to thoroughly mix solutions, but are seldom used for chemiluminescence detection.

Stieg and Nieman, plus Terry et al. have shown that white coloured materials are the most beneficial for sensitive chemiluminescence detection, as the material allows for greater reflectance of the light towards a detector. However, microfluidic chips are often prepared using transparent glasses or polymers, which have been shown to greatly reduce the sensitivity of chemiluminescence reactions. Yet, despite the ability for industry to readily produce coloured polymers and glasses which can be used in the manufacture of microfluidic devices, no research has explored the impact of coloured materials on chemiluminescence emissions.

This chapter aims to explore the effect of smaller channel dimensions, which are more suitable to a microfluidic application, on chemiluminescence detection sensitivity. Further to this, the effect of coloured materials on the reaction emissions and sensitivity will be explored for four model chemiluminescence reagents of varying emission wavelengths: enhanced potassium permanganate (λ_{max} 689 nm), [Ru(bpy)_3]^{2+} (λ_{max} 610 nm), luminol (λ_{max} 425 nm), and a purple
luminol-rhodamine B hybrid reagent (L+RB) (\(\lambda_{\text{max}}\) 425 nm and 590 nm). A novel image analysis technique will also be used to compare emission wavelengths and intensities across the coloured materials. Finally, a single-sided serpentine channel configuration, based on microfluidic S-shaped mixers, will be evaluated as an alternative to the commonly used serpentine channel configuration, to determine its potential as a mixing zone in a microfluidic device, and its impact on mixing efficiency and sensitivity of the model chemiluminescence reactions.
2 Experimental

2.1 Chemicals

Unless otherwise stated, all solutions were prepared in Milli-Q filtered deionised water (0.45 µm). Tris(2,2’-bipyridyl)ruthenium(II) was purchased from Strem Chemicals (Newbury Port, MA, USA). Ofloxacin, cerium(IV) sulfate, sodium polyphosphate, sodium thiosulfate, and luminol were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Morphine was supplied by SunPharma (Port Fairy, VIC, Australia). Potassium permanganate was purchased from Chem Supply (Gillman, SA, Australia). Sulfuric acid was obtained from Merck (Bayswater, VIC, Australia). Sodium hydroxide was purchased from Ajax Chemicals (Sydney, NSW, Australia). Rhodamine B was purchased from BDH Chemicals (Poole, England). Commercial bleach and food dye (Queen Fine Foods, Alderley, QLD, Australia) were purchased at a local supermarket.

2.2 Reagents

2.2.1 Enhanced potassium permanganate

Enhanced potassium permanganate was prepared daily using potassium permanganate (1.9 mM) in sodium polyphosphates (1% w/v) and deionised water. The solution pH was adjusted to 2.5 through drop-wise addition of concentrated sulfuric acid and then sodium thiosulfate (0.6 mM) was added.

2.2.2 Tris(2,2’-bipyridyl)ruthenium(III)

Tris(2,2’-bipyridyl)ruthenium(II) chloride hexahydrate solutions (1 × 10⁻³ M) were prepared daily by dissolving [Ru(bpy)₃]Cl₂.6H₂O crystals in 0.05 M sulfuric acid. 1 mM cerium(IV) sulfate was also prepared in 0.05 M sulfuric acid, and was reacted
with Ru[(bpy)]$_3^{2+}$ on-line to form the oxidised [Ru(bpy)$_3^{3+}$ species. The oxidised ruthenium reagent was reacted with ofloxacin, prepared as 1.0 mM stocks, and diluted in 0.05 M sulfuric acid as required.

2.2.3 Luminol

The luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) reagent (2.6 mM) was prepared in 0.1 M sodium hydroxide, and sonicated until dissolved. Sodium hypochlorite solutions ($2.8 \times 10^{-6}$ M – $2.8 \times 10^{-7}$ M) were prepared by dilution of a commercial bleach solution (42 g/L sodium hypochlorite, 9 g/L sodium hydroxide) in the 0.1 M sodium hydroxide stock.

2.2.4 Luminol + Rhodamine B

For the preparation of the luminol + rhodamine B (L+RB) reagent, rhodamine B (1.7 mM) was added to the 2.6 mM luminol reagent. The sodium hypochlorite reagents prepared for the luminol reactions were also used with the L+RB reagent system.

2.2.5 Dye solutions

Various dye solutions were prepared for the testing of the serpentine flow-cells. To examine mixing within the serpentine flow-cells, red and blue dye solutions were prepared by dilution of 1.0 mL of food dye into 25 mL of deionised water.

2.3 Flow Injection Analysis

The flow injection analysis (FIA) manifold used for chemiluminescence detection consisted of a Harvard Instruments PHD Ultra syringe pump, black manifold tubing (0.76 mm i.d.) and a six-port injection valve (Vici 04 W-0192L Valco Instruments, Houston, Texas, USA) with a 70 µL injection loop. Flow-cells were mounted against
the window of an extended range photomultiplier tube (PMT) (Electron Tubes P30A-05, ETP, NSW, Australia) using a custom-built flow-cell holder\(^{215}\) within a light-tight housing. Data output was collected using an eDAQ e-corder 410 data acquisition system (eDAQ, NSW, Australia).

For reactions with the enhanced potassium permanganate and luminol reagents, the analyte standards (morphine and sodium hypochlorite) were injected into a deionised water carrier stream, which merged with the chemiluminescence reagent within the flow-cell (see Figure 4.1).

For the coloured flow-cells work with \([\text{Ru(bpy)}_3]^{2+}\), the ofloxacin analyte was injected into the cerium(IV) sulfate carrier stream, which merged with the \([\text{Ru(bpy)}_3]^{2+}\) reagent within the flow-cell.

To oxidise the \([\text{Ru(bpy)}_3]^{2+}\) complex to the excited \([\text{Ru(bpy)}_3]^{3+}\) state for the serpentine flow-cell comparison, a reverse FIA system was used, where \([\text{Ru(bpy)}_3]^{2+}\) was injected into a cerium(IV) sulfate oxidant carrier stream, which merged with the ofloxacin analyte within the flow-cell. This method allowed for oxidation of the \([\text{Ru(bpy)}_3]^{2+}\) reagent prior to merging with the analyte, and therefore enhancing the signal intensities achievable in comparison with normal-FIA methodologies.
Figure 4.1: Flow injection manifold setup, using a syringe pump. Potassium Permanganate FIA: A- KMnO₄, B-Deionised Water, C- Analyte. [Ru(bpy)₃]²⁺ reverse-FIA: A- Analyte, B- Cerium(IV) Sulfate (in 0.05 M H₂SO₄), C – [Ru(bpy)₃]²⁺ reagent, in 0.05M H₂SO₄. Luminol FIA: A- Luminol in 0.1 M NaOH, B- Deionised Water, C- Analyte in 0.1 M NaOH.

2.4 Flow-Cell Manufacture

Acrylic sheets (4.5 mm thick) (clear, white, black, red and blue) were purchased from Showcase Plastics (Melbourne, VIC, Australia) and cut into 34 × 46 × 4.5 mm chips, into which channels (0.4 mm wide × 0.2 mm deep) were machined. Three designs for the flow-cell reaction zone were used: a simple spiral (channel length 325 mm; channel volume: 26 µL); a spiral serpentine, consisting of 116 reversing turns, similar to previous designs⁷⁴-⁷⁵ (channel length: 282 mm; channel volume: 23 µL); and a linear serpentine, based on the S-shaped serpentine mixers used in microfluidic devices⁷⁴ (channel length: 344 mm; channel volume: 28 µL). Three-dimensional representations of the flow-cells were drawn in SolidWorks 2015 (Dassault Systèmes, S.A., Vélizy, France), and from these models, the machining G-code was generated using NX 10.0 software (Siemens, Munich, Germany). The channels were machined using a Datron M7 HP CNC milling machine (Datron,
Mühlthal, Germany) with a 0.4 mm diameter, two fluted end mill (Datron) at a spindle speed of 48,000 RPM and linear feed of 200 mm/min in a single pass, with a cut depth of 0.2 mm.

The spiral and spiral serpentine flow-cell designs used for the comparison of the coloured flow-cells were sealed using polyolefin adhesive qPCR sealing tape (Sarstedt, Mawson Lakes, SA, Australia) on both the front and back faces, cut to approximately 2 × 2 cm squares, and laminated onto the flow-cells using a Drytac Jetmounter Laminator (Richmond, VA, USA).

The spiral and linear serpentine used in the channel design comparison were sealed with clear 2 mm thick acrylic sheets, similar to sealing methods used for microfluidic devices. This was done to ensure that channels would be properly sealed when incorporated into a device. The clear acrylic cover sheet was laminated with double sided tape (Tesa 4965 205 µm double sided transparent tape, Eastern Creek, NSW, Australia), and cut using a laser cutter (Trotec SP500 Laser, Sunshine West, VIC, Australia) with through holes for the two inlet and one outlet tubes also cut by laser. The clear acrylic top plates were then laminated onto the white acrylic flow-cells. Pieces of 0.5 mm i.d. clear PTFE tubing (DKSH, Hallam, VIC, Australia) were cut to length (~ 4 cm) and sealed into the pre-cut top plate holes using epoxy adhesive (Parfix, Padstow, NSW, Australia). The back of the spiral serpentine flow-cell was sealed with a 2 × 2 cm square of polyolefin adhesive qPCR sealing tape.
Figure 4.2: Flow-cell configurations used throughout this work. All flow-cells are 34 × 46 × 4.5 mm in size. A-C Configurations of flow-cells used in the Coloured Flow-Cells experiments. (a) spiral front face, (b) spiral serpentine front face, (c) back face of both spiral and spiral serpentine cells. D-F Configurations of flow-cells used for serpentine comparisons. (d) Spiral Serpentine front face; (e) spiral serpentine back face; (f) linear serpentine front face.

2.5 COMSOL® Simulations

Simulations of solution flow within the serpentine channels was performed in order to analyse the mixing ability of the different channel configurations. A software package, COMSOL® Multiphysics (Stockholm, Sweden) was used for the simulations, which was capable of performing time-dependent studies of solution
mixing. For the purposes of COMSOL® simulations of flow properties through the channels, the aforementioned three-dimensional flow-cell designs were modified in SolidWorks® to form a solid body of the channel for importation into COMSOL Multiphysics® Software. Mesh densities were generated and modified to maximise solving efficiency and accuracy. Laminar flow and diluted species studies were performed in the software to observe pressure, velocity and solution mixing within each of the flow-cell channel designs.

2.6 Image Analysis

To obtain photographs of the chemiluminescence reactions within each of the coloured flow-cells and flow-cell designs, chemiluminescence reagents were continuously merged with high concentration analytes (Figure 4.4) and photographed using a Canon EOS 6D Digital SLR camera (Tokyo, Japan) (ISO-3200, f-stop 2.8, exposure: KMnO₄ and luminol: 10 s; [Ru(bpy)₃]²⁺: 30 s). ImageJ (NIH, USA), a public domain image processing and analysis software, was used to analyse all chemiluminescence images.

For analysis of the coloured flow-cells, RGB Analysis software in ImageJ was used, and a circular zone comprising the entire channel area was selected for analysis (see Figure 4.3). To gain further insight into the intensity profiles, white and black spiral flow-cell images were analysed using a straight line across the centre of the flow-cell and the ‘Plot Profile’ tool. Data was then extracted, and intensity was plotted against the corresponding channel length, and time.
Figure 4.3: Selected areas on flow-cell images for image analysis (yellow circles); and ‘Plot Profile’ analysis line (green line).

For serpentine channel design comparison, photographs were analysed using ImageJ software to determine the point of highest light intensity, using the in-built ‘Plot profile’ tool. Points were plotted on the SolidWorks model, and path length measured using the in-built measuring tool in SolidWorks to determine distance to maximum emission.

Figure 4.4: Chemiluminescence reaction image capture setup within a dark room. Reagent and analyte solutions were continuously merged within the flow-cell, with a camera focussed at the flow-cell. In order to oxidise the $[\text{Ru(bpy)}_3]^{2+}$, the 1 mM reagent solution was mixed with 1 mM Ce(IV) sulfate off-line prior to imaging. This setup was also utilised to photograph dyes merging within the spiral and linear serpentine flow-cells.
2.7 Chemiluminescence spectra and CIE plots

The emission spectra for chemiluminescence reactions were collected using an Ocean Optics QE65000 Pro spectrometer (Quark Photonics, Waverley, VIC, Australia), configured with an open slit. The spectrometer was interfaced with the chemiluminescence flow-cell using a 0.5 m, 1000 µm core diameter fibre and a 30 mm collimating lens (Ocean Optics COL-30-UV) and custom flow-cell holder. Spectra were collected with a 10 s acquisition time, and a continuous flow method using a peristaltic pump as described above. CIE (1931) plots were used to visually represent the perceived colour changes caused by each flow cell absorbing or reflecting different wavelengths of light. These plots were produced using OriginLab Pro with the Origin Chromaticity Diagram Template (OriginLab Corporation, Northampton, MA, USA). CIE (1931, 2°) coordinates for each reaction were calculated from interpolated emission spectra using CIE standard observer colour matching functions.
3 Results and Discussion

3.1 Smaller Channel Dimensions

Microfluidic devices are used in applications where minimal sample volume is available, and where minimising reagent consumption is optimal. Conventional machined flow-cells commonly use relatively large channel dimensions, such as 0.8 × 0.8 mm or 0.7 × 0.7 mm (w × d), making the design unsuitable for direct application to a microfluidic device. Therefore, preliminary studies were undertaken to explore the use of smaller channel dimensions in spiral and serpentine flow-cell designs.

Channels of 0.4 × 0.2 mm (w × d) were machined into white acrylic plastic, and chemiluminescence intensities were compared using FIA, in which the enhanced potassium permanganate reagent was reacted with standard solutions of morphine, as is commonly performed by our research group for flow-cell comparisons. An equivalent linear velocity to that used in the larger channel dimensions for chemiluminescence reactions was required in order for the reaction kinetics to be similar within the smaller channel dimensions. The calculation is shown below in Equation 4.1.
linear velocity = \frac{flow rate}{channel area}

v = \frac{f}{A}

v = \frac{3.5 \text{ mL/min}}{0.7 \times 0.7 \text{ mm}}

v = 3.5 \div 0.49

v = 7.14 \text{ m/min}

7.14 = \frac{f}{0.4 \times 0.2 \text{ mm}}

f = 7.14 \times 0.08

f = 0.57 \text{ mL/min}

Equation 4.1: Calculation for flow rate for 0.4 × 0.2 mm channel dimensions with the same linear velocity as the 0.7 × 0.7 mm channels. Where v is the linear velocity, f is the flow rate, and A is the channel area.

It was determined that a flow rate of 0.57 mL/min per line was required within the smaller channels in order to maintain the linear velocity desirable for the chemiluminescence reaction kinetics.

Despite the new channel dimensions having a cross-sectional area 16% of the commonly used 0.7 × 0.7 mm channel cross-sectional area,\textsuperscript{174-175} chemiluminescence intensities for the enhanced potassium permanganate reagent were only decreased by ~66% with 1 × 10\textsuperscript{-7} M morphine in the spiral serpentine configuration, which was comparative with literature values.\textsuperscript{177}
Figure 4.5: Comparison of reduced channel dimensions for spiral flow-cells (black columns) and spiral serpentine flow-cells (grey columns). Enhanced potassium permanganate (KMnO₄) (1.9 mM with 1% polyphosphates) was reacted with 1 × 10⁻⁷ M Morphine. 1σ error shown, n=5. 0.7 × 0.7 mm spiral serpentine comprises 114 reversing turns; 0.4 × 0.2 mm spiral serpentine comprises 116 reversing turns.

The results, shown in Figure 4.5, demonstrated a similar trend as previously observed between spiral and spiral serpentine channel configurations.¹⁷⁴⁻¹⁷⁵ The spiral serpentine configuration produced chemiluminescence signal intensities marginally better than the spiral configuration, and a slightly better limit of detection for morphine was observed with the spiral serpentine flow-cell, as shown below in Table 4.1. These limits of detection are similar to previously reported limits of detection for morphine in the literature.²²³
Table 4.1: Limits of detection and correlation for enhanced potassium permanganate reacted with morphine within white acrylic spiral and spiral serpentine configuration flow-cells with the smaller 0.4 × 0.2 mm channel dimensions. Calibration function derived from log-log plot of concentration and intensity.

<table>
<thead>
<tr>
<th>Flow-Cell</th>
<th>Calibration Function</th>
<th>$R^2$</th>
<th>LOD (M)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spiral</td>
<td>$y = 0.9999x + 8.5021$</td>
<td>0.999</td>
<td>$3.5 \times 10^{-9}$</td>
<td>0.525</td>
</tr>
<tr>
<td>Spiral Serpentine</td>
<td>$y = 0.9580x + 8.2631$</td>
<td>0.999</td>
<td>$2.7 \times 10^{-9}$</td>
<td>0.295</td>
</tr>
</tbody>
</table>

One of the major advantages of the smaller channel design is the reduction in waste due to the slower flow rate used. Therefore, despite the lower signal intensities generated, and slightly worse limits of detection, the smaller channel dimensions were chosen to be utilised for all later experiments, due to their greater compatibility with integration into a microfluidic device.

3.2 Coloured Flow-Cells

Material choice is an important factor for chemiluminescence detection flow-cells, as demonstrated in the studies of Terry and co-workers. Stieg and Nieman’s research in the early days of flow injection analysis had compared a white and dark polymer material for use as a flow-cell (in their case, a reaction vessel, where reactants merged and mixed within a void in a piece of plastic). A dark brown polymer was used as the dark polymer, instead of a black material, and their results showed that a white polymer material would produce signal intensities two times greater than those achievable with the darker flow-cell, due to the greater reflectance of the light to the detector by the white material. Beyond this exploration, no previous research had to-date explored the effect of coloured polymers on chemiluminescence emission intensities.
Coloured acrylic materials, which were reflective at similar wavelengths to common chemiluminescence emissions, were selected to analyse the effect of material colour on chemiluminescence intensities. For these experiments, clear, white, black, red and blue polymer sheets were used, into which both the spiral and spiral serpentine channel configurations were machined (see Figure 4.2), however, only the results for the spiral serpentine flow-cells are shown, due to their superiority in terms of signal intensities and limits of detection, as discussed in the previous section. A mirror backing was also given to the clear flow-cells for comparison, to reflect more light back to the detector, a commonly utilised method in the literature.139, 174-175, 215, 218, 350-351

3.2.1 Chemiluminescence Reactions

To examine the coloured flow-cells, we chose chemiluminescence reactions with differing emission wavelengths that matched the colours of the materials chosen. As seen in Figure 4.6, the four chemiluminescence reactions span most of the visible spectrum, with differing emission maximum wavelengths ($\lambda_{\text{max}}$). Interpolation of the emission spectra data allows the emission to be plotted on a CIE plot, a visual representation of observed emission colours, as seen in Figure 4.6b.
Figure 4.6: (a) Normalised spectra for chemiluminescence reactions with the four reagents: luminol (blue line), L+RB (purple line), tris(2,2'-bipyridyl)ruthenium(II) (orange line), permanganate (red line), performed in the white spiral serpentine-configuration flow-cell. (b) CIE Plot of the overall emission colour of each chemiluminescence reaction, with coordinates for each reaction emission determined through extrapolation from spectral data.

Two emission peaks were observed in the spectra for the L+RB reagent (Figure 4.6a), resulting from the addition of the Rhodamine B, causing the peak at around 600 nm. As shown in Figure 4.7, these four reactions also have differing kinetic
profiles. Using data extracted from ImageJ ‘Plot Profile’ analysis of the reactions within a white spiral flow-cell, it was shown that the photographic analysis technique was able to produce an emission profile similar to those attainable using stopped-flow analysis.169, 352-354

Figure 4.7: Emission intensity profile of (a) enhanced potassium permanganate; (b) \([\text{Ru(bpy)}_3]^{2+}\); (c) luminol; and (d) L+RB. Profiles were prepared using data extrapolated from photographs of the reactions within the white spiral flow-cell, using the ‘Plot Profile’ tool in ImageJ. Raw data values (red dots) and general trend lines (black lines) are shown.

Enhanced potassium permanganate is a very fast chemiluminescence reaction, reaching peak intensity very quickly, before fading, as seen in Figure 4.7a. \([\text{Ru(bpy)}_3]^{2+}\), luminol and the L+RB reactions are all slower kinetically, however it is an established fact that the emission lifespan of \([\text{Ru(bpy)}_3]^{2+}\) is much shorter than that of luminol.353
3.2.1.1 Enhanced potassium permanganate

Figure 4.8: Chemiluminescence from the reaction of the enhanced acidic potassium permanganate reagent with morphine (1 × 10⁻⁵ M), within the: (a) white, (b) red, (c) blue, (d) black, (e) clear and (f) clear+mirror flow-cells with spiral serpentine channel configuration. (g) Comparison of chemiluminescence emission intensity from each spiral serpentine flow-cell by analysis of digital photographs (white columns, left axis) and measured using a PMT under FIA conditions (grey columns, right axis). 1σ error shown, n=5.
With the enhanced potassium permanganate reaction (Figure 4.8), the red flow-cell produced the second greatest emission intensity, approximately 65% of the intensity of the white flow-cell. Red pixel analysis of the channel area using the RGB Analysis software in ImageJ showed a similar trend of emission intensities across the flow-cells compared to the emissions observed with flow injection analysis. The greater reflectance of the emission by the white and red flow-cells, as seen in Figure 4.8, enables the reaction to be observed throughout most of the cell, whereas the darker materials that absorb the light make it appear as though the emission is much shorter lived than what it actually is. This is important when considering material choices for preparation of any chemiluminescence detection zone, including those in a microfluidic device, as a more reflective material will enable greater sensitivity, especially with such small channels and sample volumes.

Limits of detection were determined for morphine with the enhanced potassium permanganate reagent (Table 4.2, below), within each of the coloured flow-cells, with the lowest limits being observed with the white and red flow-cells, in the order of nanomolar (nM) concentration of morphine for both the spiral and spiral serpentine flow-cell configurations. Better detection limits within each coloured flow-cell were observed with the spiral serpentine cell.
Table 4.2: Limits of detection for enhanced potassium permanganate and morphine, with each coloured flow-cell.

<table>
<thead>
<tr>
<th>Flow-Cell</th>
<th>Limit of Detection (M)(^a)</th>
<th>Spiral Configuration</th>
<th>Spiral Serpentine Configuration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clear</td>
<td>2.8 × 10(^{-8})</td>
<td>1.8 × 10(^{-8})</td>
<td></td>
</tr>
<tr>
<td>Clear + Mirror</td>
<td>1.4 × 10(^{-8})</td>
<td>1.4 × 10(^{-8})</td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>3.5 × 10(^{-9})</td>
<td>2.7 × 10(^{-9})</td>
<td></td>
</tr>
<tr>
<td>Black</td>
<td>3.1 × 10(^{-8})</td>
<td>3.1 × 10(^{-8})</td>
<td></td>
</tr>
<tr>
<td>Red</td>
<td>7.2 × 10(^{-9})</td>
<td>6.0 × 10(^{-9})</td>
<td></td>
</tr>
<tr>
<td>Blue</td>
<td>4.3 × 10(^{-8})</td>
<td>1.5 × 10(^{-8})</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Limit of detection calculated using 3\(\sigma\) of the blank
3.2.1.2 Tris(2,2′-bipyridyl)ruthenium(II)

Figure 4.9: Chemiluminescence from the reaction of 1 mM tris(2,2′-bipyridyl)ruthenium(II), 1 mM cerium(IV) sulfate and $1 \times 10^{-5}$ M ofloxacin, within the: (a) white, (b) red, (c) blue, (d) black, (e) clear and (f) clear+mirror flow-cells with spiral serpentine channel configuration. This combined image (a-f) has been adjusted (brightness 100, exposure 1) using Photoshop for visualisation purposes only. (g) Comparison of chemiluminescence emission intensity from each spiral serpentine flow-cell by analysis of digital photographs (white columns, left axis) and measured using a PMT under FIA conditions (grey columns, right axis). $1\sigma$ error shown, $n=5$. 
Although no significant improvement of emission intensities was observed with any of the coloured flow-cells (i.e. red or blue) with the ruthenium reagent (Figure 4.9), the red flow-cell was able to produce slightly better emissions than the Clear+Mirror flow-cell. The limits of detection (LoD) were marginally better with the red flow-cell, however for all flow-cell colours, limits of detection were within one order of magnitude of the white flow-cell LoD, as seen in Table 4.3. It is hypothesised that the use of an orange coloured flow-cell may be able to enhance the emission from the reaction of [Ru(bpy)$_3$]$^{3+}$ with ofloxacin, but experimentation would be required to confirm this.

Table 4.3: Limits of detection for the reaction of 1 mM [Ru(bpy)$_3$]$^{3+}$ with ofloxacin, within each coloured flow-cell.

<table>
<thead>
<tr>
<th>Flow-Cell</th>
<th>Limit of Detection (M)$^a$</th>
<th>Spiral Configuration</th>
<th>Spiral Serpentine Configuration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clear</td>
<td>4.3 × 10$^{-7}$</td>
<td>4.1 × 10$^{-7}$</td>
<td></td>
</tr>
<tr>
<td>Clear + Mirror</td>
<td>3.1 × 10$^{-7}$</td>
<td>1.9 × 10$^{-7}$</td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>8.6 × 10$^{-8}$</td>
<td>7.9 × 10$^{-8}$</td>
<td></td>
</tr>
<tr>
<td>Black</td>
<td>5.7 × 10$^{-7}$</td>
<td>5.2 × 10$^{-7}$</td>
<td></td>
</tr>
<tr>
<td>Red</td>
<td>2.3 × 10$^{-7}$</td>
<td>1.0 × 10$^{-7}$</td>
<td></td>
</tr>
<tr>
<td>Blue</td>
<td>6.7 × 10$^{-7}$</td>
<td>4.7 × 10$^{-7}$</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Limit of detection calculated using 3σ of the blank
3.2.1.3 Oxidation of luminol with hypochlorite

Figure 4.10: Chemiluminescence from the reaction of luminol (2.6 mM) with sodium hypochlorite (5.6 mM) in alkaline solution, within the: (a) white, (b) red, (c) blue, (d) black, (e) clear and (f) clear+mirror flow-cells with spiral serpentine channel configuration. (g) Comparison of chemiluminescence emission intensity from each spiral serpentine flow-cell by analysis of digital photographs (white columns, left axis) and measured using a PMT under FIA conditions (grey columns, right axis). 1σ error shown, n=5.
Absorption of the blue emission of luminol by the red flow-cell can be seen in Figure 4.10b, compared to Figure 4.10c, where a brighter emission is seen with the blue flow-cell. Sharp images with clearly defined channels were obtained with the red, black and clear flow-cells, due to absorption of the light by the material and surrounding environment (in the case of the clear flow-cell). The clear+mirror and blue flow-cells are able to reflect a greater amount of light back towards the detector, creating a ‘fuzzy-looking’ image, with less definition due to light scattering.\textsuperscript{174, 177, 215} This suggests that for photographing chemiluminescence reactions, or flow-cell configurations to look at channel details, a clear or darker coloured flow-cell is favourable, as the intense emission emanating from the white flow-cell can cloud the channel detail, and the evolution of the chemiluminescence reaction. Closer examination of Figure 4.10b (the reaction within the red coloured flow-cell) also shows the evolution of nitrogen gas as part of the oxidation of luminol with hypochlorite. This was previously observed in the exploration of square-serpentine channels, where gas bubbles were captured within the corners of the square-serpentine channels, affecting the flow of the solutions within the flow-cells.\textsuperscript{176}
3.2.1.4 Oxidation of luminol in the presence of Rhodamine B

![Chemiluminescence spectra for the oxidation of luminol (blue line) and oxidation of luminol in the presence of rhodamine B (purple line).](image)

Normalising the emission spectrum intensity of the Luminol + Rhodamine B (L+RB) reagent against that of the luminol emission, a 50% reduction in the intensity of the L+RB emission is visible, due to the partial energy transfer to the luminophore, rhodamine B, from the luminol system. Photograph analysis was able to provide insight into the mechanisms of wavelength absorption by the different coloured polymers.
Figure 4.12: Chemiluminescence from the reaction of the L+RB reagent with sodium hypochlorite (5.6 mM) in alkaline solution, within the: (a) white, (b) red, (c) blue, (d) black, (e) clear and (f) clear+mirror flow-cells with spiral serpentine channel configuration.

Careful examination of the spiral serpentine photographs demonstrates significant colour changes in the emissions, caused by the absorption of specific wavelengths by the material. The white flow-cell provides a “pure” emission colour – the total light is reflected back towards the detector, providing the actual colour of emission, as shown previously in Figure 4.6b. In Figures 4.12b and 4.12c, we see a transition into a darker purple emission with the red flow-cell, and a more blue emission with blue flow-cell, due to absorption of specific wavelengths by these materials. Similar trends are observable with the black, clear and clear+mirror flow-cells, but not to the same extent.

Plotting the RGB values on the CIE (1931) Plot allowed for a clearer visual representation of the changes in emission colour, as seen in Figure 4.13b.
Figure 4.13: (a) Chemiluminescence spectra for the reaction of the L+RB reagent with sodium hypochlorite in the white (grey line), blue (blue line) and red (red line) spiral serpentine configuration flow-cells. (b) CIE (1931) Plot of L+RB emissions within different coloured flow-cells. Two distinct groupings are visible, a more purple-pink colour grouping with the white, red and clear+mirror flow-cells, and a bluer grouping with the black, blue and clear flow-cells.

Examining the emission spectra of the L+RB system in the white, red, and blue flow-cells (Figure 4.13a) also highlights the mechanism of wavelength absorption of the flow-cells. The red flow-cell produces a significantly diminished emission.
intensity in comparison to the emission within the white flow-cell, due to the shorter wavelength emission absorption by the red coloured flow-cell material, with a more equivalent red-blue intensity, which is observed as a bathochromic shift in Figure 4.12. The blue flow-cell, while also decreasing overall emission intensity shows a much greater degree of longer wavelength absorption by the flow-cell material, which causes an observed hypsochromic shift in emission wavelengths, seen in Figure 4.12c. Groupings of more purple emissions are seen on the right of Figure 4.13b, where the longer wavelengths are better reflected by the materials, while more blue emissions are grouped towards the left of Figure 4.13b. The “darker” materials (blue, black and clear flow-cells) create environments which absorb the longer wavelengths, producing the bluer emissions seen.

### 3.2.2 Light transfer methods

This research into the effects of coloured materials on emissions also highlighted the methods of transmission of light to a photodetector. A photodetector measures the light emitted from a chemiluminescence reaction, which can be reflected to the photodetector via three pathways: (1) direct transfer of photons from the reaction to the photodetector; (2) the reflection of photons from near the surface of the flow-cell material; and (3) photons that travel through a significant portion of the flow-cell before being reflected to the detector by either the polymer or the flow-cell housing. These mechanisms of light transfer are illustrated in Figure 4.14 below. The variation in chemiluminescence intensities across the different coloured flow-cells is influenced by photons reflected from the flow-cell itself.
Figure 4.14: Mechanisms of light transfer to the photodetector from a chemiluminescence reaction. Pathway 1: photons travelling directly from the reaction to the detector; pathway 2: photons reflected to the detector from near the surface of the flow-cell material; pathway 3: photons which travel through a significant portion of the flow-cell before being reflected towards the detector.

By examining intensities from the clear and black flow-cells, which demonstrate light detected from the direct transfer of photons from the reaction to the photodetector through the clear window, only ~20% of the light is from this pathway, in comparison to all methods of reflection shown with a white flow-cell. The other ~80% of light detected comes from reflectance of the light from the flow-cell material and surrounding housing, demonstrating the importance of using a reflective, opaque material for chemiluminescence detection to improve sensitivity.

While the red and blue coloured flow-cells were able to increase light transfer to the detector in comparison to the commonly used clear and clear+mirror flow-cells, they were unable to reflect as much light as the white flow-cell for any of the chemiluminescence reactions explored in this work.

3.3 Spiral Serpentine vs Linear Serpentine

Previous research\textsuperscript{174-177, 215, 355} has shown that a spiral serpentine configuration is ideal to achieve maximum mixing efficiency, generating higher emission intensities,
and therefore lower limits of detection. However, the design requires machining on both sides of the material, to enable the initial mixing to occur in the centre of the photomultiplier tube, where it is most sensitive, making manufacture a more onerous task. When considering the design of a microfluidic device, fast manufacturing processes are favourable, and as such, multi-sided designs are often avoided.\textsuperscript{340} It is also important to note, that the more steps in a device manufacturing process there are, the more possible points of failure there will be. It was also observed when performing the coloured flow-cell comparison, that the use of commercial finger-tight screw-in fittings in the house-made flow-cells for the reagent inlets often proved problematic, with leakages sometimes occurring, and Teflon sealing tape required to overcome some leakages.

With these observations in mind, the most ideal chemiluminescence mixing-detection zone for a microfluidic device would be; (a) single-sided, with no tedious double sided machining; (b) easy to design and manufacture, to enable mass-production; and (c) still maintain good mixing efficiency; and for the device to be sealed in a manner which could enable reagent inlets which did not require screw-in fittings.

S-shaped serpentine channels are often used in microfluidic devices to promote solution mixing,\textsuperscript{270, 340-343} and could potentially be utilised as chemiluminescence mixer-detection zones. A modification to the spiral serpentine was made, reflecting these S-shaped mixers, as can be seen in Figure 4.15, and will herein be referred to as the linear serpentine design.
The linear serpentine design, though not as tight-knit as the spiral serpentine, reflected the use of reversing turns to maximise mixing efficiency. The simpler design requires machining on only one side of the flow-cell, with no through-holes or complex fittings required to connect with the flow-manifold. The overall channel length is 344 mm, slightly longer than that of the spiral serpentine flow-cell used for these experiments (282 mm), shown in Figure 4.15. It is important to note that the linear serpentine design is significantly easier to produce using computer aided design (CAD), due to the use of simple design elements such as straight lines and semi-circles. Such a design would also be more favourable for mass-production methods, such as injection moulding, due to being a single-sided design.

The serpentine flow-cells were also sealed using a technique commonly employed for microfluidic devices, where a thin sheet of clear acrylic is laminated onto the cell’s surface with adhesive tape, and inlets and outlets are fitted through pre-cut holes and glued in place with leak-proof epoxies.
To better understand the mechanisms of solution mixing within each design, simulation software was used to model mixing and observe physical forces at work within each of the flow-cell configurations.

Figure 4.16: Velocity profiles within the first reversing turns of the spiral and linear serpentine flow-cells.

Areas of similar high and low velocity were observable within each of the channel designs, with the maximum velocity around 0.25 m/s at the apex of each turn. This maximum velocity matches closely with the determined linear velocity per inlet in Equation 4.1, of 7.14 m/min, or 0.12 m/s. The use of two inlets doubles the observed linear velocity, which is shown here in Figure 4.16.

Upon closer inspection, as the solutions moved around each turn within the channels, an uneven distribution of velocity was observed, where velocity increased around the corner, and then decreased again immediately following. This relates to centripetal forces, where the solution on the outside of the curve (the larger diameter) has a lower pressure and moves slower than that going around the inner curve with a smaller diameter. Since both flow cell designs have the same channel diameter, but different arc diameters for their reversing turns, slightly different velocity gradients are observed, with a more uniform distribution of velocity observed with
the linear serpentine and its less rapid change in direction. This change in velocity allowed the better mixing of solutions within the spiral serpentine flow-cell.

When observing the simulated mixing within the linear serpentine flow-cell design (Figure 4.17b), it quickly became apparent that the long stretches of channel without turns had no positive effect on the mixing of the solutions. The solutions were fully mixed within three turns of the linear serpentine, but due to the long straight stretches of channel, which had no significant effect on mixing, the distance to solution homogeneity was much greater than that of the spiral serpentine (8.8 mm vs 16.7 mm). This could be overcome in future iterations by reducing the length of straight stretches and creating grids/columns of closer reversing turns.
Figure 4.17: Mixing simulations of solutions within the spiral (a and c) and linear (b and d) serpentes using COMSOL® software. Full mixing profiles are shown in Figures (a) and (b); and zoomed in profiles where the solution fully mixed are show in (c) and (d). Figure d shows the second fully reversing turn of the linear serpentes.

The solutions within the spiral serpentine were mixed in a much shorter distance compared to the linear serpentine, and within the first fully reversing turn of the front surface. However, the solutions began mixing on the back surface of the flow-cell where they met, and in the through-hole from the back to front surfaces, equating to three turns in total, similar to the linear serpentine.

The COMSOL® simulations were able to provide insight into the methods of mixing within the flow-cell channels; however, they cannot account for effects such as
turbulence from connectors or the pump, or any minor channel obstructions that may be left behind from the machining process. To observe the mixing in a real world scenario, images were taken of coloured dye solutions mixing within the channels, so a better understanding of the real-world mixing mechanisms could be gained.

![Image](image.png)

**Figure 4.18: Photographs of dyes merging within the spiral and linear serpentine configuration flow-cells. 0.57 mL/min per line flow rate was used.**

Similar results to the COMSOL® simulations were observed when photographing the dyes at the same linear velocity used with the chemiluminescence reactions, shown in Figure 4.18. Distance to solution homogeneity within the spiral serpentine was quite short, around 17 mm, including the distance through the flow-cell from the back to the front face. The distance for the linear serpentine was 149 mm, almost 10 times the distance compared with the spiral serpentine, due to the long straight stretches of channel, as previously discussed.
Figure 4.19: Chemiluminescence reaction emissions within the spiral serpentine and linear serpentine flow-cells. 1.9 mM enhanced potassium permanganate was reacted with 1 mM morphine (a & d); 1 mM [Ru(bpy)$_3$]$^{3+}$ was reacted with 1 mM ofloxacin (b & e); and 2.6 mM luminol was reacted with 2.8 µM sodium hypochlorite (c & f). Green circles indicate the determined points of maximum emission.

The distances to maximum chemiluminescence emission were also examined, using each of three model chemiluminescence reactions: enhanced potassium permanganate with morphine, [Ru(bpy)$_3$]$^{3+}$ and ofloxacin, and luminol with sodium hypochlorite (Table 4.4).
Table 4.4: Calculated distances to maximum emission within the spiral serpentine and linear serpentine flow-cells. n=3.

<table>
<thead>
<tr>
<th></th>
<th>Distance to Maximum Emission (mm)</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Spiral Serpentine</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enhanced KMnO₄</td>
<td>17</td>
<td>125</td>
</tr>
<tr>
<td>[Ru(bpy)₃]²⁺</td>
<td>29</td>
<td>152</td>
</tr>
<tr>
<td>Luminol</td>
<td>104</td>
<td>152</td>
</tr>
</tbody>
</table>

* Spiral serpentine distance to maximum emission includes the distance from the back of the cell through to the front surface.

The distance to maximum emission is related to both the channel design and the reaction kinetics. The faster reactions, enhanced potassium permanganate and [Ru(bpy)₃]²⁺, showed the biggest variation between cells. However, as can be seen in Figure 4.19, the delay in emission by the linear serpentine causes the highest intensity light to be produced towards the centre of the cell, which may benefit detection with a PMT. The longer lasting luminol reaction was less affected by the slower mixing of the linear serpentine, with only a 1.5 fold increase in distance to maximum emission observed. RGB analyses of the reaction images in Figure 4.19 reflected these results in terms of difference between the cells following the order of fastest reaction showing the greatest difference, and the slower luminol reaction exhibiting a small difference in intensity between the cells.

The model chemiluminescence reactions were then compared within each of the two serpentine flow-cells using flow injection analysis, and intensities detected with a photomultiplier tube.
Figure 4.20: Comparison of chemiluminescence intensities achieved with each cell configuration. Enhanced potassium permanganate (KMnO₄) (1.9 mM) was reacted with $1 \times 10^{-5}$ M morphine (red bars). [Ru(bpy)₃]²⁺ (1 mM) was oxidised online with 1 mM cerium(IV), and then reacted with $1 \times 10^{-5}$ M ofloxacin, all prepared in 0.05 M sulfuric acid (orange bars). Luminol (2.08 mM), dissolved in 0.1M NaOH, was reacted with 0.01% commercial bleach solution (42 g/L sodium hypochlorite) (blue bars). 1σ error shown, $n=5$.

Initial examination of chemiluminescence intensities within the two serpentine configuration flow-cells, shown in Figure 4.20, demonstrated a minor decrease (20%) in the emission intensities with the linear serpentine compared to the spiral serpentine, for all three reagent systems. It was expected, based on previous research,¹⁷⁴-¹⁷⁶ that the spiral serpentine would perform better than the linear serpentine, but the difference between the intensities achievable with the two flow-cells was expected to be much greater. A difference of only 20% makes the linear serpentine a more viable option for incorporation into a detection zone for a microfluidic device, as minor differences in intensities at higher concentrations often translate to very small differences in limits of detection. This decrease in intensity
could also be related to the area of channel exposed to the photomultiplier tube. While the linear serpentine has a longer channel length overall than the spiral serpentine (344 mm and 282 mm, respectively), the depth of the through-hole in the spiral serpentine offered a concentrated, more intense emission from the small area of channel, positioned at the centre of the photomultiplier tube (previously shown in Figure 4.19 a-c), a feature that is not achieved with the linear serpentine design. While this through-hole offers high intensity, it is very brief as the reaction progresses through the remainder of the flow-cell.

In order to compare the sensitivity afforded by each cell, a calibration series was run for each reaction, comprising six standards, and the limits of detection were determined, the results of which are summarised in Tables 4.5, 4.6 and 4.7 below.

Table 4.5: Analytical figures of merit obtained for morphine with the enhanced potassium permanganate reagent, in the spiral and linear serpentine flow-cells.

<table>
<thead>
<tr>
<th>Serpentine Flow-Cell</th>
<th>Calibration Function (peak height, mV)</th>
<th>( R^2 )</th>
<th>LOD (M)(^a)</th>
<th>RSD %*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spiral</td>
<td>( y = 8.815 \times 10^7x + 0.045 )</td>
<td>0.999</td>
<td>( 3.5 \times 10^{-9} )</td>
<td>0.53</td>
</tr>
<tr>
<td>Linear</td>
<td>( y = 5.027 \times 10^7x + 0.081 )</td>
<td>0.999</td>
<td>( 3.7 \times 10^{-9} )</td>
<td>1.57</td>
</tr>
</tbody>
</table>

\(^a\) Calculated as 3σ of the blank
\(^*\) 5.0 \times 10^{-8} \text{ M morphine (n=5)}

Table 4.6: Analytical figures of merit obtained for ofloxacin with the \([\text{Ru(bpy)}_3]^{2+}\) reagent, in the spiral and linear serpentine flow-cells.

<table>
<thead>
<tr>
<th>Serpentine Flow-Cell</th>
<th>Calibration Function (peak height, mV)</th>
<th>( R^2 )</th>
<th>LOD (M)(^a)</th>
<th>RSD %(^^)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spiral</td>
<td>( y = 2.362 \times 10^9x + 2.112 )</td>
<td>0.999</td>
<td>( 1.5 \times 10^{-9} )</td>
<td>0.65</td>
</tr>
<tr>
<td>Linear</td>
<td>( y = 2.608 \times 10^9x + 3.843 )</td>
<td>0.993</td>
<td>( 1.6 \times 10^{-9} )</td>
<td>1.03</td>
</tr>
</tbody>
</table>

\(^a\) Calculated as 3σ of the blank
\(^^\) 1.0 \times 10^{-8} \text{ M ofloxacin (n=5)}
Table 4.7: Analytical figures of merit obtained for the sodium hypochlorite standard with the luminol reagent, in the spiral and linear serpentine flow-cells.

<table>
<thead>
<tr>
<th>Serpentine Flow-Cell</th>
<th>Calibration Function (peak height, mV)</th>
<th>$R^2$</th>
<th>LOD (M)$^a$</th>
<th>RSD %$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spiral</td>
<td>$y = 7.217 \times 10^4x$</td>
<td>0.999</td>
<td>$1.2 \times 10^{-7}$</td>
<td>0.85</td>
</tr>
<tr>
<td>Linear</td>
<td>$y = 3.007 \times 10^4x$</td>
<td>0.999</td>
<td>$3.4 \times 10^{-7}$</td>
<td>1.68</td>
</tr>
</tbody>
</table>

$^a$ Calculated as 3σ of the blank
$^b$ 4.2 $\times$ 10$^{-5}$ M sodium hypochlorite (n=5)

Based on the information shown in the tables above (Table 4.5-4.7), only marginal differences in sensitivity were observed between the two flow-cell designs. The largest difference observed for limits of detection was with the luminol reaction detecting sodium hypochlorite (Table 4.7), where the spiral serpentine was approximately 3 times more sensitive than the linear serpentine. Despite smaller channels being utilised, excellent limits of detection were achieved for both flow-cells with the permanganate-morphine and [Ru(bpy)$_3$]$^{2+}$-ofloxacin reactions, comparable with literature values.$^{147, 154, 357}$

Previously reported limits of detection for the determination of ofloxacin using the [Ru(bpy)$_3$]$^{2+}$ and cerium(IV) sulfate method were $1.1 \times 10^{-9}$ M ofloxacin,$^{154}$ using flow-cell channels approximately $0.7 \times 0.8$ mm (w x d),$^{157}$ significantly larger than the channel dimensions used in this work. The low limit of detection achievable here with the spiral serpentine for this system was surprising, given the smaller channel dimensions used, but can be attributed to the comparatively larger sample-to-channel volume ratio, mentioned earlier in this section.

From the information gained through this research, it can be concluded that the linear serpentine design is an excellent candidate for use as a chemiluminescence detection zone within a microfluidic device, as it is simpler to design and manufacture, and has
been shown to have little difference in overall sensitivity compared to highly efficient designs, like the spiral serpentine, despite having poorer mixing efficiency.
4 Conclusions

This chapter demonstrated the ability to use smaller channels and a more ‘simple’ design with only minor sacrifice to sensitivity. The linear serpentine design showed less mixing efficiency, with each reaction taking longer to reach maximum emission. However, only minor differences in limits of detection were observed for each reaction, possibly due to the linear serpentine’s delay allowing maximum emission to occur within the centre of the PMT for each reaction.

The coloured flow-cell experiments showed the importance of material choice for chemiluminescence reactions. The white polymer flow-cell produced the best signals, but reasonable responses were achieved if the cell colour matched the emission colour.

This work also demonstrated that image analysis correlates to FIA results, so can be applied as a simpler way to compare flow-cells.

The findings of this chapter can facilitate the design of devices that allow for easy manufacture of detection zones, with potential mass production, without completely sacrificing sensitivity.

Some of the findings of this work have been published in a peer-reviewed journal.355
CHAPTER FIVE

STUDIES ON MICROFLUIDIC DEVICE DESIGN,
PREPARATION AND APPLICATION

1. Introduction
2. Experimental
3. Results and Discussion
4. Conclusions
1 Introduction

Device design is an important part of preparing a microfluidic device, or lab-on-a-chip system. Depending on the requirements of the device, careful engineering and design can help prevent failures caused by manufacturing and assembly processes. For a separation and chemiluminescence detection device, several aspects are required for the device to fully function and meet the consumer’s needs: sample/mobile phase introduction; separation media/zone; chemiluminescence reagent introduction; mixing/detection zone; and a waste outlet or reservoir.

Previous chapters have addressed the separation and detection aspects of the device, but alternative reagent introduction methods have not yet been explored as part of this work. Fluid storage reservoirs, such as blister packs, offer a secure, light-tight reagent storage option for microfluidic devices. The use of pumps and connecting tubing complicate a device, and open it to contamination, leakages and errors. While blister packs are utilised in other microfluidic devices, their use as chemiluminescence reagent storage has limited published research, though is mentioned in many patents spanning the past few decades, indicating interest in utilising blister reagents for on-chip storage of chemiluminescence reagents.

For a device to be marketable, it needs to be designed with mass production in mind, for easy assembly, and also designed for ease of use by the end-user. Injection moulding is a viable mass-production technique, despite high start-up costs (e.g. equipment, tools, producing the mould insert), the cost per device reduces significantly when a greater number of devices are produced. This device’s size was chosen to be compatible with our injection moulding machine’s plate insert size.
(Sumitomo Demag 100/420-200C, 1000 kN clamping force, plate size: 110 × 200 × 23 mm) and we required the device to be able to perform all required functions listed above. The device should be optimised to require as little external equipment as possible, particularly pumping and detection equipment, like photomultiplier tubes (PMTs), which will prevent a ‘chip-in-a-lab’ scenario\textsuperscript{243-245} from unfolding.

This chapter explores device design as a whole, incorporating sample and reagent introduction, separation in one and two dimensions, as well as analyte detection. The potential of blister reagent storage for chemiluminescence reagent introduction was also evaluated.
2 Experimental

2.1 Chemicals

All solutions, unless otherwise stated, were prepared in Milli-Q filtered deionised water.

Tris(2,2ʹ-bipyridyl)ruthenium(II) chloride ([Ru(bpy)₃]Cl₂·6H₂O) was purchased from Strem Chemicals (MA, USA). Potassium permanganate was purchased from ChemSupply (Gillman, SA, Australia). Sulfuric acid was obtained from Merck (Bayswater, VIC, Australia). Morphine was supplied by SunPharma (Port Fairy, VIC, Australia) and prepared as a 1 mM stock solution and diluted as required. Cerium(IV) sulfate, luminol, ofloxacin, sodium polyphosphate and sodium thiosulfate were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Sodium hydroxide was obtained from Ajax Chemicals (Sydney, NSW, Australia). Commercial bleach (42 g/L sodium hypochlorite) and food dye (Queen Fine Foods, Alderley, QLD, Australia) was purchased from a local supermarket.

2.2 Reagents

The enhanced potassium permanganate reagent was prepared as previously described in Chapter 4 by dissolving potassium permanganate (1.9 mM) in 1% (m/v) sodium polyphosphate. The solution was adjusted to pH 2.5 with sulfuric acid before the addition of sodium thiosulfate (0.6 mM).

The tris(2,2ʹ-bipyridyl)ruthenium(II) ([Ru(bpy)₃]²⁺) reagent was prepared to 1.0 mM by dissolving tris(2,2ʹ-bipyridyl)ruthenium(II) chloride hexahydrate ([Ru(bpy)₃]Cl₂·6H₂O) in 0.05 M sulfuric acid. The reagent was oxidised online to tris(2,2ʹ-bipyridyl)ruthenium(III) ([Ru(bpy)₃]³⁺) by a stream of 1.0 mM cerium(IV)
sulfate prepared in 0.05 M sulfuric acid. Ofloxacin was prepared as a 1 mM stock solution and diluted as required with 0.05 M sulfuric acid.

The luminol reagent was prepared by dissolving luminol (2.6 mM) in 0.1 M sodium hydroxide. The sodium hypochlorite sample (5.6 µM) was prepared by dilution of a commercial bleach solution (42 g/L sodium hypochlorite) in 0.1 M sodium hydroxide.

2.3 Blister pack reagent longevity

A Sepha EZ-Blister blister packaging machine (Sepha Ltd, Belfast, Ireland) was used to produce the blister packs utilised for reagent storage longevity tests. Blister packaging materials (consisting of aluminium foil and plastic) were purchased from Techni-Chem Australia Pty Ltd (Preston, VIC, Australia). Blister packs were cold formed on a 10 blister die (8 × 300 µL blisters, 2 × 770 µL blisters) for 3.5 s using a pressure of 0.8 MPa. Reagent solutions were manually pipetted into each blister – 300 µL in small blisters, and 600 µL in the large blisters. Blisters were then sealed at 160°C for 3.5 s using a pressure of 0.8 MPa, and a knurled tool to increase surface area. Blister packs were then cut to size using the cutting tool and a pressure of 0.6 MPa for 1.0 s. Final 10 blister pack measurements were 100 × 50 mm.

Blister packs containing the different reagent solutions were stored in three environments: in a refrigerator at 4°C; in a cupboard at ambient temperature; or in an oven at 37°C.

2.4 Device Design and Fabrication

The microfluidic device was designed in SolidWorks 2015 (Dassault Systèmes, S.A., Vélizy, France). The chosen device size was 142 × 72.5 × 2 mm (see Figure 5.1), for compatibility with the plate insert size of the injection moulding machine (Sumitomo
Demag Systec 100/420-200C Injection Moulder, PBE Roboplas, Seven Hills, NSW, Australia) (plate size 110 × 200 × 23 mm). The design was smaller than the plate size to allow for runners and gates to be fitted around the device for filling. The required components for the device were sample and reagent introduction zones, separation zone (i.e. monolith), and a mixing/detection zone for the chemiluminescence reactions. Dependent on the analyte being detected, a second reagent introduction may be required for the reaction, such as in the detection of tertiary amines (such as codeine) by [Ru(bpy)$_3$]$^{2+}$, in which the reagent is required to be oxidised prior to reaction with the analyte, in order to produce the chemiluminescence emission.

For the first iteration of this device, only a single-dimension separation was required, simplifying the device significantly in terms of design and operation. A base design was prepared in SolidWorks, to which future modifications could be made if necessary. The base design is shown in Figure 5.1.
Figure 5.1: Solidworks model of the device base design, suited for injection moulding.

2.4.1 Laser Prototyping

Prototypes of device designs were laser cut for visualisation using a Trotec Speedy 100 laser cutter and engraver (Trotec Laser Pty Ltd, Sunshine West, VIC, Australia). Solidworks designs were saved as DXF files and imported into Adobe Illustrator (Adobe Systems Incorporated, San Jose, CA, USA). Engraving/etching was performed at power 75%, speed 90% and frequency 500 Hz. Cutting was performed at power 75%, speed 1.4% and frequency 1500 Hz.
3 Results and Discussion

For better portability of our device, this study focussed on alternative reagent introduction methods to standard syringe pumps, which can utilise smaller volumes and which require less bulky pumping systems.\textsuperscript{342} Many alternative methods for moving solution through a chip are described in the literature including, but not limited to, capillary pumping,\textsuperscript{337, 383-386} magnetic particles,\textsuperscript{387-390} and fluid storage reservoirs (blister packs).\textsuperscript{363-381} For our reagents, the latter is the most viable option, as light tight blister packs can be created, which will prevent light degradation of some chemiluminescence reagents.\textsuperscript{391-392}

3.1 Blister pack reagent storage

Blister packs are utilised for on-chip storage of reagents for microfluidic and lab-on-a-chip devices, and can be utilised as the propulsion mechanism instead of pumps.\textsuperscript{393} Chemiluminescence reagents were stored in blisters, and their stability and longevity examined. Recoverability of reagent volume by needle and syringe was determined, as well as durability of the blister packaging itself to the reagents, and finally short- and long-term chemiluminescence reagent stability was explored.

3.1.1 Recoverability

When using blister packages for reagent introduction, theoretically, almost all of the packaged reagent would be introduced into the channels through blister compression. However, in order to be able to compare results obtainable within a device, to those detected externally, a control needed to be performed with the reagents from the blisters. As such, a regular flow injection analysis manifold was utilised for a comparison, and reagents recovered from the blisters using a needle and syringe. In the flow injection analysis system used in this work, a 70 µL injection loop was
used, so at least 70 µL were required to be recovered from each blister in order for comparisons to be made.

![Image](image.png)

Figure 5.2: Recoverable volume of reagent (the permanganate reagent is shown here) from a single blister using a needle and syringe. Approximately 200-300 µL was retrievable from each blister.

Of the 300 µL of potassium permanganate reagent solution within each of the blisters, around 275-300 µL was able to be retrieved from each blister for flow injection analysis testing using a needle and syringe, as shown above in Figure 5.2, enough for 2-3 injections ensuring the loop is filled with reagent. Due to the length of the needle tips, and the rounded shape of the blisters, it was almost impossible to retrieve every last drop of the reagent, however, a burst compressed blister should be able to expel all reagent contained within it. No change in recoverability was seen over the course of a four week testing period for the permanganate reagent.
3.1.2 Blister packaging durability

To determine the durability and robustness of the blister packaging, reagents were stored in the blisters, and changes in mass and appearance were recorded. Blister packs containing enhanced potassium permanganate (pH 2.5), [Ru(bpy)$_3$]$^{2+}$ in 0.05 M sulfuric acid, cerium(IV) sulfate in 0.05 M sulfuric acid, and luminol in 0.1 M sodium hydroxide chemiluminescence reagents were each stored for a period of up to 7 days. Blister packs were stored in the refrigerator (4°C) and in a cupboard at ambient temperature for the duration of the experiment.

Figure 5.3: Changes in blister pack masses (n=3) over 7 days for four different chemiluminescence reagents, under different storage conditions: at 4°C (blue lines), and at ambient temperature (green lines). (a) tris(2,2ʹ-bipyridyl)ruthenium(II) in 0.05 M H$_2$SO$_4$; (b) potassium permanganate (pH 2.5); (c) cerium(IV) sulfate in 0.05 M H$_2$SO$_4$; (d) luminol in 0.1 M sodium hydroxide.
Little change was seen in mass and appearance of the permanganate, ruthenium reagent and cerium(IV) sulfate reagent blisters throughout the duration of the study (Figure 5.3a-c, note y-axis scales), indicating that the acidic solutions caused no damage to the blister pack materials, and no solution had been lost. However, the luminol reagent packaging began exhibiting degradation within 2 days, and a large mass had been lost by 7 days, more so for the cupboard stored blisters, around 1.5 g.

Figure 5.4: (a) Partial corrosion of the blister package (14 days), with a white residue present on the outside of the blister package. Blister 4 shows some bubbling, where reagent has been able to leak from the blister. (b) Full corrosion of some of the blister packs (blister 10) (21 days). Blisters 7 & 8 were uncompromised and still contained the luminol reagent for analysis, while every other blister had been compromised in some way.
Figure 5.4 shows damage to blisters containing the luminol in 0.1 M sodium hydroxide solution, which were stored for longer than 7 days. Corrosion to the blisters is evident in both the 14 day and 21 day blister packs, which resulted in lost solution. Due to this damage, there was great difficulty in recovering enough of the reagent to perform an analysis.

It is known that alkaline solutions dissolve solid aluminium, the main component of the blister package foil, and therefore the alkaline sodium hydroxide was a major contributor to the corrosion of the blister packs. A further experiment was conducted to determine all of the contributing factors to the damage: if it was solely the 0.1 M sodium hydroxide, the luminol, or the combination of both. Four sets of blister packs were prepared: 1) 0.1 M sodium hydroxide; 2) luminol in 0.1 M sodium hydroxide; 3) luminol in deionised water; and 4) luminol sodium salt in deionised water. The luminol sodium salt was included as it is water soluble without the addition of acids or bases, and therefore is a viable option for use in the future if (a) both the sodium hydroxide and luminol were contributing factors to the damage, and (b) if the luminol sodium salt caused no damage to the blister packs itself. Of these four sets, 6 blister packs of 10 blisters each were prepared, with three of these kept in a cupboard at room temperature, while the other three were refrigerated at 4°C. Daily mass recordings were taken, for a period of 7 days.
Figure 5.5: Average changes in mass of blister packs (n=3) over the course of seven days, stored in a cupboard at ambient temperature (green triangles) or in a refrigerator at 4°C (blue circles). Blister packs contained (a) 0.1 M NaOH; (b) 2.6 mM luminol in 0.1 M NaOH; (c) 2.6 mM luminol in deionised water; and (d) 2.6 mM luminol sodium salt in deionised water. Masses were recorded each day over the seven day period.

Figure 5.5a demonstrates a loss in mass after 2 days of storage at ambient temperature, and a loss of close to 2 g after 7 days. The refrigerator stored packages also showed loss, but was less severe. For the luminol in sodium hydroxide solution, Figure 5.5b, losses were seen for both storage conditions, but the change in mass was less than that observed for the sodium hydroxide solution, indicating that the luminol was not a likely contributor to the corrosion of the blister packages. The smaller loss observed with the refrigerator kept packs indicates that some of the loss of solution and mass is through evaporation of the solution if the blisters were stored at room temperature. Little change in mass was recorded for both the luminol in deionised water.
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water (Figure 5.5c), which did not dissolve, and the luminol sodium salt in water (Figure 5.5d).

The studies demonstrated that the 0.1 M NaOH was main contributor to the corrosion of the blister packaging, so a water soluble luminol alternative would be favourable for the continued use of blister storage of a luminol-type reagent.

3.1.3 Short-term chemiluminescence study

Short and long term chemiluminescence reagent storage studies (7 and 28 days, respectively) were undertaken to determine the viability of long-term reagent blister storage. A control environment, where the reagent was kept in a Schott bottle in a cupboard, was utilised for the short-term studies to provide a non-blister environment control. Only the enhanced potassium permanganate and [Ru(bpy)₃]²⁺ reagents were examined in the short-term study. Reagents were subjected to four different environments: control (no blister packaging), stored in a Schott bottle in a cupboard at ambient temperature; in a refrigerator, at 4°C; in a cupboard at ambient temperature, with the control; and in an oven at 37°C. The chemiluminescence intensity of each reagent was tested using FIA on Day 0, following packaging in the blisters and 60 minutes in their designated environment, to determine if the temperatures used to seal the blisters (160°C) would immediately affect the reagents in comparison to the control.
Figure 5.6: Chemiluminescence emission intensities for the reaction of [Ru(bpy)$_3$]$^{2+}$ (from the blisters) reacted with fresh Cerium(IV) sulfate and $1 \times 10^{-7}$M ofloxacin. Results for control (grey circles), refrigerator (blue triangles), cupboard (green diamonds) and oven (orange squares) are shown. 1σ error bars are shown, $n=5$.

The results demonstrated that storage within any blister package, regardless of environment, appears to have enhanced the [Ru(bpy)$_3$]$^{2+}$ by around 7% over the first 3 days, when compared to the non-blister control (grey circles, Figure 5.6). FIA experiments were completed during summer months, and were not performed in a climate controlled environment, so room temperature fluctuation caused by increased summer temperatures is the likely cause of this small variation.
Figure 5.7: Chemiluminescence intensities for the reaction of enhanced potassium permanganate (from the blisters) reacted with $1 \times 10^{-7}$M morphine. Results for control (grey circles), refrigerator (blue triangles), cupboard (green diamonds) and oven (orange squares) are shown. $1\sigma$ error bars are shown, $n=5$.

Degradation of the permanganate reagent after three days in the oven was seen (Figure 5.7, orange squares), with a ten-fold decrease in intensity after seven days in the oven, in comparison with the other blister packs and control. Storage in the refrigerator versus cupboard made little difference on emission intensity, and it appears that blister storage has no overall negative or positive impact on the reagent emission intensities when compared to glassware storage. Therefore, a permanganate reagent solution could be stored within blister packs within a cupboard with no detrimental effects to the reagent over a short-term period.

3.1.4 Long-term chemiluminescence study

Since blister packs are designed for long-term storage of reagents and samples, longer term blister storage studies were performed on several reagents, including enhanced potassium permanganate, [Ru(bpy)$_3$]$^{2+}$, Ce(IV) sulfate and luminol. The
reagents were prepared fresh, analysed and stored in the blisters in ambient or refrigerated climates. No increased temperature environments were explored for this study, as the previous work in Section 3.1.3 had proven detrimental to emission intensities for the permanganate reagent, and is a costly and unnecessary storage solution for the ruthenium reagent.

Figure 5.8: Chemiluminescence emission intensities of reactions (n=5) (a) 1.9 mM enhanced potassium permanganate (in 1% (m/v) polyphosphates) reacted with 0.1 µM morphine; (b) 1 mM [Ru(bpy)₃]²⁺ reacted with 1 mM cerium (IV) sulfate and 0.1 µM ofloxacin; (c) 1 mM cerium(IV) sulfate reacted with 1 mM [Ru(bpy)₃]²⁺ and 0.1 µM ofloxacin; (d) 2.6 mM luminol reacted with 5.6 µM sodium hypochlorite. Blister packs were stored in a cupboard at ambient temperature (green triangles) or in a refrigerator at 4°C (blue circles).

As seen in Figure 5.8a, the potassium permanganate reaction reduced in emission intensity across the 28 days when stored in the cupboard, but exhibited a slight emission intensity increase when stored in the refrigerator over the same period.
Storage conditions had a large impact on longevity of the reagent beyond 7 days, with over a 10% increase for the refrigerator stored reagent, and over 60% decrease for the cupboard stored reagent. It has previously been observed that permanganate solutions left to degrade for long periods of time can produce superior emission intensities with specific analytes,\textsuperscript{357} which may account for the observed increase over time with the refrigerator stored reagent, shown in Figure 5.8.

The ruthenium reagent reaction’s emission intensity peaked at 7 days (Figure 5.8b), before slowly decreasing to levels similar to 0 days for both storage environments. Little difference between cupboard and refrigeration storage was seen for the ruthenium reagent, indicating that if blister package reagent storage were to be used, refrigeration storage, at least over a 28 day period, would not be necessary.

Degradation of the cerium(IV) sulfate reagent occurred rapidly across 0-7 days, indicated by the decrease in emission intensities achievable when reacted with the ruthenium reagent and ofloxacin (Figure 5.8c). It is well known that this oxidant solution should be prepared fresh daily in order to avoid differing results due to degradation.\textsuperscript{153, 160}

Refrigerator storage was able to marginally delay complete degradation in comparison to the cupboard stored cerium(IV) sulfate, but a significant decrease in emission intensities was still observed at 7 days. After just 14 days, there was no distinguishable signal from the blister packaged cerium(IV) sulfate cupboard reagent, however the refrigerator reagent was still able to oxidise the [Ru(bpy)$_3$]$^{2+}$, although the signal produced was one fifth of that of the control with fresh reagents. After seven days, the refrigerator cerium(IV) sulfate signal had been reduced by 75%, though degeneration over the subsequent days and weeks was minimal, around
4-5% reduction per following week. In the literature, cerium(IV) sulfate solutions are generally prepared fresh daily for chemiluminescence experiments, and the data shown here (Figure 5.8c) confirm that blister package storage of this oxidising agent is undesirable, and fresh cerium(IV) solutions should be prepared for use in any microfluidic device.

A slight emission intensity increase was observed over the first seven days for the luminol reaction, and then a gradual decreased observed over the following 14 days until Day 21 (Figure 5.8d). Little difference between storage conditions was seen for the luminol reagent, however degradation of the packaging was evident due to the high alkalinity of the solution dissolving the aluminium casing, as previously discussed. A spike in intensity at 28 days was seen, with detector response reaching maximum, but was not able to be investigated due to (a) no in-tact blister packs remaining for testing with due to reagent consumption; and (b) the loss of luminol within the original blister packs due to damage.

The results of this long-term storage study shown here indicate that blister storage of enhanced potassium permanganate and the ruthenium reagent is a viable option, dependent on the storage conditions for the permanganate reagent.

### 3.2 Device Computer Aided Design

Computer Aided Design (CAD) software (SolidWorks 2015) was utilised to create theoretical designs of an overall microfluidic device. Unfortunately, due to time constraints, the devices were not able to be produced or tested.

As previously mentioned, the devices were designed with future mass production in mind, so the dimensions chosen (142 × 72.5 mm) were to suit fitting on an injection moulding plate, which in this case matched the maximum plate size which would fit
inside our injection moulding machine. This limited the number of designs feasible with all the requirements needed for this device, including separation and detection.

A three-layered multi-dimensional device was first conceived, with two separation dimensions, and two separate columns of different functionality were included in the second dimension. However, initially, single dimension design iterations were created in the design software from the base design (shown previously in Figure 5.1), before moving onto multi-dimension and multilayer designs.\textsuperscript{340}

For all of the designs described herein, sample and solution introduction would be performed using pumps or injection ports, but designs could be modified for blister packaged reagent introduction.

3.2.1 Single Dimension Devices

3.2.1.1 Single reagent, single dimension separation

In Chapters 2 and 3, monolith columns 50 mm in length and 4.6 mm in diameter were used for liquid chromatography separations. The same dimensions were used for the column within the single dimension device, as shown below in Figure 5.9. Despite work in Chapter 2 exploring a monolith resin encapsulation method, the designs here would not utilise the resin method for encapsulation. It is thought that injection moulding would be used to encapsulate the monoliths within the devices, eliminating the need for resin to be used. The molten plastic used in the injection moulding process would also be able to penetrate the outer layers of the monolith, similarly to the resin encapsulation method.

Sample and reagent inlets, and the waste outlet, were kept the same as in Chapter 4 for this first design, however they can easily be modified in the future to suit
alternative introduction methods, such as blister packaged reagents. The first design iteration is shown in Figure 5.9.

Figure 5.9: Single-Dimension, 2 layer device is shown. Clear plastic top layer, and white plastic bottom layer, to enhance chemiluminescence emission reflection to the detector.\textsuperscript{177, 355} Single-dimension design includes a 50 mm column.

Due to limitations in assembly, the column cannot be positioned any closer to the detection zone without compromising the ability to properly seal the top and bottom plates together. In this design, the end of the column is positioned approximately 1 cm away from the reagent and column eluent merging point.

Standard photomultiplier tubes used in chemiluminescence detection have detector windows around 20 mm in diameter, and it was therefore decided that the detection zone would match this for the current design, despite smaller photodetectors, such as silicon photomultiplier tubes (SiPMs, around 3 mm × 3 mm) being available.\textsuperscript{400} Serpentine mixers are often used in microfluidic devices to enhance solution mixing,\textsuperscript{340} and the results from Chapter 4 demonstrated that they were able to mix chemiluminescence reagents and analytes efficiently. As such, the linear serpentine design from Chapter 4 was incorporated into the detection zone of the device, and modified to fit within the 20 mm diameter circular detection zone.
The device top-plate is shown to have three inlet holes and one outlet hole. This allows it to be interchangeable for both single-dimension designs and multi-dimension designs with two or three inlets, where the side chosen to be taped for adhesion does not matter due to the symmetry of the design. It has been discussed in the literature that designing devices for ease of manufacturability is favourable.243, 249, 358-359 This can be done through producing interchangeable parts so it does not matter which way the device is assembled, or by ensuring in the design that a device can only be assembled one way, so as to eliminate waste by assembly error.249

3.2.1.2 Two different reagents, single dimension separation
This design (Figure 5.10) was modelled on the two-split flow-cell design published in 2014, where a column eluent was split into two and detected using two different chemiluminescence reagents.215 The research had shown that an even split was achievable, and the reagent streams did not produce enough back pressure to flow back towards the column.215 This design would be useful in the simultaneous detection of the opiate alkaloid mix from Chapter 3 using the permanganate reagent and the ruthenium reagent. In this case, the ruthenium reagent would need to be oxidised prior to entering the device, but could easily be achieved with a Y- or T-piece merging an oxidant and ruthenium reagent streams just prior to the device.
Figure 5.10: Dual reagent single dimension device design.

### 3.2.2 Multidimension Devices

#### 3.2.2.1 Two reagent, multidimension separation

Figure 5.11: Multidimensional device, two reagents. Two reagents merge within the middle layer and travel through a serpentine mixer to ensure they are well mixed, prior to merging with the column eluent. Valves are positioned over the channels in the bottom layer to control column 1’s eluent direction. The centre channel flows directly to the outlet, and the two outer channels flow to two columns for the second dimension.
In this design (Figure 5.11), shorter 25 mm columns were incorporated, to ensure that all device components would fit. Valves have been included so as to control the first column eluent, and direct it towards either of the two second dimensions columns. These valves could be mechanically actuated, and at this stage would make use of PDMS cast valves plugs. A diagram of the basic valve design is shown in Figure 5.12, below. The second dimension columns could have the same or different functionalities as each other, depending on the samples to be analysed.

![Diagram of valve design](image)

**Figure 5.12: Example of valve depression design. Valves were of a conical design, prepared in SolidWorks using a cone tool. Depth was 2 mm, and penetrated through the layer of PMMA plastic to join with channels in lower layers.**

A close knit serpentine mixer was used in this design based on the results from the serpentine comparison in Chapter 4, ensuring efficient mixing within a small space would be achieved. The serpentine mixer would ensure that the two reagents would be well mixed prior to merging with the column eluent stream. Figure 5.13a better demonstrates the serpentine reagent mixer.
Figure 5.13: Multi-dimension, two reagent design: (a) top-down view; (b) exploded view. The bottom layer is displayed as an opaque white plastic, which would be utilised to enhance reflection of the reaction emission back to the detector.

This design could also utilise a single reagent, such as potassium permanganate, entering the device through both of the reagent inlets, without needing to produce a second device. This design is ideal for chemiluminescence reagents which require a
second reagent to activate or enhance the reaction, whether it be oxidation, as is the case with ruthenium(II)\textsuperscript{153, 160-161, 316} and iridium(III)\textsuperscript{154, 176, 396, 398-399, 403-406} complexes, or in the reaction of luminol with potassium hexacyanoferrate(III)\textsuperscript{174, 407-408}.

Basic testing of PDMS valves was conducted to determine the depression diameter required to ensure a good seal to block solution passage through to other channels and columns, shown below in Figure 5.14. Valve depressions were 2 mm deep, with the diameters varied slightly from 10 mm ± 1-2 mm to see if a larger or smaller diameter would better control solution flow. The results showed no observable difference in level of solution flow control, regardless of valve diameter, so a 10 mm diameter was chosen for all future designs.

\textbf{Figure 5.14: Device design for testing valve diameters. Five different diameters were tested, using finger pressed PDMS valves. Valves are approximately 10 mm in diameter, varying by 1-2 mm, with a depth of 2 mm.}
It should be noted that PDMS is a rather undesirable material in microfluidics, due to its absorption of hydrophobic molecules from solution,\(^{409-410}\) as well as being unattractive for the mass-production of devices.\(^{410-411}\) Therefore, no further testing was conducted with PDMS as a valve material for the device.

### 3.2.2.2 Two different reagents, multidimension separation

A second iteration of the multidimensional, multilayer design incorporated the use of two different reagents, as shown in Figure 5.15, which would allow for the simultaneous detection of complex samples, such as the opiate mixture utilised in Chapter 3, using the common chemiluminescence reagents \([\text{Ru(bpy)}_3]^{3+}\) and potassium permanganate, where the permanganate reagent would detect phenolic opiates such as morphine and oripavine, and the ruthenium reagent would detect non-phenolic compounds, such as codeine and thebaine.
Figure 5.15: Multidimension device, accommodating two different reagents for detection. (a) top down view; (b) exploded view. Valves are utilised to control the first dimension column eluent, in order to perform the second direction separation.

In the case of the opiate separation mentioned above, the second dimension could be the same as the first dimension, to allow more resolution from co-eluting peaks, such
as morphine and codeine, seen in the opiate separation in Chapter 3. An assembled view of the device is shown in Figure 5.16.

Figure 5.16: Multidimension, multireagent device design, assembled view.

### 3.3 Device Discussion and Future Directions

As previously discussed, each of the device designs was created with mass production injection moulding in mind. The basic plate design (142 × 72.5 mm) is around the largest sized device that could be fitted on the injection moulding plates for our machine. Some of the earlier iterations (single layer designs) could be fitted onto much smaller chips, both length and width-wise, especially if the shorter column (30 mm) was to be utilised. As discussed by Becker, back-end processes of chip manufacture can be the hindrance of a design or prototype reaching the market. Though many of the back-end processes listed by Becker are irrelevant to the purpose of the device for this project, the author does discuss that injection moulding can be beneficial in eliminating some of the back-end processes of chip
manufacture, particularly eliminating the need to drill or cut inlet holes post-production.\textsuperscript{242} As can be seen in all of the designs shown previously (Figures 5.8-5.16) each design incorporates inlet and outlet holes to enable solution introduction. Preparation of the master mould designs in SolidWorks from each of these designs will therefore include the inlet and outlet holes, further enabling easy manufacture of the device. Another aspect of design discussed by Becker is designing so a device can be assembled only one way, or can be assembled regardless of directionality. An amendment to each of the device designs to overcome this would be to include three inlet/outlet holes at each end of the device’s top-plate, which would eliminate potential failed devices from miss-assembly. Other ways of overcoming this are to include registrations which ensure correct alignment of parts.\textsuperscript{412-414} This is easier to achieve with injection moulding than some other manufacture methods (i.e. laser etching), as the mould can incorporate extruded or intruded parts to manufacture registrations.

At this stage, external syringe pumps were used to propel solutions through channels in Chapter 4, and would still be required to propel solutions through the device, which would ultimately complicate the device, and potentially lead to a chip-in-a-lab scenario.\textsuperscript{243-245} There are alternative pumping methods for microfluidic devices which have been published, including but not limited to: magnetic particle propulsion,\textsuperscript{387-390} capillary pumps,\textsuperscript{337, 383-386, 415} surface modification,\textsuperscript{416-421} and the already discussed fluid storage reservoirs (blister package reagents).\textsuperscript{363-364, 367-381} It should be noted some of the listed pumping solutions, such as magnetic particles, may be hindered by the monolithic column/s within the device, and so should be avoided. As the device is still in early stages of design and prototyping, utilising external pumping solutions such as syringe pumps is still required, however, ideally

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would be phased out in later stages of testing for alternative methods of solution pumping and manipulation.

Detectors like silicon photomultipliers (SiPMs) are available, with the sensor active area covering areas as small as $1 \times 1 \text{ mm}^2$, and still being relatively sensitive to chemiluminescence emissions, including red-emitting species.\textsuperscript{400} While these SiPMs would still require electronic components and a power source, their size is much more manageable for on-chip detection than conventional PMTs\textsuperscript{422-424} and do not require bulky high voltage power supplies.\textsuperscript{400}

Although as part of this work a final microfluidic device was unable to be created and tested, the various parts to be applied have been tested in the previous chapters of this thesis. The monolith columns were able to be produced, tested, and have successfully separated analytes of importance, and the poorer resolution compared to commercial counterparts was improved with post-column chemiluminescence detection. Chemiluminescence detection with miniaturised channel dimensions was performed, and the linear serpentine channel design also proved successful in previous chapters, and has been applied here as the detection zone. Based on these parameters tested throughout the previous chapters, the devices designed in this chapter could be produced and perform successfully with minimal optimisation.
4 Conclusions

Blister package reagent storage was evaluated for its potential as on-chip reagent storage. The luminol solution was not stable within the blister packaging, causing degradation within days of packaging. However, the luminol sodium salt, which does not require an alkaline environment for dissolution, showed no damage to the blister packaging, and is a viable alternative to regular luminol in sodium hydroxide. Over the course of 28 days, the ruthenium reagent showed good stability in both room temperature and cooled environments, with little difference between the two. Refrigerated potassium permanganate also showed good stability over the same period, however the room temperature blister packs showed decreases in emission intensities. Cerium(IV) sulfate degradation occurred rapidly, regardless of storage conditions, demonstrating that it should be prepared fresh for use in a microfluidic device, or suspended within the device in solid form.

Over the 7 day testing period, heating was the only parameter to result in a decrease in emission intensity for the permanganate reagent. No storage conditions vastly affected ruthenium reagent emission intensities over the same period of time.

Several device designs were explored in this chapter, incorporating single and multi-dimension separation, and single or multi-reagent introduction for detection. Though unable to be tested for analyte separation and detection, based on the results of previous chapters, the device should function as desired, with several potential improvements suggested for future iterations of the device to improve portability and reduce manufacture failures.
CHAPTER SIX

CONCLUSIONS AND FUTURE WORK

1. Conclusions

2. Suggestions for Future Work
1 Conclusions

Silica monolith columns were prepared as part of this work, and were characterised and optimised so as to have pore characteristics desirable for chromatography applications. Method optimisation in terms of careful monitoring of reaction times and temperatures allowed for better reproducibility across batches of monoliths. A monolith encapsulation procedure using a casting resin was created as a means of securely encapsulating monolith columns, which was solvent resistant and was able to prevent wall-effects from occurring. The encapsulation procedure resembles encapsulation via injection moulding, where liquid polymer penetrates the outer layer of the monolith before solidifying.

Encapsulated monoliths were successfully functionalised with a C\textsubscript{18} stationary phase using two methods (batch functionalisation, and post-encapsulation functionalisation). An opiate alkaloid separation was performed on the batch functionalised monolith, and when combined with chemiluminescence detection, offered good resolution between peaks of the four opiate alkaloids, with little optimisation. A lubricin glycoprotein, known to adhere easily to silica, was trialed as a stationary phase, but yielded little useful results. Tanaka Testing indicated a likely anion exchange property for the stationary phase, but due to the faster elution of analytes compared to a bare silica column, further examination of the stationary phase was not performed.

Chemiluminescence detection of important analytes was performed using conventional flow injection analysis in order to optimise detection zone configurations for a microfluidic device. Smaller channel dimensions compared to flow-cells were able to provide suitable limits of detection, and a linear serpentine
configuration was able to fully mix reagent streams within the detection zone within three reversing turns, whilst being much easier to manufacture than commonly utilised spiral or spiral serpentine flow-cell designs. Research also demonstrated that while a white polymer backing for a flow-cell reflects the greatest amount of light from a chemiluminescence reaction back to the detector, coloured plastics corresponding to the emission wavelengths of reactions performed better than clear plastics, which are utilised in many microfluidic devices; or clear+mirror flow-cells, which are often used in chemiluminescence detection manifolds.

A design for a microfluidic device for the separation and detection of analytes of interest using chemiluminescence reagents was created. Various iterations were designed, suitable for simple or complex sample separation and detection. Blister packs were analysed for their potential as long-term, external reagent storage for chemiluminescence reagents. Results showed that the aluminium-plastic blisters were suitable for storing [Ru(bpy)$_3$]$_{2+}$ and potassium permanganate for extended periods of time with no ill-effect, and so would be suitable for on-chip storage.

In conclusion, the various components of a microfluidic device for the separation and detection of analytes of interest were explored, with each component for separation and detection optimised in order to produce an efficient device. The device itself, though not tested, has several different designs which all encompass the same basic components, and is ready for testing once produced.
2 Suggestions for Future Work

Tanaka testing demonstrated that the lubricin stationary phase may have anion exchange properties. Due to the unavailability of proper instrumentation, further testing of its stationary phase properties was unable to be performed. Further exploration should confirm the stationary phase properties, if any, and should then be applied to separating samples of importance.

Multiple microfluidic device designs were produced in SolidWorks as part of this research, however, only laser cut prototypes were produced, and were not for full-scale testing of the device. CNC machined prototypes should be produced and further tested, including incorporating monolith columns with differing stationary phase properties. Further to this, injection moulding plates should be prepared to enable mass production of a suitable design.

Although blister packaged chemiluminescence reagents were explored with this work, the long-term study only examined the reagents over a 28 day period. A further longevity study of 6 to 12 months should be conducted to determine the maximum length of time that reagents could be stored within blister packages. Despite chemiluminescence testing of the blister packaged reagents in Chapter 5, on-chip chemiluminescence detection with said blisters was not performed as part of this research, and so should be tested to ensure reasonable limits of detection are still achievable, and to validate the use of such a method. Other researchers have developed a bursting mechanism for blister pack reagents, in order to deliver precise volumes of reagents,\textsuperscript{364} which could be adapted to our device and utilised to test blister bursting and solution dispersion and mixing within the device.
Should a fully functioning multidimensional device be produced, real-world complex samples of importance (such as drug seizure samples) should be separated and detected using the device, and compared to established methods.
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APPENDICES

1 Appendix A

Preparation methods for the immobilised $[\text{Ru(bpy)}_3]^{2+}$-sol gels described in Chapter 3.

KS002

A modification of the monolith preparation method described by Fletcher et al.\textsuperscript{51} 0.432 g of Pluronic F127 polymer and 40 mg Ru(bpy)$_3$Cl$_2$.6H$_2$O was dissolved in 0.02 M acetic acid (4 mL), while stirred in an ice batch for 40 minutes. 2 mL TMOS was added and stirred for a further 40 minutes. Monolith solution was transferred to a mould and incubated in an oven at 40°C for 72 hours. Monoliths underwent base treatment (1.0 M ammonium hydroxide, 90°C, 18 hours) following incubation.

KS003

Following Greenway and coworkers\textsuperscript{314} method, 2 mL of TMOS was stirred with 0.1 M HCl (5 mL) in an ice bath for 40 minutes. 0.14 g Ru(bpy)$_3$Cl$_2$.6H$_2$O and 2 mL of 10 mM NaOH were added, and the solution stirred for a further 40 minutes. The solution was pipetted into moulds and allowed to incubate in an oven at 40°C for 72 hours.

KS004

A modification of KS003, where Pluronic F127 polymer was added to the solution with the Ru(bpy)$_3$Cl$_2$.6H$_2$O, and allowed to incubate. These monoliths underwent base treatment in 1 M ammonium hydroxide, and were calcined at 250°C for 18 hours.
KS005

An attempt to follow Collinson and co-workers’ methodology\textsuperscript{311} was made, utilising tetramethyl orthosilicate (TMOS), water, 0.1 M hydrochloric acid and [Ru(bpy)\textsubscript{3}]\textsuperscript{2+}. 12 mL of TMOS was stirred for 2 hours at room temperature with 4.8 mL deionised water and 1.6 mL of 0.1 M hydrochloric acid. [Ru(bpy)\textsubscript{3}]Cl\textsubscript{2}.H\textsubscript{2}O was added to the mixture. 10 mM phosphate buffered saline (PBS) was mixed with the [Ru(bpy)\textsubscript{3}]-sol solution in a ratio of 2:1 before gelation in a desiccator.

KS006

[Ru(bpy)\textsubscript{3}]\textsuperscript{2+} doped solution from method KS005 (before PBS addition) was treated with 10 mM sodium hydroxide (NaOH, 2 mL), before being pipetted into moulds and placed in a desiccator, similar to the method of base catalysed gelation described by Greenway \textit{et al.}\textsuperscript{314}.

KS007

Prepared as described by Gorman\textsuperscript{316}. 15 mL TMOS, 7.5 mL deionised water and 2.1 mL of 0.1 M HCl were mixed for 2 hours at room temperature. 40 mg of Ru(bpy)\textsubscript{3}Cl\textsubscript{2}.6H\textsubscript{2}O was added and stirred before the addition of 5 mL PBS. The solution was pipetted into moulds, and air dried until the solution gelled.

KS008

A modification of the method described by Gorman,\textsuperscript{316} 0.432 g of Pluronic F127 polymer was mixed with 2.5 mL deionised water and 0.7 mL of 0.1 M HCl for 40 minutes. 40 mg of Ru(bpy)\textsubscript{3}Cl\textsubscript{2}.6H\textsubscript{2}O and 5 mL TMOS were added to the solution.
and mixed for a further 40 minutes. 1 mL PBS was added just prior to transfer to mould, and incubated at room temperature in a desiccator.

**KS009**

The solution from KS008 was incubated at 40°C in an oven. When gelled, the monoliths underwent base treatment and were calcined at 250°C.

**KS010**

Modification of method KS005, from Collinson et al., with the addition of the Pluronic F127 polymer. 0.432 g Pluronic F127 polymer was stirred with 4.8 mL deionised water, 1.6 mL of 0.1 M hydrochloric acid and 40 mg Ru(bpy)$_3$Cl$_2$.6H$_2$O at room temperature for 2 hours. 12 mL TMOS was added to the mixture, and then stirred for a further 2 hours. The solution was separated into four parts, each containing 5 mL of the solution. Solution KS010A was pipetted into moulds and incubated in an oven at 40°C for 72 hours. KS0101B had 2.5 mL PBS added (2:1, as discussed in method KS005), then was pipetted into moulds and incubated in an oven at 40°C for 72 hours. KS010C had the PBS added (2:1), and was incubated in a desiccator at room temperature for 72 hours. KS010D was pipetted into moulds and incubated in a desiccator at room temperature for 72 hours.

**KS012**

A modification of method KS009 with a higher polymer content. 0.7 mL of 0.01 M HCl was mixed with 3.5 mL deionised water, 0.6 g of Pluronic F127 polymer and 40 mg of Ru(bpy)$_3$Cl$_2$.6H$_2$O in an ice bath for 40 mins. 5 mL of TMOS and 2 mL of 10 mM PBS (pH 7.0) were added to the solution and mixed for a further 40 minutes. The solution was transferred to a mould and incubated in a desiccator for 72 hours until solidified.
KS013

Prepared as in method KS012, but solution contained a lower water content. 0.7 mL of 0.01 M HCl was mixed with 2.5 mL deionised water, 0.6 g of Pluronic F127 polymer and 40 mg of Ru(bpy)$_3$Cl$_2$.6H$_2$O in an ice bath for 40 mins. 5 mL of TMOS and 2 mL of PBS (pH 7.0) were added to the solution and mixed for a further 40 minutes. The solution was transferred to a mould and incubated in a desiccator for 72 hours until solidified.

KS014

Modification of method KS002, with hydrochloric acid (HCl) substituted for acetic acid. 0.432 g of Pluronic F127 polymer was mixed in an ice bath for 40 minutes with 4 mL of 0.1 M HCl and 40 mg Ru(bpy)$_3$Cl$_2$.6H$_2$O. 2 mL of TMOS was added and the solution stirred for a further 40 minutes. The solution was transferred to moulds and incubated in an oven at 40°C for 72 hours, before rinsing in deionised water.

KS015

[Ru(bpy)$_3$]$_{2}^{2+}$ monoliths were prepared as per KS002, but were not stirred in an ice bath. 0.432 g of Pluronic F127 polymer was mixed with 0.02 M acetic acid (4 mL) and 40 mg Ru(bpy)$_3$Cl$_2$.6H$_2$O at room temperature for 40 minutes. 2 mL of TMOS was added and the solution stirred for a further 40 minutes. Monoliths were incubated in an oven at 40°C for 72 hours.

KS016

Method KS005, but prepared in an ice bath. 12 mL of TMOS was stirred for 2 hours in an ice bath with 4.8 mL deionised water and 1.6 mL of 0.1 M hydrochloric acid.
Ru(bpy)$_3$Cl$_2$.6H$_2$O was added to the mixture. 10 mM phosphate buffer was mixed with the [Ru(bpy)$_3$]-sol solution in a ratio of 2:1 before gelation in a desiccator.

**KS017**

This follows the method KS007, but solutions were mixed in an ice bath. 15 mL TMOS was mixed with 7.5 mL deionised water and 0.1 M HCl (2.1 mL) in an ice bath for 40 minutes. 40 mg of Ru(bpy)$_3$Cl$_2$.6H$_2$O and 5 mL PBS (pH 7.0) were added and the solution stirred for a further 40 minutes. The solution was transferred to moulds and incubated in a desiccator for 72 hours. The dried sol-gels were rinsed in deionised water and then dried in an oven at 60°C for 24 hours.